VOLUME 11
SUPPLEMENT 2
JULY 2013
ISSN 1538–7933

jth

journal of thrombosis and haemostasis

Journal of the
International Society on Thrombosis and Haemostasis

ABSTRACTS OF THE XXIV CONGRESS OF THE INTERNATIONAL SOCIETY ON THROMBOSIS AND HAEMOSTASIS

JUNE 29 - JULY 4, 2013

Editors in Chief: Pieter Reitsma and Frits Rosendaal

WILEY-BLACKWELL

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ABSTRACT SYMPOSIA

AS 01 – Cardiovascular Disease

AS 01.1

Von Willebrand factor regulates physiological and pathological angiogenesis through vascular endothelial growth factor receptor-2 signalling

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Von Willebrand Factor (VWF) is a multimeric glycoprotein, which mediates platelet adhesion to damaged endothelium and stabilises coagulation factor VIII. VWF is synthesized by endothelial cells (EC) and stored in Weibel-Palade bodies (WPB), which also contain regulators of angiogenesis and inflammation. A qualitative or quantitative deficiency of VWF causes Von Willebrand disease (VWD), the most common congenital bleeding disorder, which can be associated with vascular lesions (angiodysplasia) causing severe intractable gastrointestinal bleeding. Angiodysplasia is characterized by a fragile vascular network with disrupted architecture, increased permeability and susceptibility to rupture. The pathogenesis has been linked to dysregulated angiogenesis. We have shown that VWF regulates angiogenesis through pathways involving VEGFR2 signalling and the integrin $\alpha v\beta 3$ (Starke et al., Blood 2011; 117:1071–1080). Here we investigate the role of VWF in two *in vivo* models of angiogenesis and arteriogenesis, and provide new evidence on the angiogenic signalling pathways regulated by VWF in EC.

To investigate the role of VWF in the response to ischemia, we used the murine model of hindlimb ischaemia and VWF-deficient mice (Denis et al., Proc. Natl. Acad. Sci. U.S.A 2001; 98:4072–4077). The femoral artery was ligated (or sham-operated) in littermate control or VWF-deficient mice and blood flow was measured in the paws by Laser Doppler analysis at different time points. At day 3 postligation, VWF deficient mice showed a significant decrease in blood flow compared to controls ($P < 0.05$). In the lower leg (gastrocnemius [GN]), H&E and CD31 staining showed increased angiogenesis ($P < 0.05$) and tissue damage ($P < 0.05$) in VWF-deficient mice compared to controls. Quantitative RT-PCR analysis showed reduced β3 integrin levels in VWF-deficient mice compared to controls, in line with our previous *in vitro* findings. In the adductor muscle, VWF deficient mice showed reduced leukocyte infiltrate and vascular proliferation, suggesting decreased arteriogenesis in the absence of VWF. These results confirm that VWF controls angiogenesis and provide the first evidence that VWF is involved in restoring blood flow by regulating arteriogenesis.

Next we evaluated the role of VWF in physiological angiogenesis in the mouse post-natal retina model. Retinas from VWF-deficient mice or littermate controls were isolated at day 6 post-partum and stained for isolectin B4 and VWF. VWF was expressed in all EC, with higher expression nearer the vascular front, where tip cells lead the angiogenic sprout. In VWF-deficient retinas, vascularisation was increased, as measured by increase in vascular density near the optic nerve region and increase in the number of endothelial tip cells at the vascular front. These data support the evidence for VWF's role in regulating tip cell formation and sprouting angiogenesis.

We also carried out *in vitro* studies on the mechanism of VWF regulation of angiogenesis. Using VWF-specific siRNA in human umbilical vein EC, we show that VWF regulates EC proliferation through a pathway involving VEGF receptor-2 and ERK phosphorylation.

In conclusion, our data provide further evidence that VWF regulates both developmental and pathological angiogenesis, and add new insight into the pathways involved. These findings may help to identify novel targets for the treatment of angiodysplasia in patients with VWD.

AS 01.2

Sign and shape: correlation of clinical findings and clot ultrastructure in arterial thrombi

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Background: Thrombus architecture is an important determinant of thrombus stability and affects the outcome of preventive and therapeutic interventions in acute myocardial infarction, stroke and peripheral arterial disease, but it is hardly accessible for evaluation in the everyday clinical practice. Here we address the potential correlations between routinely available clinical data and structure of thrombi removed with percutaneous coronary intervention (PCI) or thrombendarterectomy of large arteries.

Methods: Thrombus samples removed by PCI-thrombus aspiration following acute myocardial infarction ($n = 101$) or surgical open repair ($n = 50$) in a heterogeneous group of patients (age range 36–98 years, male-female ratio 6:4) were processed in two parallel ways: glutaraldehyde-fixation for scanning electron microscopy or freezing at −80 °C for cryosections and indirect immunostaining for fibrin and platelet receptor GpIIb/IIIa. Ten to fifteen images were taken of each thrombus with both microscopic techniques, and then analyzed morphometrically to determine fibrin fiber diameter, relative occupancy by fibrin, platelet, red blood cells (RBC), white blood cells (WBC). The correlation between the measured ultrastructural characteristics and selected clinical parameters (age, sex, location of vascular lesion, blood cell counts, haematocrit, C-reactive protein (CRP) in plasma, ECG findings, anti-platelet medication, accompanying diseases) was assessed using multiple hypothesis testing and regression analysis.

Results: Fibrin content of peripheral thrombi showed positive correlation with CRP and male sex ($P = 0.014$ and $P = 0.04$, respectively), but no such dependence was observed in coronary thrombi. Platelet content of thrombi correlated stronger with the hematocrit ($P = 3 \times 10^{-12}$, $R^2 = 0.75$ coronary; $P = 0.02$, $R^2 = 0.238$ peripheral) than with the platelet count in blood ($P = 2 \times 10^{-4}$, $R^2 = 0.20$). Aspirin premedication reduced the role of local factors seen as increased dependence of thrombus platelet content on systemic platelet count ($P = 4 \times 10^{-7}$, $R^2 = 0.54$) and stronger dependence of fibrin structure on RBC count in blood. Fiber diameter of peripheral thrombi decreased at higher RBC counts ($P = 0.009$, $R^2 = 0.16$) and the dependence was significantly stronger in the aspirin-treated group ($P = 0.003$, $R^2 = 0.29$). No such effect was found for clopidogrel. Sorting thrombi by their vessel of origin revealed a marked difference in fibrin-platelet ratio with lower values in the coronaries than in the ilio-femoro-popliteal arterial region ($P < 0.023$ by Kuiper's test for various combinations of subgroups). In line with this observation, platelet content was significantly higher in left anterior descending coronary thrombi than in the ilio-femoral subgroup ($P = 0.037$). In terms of platelet content and fibrin-platelet ratio coronary thrombi were similar to those of aortic origin. Neither the registered ECG findings, nor the accompanying diseases proved to be important determinants of thrombus structure on their own, but complex regression models revealed effects of combination of factors. For example, age at operation and CRP value had an additive effect on WBC content of thrombi in patients with atherosclerosis ($P = 0.003$, $R^2 = 1$) or hypertonia ($P = 0.03$, $R^2 = 0.95$).

Conclusion: The fibrin and platelet content of arterial thrombi as well as their fibrin structure are differentially affected at different vascular

locations and by systemic blood cell counts. Conventional anti-platelet drugs differ in their impact on thrombus structure. Improved individually tailored strategies for prevention of acute thrombotic events could be developed on the basis of these findings.

AS 01.3

Joint association of elevated factor VIII and low protein C with stroke and coronary heart disease: the reasons for geographic and racial differences in stroke (REGARDS) study

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Background: Low protein C (PC) and high factor VIII (FVIII) have been inconsistently associated with risk of stroke and coronary heart disease (CHD) in observational studies. We hypothesized that high FVIII and low PC are associated with increased risk of incident stroke and CHD. Further, as PC inactivates FVIII, we also hypothesized that the association of high FVIII with stroke and CHD will be stronger in those with low PC.
Aims: To study the associations of high FVIII and low PC on stroke and CHD risk in the REasons for Geographic and Racial Differences in Stroke (REGARDS) study.
Methods: REGARDS recruited 30,239 black and white US participants ≥ 45 years old between 2003 and 2007. FVIII and PC were measured in a case-cohort sample of 646 stroke cases, 746 CHD cases (defined as incident nonfatal myocardial infarction or fatal CHD), and a 1104 person cohort random sample with follow up for 4.5 years. High FVIII was defined as the top 20% and low PC as the bottom 5% of the biomarker distribution in the cohort random sample. The hazard ratio (HR) for high FVIII in the presence or absence of low PC was estimated using Cox proportional hazards models. We adjusted for Framingham stroke risk factors for the stroke outcome (age, sex, race, systolic blood pressure, anti-hypertensive medication use, diabetes, smoking, heart disease, atrial fibrillation, and left ventricular hypertrophy) and Framingham CHD risk factors for the CHD outcome (age, sex, race, systolic blood pressure, anti-hypertensive medication use, diabetes, smoking, total and high density lipoprotein cholesterol, and lipid-lowering medication use).
Results: FVIII and PC were not correlated ($R^2 = -0.01$, $P = 0.77$). High FVIII was associated with increased risk of both stroke (HR 1.42; 95% CI 1.02, 1.99) and CHD (HR 1.78; 95% CI 1.21, 2.61). Low PC was non-significantly associated with increased risk of both stroke (HR 1.65; 95% CI 0.95, 2.84) and CHD (HR 1.74; 95% CI 0.94, 3.20). For stroke, there was no evidence for an interaction between high FVIII and low PC (p for interaction 0.70). For CHD however, the adjusted HR for high FVIII was significantly greater in those with a low PC (HR 5.11; 95% CI 1.62, 16.20) than normal PC (HR 1.59; 95% CI 1.07, 2.39; *P*-interaction = 0.06).
Summary/Conclusion: High FVIII was associated with both CHD and stroke risk, while low PC was non-significantly associated with increased stroke and CHD risk. The association of high FVIII with CHD was higher in participants with low PC, but was not different for stroke. These data demonstrate that risks conferred by adverse levels of one biomarker may be augmented by levels of another biomarker. These findings suggest that there may be differences in the role of hemostasis in stroke and CHD pathophysiology.

AS 02 – Assays for Antithrombotic Drugs

AS 02

Assays for antithrombotic drugs

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The first generation of antithrombotic drugs, warfarin and heparin, require continuous monitoring of drug levels using the PT/INR for warfarin and PTT or anti-Xa activity for heparin. Second generation antithrombotic drugs like low molecular weight heparin (LMWH) and pentasaccharides do not require routine monitoring, they are measured only when questions arise associated renal function, overdose, bleeding, elimination of the drug prior to surgery, or patients not approved for use. Levels of LMWH and pentasaccharides are measured using anti-Xa activity assays. The latest oral antithrombotic drugs to be introduced, dabigatran (direct thrombin inhibitor), and rivaroxaban and apixaban (anti-Xa inhibitors), do not require routine monitoring. Treatment is based on fixed doses with changes in dose associated with reduced renal function, patient weight and age in some cases. Monitoring of these medications may be needed in some clinical situations, including changes in renal function, concern about overdose in patients that are bleeding or thrombosis while on the drug, elimination of the drug prior to surgery, adherence to therapy, and patients not approved for use (e.g. children, pregnancy). Important considerations related to monitoring these new drugs include (i) therapeutic ranges, (ii) calibrators, (iii) assay to be used and (iv) assay availability, stat vs. routine. A major issue with monitoring of these new drugs is establishing a therapeutic range, regardless of the assay used for monitoring. These drugs were approved for use without monitoring, there are no approved therapeutic ranges based on clinical trial data. Levels in patients taking the drugs can be estimated from pharmacodynamic studies, but these are not official ranges, and the levels vary substantially during the day. Timing of when the sample is taken (peak vs. trough) is important for interpreting results. A second issue is calibrators for these assays. Dabigatran assays must be calibrated with the active form dabigatran and not the inactive parent drug dabigatran etexilate. Rivaroxiban and apixaban assays can be calibrated with parent drug and some companies are developing calibrators for future assays. For dabigatran (and older parenteral direct thrombin inhibitors), the standard thrombin time can be used to determine if the drug has cleared from the blood prior to surgery or in bleeding patients, but the assay is too sensitive for monitoring of drug levels in the therapeutic range. Dabigatran can be monitored with a PTT, but the degree of prolongation varies with different reagents and the test overestimates dabigatran levels in many patients. A plasma diluted thrombin time has been developed for monitoring that is rapid, uses standard thrombin time reagents and is more accurate than the PTT. Ecarin clotting times can also be used to monitor dabigatran, but are more expensive and not available in many laboratories. For oral anti-Xa drugs, the PTT is not sensitive enough and while the PT does prolong, the degree of prolongation is highly variable between reagents. The optimum test for oral anti-Xa monitoring is likely to be a modified anti-Xa assay with calibrators specific for the drug being measured.

AS 02.1

Comparison of the HAS-BLED, ATRIA and HEMORR2HAGES scores to the CHADS2 score for bleeding risk in atrial fibrillation patients on warfarin therapy: the MAQI2 experience

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Background: Bleeding risk scores have the potential to impact anticoagulation care decision making in patients with atrial fibrillation (AF). Recent studies have demonstrated bleeding risk increases with increasing $CHADS_2$ score as well. Some clinicians have begun using the $CHADS_2$ score as a quick estimate of bleeding risk for AF patients. However, a comparison of these validated bleeding risk scores to the $CHADS_2$ score has not been performed.

Aims: To compare the HAS-BLED, ATRIA and $HEMORR_2HAGES$ bleeding risk scores to the $CHADS_2$ score for predicting major bleeding in AF patients on warfarin.

Methods: Two thousand five hundred and seventy-seven AF patients with at least 2 INR values were followed for a mean 1.0 year (SD 0.8) at seven anticoagulation management centers in Michigan, USA. Data was collected by trained abstractors with random audits to ensure accuracy. Demographic and comorbidity data were used to calculate HAS-BLED, ATRIA, $HEMORR_2HAGES$ and $CHADS_2$ scores for each patient. Genetic information was not available for use in $HEMORR_2HAGES$ score calculation. Major bleeding rates with 95% CI, as defined by the ISTH definition, were calculated for each risk score. ROC curves were generated and compared using the c-statistic with 95% CI. Statistical analysis was performed using Chi-squared, Wilcoxon Rank Sum, Log-rank and Fisher's exact tests.

Results: One hundred and sixteen major bleeding events occurred during 2580 patient-years of follow-up. Major bleeding risk occurred more commonly in patients with higher HAS-BLED, ATRIA and $HEMORR_2HAGES$ scores ($P < 0.0001$ for trend) but not with higher $CHADS_2$ scores ($P = 0.08$ for trend). Patients with low (0), moderate (1–2) and high (3+) bleeding risk according to the HAS-BLED score experienced 1.9 (0.05–10.4), 1.6 (0.9–2.7) and 6.2 (5.1–7.6) major bleeds per 100 patient-years, respectively. Patients with low (0–3), moderate (4) and high (5+) bleeding risk according to the ATRIA score experienced 2.3 (1.7–3.2), 7.4 (4.6–11.3) and 9.0 (6.8–11.7) major bleeds per 100 patient-years, respectively. Patients with low (0–1), moderate (2–3) and high (4+) bleeding risk according to the $HEMORR_2HAGES$ score experienced 1.6 (0.7–3.2), 3.6 (2.7–4.8) and 8.6 (6.5–11.1) major bleeds per 100 patient-years, respectively. Patients with low (0–1), moderate (2) and high (3+) risk $CHADS_2$ scores experienced 3.3 (2.2–4.7), 4.8 (3.5–6.4) and 5.6 (4.0–7.6) major bleeds per 100 patient-years, respectively. Mean TTR was 54.6% for those with major bleeding and 59.5% for those without major bleeding ($P = 0.0018$). The c-statistic was 0.72 (0.67–0.77) for HAS-BLED, 0.69 (0.64–0.74) for ATRIA, 0.69 (0.64–0.73) for $HEMORR_2HAGES$ and 0.59 (0.54–0.64) for $CHADS_2$. The c-statistic was not significantly different between HAS-BLED, ATRIA and $HEMORR_2HAGES$ ($P = 0.053$–0.72 for direct comparisons). HAS-BLED, ATRIA and $HEMORR_2HAGES$ each had significantly higher c-statistics than the $CHADS_2$ score ($P < 0.0001$ for each direct comparison).

Conclusion: In our observational cohort of AF patients, the HAS-BLED, ATRIA and $HEMORR_2HAGES$ bleeding risk scores performed equally well to predict major bleeding events. The $CHADS_2$ score's predictive value for major bleeding was significantly less than the HAS-BLED, ATRIA or $HEMORR_2HAGES$ scores. Use of the $CHADS_2$ score in place of a formalized and validated score, such as HAS-BLED, ATRIA or $HEMORR_2HAGES$, to predict major bleeding risk is not advised.

AS 02.2

Effects of dabigatran on routine tests of haemostasis as determined with a full range of reagents: results of a UK NEQAS exercise

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Background: Dabigatran is known to influence the results of some routine tests of haemostasis. Published studies include data on only a few reagents and methods. Some guidelines (Baglin et al. BJH 2012:159:427–9) recommend that laboratories should be aware of the sensitivity of their methods to the presence of dabigatran. Data are needed on the full range of reagents in routine use worldwide.

Aims: To assess the effects of a range of dabigatran concentrations including likely peak trough and accumulation levels on prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin time (TT) and Clauss fibrinogen (Fib) results.

Methods: The same pooled normal plasma was spiked with 0, 50, 200, 500 and 1000 ng/mL dabigatran (a kind gift of Boehringer Ingelheim), lyophilised in 1 mL aliquots, lyophilised and despatched to all centres registered with UK National External Quality Assessment Scheme (NEQAS) for Blood Coagulation for any combination of PT, APTT, TT and Fib. Results were grouped according to reagent used and the median ratio calculated (test/mid point of normal range).

Results: Results were received from 570 centres (~60%UKand 40% nonUK) who used 17–25 different reagent sets, the majority performed PT APTT and Fib, and 290 returned TT results. 168 centres reported that patients in their centre had received dabigatran therapy, 162 had not and 201 centres did not know. The sample containing no dabigatran had normal overall median PT, APTT, TT and Fib results. PT reagent median ratios: 50 ng/mL – most reagent medians essentially normal at < 1.2 (with 2 medians around 1.3); 200 ng/mL – median ratios ranged from 1.22 to 1.67; 500 ng/mL 1.51–2.42; 1000 ng/mL – range 2.63–6.65. APTT reagent median ratios: 50 ng/mL – all reagent median ratios prolonged (range 1.3–1.69); 200 ng/mL – range 1.78–2.46; 500 ng/mL – range 2.36–3.46; 1000 ng/mL – range 3.32–4.73. TT results: At 50 ng/mL ~25% of centres were unable to detect a measurable clotting time, with a median TT ratio of 7.03 and reagent medians for the commonly used reagents ranging from 5.3 to 11.9; 200 ng/mL – only 10% of centres could record a clotting time.

Fib results: Clauss fibrinogen results were unaffected by 200 ng/mL dabigatran for most methods. At 500 ng/mL there was underestimation by up to ~20% except for one method (in which test plasma is not prediluted in contrast to most other techniques) with ~60% underestimation. At 1000 ng/mL there was 50–80% underestimation in three methods, though most had < 20% underestimation.

Conclusions: All PT and APTT reagents were affected by dabigatran. PT was less affected by low levels and showed more variability in reagent sensitivity. TT was unclottable even at 50 ng/mL in 75% of centres, and in most centres at higher drug concentrations. Fibrinogen was underestimated by up to 20% in most assays but some methods were markedly affected at higher concentrations.

AS 02.3

How to monitor Dabigatran- when needed: comparison of coagulation laboratory methods an Dabigatran concentrations in plasma

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Dabigatran (Pradaxa®, Boehringer Ingelheim) is an oral direct thrombin inhibitor for which routine laboratory monitoring is currently not recommended. However, there are situations in which measurements of the drug and its effect are desirable (e.g. preparation for surgery; major bleeds, to check compliance and/or effect). Direct measurement of dabigatran plasma concentrations may be considered the method of choice. However, this is not widely available and standardized coagulation assays may be used by the routine 24/7 laboratory instead. We have compared some coagulation tests with dabigatran measurements in plasma.

Methods: Plasma samples were obtained from 70 patients treated with dabigatran. Plasma concentrations were measured using liquid chromatography coupled with mass-spectrometry (LC-MS/MS) and were compared with new methods for indirect determination of dabigatran concentrations (Hemoclot thrombin inhibitors [HTI], Hyphen) and Ecarin clotting assay (ECA; Stago), as well as with PT-INR (Owren reagent SPA+, Stago) and APTT (Silica reagent, Automate, Stago) and the endogenous thrombin potential (ETP; Siemens).

Results: Dabigatran concentrations by LC-MS/MS varied considerably between patients (0–586 ng/mL, median 45, 95% CI 48–111). Correlations between directly measured and estimated concentrations were excellent for both HTI and ECA (R^2 = 0.97 and 0.96 respectively, $P < 0.0001$), and the same was true for HTI vs. ECA (R^2 = 0.94). The lowest calibrator point for HTI was 30 ng/mL (with dilution to 15 ng/mL the CV was > 30% at those low values), while ECA had null calibration. There were significant correlations between dabigatran levels and both APTT (R^2 = 0.58) and ETP (R^2 = 0.69). However, APTT was within the normal range (< 40 s) even with dabigatran levels of 60 ng/mL, and ETP decreased below 80% only at dabigatran concentrations above 150 ng/mL. There was no correlation between dabigatran concentration and PT-INR; the only abnormal PT-INR value was observed with a dabigatran concentration of 500 ng/mL.

Interpretation: This is the first comparison of the plasma concentrations of dabigatran and coagulation assays in samples obtained from patients treated in real clinical settings. It seems that both the HTI and ECA assays may be used to estimate the intensity of dabigatran anticoagulation and drug levels in clinical samples. However, the interpretation of low values should be cautious, especially for HTI. The Owren PT reagent is insensitive to dabigatran, and ETP is not useful. The APTT assay used in our laboratory is relatively insensitive to dabigatran and normal APTT results may be observed even if dabigatran concentration is slightly above 60 ng/mL. The APTT could be useful for the assessment of peak effects shortly after taking the drug or in connection with bleeding associated with high concentrations of dabigatran. In conclusion, determinations of plasma concentrations of dabigatran by LC-MS/MS is the gold standard when monitoring of dabigatran is needed. If this is not available, HTI and ECA are the assays of choice. However, neither of those assays can be used to monitor low levels or infer the total absence of dabigatran.

AS 03 – Platelet Interactions

AS 03.1

Investigation on the role of protein S in platelets

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Background: Anticoagulant protein S (PS) is essential for maintaining the haemostatic balance. In humans, although most of the PS in plasma is thought to be mainly synthesized by hepatocytes, the PS gene (*ProS*) is also expressed by several other cell types, including megakaryocytes (MK). Notably, about 2.5% of PS in circulating blood is stored in platelets and released upon stimulation. So far, little is known about the functionality and importance of platelet PS. Our previous characterization of PS deficiency in mouse models has shown similarities with the human phenotypes as heterozygous PS-deficient mice had increased susceptibility to thrombosis and homozygous PS deficiency was incompatible with life.

Aims: To explore the functional roles of PS secreted from platelets.

Methods: Mice carrying a conditional *ProS* knockout allele in the MK lineage, were established using the *Pf4* promoter as Cre driver ($ProS^{Lox/Lox}Pf4Cre^{+}$). The characterization of the resulting phenotype was performed by evaluating the thrombotic risk *in vivo*, as well as platelet function by using the Platelet Function Analyzer-200 and light transmission aggregometry (collagen and ADP were used as agonist). In addition, PS cofactor activity for tissue factor pathway inhibitor α (TFPI α) was measured by a Factor Xa amidolytic activity assay.

Result: $ProS^{Lox/Lox}$ $Pf4Cre^{+}$ mice did not have detectable PS levels in platelet lysates (n = 5) but did have normal levels in plasma (88.9 ± 4.6%, n = 19), as assessed by antigenic assays. Analysis of complete blood counts established that these animals retained a normal blood cell profile compared with wt mice. Moreover, no difference in platelet function was seen in $ProS^{Lox/Lox}$ $Pf4Cre^{+}$ mice compared to control wt mice. In contrast, a tail clipping test showed significantly decreased bleeding times for animals lacking PS in platelets, as compared to wt mice (P = 0.021). When challenged in a tissue factor-induced thromboembolism model, $ProS^{Lox/Lox}$ $Pf4Cre^{+}$ mice (n = 15) exhibited a notable thrombotic phenotype (survival rate 46.7% compared to 85.8% for $ProS^{Lox/Lox}$ mice, n = 14; $P < 0.05$).

Beside its anticoagulant cofactor role for activated protein C, PS acts as cofactor for TFPI α in the inhibition of factor Xa. The complete deletion of *ProS* gene in MK lineage did not affect antigenic TFPI α levels in platelet lysates. Preliminary data on cofactor activity of PS for TFPI α indicate that the absence of PS in platelet lysates abolished the capacity of TFPI α to inhibit factor Xa amidolytic activity.

Summary/Conclusion: These data suggest that platelet PS is delivered at the site of thrombosis to counterbalance procoagulant activities on platelets, thus inhibiting thrombus growth. Upon release, platelet PS may function as an anticoagulant protein by enhancing not only activated Protein C but also critically the TFPI-dependent inhibition of factor Xa thereby blocking prothrombinase activity.

AS 03.2

Toll-like receptor 2 is critical for platelet adhesion to the injured arterial wall

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Innate immune signaling and thrombosis are closely intertwined. Several mechanisms have evolved that connect innate immunity with thrombus formation. In spite of recent reports on functional Toll-like receptor 2 (TLR2) signaling in platelets and endothelial cells it remains unclear whether TLR2 is required for normal hemostasis and it is not resolved if TLR2 signaling plays a role in experimental thrombus formation. Therefore, we set out to explore the role of TLR2 on platelet adhesion to the subendothelial matrix in a murine *arteria carotis communis* ligation model using high-speed intravital epifluorescence microscopy.

In *Tlr2−/−* mice platelet adhesion was vastly impaired at the ligation injury site compared with *WT* controls. No difference in whole blood platelet counts or average tail bleeding times could be detected between both groups. Interestingly, platelet adhesion was similar in *WT* mice and *Myd88−/−*mice suggesting that the involvement of TLR2 in platelet adhesion is independent of its signaling function. Transfusion experiments with fluorescently labeled *WT* platelets into *Tlr2−/−* mice and fluorescently labeled *Tlr2−/−* platelets into *WT* mice were performed to pinpoint whether the observed effects are originating from the vessel wall or from the platelets. Both groups (*Tlr2−/−* platelets in *WT* mice and *WT* platelets in *Tlr2−/−* mice) showed a decreased platelet adhesion compared with *WT* controls (*WT* platelets in *WT* mice). This indicates that both, TLR2 in the vessel wall and in circulating platelets, is required for platelet adhesion. *WT* mice that were infused with a functional inhibitory TLR2-antibody showed significantly decreased platelet adhesion to the injury site compared with the isotype control group. Furthermore, under static conditions the adhesion of washed platelets from *Tlr2−/−* mice to collagen and laminin matrices was strongly impaired compared with *WT* controls. First experiments with whole blood from $Tlr2^{-/-}$ compared with WT mice under flow conditions confirm our previous results of impaired platelet adhesion to collagen and laminin matrices. Deficiency of TLR2 did not result in altered expression levels of the major platelet adhesion molecules integrin α_2, integrin β_1, glycoprotein VI, glycoprotein Ib or plasmatic von Willebrand Factor. This further supports a direct role of TLR2 in platelet adhesion.

Here, we report that TLR2 from circulating platelets and the vessel wall promotes primary platelet adhesion under arterial flow. This effect is independent of the TLR2 adapter molecule MyD88 excluding the involvement of downstream signaling events. We provide first evidence that targeting of TLR2 could be used as a novel therapeutic strategy to inhibit arterial thrombus formation.

AS 03.3

The activated protein C cofactor activity of protein S is enhanced through extracellular phosphorylation by platelet kinases

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Background and Aims: Protein S is a multifunctional plasma protein of the hemostatic and inflammatory pathways, although mechanisms for its regulation are poorly understood. Anticoagulant reactions of protein S likely occur on activated platelets. Since certain plasma proteins are regulated through extracellular phosphorylation, we investigated whether the anticoagulant activity of protein S is regulated through phosphorylation by platelet-secreted kinases.

Methods and Results: Protein S was phosphorylated upon exposure to activated platelets or their releasates, as judged by immunoblotting for phospho-amino acids and for protein S. The responsible kinases showed casein kinase-1 and casein kinase-2-like properties, since protein S phosphorylation was reduced by specific inhibitors of these casein kinases (10 μM D4476 and 100 μM CK2-inhibitory peptide YNLKSKSSEDIDESS). Involvement of casein kinases in protein S phosphorylation was confirmed using purified kinases. Phosphorylation of protein S by purified casein kinase-1 did not affect its activated protein C cofactor activity in Activated Partial Thromboplastin Time (APTT) assays in protein S-depleted plasma. In contrast, phosphorylation of protein S by purified casein kinase-2 or by casein kinase-1 and casein kinase-2 combined increased protein S cofactor activity ~1.6-fold (158.7 ± 4.8%, $P < 0.01$) or ~2-fold (191.5 ± 6.4%, $P < 0.0001$), respectively. The activated protein C cofactor activity of protein S in protein S-depleted plasma exposed to platelet-secreted kinases was enhanced, while inhibitors against casein kinase-1 and casein kinase-2 reduced this cofactor activity. Proteomic studies of protein S exposed to platelet releasate revealed phosphorylated sites for casein kinase-1 and casein kinase-2 in the EGF2-like domain (Thr137) and Gla-domain (Thr37), respectively. Alignment of the primary sequence of human protein S with those of five other species revealed that Thr37 and Thr137, together with their casein kinase-1 and casein kinase-2 consensus sequences, are completely conserved.

Conclusions: These findings identify platelet-mediated extracellular phosphorylation of protein S as a potential mechanism by which its activity is regulated.

AS 04 – Inflammation and Coagulation in Atherosclerosis

AS 04.1

Increased levels of circulating platelet-monocyte complexes and platelet-neutrophil complexes are associated with myocardial infarction

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Background: Inflammation plays a key role in the development of coronary artery disease (CAD). Platelet-leukocyte complexes contribute to the progression of CAD and have been proposed to be reliable markers of inflammation. For this reason we investigated if circulating platelet-leukocyte complexes can serve as a biomarker for the diagnosis and/or prognosis of CAD.

Methods: We conducted a prospective multicenter cohort study in which 263 CAD patients were admitted for percutaneous coronary intervention (PCI) to four Dutch medical centers: Catharina Hospital in Eindhoven, University Medical Center in Maastricht, University Medical Center in Leiden and the University Medical Center in Utrecht. Citrate and EDTA anti-coagulated whole blood samples were drawn from patients directly before PCI. Monocytes, neutrophils and lymphocytes were identified by scatter gating and CD14 labeling. Platelet-monocyte, platelet-neutrophil and platelet-lymphocyte complexes were quantified by the percentage of cells positively stained for the platelet marker Gp1b within the respective gates. The number of platelets bound per leukocyte was quantified by the mean fluorescence intensity of platelet marker Gp1 per bound leukocyte. Patients were followed for 9 months after intervention for the composite end point major adverse cardiovascular events (MACE), being myocardial

infarction, another PCI, coronary artery bypass graft, or death by cardiovascular disease.

Results: We found significantly elevated levels of platelet-monocyte complexes (33.9 ± 3.6% vs. 26.1 ± 1.3%, mean ± SEM, $P < 0.05$) and platelet – neutrophil complexes (20.5 ± 2.8% vs. 15.0 ± 0.8%, mean ± SEM, $P < 0.05$) in citrate anticoagulated blood of patients that were diagnosed with non-ST elevation myocardial infarction (NSTEMI) ($n = 26$) compared to patients with stable angina ($n = 209$). Comparable results were found in EDTA anticoagulated blood. No differences were found in the number of platelets bound per leukocyte between patient with NSTEMI compared to stabe angina. Levels of platelet-leukocyte complexes and platelets bound per leukocyte were not associated with future MACE ($n = 27$).

Conclusions: Circulating platelet-monocyte complexes and platelet-neutrophil complexes might serve as biomarkers for the diagnosis of CAD, but do not predict future adverse cardiac events.

AS 04.2

Small molecule inhibitors of CD40-TRAF6 interaction reduce atherosclerosis by targeting its inflammatory nature

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Backround: Atherosclerosis is a chronic inflammatory disease of the arterial wall. A pivotal role in the pro-inflammatory activation is played by the co-stimulatory CD40-CD40L dyad. Previously, it was discovered that inhibition of CD40 in hyperlipidemic mice drastically reduces atherosclerosis; however its long-term inhibition results in immunosuppression and/or thromboembolic events. Therefore more downstream inhibition of the CD40L-CD40 dyad should present better targets for pharmacological therapy. CD40 lacks intrinsic signalling activity and thus to elicit intracellular signalling upon activation, CD40 needs to recruit adaptor proteins: the tumour necrosis factor receptor-associated factors (TRAFs). Further, using mice with a mutation in the CD40-TRAF binding site, it was shown that CD40-TRAF6 but not CD40-TRAF2/3/5 interaction is crucial for the atherosclerotic plaque reduction.

Aims: We aimed at identification of small drug-like molecule inhibitors of the CD40-TRAF6 interaction which will interfere specifically with the inflammatory nature of atherosclerosis.

Methods/Results: For the discovery of CD40-TRAF6 inhibitors we used an *in silico* structure-based virtual ligand screening (VLS) approach with a small molecule collection (ChemBridge, ~400,000 compounds). To screen only for drug-like molecules, the compound collection was filtered based on predicted absorption, distribution, metabolism, excretion, and toxicity (ADME/Tox) properties. Next, we applied a hierarchical protocol that combines rigid and flexible docking methods. The 800 most likely binders after *in silico* selection were analyzed *in vitro*, using a cell-based NFκB reporter gene assay. This resulted in the identification of seven hits that reduced NFκB activation in RAW cells in a dose-dependent way (IC50 in a range 0.1–16.1 μM). Moreover, the seven hit compounds suppressed CD40-induced production of IL1β and IL6 in primary bone marrow-derived macrophages in a dose-dependent way. In order to analyze effects of these molecules on atherosclerosis we selected two compounds for *in vivo* analysis in *Apoe*−/− mice with developed initial lesions, which were treated with compounds 1 or 2 at 10 μmol/kg/day for 6 weeks. Total plaque area per aortic arch was reduced by 47.1% and 66.8% in mice treated for compound 1 and 2, respectively. Moreover, aortas of treated mice contained more initial lesions (intimal xanthoma and pathological intimal thickening) and exhibited a decrease in advanced atherosclerosis (fibrous cap atheromata). Compounds 1 and 2 reduced the number of leukocytes per plaque by 43.1% and 52.6%, respectively. Intravital microscopy showed that recruitment of leukocytes, especially monocytes and neutrophils to the endothelium was decreased in compound treated mice. As a result, compound 1 and 2 reduced monocyte adhesion by 40.1% and 51.2% respectively, and neutrophil adhesion by 40.2% and 51.2%, respectively. We verified the direct binding of our hit compounds by SPR and ITC-based direct binding assays in which small compounds bound dose-dependently to the recombinantly expressed target domain.

Conclusion: We have discovered and characterized a group of druglike compounds that inhibit CD40-TRAF6 interaction, which result in reduction of atherosclerosis progression by inhibition of chemokine-mediated leukocyte influx into the arterial wall. These results indicate the possibility of long-term therapeutic inhibition of atherosclerosis and its haemostatic complications as well as of other inflammatory diseases.

AS 04.3

Platelet CD40 exacerbates atherosclerosis by transcellular activation of endothelial cells and leukocytes

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Background: Beyond an eminent role in hemostasis and thrombosis, platelets are important mediators of inflammation and protagonists of atherogenesis. Here we investigated the inflammatory propensity of platelet-specific expression of the CD40 receptor, an integral membrane protein of the tumor necrosis factor receptor (TNF-R) family. Besides its presence on immune and other cell types the CD40 receptor is constitutively expressed on platelets where its function remains unknown.

Aim: We therefore investigated the inflammatory propensity of platelet CD40 co-stimulation, as well as its underlying mechanisms in atherosclerosis.

Methods: Platelets were isolated from *Apoe*−/− or *Cd40*−/−*Apoe*−/− mice, activated with thrombin, and injected (3×10^7 platelets, i.v.), into 17-week-old *Apoe*−/− mice every 5 days for 12 weeks. At week 29, atherosclerosis was quantified in the aortic arch. Platelet-leukocyte and platelet-endothelium interactions were quantified with flow cytometry and intravital microscopy.

Results: Compared to infusion of activated *Apoe*−/− (wild type) platelets, injection of activated *Cd40*−/−*Apoe*−/− platelets caused > 2-fold decrease in plaque size (*Apoe*−/− platelets $18.3 \times 10^4 \pm 2.7 \times 10^4$ μm^2 vs. *Cd40*−/−*Apoe*−/− platelets $6.7 \times 10^4 \pm 2.1 \times 10^4$ μm^2, Vehicle $9.8 \times 10^4 \pm 2.8 \times 10^4$ μm^2, $P < 0.05$) in the aortic arch. Absence of CD40 on platelets reduced the absolute number of plaque macrophages (Mac-3) by 39% but did not affect the content of $CD45^+$ cells and $CD3^+$ T lymphocytes. Flow cytometric analysis revealed an elevated number of circulating $Ly6G^+$ neutrophils (+32%) upon injection of activated platelets. However, this increase was absent when CD40-deficient platelets, were injected highlighting the inflammatory potential of platelets and a central role for CD40 in this process. In addition, we detected a significant decrease in the formation of platelet-leukocyte aggregates *in vitro* while intravital microscopy in carotid arteries showed a two-fold decrease of platelet adhesion to the vessel wall of mice, injected with activated *Cd40*−/− platelets.

Conclusions: In sum, this study reveals that platelet CD40 promotes atherosclerosis by interaction with both neutrophils and endothelial cells, thereby amplifying leukocyte recruitment to sites of vascular injury.

AS 05 – Hormones and Thrombosis

AS 05
Exogenous hormones and thrombosis

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Exogenous hormones are used in women for contraception and treatment of menopausal symptoms. Both treatments are associated with increased vascular risks, most notably venous thrombosis. Regarding postmenopausal hormones (PHT), conjugated equine estrogens (CEE) plus medroxyprogesterone acetate (MPA) double the risk of deep vein thrombosis (DVT) and pulmonary embolus (PE), and this risk is larger in older women and those who are obese. The absolute risk in obese women is 0.5% per year. In women with factor V Leiden this risk is 0.8% per year in general and 2.9% per year if they have a family history of factor V Leiden and venous thrombosis (VT). Mechanisms for an increased risk of VT with PHT likely include reductions in antithrombin and protein S, and induction of activated protein C resistance. In unpublished data we showed that women with higher D-dimer prior to prescription are at high risk, with a risk similar to that in women with factor V Leiden. Limited evidence suggests that micronized progesterone may not increase the risk of VT with PHT. Further, in observational studies transdermal estrogen was not associated with increased risk and some societies suggest this is reasonable treatment for PHT use in women with prior VT or at risk of VT.

Risk of VT is similarly increased by about two-fold in women using oral contraceptives (OC), with the highest risk in the first year of use. The relative risk is especially high in women with other risk factors such as factor V Leiden, obesity and older age of use. Fortunately due to their younger age, the absolute risk of VT with OC remains low (e.g. about 3.5/1000 in young women with factor V Leiden heterozygosity). Risk depends on the pill type and dose. Available progestin only pills do not seem to increase risk. Pills containing 3rd generation progestins, such as desogestrel and gestodene, and those containing drospirenone, have higher risks than those with 2nd generation progestins, such as levonorgestrel. There are misconceptions among some clinicians that non-oral contraceptives have a lower risk. Vaginal ring ethinyl estradiol plus etonogestrel, depo-MPA, and transdermal ethinyl estradiol plus norgestimate all seem to increase risk of VT. The mechanisms of increased risk for contraceptives are probably similar to PHT; namely activated protein C resistance and reductions in antithrombin and protein S. The clinical approach to use of these medications in general and in women at risk of thrombosis will be discussed.

AS 05.1
The risk of venous thrombosis in oral contraception users with a history of superficial thrombophlebitis

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Background: Women using oral contraception have a 3–5-fold increased risk of venous thrombosis compared with non-users. Individuals with superficial thrombophlebitis have a 5–6-fold increased risk of a subsequent venous thrombosis. There is consensus that women should discontinue the use of oral contraceptives after an episode of venous thrombosis, but no such consensus exists for oral contraceptive use after superficial thrombophlebitis.

Aim: The aim of our study was to assess the risk of venous thrombosis in oral contraception users with a history of superficial thrombophlebitis.

Methods: From the MEGA study, a large case-control study on risk factors for venous thrombosis, 1428 female patients with a first venous thrombosis and 1755 female control subjects without venous thrombosis were included, all aged < 50 years. Odds ratios (OR) and 95% confidence intervals (95% CIs) for venous thrombosis were calculated in oral contraception users, in women with a history of superficial thrombophlebitis and in women with both oral contraception use and a history of superficial thrombophlebitis, compared with women with neither of these risk factors. The outcomes deep vein thrombosis, pulmonary embolism and deep vein thrombosis with concomitant pulmonary embolism were combined in the overall analyses but also considered separately. Risk estimates were adjusted for age, body mass index, smoking and family history of venous thrombosis. The study was approved by the Medical Ethics Committee of the Leiden University Medical Center and all participants provided written informed consent. Funding: Netherlands Heart Foundation (grant NHS 98.113), the Netherlands Organisation for Scientific Research (grant 912-03-033|2003), Dutch Cancer Foundation (grant RUL 99/1992).

Results: The ORs for venous thrombosis were 6.3 (95% CI, 5.2–7.6) for women using oral contraception and 6.6 (95% CI, 3.4–12.8) for women with a history of superficial thrombophlebitis compared with women with neither of these risk factors. When both factors were present together the OR increased to 51.5 (95% CI, 20.5–129.3). Oral contraception users with a history of superficial thrombophlebitis had a particularly high risk of deep vein thrombosis: OR 86.9 (95% CI, 23.5–321.0) with concurrent pulmonary embolism and OR 71.6 (95% CI, 27.6–185.5) without concurrent pulmonary embolism. The risk of pulmonary embolism alone was also increased, but to a lesser extent: 30.1 (95% CI, 11.1–82.1). When the baseline incidence in this age group is set at 1 per 10,000/year, it follows that 250 women with a history of superficial thrombophlebitis should refrain from oral contraceptive use to prevent one venous thrombotic event per year of non-use.

Summary/Conclusion: In our study we found a markedly increased risk of venous thrombosis, especially deep vein thrombosis, in oral contraception users with a history of superficial thrombophlebitis. Our findings suggest that oral contraceptive use in women with a history of superficial thrombophlebitis may be ill-advised.

AS 05.2
Progestin-only contraception after venous thromboembolism: cohort study

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Background: Contraception is a main problem in women at risk for venous thromboembolism (VTE). Combined oral contraception is contra-indicated in women with a personal history of VTE. Progestin-only contraception may be a good option, but there is little data about risk of recurrent thrombosis, efficacy and side effects associated with progestin-only use in these women.

Aims: We aimed to determine the risk of recurrence of VTE in young women exposed to progestin-only contraception after a first VTE.

Methods: We studied the risk of recurrent VTE during progestin-only contraception in a cohort of young women under 50 years with first proved VTE, admitted from 1992 to 2011 in BrestuniversityHospital. Follow-up information was collected annually about medical events

and thrombosis risk factors, including pregnancy and contraception use. Progestin-contraception users were asked about efficacy and side effects of the treatment. We compared the incidence of recurrent VTE (after stopping anticoagulants) during periods of exposition to progestin-only contraception, combined contraception, pregnancy and no hormones.

Results: Of the 338 women under 50 years with a first VTE, 122 used progestin-only contraception during follow-up. Mean duration of follow-up was 7.5 years, total duration of progestin-use was 476 women-years. We observed six recurrent VTE, two in progestin users, one in combined contraception users, three during post-partum. The incidence for recurrent VTE was 9.8/1000 women-year, and 4.8/1000 women-years during progestin contraception. The incidence of unplanned pregnancies was 0.63/1000 women-year. Bleeding and amenorrhea were frequent. Anyway, 64% of women used progestin-only contraception at least 3 years and 74% of women tolerated it as well as previous combined contraception.

Conclusion: Risk of recurrent VTE after a first event during prolonged use of progestin-only contraception is low. These results are close to the results of another study in women at risk using chlormadinone acetate contraception. Side effects were frequent but did not affect long-term use of progestin-only contraception.

AS 05.03

Heparanase procoagulant activity is elevated in women using oral contraceptives

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Background: Estrogen therapy, known to increase the risk of thrombosis, was previously found to up-regulate heparanase expression. Heparanase, known to be involved in angiogenesis and metastasis, was shown to form a complex with tissue factor (TF) and to enhance the generation of factor Xa (Nadir et al., Haematologica, 2010).

Aims: In the present study the effect of estrogen on heparanase procogulant activity was studied.

Methods: Estrogen receptor – positive (MCF-7) and negative (MDA-231) cell lines were incubated with estrogen, tamoxifen and ICI – a pure estrogen receptor antagonist. The cells medium was evaluated for TF/heparanase complex activity, TF activity and heparanase procoagulant activity by chromogenic substrate. Plasma samples of 34 healthy women taking oral contraceptives (OC) and 41 control women not on hormonal therapy were investigated. TF/heparanase activity, TF activity, heparanase procoagulant activity and factor Xa levels were studied using chromogenic substrate. Heparanase and thrombin-antithrombin (TAT) levels were analyzed by ELISA.

Results: Estrogen and tamoxifen increased heparanase procoagulant activity in the medium of estrogen receptor positive (MCF-7) cells. TF/heparanase activity, TF activity, heparanase procoagulant activity and factor Xa were significantly higher in the OC group compared to the control group. The most dramatic difference was observed in heparanase procoagulant activity, reaching a 3.3-fold increase ($P < 0.0001$). Levels of heparanase and TAT measured by ELISA did not statistically differ among the study groups.

Conclusions: Estrogen increases heparanase procoagulant activity. The findings of the present study suggest a new potential mechanism of hypercoagulability in OC users.

AS 06 – Post-Thrombotic Syndrome

AS 06

Post-thrombotic syndrome: evidence-based update on prevention and treatment

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This presentation will review the prevention and treatment of late sequelae of venous thrombosis such as the post-thrombotic syndrome. Post-thrombotic syndrome (PTS) is the most frequent complication of deep-vein thrombosis (DVT). After a proximal DVT, 20–50% of patients will develop PTS and 5–10% of patients will develop severe PTS, which reduces quality of life and has important socio-economic consequences. A number of risk factors for PTS have been identified; of these, proximal location of DVT and a previous ipsilateral DVT are the most consistently predictive. PTS is diagnosed on clinical grounds, based on the presence of signs and symptoms of venous insufficiency in the leg ipsilateral to DVT. Therapeutic options for PTS are limited and mainly rely on (i) prevention of DVT with use of thromboprophylaxis in high risk surgical and medical patients; (ii) prevention of PTS with compression stockings (new data from the SOX Trial will be presented) and, possibly, in select cases, catheter-directed thrombolysis of iliofemoral DVT if bleeding risk is low; (iii) prevention of DVT recurrence with the use of optimal and/or prolonged duration of anticoagulation; (iv) treatment of moderate to severe PTS with various compressive devices. Areas for future research in this important aspect of venous thrombosis will be highlighted.

AS 06.1

The good, the bad, and the ugly: post-thrombotic syndrome in children with upper extremity deep vein thrombosis

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Background: Thirty to 50% of pediatric deep vein thrombosis (DVT) involves the upper extremity (UE), reflecting the role of central venous lines (CVL) as its major risk factor. Children with UE-DVT can develop post-thrombotic syndrome (PTS), a chronic and potentially devastating complication. Despite its relatively estimated high frequency, the characterization of pediatric UE-PTS is still lacking.

Aims and Methods: We wished to investigate the occurrence, characteristics, and predictors of UE-PTS in a cohort of children with objectively confirmed UE-DVT followed-up at Sickkids, Toronto, between Apr/1996 and Jun/2011. PTS was determined by the Modified Villalta Scale. Patients were analyzed in three groups according to whether DVT was unprovoked (G1) or provoked (neonates [G2A] vs. non-neonates [G2B]). This study was approved by the ethics review board. Informed consent was waived. Descriptive statistics, univariable and multivariable logistic regression model were applied.

Results: One hundred and sixty-five children (G1 $n = 23$; G2A $n = 32$; G2B $n = 110$) with a median age (interquartile range [IQR]) of G1: 16.1y (14.9–16.9 years); G2A: 17 days (9.5–23 days); G2B: 1.8 years (87 days–10.1 years), $P < 0.001$, were included. Overall, the M/F ratio was 1.5/1.0, with a similar sex distribution among groups ($P = 0.1$). The most common identified risk factors were: G1 (effort-related [82%], idiopathic [18%]); G2A (CVL [100%]); G2B (CVL [95%]; mass effect [5%]). PTS was identified in G1: 92% (mild: 72%, moderate: 28%); G2A 21% (mild: 100%), and G2B 41% (mild: 95%, moderate: 5%) of patients with at least two subsequent measurements > 180 days after DVT, $P < 0.001$.

The last clinic follow-up occurred at a median of 3.25 years (IQR 1.8–5.8 years). The mean PTS-score was assessed at different time-points

(6–18, 19–36, 37–54, and 55–72 months); distribution was: G1: 2.0/2.0/2.5/no f/u; G2A: 0.5/0.0/0.5/0.0; G2B 1.0/1.0/1.0/1.0. The highest scores per group were G1: 4–5 (n = 7/23), G2A: 1–2 (n = 6/32), and G2B: 4–7 (n = 4/110). The most common findings were increased limb circumference and pain (G1) and collaterals and increased limb circumference (G2A/G2B).

Treatment included: G1, heparin/LMWH 65%, lysis 35%; G2A, 97% heparin/LMWH; G2B, 87% heparin/LMWH, 10% not treated, 3% lysis.

We constructed a model to predict the occurrence of ≥ 2.0 points PTS score in 109 patients with at least one clinical follow-up, at least 180 days after DVT diagnosis. Potential predictors considered in univariable analysis were: treatment modality/length, delay in starting therapy, sex, group (G1/G2A/G2B), resolution status, number of segments affected, subclavian involvement, and DVT-recurrence. The final model included resolution status and group: the odds ratio (OR) for the outcome was 7.8 (95% CI 1.9–32.4, P = 0.005) when comparing DVT extension vs. complete resolution; 1.7 (95% CI 0.5–5.5, P = 0.34) for partial vs. complete resolution; and 3.3 (95% CI 0.6–17.3, P = 0.15) for no vs. complete resolution. OR for the outcome was 47.6 (95% CI 4.8–472.8; P = 0.001) in G1 vs. G2A, and 8.4 (95% CI 2.7–26.1; P = 0.002) for G1 vs. G2B.

Conclusion: Pediatric UE-PTS frequency and severity likely depends on UEDVT nature (primary vs. secondary) and on patient's age. Line-related UE-PTS likely has a more benign course, particularly in neonates. Risk stratification seems warranted for tailored treatment of pediatric UE-DVT.

AS 06.2

Post-thrombotic syndrome after central venous catheter removal in childhood cancer survivors is associated with decreased quality of life

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Background: Although the use of central venous catheters (CVCs) has greatly improved the quality of care of children with cancer, these catheters may cause deep vein thrombosis (DVT). The potential long-term complication of CVC-related DVT (symptomatic and asymptomatic) is the development of the post thrombotic syndrome (PTS). A consensus paper from the Pediatric/Neonatal Hemostasis and Thrombosis Subcommittee of the ISTH recommends that upper venous system PTS assessment should include physical, functional and quality of life (QoL) domains.

Aim: To study PTS post-CVC removal using these three domains in childhood cancer and bone marrow transplantation (BMT) survivors.

Methods: We conducted a prospective study in a consecutive cohort of childhood cancer and BMT survivors with a history of CVC use. All participants were evaluated for PTS during their follow-up visit at the After-cancer Clinic at Hadassah Medical Center. The two available pediatric PTS assessment tools, the Modified Villalta Score (MVS) and the Manco-Johnson Instrument (MJI), were used. Patient and CVC related data, as well as health related quality of life (QoL) (using the PedsQL™ questionnaire) were assessed and associated with occurrence of PTS.

Results: A total of 158 children were enrolled at a median of 41 (4–149) months from CVC removal. Mean ± standard deviation (SD) of age at enrollment was 12.30 ± 5.8 years. Signs and symptoms ipsilateral to the side of CVC (or CVC-related DVT) were considered in determining PTS. PTS was present in 34% (95% confidence interval [CI] 27–43%) of the study population with the MVS criteria and 28% (95% CI 21–35%) with the MJI criteria. Pain was the most common symptom reported in the PTS assessment, which occurred in 13.2% of participants. Higher rates of PTS were found among children with a history of CVC occlusion. In a univariate logistic regression model, the odds ratio (OR) for PTS in children with a history of CVC occlusion was 2.2 (95% CI 1.1–4.4) using the MVS and 2.6 (95% CI 1.2–5.5) using the MJI. PTS was not associated with age at diagnosis, time from CVC removal or history of infection. Higher rates of PTS were found among children with a history of DVT, although the sample size was small (six of the eight participants with a history of DVT). Interestingly, patients who had signs and symptoms of PTS ipsilateral and/or contralateral to the CVC had lower average PedsQL scores compared to those without signs and symptoms of PTS. The mean difference for PedsQL score was 5.64 using the MVS (P = 0.029) and 6.26 using the MJI (P = 0.018). The physical-function domain of the PedsQL score showed similar discrepancies between those with and without PTS.

Conclusion: PTS post-CVC removal, associated with pain in over 10% of cases, is not a rare event. The association between PTS and the history of CVC occlusion confirms earlier findings, and suggests that CVC occlusion may indicate asymptomatic DVT. The lower QoL scores among children with signs and symptoms of PTS indicate the detrimental effects of PTS post-CVC removal and suggest the need to study preventative measures.

AS 06.3

Risk factors for severe post-thrombotic syndrome: VTE Epidemiology Group (VEG) Study

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Background: Severe post-thrombotic syndrome (PTS) with venous ulceration results in significant morbidity and cost. PTS occurs following DVT and PE and the recognition of risk factors may help to evaluate and guide therapy type and duration. To simplify decision making these factors for PTS must be assessed allowing for the presence, quality and absence of anticoagulant therapy.

Aim: To explore potential risk factors for PTS.

Methods: Patient data were retrieved from the subset of general practices in England contributing to the Clinical Practice Research Database (CPRD) that has been linked to data from the Hospital Episodes Statistics (HES) and to the Office for National Statistics (ONS). From January 2001 to October 2011, all VTE cases in the CPRD were verified with an algorithm based on review of medical records and notes, hospital diagnoses, cause of death and anticoagulation therapy. We conducted a nested case-control study in patients with a first VTE. Incident events of venous ulcer associated PTS, defined as severe PTS, during the entire observational period were defined as cases and the date of severe PTS as the index day. For each case we selected five patients free of PTS at random by matching on the index day and with the same duration of observation as the respective case. The independent association between a potential risk factor and severe PTS was derived from conditional multivariate logistic regression models and presented as adjusted odds ratio (OR) with 95% confidence intervals (CIs). Adjustment included age, gender, socioeconomic risk factors, components of the Charlson index, and the type of antithrombotic therapy (VKA, low molecular weight heparin or antiplatelet therapy).

Results: Of 35,373 patients with first VTE 29,550 patients survived more than 7 days and had 81,906 person-years of observation were at risk of PTS. Over the observational period and after at least 90 days following first VTE 486 cases of severe (venous ulcer associated) PTS were identified.

The hazard of VTE recurrence was greater in the following risk factors: the elderly, increasing each decade from the 50s' maximal for those aged 90+, OR 5.27 (2.55–10.88), males OR 1.25 (1.00–1.58), following DVT compared with PE, OR 1.80 (1.38–2.35), following recurrent DVT, OR 1.94 (1.37–2.74), following recurrent PE, OR 2.26 (1.42–3.59); in unprovoked cases OR 1.41 (1.12–1.78); current smokers

OR 1.60 (1.18–2.17); and in those with varicose veins, OR 4.98 (3.10–8.01); no relation was seen with time in therapeutic range (TTR).
Summary/Conclusions: The recognition of risk factors for PTS should facilitate its prevention or early detection and the decision for appropriate therapy including the extension of anticoagulant therapy.

AS 07 – Upcoming Anticoagulants

AS 07
Rational design of future low molecular weight anticoagulant medications: can we get assistance from novel computational drug discovery methods?

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Drug discovery is a remarkably complicated process that requires different skills and appropriate technologies. Several steps are needed with most often the need to identify a potential therapeutic target or a novel molecular mechanism involved in the disease state. In the next step, a small chemical compound that modifies the function of this selected target has to be found. This small molecule can be used to further validate the target or as a starting point for hit to lead and lead optimization. Indeed, hit molecules have to be optimized in term of potency, selectivity while paying attention to ADMET properties since ultimately the compounds will be given to human. One would also like to know if the bioactive small molecules acting on the selected therapeutic target could also bind to off-targets and potentially induce toxicity. Several cutting edge in silico approaches and databases have been developed to assist the drug discovery process. We will go through several of these methods and examples of applications will be commented upon with a special emphasis on blood coagulation proteins.

AS 07.1
EP217609, a neutralizable synthetic dual-action FIIa/FXa anticoagulant, has a superior antithrombotic effect compared to its parent compounds in murine models of arterial thrombosis

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Background: EP217609 is a synthetic dual-action anticoagulant combining a direct thrombin inhibitor (α-NAPAP), an indirect factor Xa inhibitor (fondaparinux) and a biotin moiety. It has been reported that this molecule exhibits an unprecedented pharmacologic profile with high bioavailability, long plasma half-life, and potent anticoagulant activity (Olson et al., Blood 2012). Moreover, EP217609 is potentially rapidly neutralized by an avidin injection.
Aims: The aim of the study was to evaluate the antithrombotic potential of EP217609 compared to its parent compounds in two models of localized arterial thrombosis.
Methods: The anticoagulant properties of EP217609 were determined *in vitro* and *ex vivo* in mice by: i) measuring the activated partial thromboplastin time (APTT) and the thrombin clotting time; ii) measuring anti-factor IIa and anti-factor Xa activities. The effect of EP217609 in thrombosis was assessed in two mouse models of arterial thrombosis: a $FeCl_3$-injury of the carotid artery of WT mice or a mechanical injury of carotid arteries bearing atherosclerotic plaques in ApoE-deficient mice. Its impact on haemostasis was determined in a tail-bleeding assay.
Results: *In vitro*, EP217609 exhibited anticoagulant properties similar to its parent compounds. *In vivo*, it reached a 2.0 mM concentration in the plasma, 1.2 h after administration to mice, and presented an elimination half-life of 3.4 h. We determined low and moderate doses of EP217609 exhibiting similar *ex vivo* prolongation of the APTT than a-NAPAP and comparable *ex vivo* anti-FXa activity than fondaparinux, respectively. The effect of EP217609 was then evaluated *in vivo*, in comparison with the effect of the parent compounds used alone or in combination. At doses which hardly affected the APTT (1.09 times increase), EP217609 significantly reduced the thrombus area in both models as compared to a-NAPAP and fondaparinux used alone, but not when they are combined. In contrast, at doses increasing the APTT by 1.5 times, EP217609 did not present superiority as compared to either parent compound used alone or in combination. Interestingly, low doses of EP217609 did not prolong the tail-bleeding time nor the volume of blood lost. Finally, the effect of EP217609 could be neutralized *in vivo* after avidin injection in the $FeCl_3$-injury model.
Conclusion: The antithrombotic profile of EP217609, combined with its possible neutralization, makes it an interesting molecule and a potential drug candidate for indications which remain to be characterized.

AS 07.2
Identification of novel antagonists of protein disulfide isomerase for inhibition of thrombus formation

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Background: Protein disulfide isomerase (PDI) is a widely expressed oxidoreductase that is highly concentrated in endoplasmic reticulum and is required for protein folding. However, it can also be released from cells into the extracellular environment, where it catalyzes the rearrangement of intramolecular disulfide bonds in receptors and secreted proteins. Several animal models demonstrate that PDI released from platelets and endothelial cells is required for thrombus formation *in vivo*. Recently, we identified the flavonoid rutin as an inhibitor of PDI. Rutin inhibited both platelet accumulation and fibrin generation during thrombus formation in mouse models at concentrations commonly ingested as nutritional supplements, providing proof-of-principle for PDI inhibitors as a new class of antithrombotics (*J. Clin. Invest.*, 122:2104). Although quercetin flavonoids may be useful as a first generation of PDI inhibitors, they are only modestly potent for PDI (IC_{50} 6 μM), poorly absorbed, and have activities other than PDI inhibition.
Aims: Our goal was to identify potent and selective inhibitors of PDI as novel antithrombotics.
Methods: A high throughput screen of PDI activity was developed based on the ability of the enzyme to reduce the insulin β-chain, resulting in insulin aggregation. This insulin turbidimetric assay was converted to a 1536-well format and an industrial scale screen of approximately 350,000 compounds of the Molecular Libraries Small Molecule Repository was performed.
Results: The primary screen identified 443 putative PDI inhibitors, which were subsequently tested in 8-point dose curves. Forty-one compounds were identified with IC_{50}s < 10 μM. Two compounds, a piperdine (IC_{50} 0.3–0.6 μM) and a bromo indole (IC_{50} 0.6–0.8 μM), were selected for further analysis. To assess the selectively of these compounds, their activity in other bioassays performed within the NIH Molecular Libraries Probe Production Network was evaluated. The piperdine showed no confirmed activity at < 10 μM in any of the other of 380 biological assays in which it has been tested. The bromo indole had confirmed activity at < 10 μM in only two other assays out of 473 in which it had been tested. Within the thiol isomerase family, both compounds demonstrated selectivity for PDI, failing to inhibit either

ERp5, ERp57, or thioredoxin. The piperdine was soluble at 65 μM in aqueous solution and demonstrated 97% stability in GSH at 48 h, indicating that the compound did not inhibit PDI by interacting with the CxxC catalytic domain. The bromo indole was soluble at 81 μM and demonstrated 99% stability to GSH. Consistent with this observation, inhibition of PDI by the piperdine and the bromo indole was entirely reversible. Neither compound demonstrated toxicity in a HeLa cell assay at 20-fold their IC_{50}s. Organic synthesis of 31 analogs of the piperdine lead was performed and structure activity relationships determined. In preliminary *in vivo* studies, the piperdine inhibited platelet accumulation in cremaster arterioles following laser injury.
Conclusions: The compounds identified in this screen will serve as leads for the development of a second generation of PDI inhibitors as a new class of antithrombotics with improved potency and specificity compared with quercetin flavonoids.

AS 07.3

Cardioprotective effects of EWE thrombin, a selective protein C activator, in a mouse model of acute focal myocardial ischemia and reperfusion

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Background: Acute myocardial ischemia resulting from progressive thrombotic coronary artery occlusion is typically not treated with recanalization therapies before the patient arrives to the hospital due to bleeding concerns with current anticoagulant and thrombolytic therapies. A hemostatically safer antithrombotic treatment could improve survival by decreasing the 'time to needle' period.
Aims: EWE thrombin (EWE) is an *E. coli*-derived recombinant protein C-activating thrombin analog (W215A/E217A) that is potently antithrombotic without inducing hemostasis impairment or procoagulant side effects in primates. EWE treatment also reduces neurological deficits without bleeding side effects in an experimental mouse model of ischemic stroke. Here, we tested the hypothesis that EWE reduces infarct size following experimental myocardial ischemia-reperfusion in mice.
Methods: C57BL6 mice were used for all studies. To evaluate the effect of EWE on ischemic injury, the left coronary artery was ligated for 40 min, and EWE (25 μg/kg; iv) or vehicle infused during the last 15 min of occlusion. Mice were sacrificed at 2 h post reperfusion and the infarcted myocardium volumes of EWE- and vehicle-treated mice were determined. Vascular reflow was evaluated in a separate cohort, immediately following ischemia. Fluorescent microspheres were injected into the ventricular apex and 3 min later hearts were removed and sectioned. The fluorescence intensity in each section was quantified to examine EWE effects on reperfusion. To evaluate the direct cardioprotective potential of EWE, we used an ex vivo model of oxygen/glucose deprivation (OGD) and reoxygenation/glucose repletion (RGR). Adult cardiomyocytes were pre-incubated in serum-free media with EWE (0.1–10 μg/mL) or vehicle and subjected to 90 min OGD, followed by 3 h RGR and determination of cell viability with trypan blue exclusion.
Results: EWE increased vascular reflow by 23% compared to vehicle, measured immediately after ischemia. Likewise, treatment with EWE reduced myocardial infarct volume by 33%, measured 2 h post-reperfusion. *In vitro*, EWE improved cardiomyocyte viability from 45% to 73% in a concentration-dependent manner. Cardioprotection was dependent on the enzymatic activity of EWE, since an enzymatically inactive analog (S195A-EWE) produced a result similar to vehicle treatment. Pretreatment with a PAR-1 inhibitor abolished EWE mediated cardioprotection, suggesting cytoprotection was dependent on PAR-1 signaling. EWE-mediated cardioprotection *in vitro* was inhibited by 36% by anti-protein C antibodies (10 μg/mL) and by > 90% by anti-PAR-1 antibodies (20 μg/mL).
Summary/Conclusion: Altogether, treatment with EWE, a selective protein C activator, during ischemia reduced myocardial damage and improved reperfusion *in vivo*, and also preserved cardiomyocyte viability ex vivo. We conclude that treatment with EWE before recanalization therapy may confer outcome benefit in temporal myocardial ischemia.

AS 08 – Antiphospholipid Syndrome

AS 08.1

Conformation of beta2glycoprotein I and its effect on coagulation

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Background: Contradictory, the antiphospholipid syndrome (APS) is characterized by the presence of vascular thrombosis, but diagnosed by a phospholipid-dependent delay of the coagulation *in vitro*. β2glycoprotein I (β2GPI) is the major antigen in APS. In blood, b2GPI is present in a native conformation (either circular or S-shaped). After interaction with anionic surfaces it opens up (J-shaped conformation), resulting in the exposure of a cryptic epitope in domain I of β2GPI. Importantly, predominantly antibodies reactive to this cryptic epitope in domain I of β_2GPI are associated with thrombosis in APS. In literature anticoagulant properties of β2GPI have been indicated, although no consensus exists about the underlying mechanisms.
Aim: We investigated whether the conformation of β2GPI plays a role in the proposed anticoagulant activity of β2GPI.
Methods: To unravel the role of the conformation of β2GPI in its effect on the thrombin generation (TG), we used the previously described Calibrated Automated Thrombogram (CAT) assay. To study the effect of β2GPI in its open conformation, β2GPI was pre-incubated with 48 μM phospholipids for 15 min before being tested in the TG assay. Two patient-derived monoclonal antibodies P2-6 and P1-117 were tested for their effect in our system. Antibody P1-117 was previously shown to specifically react with the cryptic epitope G40-R43, exposed only in the open conformation of β2GPI, while antibody P2-6 recognizes β_2GPI irrespective of its conformation.
Results: Native β2GPI was found to have no significant effect on the TG regardless of the concentration of tissue factor (TF). On the contrary, β2GPI pre-incubated with phospholipids significantly and dose-dependently inhibited TG when triggered with low TF concentration, suggesting an effect on the intrinsic pathway of the coagulation cascade. As to the effect of the incubation period, increasing incubation times resulted in a further decrease in peak height and increase in lag time. Interestingly, the inhibitory effect of phospholipid-bound β2GPI was completely abolished by adding antibody P1-117, but not P2-6. These data illustrate that thrombosis-related antibodies reactive against epitope G40-R43 in domain I abrogate the observed anti-coagulant effect of phospholipid-bound β2GPI.
Conclusions: We have demonstrated that native β2GPI in circulation obtains its anticoagulant activity in the presence of anionic phospholipids such as activated blood cells, thereby serving as an inhibitory modulator in hemostasis. That only the open conformation of b2GPI exerts this anticoagulant effect, suggests a role for domain I. Indeed, using monoclonal antibodies we have established abrogation of the anticoagulant activity of b2GPI only by antibodies specific for the cryptic epitope on domain I. Our results may therefore provide an explanation for the prothrombotic phenotype in APS patients.

AS 08.2

Human anti-beta2-glycoprotein I autoantibodies bind to platelets but not to endothelium in a mouse model of anti-phospholipid syndrome

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Background: Antiphospholipid syndrome (APS) is defined by thrombosis, obstetrical complications, and the presence of the lupus anticoagulant, anticardiolipin antibodies, or anti-β2-glycoprotein-I (anti-β2GPI) antibodies. Anti-β2GPI antibodies have been documented as a biomarker for diagnosis of APS, and we have shown their direct role in the pathogenesis of thrombosis *in vivo* using a mouse model of arterial laser-induced injury. *In vitro*, these antibodies in complex with β2GPI have been shown to bind and activate endothelial cells and platelets.

Aims: We asked whether we could determine the cellular target of anti-β2GPI autoantibodies and plasma β2GPI *in vivo* using our thrombosis model in a live mouse and anti-β2GPI autoantibodies affinity-purified from humans with APS.

Methods: Anti-β2GPI antibodies were prepared from the serum of a patient with APS by affinity purification. $F(ab')_2$ fragments of anti-β2GPI autoantibodies, labeled with Alexa 488, and human β2GPI, labeled with Alexa 647, were infused both separately and together into wild type mice. Platelet labeling was performed using anti-CD42 antibody conjugated to Dylight 488 or Dylight 647. Cell binding of labeled β2GPI and anti-β2GPI autoantibodies at the site of injury was imaged after arterial laser-induced injury using intravital microscopy in wild type mice. In some experiments, eptifibatide, an inhibitor of platelet accumulation at the site of injury, was infused into the mouse. Endothelium activation was studied using calcium mobilization after infusion of Fluo-4 AM into the mouse circulation in the presence of eptifibatide.

Results: Alexa 488-conjugated anti-β2GPI $F(ab')_2$ autoantibodies bound specifically to the developing platelet thrombus, which were imaged using anti-CD42 conjugated to Dylight 647. In the presence of $F(ab')_2$ fragments of anti-β2GPI autoantibodies injected simultaneously, we found that Alexa 647-conjugated β2GPI bound specifically to the developing platelet thrombus, which were imaged using anti-CD42 conjugated to Dylight 488. Finally, Alexa 488-conjugated $F(ab')_2$ fragments of anti-β2GPI autoantibodies and Alexa 647-conjugated β2GPI infused simultaneously into the live mouse bound with similar kinetics to the developing thrombus. Experiments examining the binding of Alexa 488-conjugated irrelevant control $F(ab')_2$ IgG or Alexa 647-conjugated albumin showed only non-specific interaction. When platelet accumulation at the site of injury was inhibited by the infusion of eptifibatide prior to infusion of the anti-β2GPI $F(ab')_2$ autoantibodies conjugated to Alexa 488 and β2GPI conjugated to Alexa 647, no significant binding of either anti-β2GPI $F(ab')_2$ autoantibodies or β2GPI was observed. These results indicate that anti-β2GPI $F(ab')_2$ autoantibodies and β2GPI bind to platelets but not significantly to endothelial cells. Moreover, at the site of injury, there was no increase in endothelial cell activation after injection of anti-β2GPI autoantibodies when studied by monitoring calcium mobilization within the endothelium.

Conclusion: We have shown that, *in vivo*, the anti-β2GPI antibody-induced enhancement of thrombus formation is associated with platelet binding of both anti-β2GPI autoantibodies and β2GPI, presumably the anti-β2GPI/β2GPI complex. Using the human anti-β2GPI autoantibodies studied, neither endothelial cell binding nor enhanced endothelial cell activation is observed during thrombus development in this model of APS.

AS 08.3

Fibrin clot properties in patients with antiphospholipid syndrome

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Background: Antiphospholipid syndrome (APS) is an autoimmune disease that manifests as venous thromboembolism (VTE), arterial thrombosis and/or pregnancy complications in the presence of antiphospholipid antibodies (APLA). It has been suggested that patients with APS have impaired fibrinolysis. Data on fibrin clot properties in APS are scarce.

Aim: The aim of the study was to test the hypothesis that plasma fibrin clot structure/function is unfavorably altered in patients with APS after VTE and/or arterial thrombosis.

Methods: We investigated 118 patients with APS, including 88 subjects with primary APS, vs. 100 age- and sex-matched controls. Patients were analyzed in subgroups according to clinical presentation (VTE or arterial thrombosis; if both were present – first presentation was recorded). In the VTE subgroup we identified those who had deep vein thrombosis (DVT), pulmonary embolism (PE), or DVT combined with PE. We assessed lupus anticoagulant (LA), anticardiolipin antibodies (ACL) and anti-β2-glycoprotein I antibodies were measured by INOVA kits (USA). The fibrin clot structure was assessed by measurement of permeability (K_s) and turbidity (lag phase and maximum absorbency (Abs_{max}). The efficiency of clot lysis was evaluated by the assessment of clot lysis time (CLT) and maximum rate of increase of D-dimer levels released from fibrin clots along with its maximum concentrations in the effluent. Fibrinogen, tPA and PAI-1 antigens, high-sensitivity (hs)CRP, total homocysteine (tHcy) concentrations were measured.

Results: Of the 118 APS patients, eight subjects had previous myocardial infarction (MI), 26 – had stroke, 61 – symptomatic DVT without PE, 15 patients had PE alone, and 16 – DVT combined with PE. In 67 patients VTE was the first clinical presentation of APS. The patient group did not differ from the controls with regard to lipid profile, glucose, creatinine, hsCRP and tHcy. APS patients had higher fibrinogen, PAI-1 and tPA antigens than controls (all $P < 0.001$). Analysis of plasma fibrin clot variables showed that APS patients had 25.8% lower clot permeability ($P < 0.001$), 23% longer CLT ($P < 0.001$), 9.4% shorter lag phase ($P < 0.001$), 5.4% lower maximum rate of D-dimer release ($P < 0.001$) and 14.1% higher maximum D-dimer level ($P < 0.001$) than controls. Higher clot absorbance was observed in patients with positive ACL IgG ($P = 0.009$), those with negative LA ($P = 0.003$), and whose first clinical presentation was stroke or MI ($P = 0.023$). Patients who experienced PE showed higher K_s ($P = 0.027$), shorter CLT ($P = 0.008$), lower absorbance ($P = 0.006$) and higher D-dimer release rate ($P = 0.048$), regardless of the presence of concomitant DVT or not. The lowest clot permeability was observed in patients who experienced both VTE and arterial thrombosis ($P = 0.01$). Patients whose clinical presentation was only stroke or MI had longer CLT than the remainder ($P = 0.038$). Patients with SLE had less favorable fibrin clot properties with a significant difference only for D-Dimer rate ($P = 0.048$).

Conclusions: Our study demonstrates unfavorably altered plasma clot properties in APS patients, including reduced permeability and impaired fibrinolysis. There are differences in plasma fibrin clot phenotype depending on thrombotic manifestation of APS. Our findings indicate the abnormal clot characteristics represent a novel prothrombotic mechanism of thromboembolism in APS.

AS 09 – Flow and Von Willebrand Factor

AS 09
Activation of A1 domain adhesiveness in VWF by elongational force

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Multiple mechanisms may contribute to activation of VWF adhesiveness by elongational flow at sites of hemostasis, including enhancement of A1 domain exposure within VWF concatamers and conformational change within the A1 domain or its complex with GPIbα. A receptor and ligand in a single molecule (ReaLiSM) containing the A1 domain, a flexible linker, and GPIbα fused in a single polypeptide and suspended between beads using DNA handles was interrogated with a laser trap. Two pathways for unbinding representing flexed and extended states were previously reported, with the flexed, more stable state predominantly at forces above 10 pN. VWD type 2B mutations in the A1 domain selectively stabilize the extended, high affinity state whereas platelet-type VWD mutations in GPIbα stabilize both states. With ReaLiSM we can also measure the kinetics and force-dependence of receptor-ligand binding. Remarkably, we also see two on-rates for receptor-ligand binding, with the faster on-rate predominating above 10 pN and the slower on-rate predominating below 10 pN. VWD type 2B mutations in the A1 domain selectively increased the fast on-rate, whereas platelet-type mutations in GPIbα increased both on-rates. Our results support force-dependent conformational change as one of the mechanisms that activates VWF in hemostasis.

AS 09.1
Solution structure and dynamics of the major FVIII binding region on von Willebrand factor: implications for type 2N von Willebrand disease

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Background: The preservation of haemostatic integrity is secured by the activities of von Willebrand factor (VWF). Upon vascular damage, VWF acts as a molecular bridge facilitating the initial adhesion and aggregation of platelets to the site of vessel injury, and consequently thrombus formation ensues. Furthermore, VWF is the protective carrier of pro-coagulant factor VIII (FVIII) in plasma, thereby prolonging its half-life, and efficiently localising FVIII to the incipient platelet plug. Much of the function of VWF has been revealed, however, detailed insight into the molecular structure that enables VWF to orchestrate haemostatic processes, in particular FVIII stabilisation in plasma, is lacking.
Aims: Our principal aim is to determine an atomic-resolution structure of the D' region (TIL'E') of VWF, which is believed to form the primary FVIII binding site. The importance of elucidating the structure of the FVIII-binding region on VWF is two-fold. First, it would provide insights into the mechanism of FVIII docking onto VWF, and the minimal VWF unit required to stabilise FVIII *in vivo*. This would allow for the development of novel approaches to treat both haemophilia A and von Willebrand disease (VWD). Second, analysis of VWD patient mutations, against a background of both structural and functional perturbations, will provide the link between genetic pathology and clinical phenotype.
Methods: TIL'E', containing 16 cysteine residues, was uniformly $^{13}C/^{15}N$-labelled by expression in *Escherichia coli* and purified for structural and dynamic NMR studies. Native mass spectrometry, 1D NMR, and dot blot analyses with both FVIII and a conformational FVIII binding-blocking monoclonal antibody, confirmed that the recombinant TIL'E' fragment was monomeric, homogeneous and binds to FVIII.
Results: We present the high-resolution NMR solution structure of the major FVIII-binding region (D') on VWF, and report on the molecular dynamics and flexibility of its substructure. The complex disulphide-bonded D' region (99 aa) is composed of a two sub-domain architecture – TIL'E'. Domain TIL' lacks extensive secondary structure, wherein five disulphide bonds constitute the main stabilising force constraining the fold. In contrast, E', is a well-structured, all-β-sheet domain. We confirm the disulphide-bond topology of this region. The symbiosis between protein dynamics, structure and biological activity is clarified by nuclear spin relaxation data that reveal interesting motional trends in the D' region; most strikingly, that there is a readily distinguishable mobility differential between TIL' and E'. TIL' is dynamic on at least two time-scales measured by NMR relaxation experiments, and this region is coincident with the clustering of pathological mutations leading to decreased FVIII binding affinity (type 2N VWD). The most severe type 2N VWD mutations circumscribe a pocket of concentrated positive charge density, that is complementary to the negatively charged FVIII a3 domain which mediates high-affinity binding to VWF.
Conclusions: The structural and dynamic data we present strongly suggest that the conformational fluctuations and surface charge distribution of TIL' reveal the major interaction surface mediating the binding of FVIII to VWF.

AS 09.2
Enhanced binding of von Willebrand factor mutants to LRP1 may explain their reduced circulatory half-life

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Background: Von Willebrand Disease (VWD) originates from mutations in the gene encoding von Willebrand factor (VWF), which may affect different steps in the life-cycle of the protein, such as biosynthesis/secretion, function and/or clearance. At present, more than 20 different mutations have been identified that are associated with an increased clearance of VWF, which could contribute to the reduced VWF antigen levels in these patients. The mechanisms that mediate the increased clearance of the mutants have remained unsolved. Recently, we have identified the lipoprotein-receptor LRP1 as a clearance receptor for VWF (Rastegarlari et al. Blood 2012 119:2126). Interestingly, VWF only interacts with LRP1 upon exposure to increased shear stress, thereby mimicking interactions between VWF and its platelet receptor glycoprotein Ibα.
Aim: We investigated how VWD-mutations affect the interaction between VWF and LRP1.
Methods and Results: Using a static immunosorbent assay, we first investigated the binding of wild-type (wt)-VWF or fragments thereof to LRP1 in the absence or presence of ristocetin. As expected, no binding of wt-VWF in the absence of ristocetin was observed, whereas in its presence wt-VWF bound dose-dependently to LRP1. This confirms that shear stress-like changes in VWF are needed to allow LRP1 binding. Surprisingly, a number of individual recombinant VWF domains especially the D'D3 fragment displayed binding to LRP1 in the absence of ristocetin. Since several of the VWF mutations that provoke increased clearance of the protein are located in the D'D3 region of VWF, we tested if such mutations modulate binding to LRP1. Simultaneously, given the parallel between the shear stress-regulated interactions of VWF with glycoprotein Ibα and LRP1, we examined how VWD-type 2B mutations affect LRP1 binding. *In vitro* binding experiments revealed that the VWD-type 2B mutants VWF-p.R1306Q and p.V1316M as well as the VWD-type 1 clearance mutant VWF-p.R1205H (the Vicenza variant) show enhanced binding to LRP1. In contrast to wt-VWF, all three mutants interact spontaneously with

LRP1 under static conditions in the absence of shear stress or ristocetin. *In vivo* clearance experiments confirmed that mutants VWF-p.R1205H and VWF-p.V1316M are cleared more rapidly than wt-VWF. However, preliminary analysis revealed that in the presence of the LRP1-inhibitor RAP, clearance of the mutants is delayed and their survival mimics that of wt-VWF in the presence of RAP.
Conclusions: Our data indicate that several VWD-mutations relieve the shear stress-requirement for the interaction with the clearance receptor LRP1. Enhanced binding to LRP1 may provide an explanation why these mutants are cleared more rapidly and are associated with reduced VWF antigen levels in VWD patients.

AS 09.3

Fluid shear dependent regulation of von Willebrand factor (VWF) binding to platelet GpIba: Mechanism revealed by MS analysis of cross-linked VWF

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Background: The domain level organization within the largest glycoprotein in blood, von Willebrand Factor (VWF), regulates many of its functions under fluid shear conditions.
Aim: In order to identify the structural basis of such regulation, VWF was chemically crosslinked and tandem mass spectrometry (MS) was performed in order to identify specific peptides within the protein that lie in close spatial proximity. Findings emerging from such analysis were tested, with focus on the mechanism of fluid shear dependent enhancement of platelet binding to immobilized VWF.
Methods: Full-length dimeric VWF (ΔPro-VWF) reacted with the homobifunctional crosslinker bis(sulfosuccinimidyl)suberate (BS^3) was enzymatically digested and subjected to high-throughput tandem MS. A scoring strategy called XPA was developed to analyze experimental data and to identify crosslinked peptide-pairs that link more than one protein domain. Functional experiments were performed to test hypotheses emerging from such analysis.
Results: Six of the nine inter-domain VWF peptide-pairs identified by such analysis linked VWF-A1 to the VWF-D'D3 domain, including one A1-peptide that forms the VWF binding interface with the platelet receptor GpIbα. MS analysis thus suggests that the proximal location of VWF-D'D3 to -A1 may regulate VWF binding to platelet GpIbα. Functional data support this hypothesis: i. Deletion of the D'D3 domain resulted in a dimeric protein (ΔD'D3-VWF), which unlike ΔPro-VWF, bound GpIbα under static conditions even in the absence of ristocetin ii. In surface plasmon resonance studies, monomeric VWF D'D3-A1 bound immobilized GpIbα with a lower on-rate compared to VWF-A1 iii. ΔD'D3-VWF, but not ΔPro-VWF and multimeric plasma VWF (pVWF), supported platelet binding at low shear stress (1 dyn/cm^2). At higher shears (> 5 dyn/cm^2), platelet translocation velocity was ~50% lower for ΔD'D3-VWF. Abrupt reduction in shear from 10 to 1 dyn/cm^2 released platelets from ΔPro-VWF, but not ΔD'D3-VWF, bearing substrates. iv. A monoclonal antibody against the human VWF-D'D3 domain, DD3.1, reduced VWF-A1 mediated platelet aggregation by > 50%, and it also inhibited platelet thrombus formation in a parallel plate flow chamber assay.
Summary/Conclusion: The D'D3 domain of VWF attenuates the interaction between its A1 domain and the platelet receptor GpIbα. This diminished molecular recognition results in reduced platelet adhesion at low shear rates. At higher shears, platelet deformation enhances the number of A1-GpIbα interactions and this contributes to more robust cell adhesion.

AS 10 – Coagulation and Inflammation

AS 10.1

TLR3 activation is involved in venous thrombosis development

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Background: Venous thromboembolism (VTE) afflicts 117 people per 100,000 each year and is an important cause of morbidity and mortality. A number of recent reports indicate that platelets and leukocytes may sense 'pathologic ligands' accumulating in the circulation via a specific set of receptors and translate an activation signal into a prothrombotic state. These receptors may include toll-like receptors (TLR), which recognize self-molecules generated during tissue damage and inflammation in the presence or absence of infection. Endogenous RNA, released from cells under pathological conditions, has been identified as a potent pro-coagulant factor by activating factor XII of the coagulation cascade in arterial thrombosis. TLR3 senses dsRNA and has been increasingly linked to tissue damage. Endogenous RNA might induce TLR3 expression and signaling. Thus, we hypothesize that endogenous RNA might be involved in the inflammatory development of venous thrombosis after vessel injury.
Aims: The objective of the present study is to evaluate the role of endogenous RNA and TLR3 in venous thrombosis.
Methods: First, wild type (WT) mice received an intravenous injection of RNase or vehicle prior to thrombus induction with 5% $FeCl_3$. In separate experiments, WT and TLR3 deficient (−/−) mice received an intravenous injection of polyinosine polycytidylic acid, a synthetic double-stranded RNA (dsRNA) analog, or vehicle and thrombosis was also induced by 5% $FeCl_3$.
The venous wall with thrombi were removed and samples were prepared for hematoxylin and eosin and immunofluorescent staining. Human umbilical vein endothelial cells (HUVECs) or endothelial cells prepared from WT or $TLR3^{-/-}$ mice lungs were incubated with dsRNA and cytokine mRNA expression were analyzed by real time PCR.
Results: RNase treatment significantly reduced thrombus size compared with vehicle injected WT mice ($P < 0.05$). Synthetic dsRNA increases the size of thrombi after $FeCl_3$-induced inferior vena cava injury (IVC) compared to mice treated with vehicle ($P < 0.05$). In $TLR3^{-/-}$ mice, dsRNA did not induce a further increase in thrombus size compared to vehicle control. dsRNA injection was associated with an increase of neutrophil and monocytes infiltration in the thrombus in WT but not in $TLR3^{-/-}$ mice ($P < 0.05$). Leukocyte infiltration was decreased by RNase treatment ($P < 0.05$). We found that dsRNA injection was associated with increased neutrophil extracellular traps (NETs) formation. In the thrombus of WT mice, immunofluorescence staining for myeloperoxidase, neutrophil elastase and citrulinated H3, markers for neutrophils activation, were increased by dsRNA and reduced by RNase treatment in WT mice. Interestingly, in $TLR3^{-/-}$ mice, no increase of these markers was found after dsRNA injection as compared to vehicle control. In HUVECs and murine endothelial cells, dsRNA induced mRNA expression of IL-8 and CCL5. Absence of TLR3 (siRNA in HUVEC or $TLR3^{-/-}$ cells) blocked dsRNA-induced mRNA expression of IL-8, CCL5 and CCL2.
Summary/conclusions: Taken together, we found that endogenous RNA and TLR3 activation participate in thrombus formation and leukocyte recruitment in part by inducing the expression of chemoattractants in the endothelium. These results strongly suggest that TLR3 stimulation after endothelial cell injury participate in thrombus formation by inducing a pro-inflammatory response leading to the recruitment and activation of leukocytes.

AS 10.2

High sensitivity C-reactive protein, body mass index, factor VIII levels and risk of venous thrombosis: results from the MEGA study

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Background: The role of C-reactive protein (CRP) as a risk factor for venous thrombosis is debated. Moreover, genetically elevated CRP was not associated with venous thrombosis in a large population based study, suggesting that the observed relationship may be confounded or mediated by other factors. Obesity is associated with a state of chronic low-grade inflammation, and CRP levels correspond to the presence of adiposity in the body. Moreover, increased plasma levels of CRP have been shown to correlate with increasing levels of coagulation factor VIII (FVIII). Both obesity and FVIII are well-established, strong risk factors for venous thrombosis.

Aims: Since controversy exists on the causal role of CRP on venous thrombosis risk, we set out to determine whether an observed association between CRP and venous thrombosis could be explained by a concomitant procoagulant state and/or presence of obesity. To study this hypothesis, we first examined the crude association between CRP and venous thrombosis, and then assessed to what extent FVIII and BMI influenced this relationship.

Methods: High sensitivity CRP (hsCRP), factor VIII antigen (FVIII) and body mass index (BMI) were measured in the Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis-study (MEGA study), a large case-control study including 2205 cases of venous thrombosis and 2777 age- and sex-matched controls. With logistic regression models, odds ratios (OR) for venous thrombosis by percentiles of hsCRP were calculated and further adjusted for BMI and FVIII, introducing one co-variate at the time.

Results: HsCRP values increased linearly with increasing BMI and increasing FVIII levels in both genders. In age and sex-adjusted analysis, the risk of venous thrombosis increased dose-dependently across percentiles of hsCRP. Subjects with an hsCRP level above the 97.5th percentile (> 13.48 mg/L) had a 3.5-fold (OR 3.47; CI95: 2.52–4.78) higher risk of venous thrombosis compared to those with an hsCRP level below the 25th percentile (< 0.68 mg/L). However, adjustment for either BMI (OR for > 97.5th vs. < 25th percentile of hsCRP: 2.71; CI95: 1.95–3.76) or FVIII (OR 1.55; CI95: 1.10–2.21) highly attenuated the risk estimates, and the association between hsCRP and venous thrombosis was no longer present when both BMI and FVIII were included in the adjustment model (OR for > 97.5th vs. < 25th percentile: 1.26, CI95: 0.87–1.81). Similar risk estimates were found in separate analyses of unprovoked and provoked VTE, and in analyses of deep vein thrombosis and pulmonary embolism.

Conclusions: Our findings suggest that CRP is not causally related to venous thrombosis, but rather a marker of concomitant high levels of FVIII and BMI. Persistent rises in FVIII levels have been reported in major illnesses such as chronic inflammation, liver disease, hyperthyroidism and renal disease. Thus, it can be hypothesized that the observed association between CRP and venous thrombosis may be due to presence of chronic diseases that cause a concomitant rise in CRP and FVIII.

AS 10.3

Differential contribution of FXa and thrombin to vascular inflammation in a mouse model of sickle cell disease

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Sickle cell disease (SCD) is a hematologic disorder associated with activation of coagulation and vascular inflammation. We have previously demonstrated that inhibition of tissue factor (TF) with a mouse anti-TF antibody 1H1 prevents activation of coagulation (plasma thrombin anti-thrombin [TAT] levels), attenuates endothelial cell (EC) activation (plasma levels of sVCAM-1) and inflammation (assessed by plasma IL-6) in mouse models of SCD. TF inhibition also attenuated neutrophil recruitment and activation in lungs (measured by organ myeloperoxidase [MPO] levels). Interestingly, deletion of TF from ECs only reduced plasma levels of IL-6 in sickle mice.

In the current study, we investigated the contribution of FXa and thrombin to vascular inflammation in SCD. Four month old Berkley sickle cell (SS) and non-sickle (AA) littermate control mice (n = 9–11) were fed chow containing either no inhibitor (control), the FXa inhibitor rivaroxaban (1.8 mg/g) or the thrombin inhibitor dabigatran (10 mg/g) *ad libitum* for 10 days. Furthermore, we used bone marrow transplantation (BMT) to generate AA and SS mice deficient in either protease activated receptor-1 (PAR-1) or PAR-2 (n = 8–10) on all non-hematopietic cells (n-HCs). These mice were used for the study 4 months after BMT.

SS mice on control chow had increased plasma levels of TAT (2.3-fold, $P < 0.001$), IL-6 (5.6-fold, $P < 0.01$), sVCAM-1 (1.9-fold, $P < 0.001$) and MPO levels in the lungs (2.7-fold, $P < 0.001$) compared to AA controls. Rivaroxaban significantly reduced plasma TAT (3.1 ± 0.6 vs. 6.1 ± 0.6 ng/mL, $P < 0.001$) and IL-6 (7.3 ± 2.4 vs. 19.2 ± 6.7 pg/mL, $P < 0.05$) but had no effect on sVCAM-1 levels. Inhibition of FXa also moderately reduced (24.5%) lung MPO levels. Dabigatran significantly attenuated plasma TAT levels (7.5 ± 2.7 vs. 13.8 ± 9 ng/mL, $P < 0.01$) and lung MPO (70 ± 7.8 vs. 116.7 ± 16.8 ng/mg protein, $P < 0.01$), but had no effect on plasma IL-6 or sVCAM-1 in SS mice. Neither inhibitor induced bleeding or affected red blood cell numbers or hematocrit in AA and SS mice.

In wild-type mice transplanted with SS bone marrow, we observed significant increases in plasma TAT (1.7-fold, $P < 0.05$), sVCAM-1 (1.6-fold, $P < 0.0001$), IL-6 (4.8-fold, $P < 0.001$), and lung MPO (2.8-fold, $P < 0.001$) compared to AA-transplanted controls. PAR-1 deficiency in all n-HCs had no effect on these biomarkers in SS mice. Moreover, PAR-2 deficiency in n-HCs had no effect on coagulation activation (TAT) or EC injury (sVCAM-1) in SS mice. However, SS mice lacking PAR-2 in all n-HCs had significantly attenuated plasma levels of IL-6 (9.4 ± 0.9 vs. 18.9 ± 4.5 pg/mL, $P < 0.05$) and lung MPO (87.4 ± 6.4 vs. 142.4 ± 16 ng/mg, $P < 0.0001$).

This study demonstrated efficient anticoagulation of SS mice with rivaroxaban or dabigatran. However, we found that FXa, but not thrombin, contributes to systemic inflammation (IL-6), likely through TF: FVIIa:FXa-dependent activation of PAR-2 on ECs. nHCs PAR-1 appears to play no role in vascular inflammation in SS mice. Nevertheless, thrombin, and to a lesser extent FXa, contributes to the local neutrophil response in the lung, most likely via a fibrin-dependent mechanism (under investigation). The TF-dependent increase in sVCAM-1 is not mediated by FXa or thrombin. Ligands other than coagulation proteases might contribute to PAR-2-dependent increase in lung MPO.

AS 11 – New Genetic Determinants of Venous Thrombosis

AS 11
New genetic determinants of venous thrombosis: a novel mechanism of hereditary thrombosis by antithrombin resistance

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Background: Venous thromboembolism is a multifactorial disease, resulting from a complex interaction of inherited and environmental factors. To date, numerous genetic defects have been found in the families with hereditary thrombophilia, but there may be still many undiscovered causative mutations.
Aim: We investigated the possible causative gene defect in a large Japanese family with inherited thrombophilia.
Methods: We analyzed the prothrombin gene by the PCR mediated sequencing method. Informed consent was obtained and our Institutional ethics committee approved the study. We prepared recombinant prothrombins, and analyzed their characters and functions. To examine the functions of the recombinant prothrombins in plasma, we prepared reconstituted plasma by mixing prothrombin-deficient plasma with the recombinant prothrombins. The proband's plasma was not suitable for evaluation because of warfarin treatment. We performed three tests of prothrombin activity: a one-stage clotting assay, a two-stage clotting assay, and a chromogenic assay that uses S-2238. We measured thrombin-antithrombin (TAT) complex formation by ELISA. We also performed a thrombin generation assay to determine the potential procoagulant activity of the recombinant prothrombins in plasma.
Results: We found a novel missense mutation in the prothrombin gene (p.Arg596Leu) resulting in a variant prothrombin (prothrombin-Yukuhashi). The mutation cosegregated with deep vein thrombosis in the family, suggesting being a cause of hereditary thrombophilia. The mutation occurred at residue Arg596 within the Na+ binding region of thrombin molecule, which locates at one of the antithrombin binding sites. We observed that the mutant and wild-type prothrombins were fully converted to thrombins in a similar manner within 5 min by prothrombinase. However, conversion of the mutant prothrombin to thrombin appeared to be seconds slower than that of the wild-type thrombin in the clotting assays. In addition, the mutant thrombin likely had a lower catalytic activity for fibrinogen than the wild-type thrombin. Extremely low levels of TAT complex were detected in the mutant thrombin sample, suggesting that disruption of the Na+ binding region structure losing two hydrogen bonds between the thrombin Arg596 and antithrombin Asn265 may be critical for TAT formation. These findings indicate that prothrombin-Yukuhashi can be characterized as a dysprothrombin highly resistant to inhibition by antithrombin. A thrombin generation assay is a comprehensive coagulation function test using a fluorogenic substrate that enables to evaluate not only the initial phase of thrombin generation, but also the late phase of its inactivation. The thrombin generation assay data again suggested that the mutant prothrombin could be a low procoagulant, but was highly antithrombin resistant. Therefore, its active form, the mutant thrombin, would not be inactivated by antithrombin, and would continue to facilitate blood coagulation despite its low activity.
Conclusion: We identified a novel mechanism of hereditary thrombosis by antithrombin resistance, associated with a missense mutation in the prothrombin gene. The antithrombin-resistant thrombin may possess prolonged procoagulant activity *in vivo*, leading to a susceptibility to thrombosis.

AS 11.1
Genome-wide association of quantitative trait loci with levels of hemostatic factors and thrombin generation in the GIFT study

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Background: Deficiencies and elevated levels of several circulating hemostatic factors as well as elevated thrombin generation have been associated with venous thromboembolism. The observed inter-individual variation in de levels of these hemostatic phenotypes can be partly explained by sequence variations in or outside the structural gene encoding these factors or influencing these analytes.
Aim: The aim of this study was to identify new quantitative trait loci (QTL) of several hemostatic traits and to confirm known loci by using genome-wide association study (GWAS) data.
Methods: Plasma levels of coagulation factors (protein C, protein S (free+total), C4BP, antithrombin, heparin cofactor II, fibrinogen, coagulation factors II, V, VIII, IX, X, XI, and protein Z), and thrombin generation parameters under three assay conditions (1 pM tissue factor (TF), 10 pM TF, 10 pM TF+ activated protein C (APC)) were measured in 413 subjects from the Genetics In Familial Thrombosis (GIFT) study. This study consists of 201 small, unrelated thrombophilia families with affected siblings with venous thromboembolism (n = 434). Subjects treated with anticoagulants at time of blood collection (n = 142) were excluded from the analyses of the vitamin K-dependent factors protein C, protein S, protein Z, factors II, IX, X as well as for the thrombin generation parameters.
SNP genotype data were generated with the Illumina 660W-Quad Beadchip. SNPs of high quality (SNP yield > 96%; MAF > 1%; HW P-value > 10^{-6}) were used for imputation with HapMap version 2. After imputation, more than 1.9 million SNPs were in agreement with the quality control settings (imputation $R^2 \geq 90\%$; $5\% \leq$ MAF $\leq 95\%$) for current analyses. Association analysis was performed on the GWAS SNP data and the age and sex adjusted hemostatic traits by using the generalized estimating equations assuming an additive genetic model.
Results: This association analysis revealed genome-wide significant ($P < 5.0*10^{-8}$) results for plasma levels of protein Z and factor VIII, and for the endogenous thrombin potential (ETP) and lag time from the thrombin generation assay performed with 10 pM TF+APC. For protein Z, two loci were identified with a lowest P-value of $1.1*10^{-11}$ at SNP rs12876365 on chromosome 13 near the structural gene, *PROZ*. The second locus, with a lowest P-value of $2.8*10^{-9}$, was located at SNP rs1526520 on chromosome 7 in the *THSD7A* gene that encodes thrombospondin type I domain containing 7A. An association signal for FVIII levels was confirmed in the *ABO* blood group genes on chromosome 9, with a lowest P-value of $5.9*10^{-11}$. Both the ETP and lag time from the thrombin generation assay with 10 pM TF+APC were associated with SNPs on chromosome 1 in the genes *F5* and *NME7*, with P-values in the range of 10^{-9}.
Conclusion: Using GWAS data, we have confirmed association of protein Z levels with SNPs in the structural gene encoding protein Z, and we identified a possibly new quantitative trait locus at *THSD7A* on chromosome 7. A known locus for FVIII levels was confirmed, and the analysis with thrombin generation parameters revealed association with SNPs in the genes *F5* and *NME7*. These novel identified genes can be examined as candidate genes in the investigation of venous thromboembolism.

AS 11.2

Y haplogroup R1b is associated with an increased risk of recurrent venous thrombosis in men

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Introduction: The recurrence risk of venous thrombosis is 1.5–2.5 higher in men than in women. Several explanations for the inequity by sex have been proposed, such as differences in age and the inclusion of hormone associated venous thrombosis, but none proved sufficient. We hypothesized a role for the Y chromosome in recurrence risk in men.

Aim: The aim of this study was to examine the effect of variation in the male-specific region of chromosome Y (MSY) in recurrent venous thrombosis in men.

Methods: In order to determine Y haplogroups according to the Y chromosome phylogenetic tree, we genotyped 26 variants of the MSY in the male participants of the MEGA follow-up study (1872 men with a first event of whom 396 had a recurrence during follow-up). Consent and ethical approval were obtained. For each haplogroup, we compared the recurrence risk with all men in the other haplogroups by calculating hazard ratios (HR) with 95% confidence intervals (CI). Currently, we have genotype information of 815 men with a first event of whom 195 had a recurrence during follow-up. Complete results for the total study population will be available at the ISTH congress.

Results: Overall, we observed a cumulative incidence of recurrent venous thrombosis of 33.1 (95% CI 27.9–38.3) per 1000 person years. We identified 19 Y haplogroups among the 815 men, of which haplogroups R1b and I were the most prevalent, 58.5% and 21.1% respectively. The cumulative incidence of recurrent venous thrombosis was 38.4 (95% CI 31.0–45.7) per 1000 person years in R1b carriers compared with 25.8 (95% CI 18.7–32.9) in non-R1b carriers. Carriers of haplogroup R1b had a 1.5-fold higher risk (95% CI 1.1–2.1) of recurrent venous thrombosis than non-R1b carriers. Exclusion of men of non-Dutch origin did not affect this association. We observed no association between recurrent venous thrombosis and carriers of haplogroup I compared with non-I carriers (HR 0.8, 95% CI 0.5–1.1).

Conclusions: Our results show a predisposing effect of haplogroup R1b on recurrence risk of venous thrombosis in men, which may explain the inequity by sex in disease risk. Further study of haplogroup R1b will provide insight in the biology and patho-physiology of recurrent venous thrombosis.

AS 11.3

Whole exome sequencing of > 900 individuals provides insight into genetic architecture of venous thromboembolism

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Background: Presently known genetic variants, including common variants identified in GWAS studies, explain 20–30% of venous thromboembolism (VTE) events. Family and twin studies, however, indicate that genetic factors account for about 60% of the VTE risk. Thus, there is an opportunity to identify new gene variants involved in the pathogenesis of VTE. Previously implicated loci include protein coding variants in genes with common alleles of moderate effect (e.g. ABO and F5), and genes with multiple private mutations (e.g. PROC and PROS1), suggesting that a genome-wide search to identify common and rare coding variants may reveal additional loci and alleles.

Aims: We have applied whole exome sequencing to comprehensively identify common and rare coding variants and tested for association with VTE in a case-control study of > 900 individuals.

Methods: Cases who had a first VTE (n = 465) and matched controls (n = 455) were identified from a population-based, nested, case-cohort study (the Tromsø study) comprising 78% (n = 27,158) of the adult residents of Tromsø in Norway. Exome sequencing was performed to an average depth of ~100× to identify common and rare variants. Using the 1000 Genomes Project EUR individuals as reference, we imputed genotypes at SNPs and indels at minor allele frequencies (MAF) > 0.005 in the reference population. We have tested for association with VTE across all sites and are currently performing rare variant collapsing tests of association. The study was approved by the regional committee for research ethics, and all participants gave their informed written consent to participate.

Results: Using whole exome sequencing, we identified 198,868 coding variants including 116,930 missense, 2329 nonsense, and 79,609 synonymous sites. Most sites were rare, with 82% with MAF < 5%. We observed an association between severity of effect and MAF with more deleterious groups of alleles tending to be rarer. We are currently undergoing analyses to test for association between cases and controls and will be performing common variant association similar to GWAS studies and rare-variant association including collapsing variant tests. Preliminary analyses verify previously known genetic risk factors and also identify novel nominally associated variants in relevant pathways such as the coagulation system, lipid metabolism, and microparticle formation in VTE patients compared to age- and sex-matched controls. Imputation to the whole genome resulted in high quality genotype information at 3.3 M SNPs and 300 K indels throughout the genome, effectively covering ~1/3 of the genetic variation present at MAF > 0.005.

Conclusions: This is the first study to comprehensively study protein-coding genetic variation and its association with VTE. In addition to replicating previously implicated sites, we have also identified novel candidates potentially associated with VTE. Further studies will include replication in larger cohorts.

AS 12 – Late Breaking Abstracts I

AS 12.1

The Anticoagulation of Calf Thrombosis (ACT) project: results from the randomized controlled external pilot trial

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Background: There is currently little evidence defining the clinical importance of detecting and treating isolated distal deep vein thrombosis (IDDVT). Contemporary international guidelines vary regarding diagnostic and therapeutic advice. The potential benefits of anticoagulation remain poorly defined.

Aims: We sought to evaluate the feasibility of a randomized controlled trial within a modern cohort, to determine whether patients with IDDVT benefit from therapeutic anticoagulation.

Methods: A pragmatic, open label, external pilot randomized controlled trial. Consecutive symptomatic IDDVT patients were approached for inclusion, within an ambulatory thrombosis service. Participants were randomized to receive either phased therapeutic anticoagulation (intervention) or conservative management (control) from diagnosis. Both groups received grade 2 compression stockings and analgesia. All patients underwent assessor blinded color duplex imaging after 7 and 21 days, and follow up at 3 months. Principal fea-

sibility outcomes were recruitment rate and attrition, including loss to follow-up and allocation crossover. The primary clinical outcome was a composite of proximal propagation, pulmonary embolism, death attributable to venous thromboembolic disease or major bleeding. Secondary clinical outcomes included propagation to any site (including local extension confined to the calf), symptomatic progression and minor/nuisance bleeding rates. Analysis was by intention to treat. The trial protocol has been previously published.

Results: During the recruitment phase, 951 patients underwent ambulatory assessment for suspected DVT. Proximal disease was confirmed in 104 cases. Acute IDDVT was detected in 93 cases. Of these patients, 79 were subsequently deemed eligible and 70 (88.6% of those eligible) were recruited. 59/70 (84.3%, 95% CI 74.0–91.0%) patients completed the full protocol. All patients but one were followed up by direct contact after 90 days. A single patient (1.4%) was personally uncontactable: follow up occurred through medical record review and discussion with the primary care practitioner. Allocation crossover occurred in 15 (21.4%) patients.

All predefined feasibility outcomes were successfully achieved. The primary clinical outcome occurred in 4/35 (11.4%) controls and 0/35 in the intervention group (Absolute Risk Reduction [ARR] 11.4%, 95% Confidence Interval [CI] −1.5 to 26.7, $P = 0.11$, number needed to treat [NNT] of 9). There were no cases of major bleeding in either group.

Regarding secondary clinical outcomes, propagation to any site occurred in 11/35 (31.4%) of conservatively treated patients compared with 2/35 (5.7%) of those randomized to anticoagulation (ARR 25.7%, 95% CI 5.9 to 44.3%, $P = 0.001$, NNT 4). Pain scores at day 7 rose from baseline in a significantly higher number of patients treated conservatively, compared to patients treated by therapeutic anticoagulation (6/35 vs. 0/35, $P = 0.03$). Minor bleeding occurred in 3/35 (8.6%) controls and 7/35 (20.0%) anticoagulated patients ($P = 0.31$). Nuisance bleeding rates were equivocal.

Conclusion: We have established feasibility for a definitive trial on the value of therapeutic anticoagulation for IDDVT. Our pilot study currently provides the largest prospective randomized clinical dataset on this topic and demonstrates a non-significant trend towards reduction of serious complications with anticoagulation. This highlights the importance of further evaluation with an appropriately powered design.

Trial Registration – ISCTRN 75175695

AS 12.2

Apixaban for the treatment of symptomatic deep-vein thrombosis and pulmonary embolism: a randomized, double-blind trial (AMPLIFY)

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AMPLIFY Investigators, Italy

Background: Apixaban, an oral factor Xa inhibitor administered in fixed doses, may simplify the treatment of acute venous thromboembolism. In a previous study, apixaban was shown to be highly effective for the prevention of recurrent venous thromboembolism in patients who had completed 6–12 months of anticoagulant therapy and was associated with rates of major bleeding comparable to placebo [1].

Aims: To compare the efficacy and safety of apixaban alone with those of conventional anticoagulant therapy consisting of low-molecular-weight heparin followed by a vitamin K antagonist in patients with acute symptomatic venous thromboembolism.

Methods: This randomized, double-blind trial compared apixaban, 10 mg twice daily for 7 days followed by 5 mg twice daily for 6 months, with conventional therapy consisting of subcutaneous enoxaparin followed by warfarin in patients with acute symptomatic proximal deep-vein thrombosis and/or pulmonary embolism (NCT00643201). The study was designed to test the hypothesis that apixaban would be non-inferior to conventional therapy for the primary efficacy outcome. The criteria for non-inferiority required that the upper limit of the 95% confidence intervals were below pre-specified margins for both the relative risk (< 1.8) and the risk difference (< 0.035).

The primary efficacy outcome was symptomatic recurrent venous thromboembolism or venous thromboembolism-related death. The principal safety outcomes were major bleeding and major bleeding plus clinically relevant nonmajor bleeding. The diagnosis at study entry, the extent of initial deep-vein thrombosis or pulmonary embolism, and all suspected outcomes were adjudicated by an independent committee whose members were unaware of study group assignments. The trial was sponsored by Bristol-Myers Squibb Company and Pfizer Inc. The protocol was approved by the institutional review board at each participating center, and written informed consent was obtained from all patients.

Results: Five thousand four hundred patients were enrolled at 358 centers in 28 countries. The baseline characteristics of the treatment groups were similar. The diagnosis at study entry was deep-vein thrombosis in 67% of patients and pulmonary embolism with or without deep-vein thrombosis in 33%.

Summary/conclusions: Final results for the efficacy and safety outcomes will be presented.

Reference: 1. Agnelli G, Buller HR, Cohen A, et al. Apixaban for extended treatment of venous thromboembolism. *N Engl J Med.* 2013; 368: 699–708.

AS 12.3

Dalteparin sodium for the long-term management of venous thromboembolism in cancer patients. The DALTECAN Study

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Background: Venous thromboembolism (VTE) is a common and potentially life-threatening complication in patients with malignant disease. Current treatment guidelines recommend anticoagulation with a low molecular weight heparin for at least 3–6 months, or for the duration of the malignancy. Uncertainty exists whether to extend anticoagulation longer to prevent recurrent thromboembolism.

Aim: The DALTECAN Study was undertaken to determine whether extending anticoagulation with dalteparin in cancer-associated VTE beyond 6 months has an acceptable safety and adherence profile.

Methods: Patients with active cancer and newly diagnosed VTE were enrolled in a single-arm multi-center study and received treatment with dalteparin 200 IU/kg daily subcutaneously for 1 month, followed by 150 IU/kg daily for the subsequent 11 months. Bleeding and recurrent VTE were centrally adjudicated to determine their incidence rates in month 1, months 2–6, and months 7–12 following enrollment.

Results: Three hundred and thirty-four patients with VTE and active cancer were treated with dalteparin, of whom 185 (55.4%) completed 6 months of therapy and 109 (33%) completed 12 months. Overall,

92% of the patients had solid tumors, with lung (16.8%), breast (9.3%), or pancreas (9.3%) as the prevailing primary sites. Baseline ECOG performance status was 0 (29.6%), 1 (48.8%), 2 (20.7%), and 3 (0.3%), with two subjects' status unknown. Therapy adherence was 96% across the entire cohort, with a median treatment duration of 214 days. The overall frequency of major bleeding was 10.2%, observed at a rate of 1.3% per patient-month. The highest major bleeding rate was in the first month of dalteparin therapy at 3.6%, with a frequency declining to 1.1% during months 2–6, and 0.7% over months 7–12, with no statistically significant difference in rates between months 2–6 and 7–12 ($P = 0.39$). The incidence of new or recurrent VTE was 11.1% (37 patients), a rate of 1.4% per patient-month. The rate was highest for month 1 at 5.7%, falling thereafter to 0.8% per month for months 2–6 (incidence 3.4%) and 0.7% per month for months 7–12 (incidence 4.1%). Death occurred in 154 patients, of whom 115 died during the study period and had an adjudicated death. Among these 115 deaths, 105 were due to underlying cancer, four due to VTE, two due to bleeding, and the remaining four deaths were due to other causes.

Summary: Extending dalteparin therapy in patients with VTE and cancer beyond 6 months is not associated with an increase in bleeding compared to the initial period of therapy. The risks for developing major bleeding complications were greatest in the first month of therapy and decreased over the subsequent 11 months. The risk for VTE recurrence was also greatest in the first month after initiating dalteparin, decreased subsequently thereafter, and remained low during the observation period. The adherence rate was high, confirming that an extended LMWH regimen with dalteparin in cancer patients is feasible.

AS 12.4

Safety, efficacy and pharmacokinetics of nonacog beta pegol (N9-GP) for prophylaxis and treatment of bleeding episodes in patients with haemophilia B

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Background: Nonacog beta pegol (N9-GP) is a glycoPEGylated recombinant factor IX (rFIX) with a significantly longer plasma half-life (93 h) as compared to currently available plasma-derived and recombinant FIX products (18–19 h).[1] N9-GP is therefore expected to reduce the burden of treatment by decreasing dosing frequency in prophylaxis as well as controlling bleeding episodes with fewer injections. In this phase 3 trial, paradigmTM 2, two different prophylaxis dose levels of N9-GP were tested, along with an on-demand treatment arm.

Aims: The primary objective of this trial was to evaluate immunogenicity of N9-GP. Key secondary objectives were to assess therapeutic and prophylactic efficacy, pharmacokinetic (PK) properties and general safety of N9-GP.

Methods: Haemophilia B patients with ≤ 2% FIX activity, aged 13–70 years, with no history of inhibitors and at least 150 exposures days (ED) to other FIX products were included. The trial was approved by local IRB/REC and all participants signed an informed consent before any trial related activity. The aim was to have at least 25 patients completing each prophylaxis arm and at least 10 patients completing the on-demand treatment arm. Patients for the two prophylaxis regimens were randomised to once weekly N9-GP dosing of 10 or 40 U/kg, respectively, for 52 weeks. Treatment in the on-demand arm lasted for 28 weeks. Here patients were dosed with 40 or 80 U/kg for mild/moderate or severe bleeding episodes respectively. Same treatment regimen was used for prophylaxis patients experiencing breakthrough bleeding episodes. Immunogenicity was evaluated as the primary endpoint; incidence of inhibitory antibodies against FIX defined as titre ≥ 0.6 Bethesda Units (BU). Efficacy endpoints included frequency of bleeds among prophylaxis patients, haemostatic response to N9-GP infusion based on a 4-point categorical scale, as well as FIX activity as a surrogate marker. Following completion of the dosing period, all patients were offered continued treatment with N9-GP in the extension trial, paradigmTM 4.

Results: The trial is on-going, though recruitment has been completed. Results will be presented at the meeting.

Conclusions: Conclusions will be presented at the meeting.

Reference: 1. Negrier et al. *Blood* 2011; 118(10): 2695–2701.

AS 12.5

An antibody to thrombin's exosite 1 prevents thrombosis without causing bleeding

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A 53 year old female presented with a subdural haematoma following a head injury. The coagulation screen indicated a severe coagulopathy (prothrombin time 50 s, APTT 190 s, thrombin time > 100 s). An inhibitor was demonstrated by mixing studies, and was identified as an anti-thrombin IgA paraprotein at 3 g/L. The antibody was purified by Jacalin agarose, thrombin agarose and size-exclusion chromatography. The K_d was determined to be in the low nanomolar range, with a fast on- and slow off-rate (1×10^6/M/s and 1×10^{-3}/s, respectively). The antibody was completely unable to bind prothrombin. The antibody did not block the active site of thrombin as shown by cleavage of small substrates and inhibition by antithrombin. Exosite 1 binding was suggested by the ability of the antibody to inhibit fibrinogen cleavage in a purified system, and a 30% increase in rate of S-2238 cleavage. Competitive binding with fluorescently labeled hirugen confirmed exosite 1 as the binding site on thrombin. A Fab fragment was prepared from the antibody by papain cleavage and co-crystallised with human PPACK-Thrombin, and a 1.9 Å structure was solved showing an interaction between CDRH3 and exosite 1.

Whilst at first it appeared the patient had a severe bleeding disorder this was not the case. She had undergone knee surgery 6 months before presentation. A pre-operative coagulation screen had not been performed. There was no abnormal bleeding during or after surgery although it is highly likely that the paraprotein was present at the time. She also made a rapid complete recovery following the subdural haematoma with full resolution of the haematoma without rebleeding without treatment. Five years later she remains well with no spontaneous haemorrhage or bleeding after further trauma. These observations suggested that the IgA does not inhibit normal haemostasis, but that it might prevent thrombosis.

In a mouse femoral vein 10% ferric chloride injury model using fluorescein labelled fibrinogen, the antibody at concentrations > 40 nM completely abolished fibrin deposition. Up to, and including the high-

est concentration used (4 mM), tail bleeding times were not prolonged. In a murine carotid artery occlusion model, with a 5% ferric chloride injury, the antibody was able to prevent arterial occlusion and maintain blood flow. At the maximum concentration studied (400 nM), blood loss from a tail clip was not increased compared to saline controls.

We have identified an antibody that appears to prevent thrombosis without causing bleeding. A therapeutic derivative of this IgA might thus permit unlimited dose escalation of antithrombotic therapy without increasing bleeding – the Holy Grail of thrombosis research.

AS 13 – Role of FXII Activation in Mechanism of Thrombosis

AS 13.1

Histidine-rich glycoprotein binds to DNA, RNA and fXIIa with high affinity and attenuates contact-mediated coagulation in a mouse model of arterial thrombosis

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Background: Histidine-rich glycoprotein (HRG) is an abundant plasma protein that binds fibrin(ogen) and other plasma proteins, but whose function is unclear. The activated partial thromboplastin time (aPTT) is shortened in HRG deficient humans and mice; and we have previously shown that HRG modulates the contact pathway by binding factor (f) XIIa (K_d 21 nM) and attenuating its capacity to activate fXI. Because DNA and RNA serve as physiological activators of the contact pathway, the capacity of HRG to bind polyanions may endow it with a regulatory role in thrombosis.

Objective: To quantify HRG modulation of DNA- and RNA-mediated activation of the contact pathway *in vitro* and in a mouse model of arterial thrombosis.

Methods: Binding of HRG to DNA and RNA was assessed by surface plasmon resonance (SPR). The inhibitory effects of HRG on fXII activation and fXIIa and thrombin activation of fXI were assessed with chromogenic assays. The effects of HRG on DNA- and RNA-mediated activation of coagulation were assessed in clotting assays using human and mouse plasma. To explore effects *in vivo*, we compared the time to occlusion (TTO) after $FeCl_3$-induced carotid artery injury in HRG (−/−) and wild-type mice.

Results: SPR analyses reveal that HRG binds DNA and RNA (K_d values of 0.9 ± 1.3 and 1.4 ± 1.6 nM, respectively). Consistent with binding, HRG inhibits DNA-mediated (a) fXII activation by kallikrein (IC_{50} = 22 nM), (b) activation of fXI by fXIIa (IC_{50} = 460 nM), and (c) feedback activation of fXI by thrombin (IC_{50} = 3600 nM). HRG inhibits RNA-mediated activation and propagation of the contact pathway with similar potency. The concentrations of DNA and RNA required to shorten the aPTT by 50% in HRG-depleted human plasma are 8- and 24-fold lower, respectively, than in control plasma; a difference that is abolished when the deficient plasma is supplemented with 2 μM HRG. Similar results are obtained when DNA or RNA is used to activate plasma from HRG (−/−) and wild-type mice; and addition of human HRG to plasma from HRG (−/−) mice abrogates the difference. In an arterial injury model, the TTO in HRG (−/−) mice is 11.8 ± 2.8 min, whereas that in wild-type mice is over 30 min ($P < 0.0005$); this difference is abolished when HRG (−/−) mice are administered 2 μM human HRG.

Conclusions: HRG binds DNA, RNA and fXIIa with high affinity and is a potent inhibitor of contact-mediated activation of coagulation. HRG (−/−) mice exhibit accelerated thrombosis compared to controls; a difference that is abrogated with HRG administration. These data suggest that HRG attenuates contact-mediated thrombosis by modulating the prothrombotic properties of fXIIa and extracellular nucleic acids.

AS 13.2

Selective depletion of contact factors with antisense oligonucleotides attenuates catheter thrombosis in rabbits

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Background: Indwelling central venous catheters can trigger upper extremity deep vein thrombosis and pulmonary embolism. Neither low-dose warfarin nor low-molecular-weight heparin prophylaxis prevents this problem. Recently, we reported that upstream inhibition at the level of factor (f) XIIa was better than downstream inhibition at the level of fXa and thrombin to prevent catheter-related thrombosis *in vitro* and *in vivo* (Blood 2011; 118(25):6667–6674 and Acta Biomater 2012; 8(11):4092–4100). This demonstrates that the contact pathway plays an important role during catheter-induced clotting.

Aim: To further explore the role of the contact pathway in catheter thrombosis, we used antisense oligonucleotides (ASO) to knock down the levels of fXII, fXI or high-molecular-weight kininogen (HK) in rabbits and examined their effect on the time to catheter occlusion (TTO) in both an acute and a chronic model.

Methods: Rabbits (n = 6–8/group) were randomized to receive 30 mg/kg/week of control, fXII-, fXI- or HK-directed ASO for 4 weeks. Hepatic mRNA levels and plasma protein levels were quantified. Activated partial thromboplastin times (APTT) and prothrombin times (PT) were also measured. To initiate the occlusion study, a 6 F catheter was inserted into the right atrium via the jugular vein. In the acute study, blood was withdrawn from the catheter every 5 min and re-injected, and the catheter was then flushed with saline. TTO was determined as the time when blood could no longer be withdrawn from the catheter. TTO was scored as 4 h if catheter occlusion did not occur. In the chronic study, blood was withdrawn once-daily and TTO was determined as the time when saline could no longer be injected, or scored as 30 days if catheter occlusion did not occur.

Results: Specific ASO treatment reduced hepatic mRNA levels of fXII, fXI and HK by 97%, 84% and 57%, respectively, and plasma protein levels by 97%, 96% and 87%, respectively; whereas the control ASO had no effect on any parameters. FXII-, fXI-, or HK-directed ASOs prolonged the APTT in rabbits by 4.7-, 3.6-, and 3.1-fold compared with rabbits given control ASO, whereas PT in rabbits showed no difference between the ASO groups. In the acute occlusion study, the mean TTO (± standard deviation) in the control group was 40.5 ± 6.2 min. fXII-, fXI- and HK- directed ASO treatments significantly prolonged the mean TTO by 2.8-, 4.4-, and 2.6-fold, respectively ($P < 0.05$). In the chronic study, the mean TTO in the control group was 10.3 ± 6.5 days. FXII- and fXI- directed ASO treatments significantly prolonged ($P < 0.01$) the mean TTO by 2.0- and 2.5-fold, respectively, whereas the HK-directed ASO had little effect.

Summary/Conclusions: fXII- or fXI- directed ASO attenuates catheter thrombosis in both acute and chronic rabbit models. These findings support the role of the contact pathway in catheter thrombosis, thereby identifying new targets and novel methodology to prevent thrombosis in patients with indwelling central venous catheters.

AS 13.3

Factor XII promotes blood coagulation independent of factor XI in the presence of long chain inorganic polyphosphates

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Background: Inorganic polyphosphates (polyP), which are secreted by activated platelets (short chain polyP) and accumulate in bacteria (long chain polyP), support the contact activation of factor XII (FXII) and accelerate the activation of factor XI (FXI) by thrombin. FXII-deficient mice have been shown to be more resistant to $FeCl_3$-induced arterial occlusion than either factor IX (FIX) or FXI deficient mice, suggesting that FXII also contributes to thrombosis in a FXI-independent manner *in vivo*. It has been shown that the injection of polyP induces coagulopathy in mice in a FXII-dependent manner, but the role of FXI in this process remains unclear.

Aims: The aim of the present study was to determine whether FXII activation by polyP promotes blood coagulation and experimental thrombus formation in a FXI-independent manner.

Methods and Results: Both long and short polyP promoted thrombin-initiated coagulation of plasma in a concentration-dependent manner. Neither the activated factor X (FXa) inhibitor, rivaroxaban, nor antibodies that inhibit FXI activation by activated FXII (FXIIa) or FIX activation by activated FXI (FXIa) were able to inhibit the procoagulant effect of long polyP. In contrast, the FXIIa inhibitor, corn trypsin inhibitor (CTI), blocked the procoagulant effect of long polyP, suggesting that the activation of FXII by polyP promotes coagulation in a FXI-independent manner. In purified system, long polyP significantly enhanced the rate of FXII activation in presence of prekallikrein (PK) and high molecular weight kininogen (HK) and the rate of PK activation by FXIIa. We observed that long polyP enhanced the rate of FXI activation by α-thrombin. In contrast, polyP did not increase FXI activation by FXIIa. In the presence of FXII, PK and HK, long and short polyP blocked the generation of FXIa, suggesting that the activation of FXII by polyP promote kallikrein generation but not FXI activation. We found that addition of 50 μM long polyP decreased the recalcification time of FXI-deficient plasma by up to 60%. The procoagulant activity of long polyP in FXI-deficient plasma was inhibited by a blocking FIX antibody, and eliminated by the presence of CTI or rivaroxaban, suggesting that the activation of FXII by long polyP shortens the clotting time of FXI- deficient plasma in a manner that is dependent of FIX and FX. In a purified system, FXIIa was unable to activate FIX or FX but the activation of PK by long polyP promoted FIXa generation. We also observed that FXIIa could activate prothrombin in a dose dependent manner. In an *ex vivo* model of occlusive thrombus in which recalcified human blood was driven through collagen and tissue factor (TF)-coated capillary tubes, inhibition of FXIIa with CTI but not of FXI with a neutralizing antibody abolished the prothrombotic effect of long polyP.

Summary/Conclusions: We propose that in addition to its established effects on FXI activity and FXII activation, long polyP promotes FXII-mediated blood coagulation, bypassing FXI. Accordingly, some polyP containing pathogens may have evolved strategies to exploit polyP-initiated FXII activation for virulence, and selective inhibition of FXII may improve the host response to pathogens.

AS 14 – Methods and Relevance of Microparticle Detection

AS 14

Microparticle detection: why and how?

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Microvesicles (MV), exosomes and apoptotic bodies (collectively known as extracellular vesicles or EV) are becoming recognised as potentially important biomarkers in many diverse areas of clinical research. It is clear that they play previously unrecognised important roles in normal cellular physiology and provide novel intercellular communication mechanisms. Their levels in body fluids are probably an accurate reflection of health or disease of specific organs and tissues and the underlying inflammatory and activation status of the vascular endothelium and circulating blood cells. The circulating pool of EV are heterogeneous (derived from platelets, leucocytes, erythrocytes, the vascular endothelium or other tissues), small (traditionally it was thought that exosomes range from 50 to 100 nm, MV from 100 to 1000 nm and apoptotic vesicles 1–3 μ in size, but in reality there is considerable overlap between the three main types), can express antigens from their cell of origin, are often procoagulant (with expression of anionic phospholipids) and sometimes carry nucleic acids (e.g. mRNA and microRNA). Pre-analytical variables are important (e.g. blood sampling, processing, centrifugation (number of steps, time, g values etc), isolation techniques and storage of samples) as they can significantly impact upon EV measurement. The ISTH vascular biology SSC has made a number of recommendations about pre-analytical and analytical variables that will help to improve standardization of their measurement. Despite this it is clear that the centrifugation steps used to measure or isolate plasma EV will result in the loss of larger vesicles. Most traditional MV assays are based upon (i) Measurement of procoagulant potential (e.g. thrombin generation or clotting based assays), (ii) Capture using specific ligands (e.g. Annexin-V) or specific antibodies (e.g. Tissue factor) (iii) Measurement of their size (by electron microscopy) and (iv) concentration and phenotype (by flow cytometry). More recently proteomic and genomic screening of purified EV have revealed unique signatures. Although there are many methods available to measure circulating EV, most methods also exhibit significant limitations and standardization issues. For example the procoagulant and/or Annexin-V capture assays by definition only measure procoagulant EV and do not provide information on phenotype (i.e. origin) and size. Although EV size, number and phenotype can be potentially measured by flow cytometry, many instruments cannot reliably measure EV below ~500 nm, although new generation cytometers are beginning to significantly extend this threshold down to below 200 nm and beyond. Measurement of EV by flow cytometry is again an area where standardization is very important and use of calibration beads has been recommended by the ISTH vascular biology SSC to control for instrument noise and set defined size gates. A number of alternative technologies have now been investigated for measuring EV (e.g. Atomic Force Microscopy and Nanoparticle Tracking Analysis) and suggest that the number of EV in plasma may be considerably underestimated by flow cytometry. Comparison of methods using different types of calibration beads and biological standards should therefore help to confirm and fully understand these observations. Standardised and accurate measurement of EV (including their size, phenotype (origin), content and concentration) could therefore provide novel diagnostic and prognostic markers of various disease states.

AS 14.1

The activation pathway determines the properties of platelet-derived microvesicles

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Platelets release microvesicles (MVs) both from plasma membrane (microparticles, PMPs) and from endosomal route (exosomes). Changes in *in vivo* platelet-MV levels correlate to various physiological (e.g. gender, age, pregnancy, exercise) and pathological states. Therefore, we have used different activating pathways to induce MV formation and systemically compared their number, size and molecular make-up.

Platelets were isolated from healthy volunteers with modified gradient-procedure to improve sample purity and activated with physiologically relevant agonists on comparison to Ca^{2+}-ionophore (A23187). Enriched microparticle and exosome populations were isolated with differential centrifugation method. MVs number and size distribution were analyzed by nanoparticle tracking analysis (NTA) and correlated with total protein, lipids and EM, respectively. Proteomic comparison was made from pooled samples of six healthy donors with LTQ-Orbitrap-XL mass spectrometry.

Size distribution data from NTA and EM showed that 64–85% of MVs were < 250 nm depending on activation and 95–99% were < 500 nm. Only 0.5–5% of PMVs were in the 0.5–1 μm range. PMVs induced by thrombogenic/immunological or A23187 -pathways showed significant differences in the total protein and the lipid amounts and the proteomic cargo compared to their time-matched constitutively formed controls. Proteomic analysis of PMVs identified proteins of cytoplasmic (47%), surface (16%), organelle (15%) and unknown (14%) origins and 8% as secreted proteins. From 537 total proteins, 12 proteins were shared by all the exosome and PMP conditions, including CD9 and α-IIb integrin. 60% of proteins were unique to each condition, whereas Multimerin-1 was found in all exosomes but not in PMPs.

Our data show that the activation pathway significantly modulates the quantity and the molecular composition (protein/lipid) of the subsequently formed PMVs, but not so much their size distribution. The scarcity of PMVs in the 0.5–1 μm range strongly urges to re-evaluate the data obtained with the 1st generation flow cytometers. Agonist-specific proteomic profiles suggest that different PMVs should be individually tested in cell biological assays to reveal the physiological and pathological significance of PMVs.

AS 14.2

Physical interpretation of the size and concentration of extracellular vesicles measured by advanced techniques

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Background: Novel advanced techniques are utilized to determine the size and concentration of extracellular vesicles. All techniques provide data, but often the measured physical quantity or units are different, precluding comparison and validation of measurements with established techniques. We have developed a model to enable data comparison between five different techniques that are increasingly used to detect vesicles.

Aims: Facilitate data comparison between different techniques based on the underlying physical parameters of each technique and provide thorough insight in the application and limitations.

Methods: A heterogeneous mixture of beads (Thermo Fisher Scientific, Waltham, MA, USA) of known size and concentration and a standard population of vesicles from human cell-free urine (n = 5) were prepared and analyzed by single particle tracking (SPT; Nanosight NS500), resistive pulse sensing (RPS; Izon qNano), a novel flow cytometer (Apogee A50-Micro), a conventional flow cytometer (BD FACSCalibur), and transmission electron microscopy (TEM). Data interpretation and comparison is enabled by presenting size distributions on the same absolute scale using a physical model, including data from TEM and flow cytometry, which was previously impossible. The size distributions from the well-characterized mixture of beads were used to investigate the capabilities of each technique, thereby facilitating the interpretation of data obtained from the standard population of vesicles.

Results: Using the beads we obtained that, among the techniques capable of measuring in suspension, surprisingly, flow cytometry can resolve the smallest differences in size, whereas SPT detects the smallest vesicles, and RPS is most accurate in determining the concentration. All methods produce different size distributions and concentrations for the standard population of vesicles.

Conclusions: Various detection techniques produce different size distributions and concentrations for a standard population of vesicles. However, most differences can be explained based on the underlying physical parameters of the technique, which we have investigated using a well-characterized mixture of beads. Since the techniques have divergent capabilities, the most suitable technique for detecting vesicles depends on the requested information.

AS 14.3

Microparticles from blood plasma revealed by cryo-electron microscopy, receptor-specific gold labeling and flow cytometry

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Microparticles (MPs) derived from activated or apoptotic cells are found in plasma and other body fluids in physiological conditions and are present at elevated levels in various diseases, including cardiovascular diseases and cancer. MPs present surface receptors originating from their parental cells, which has promoted the concept of using MPs as biomarkers of diseases. However, research on MPs is rendered difficult by their small size and by the limitations of analytical methods currently in use.

Our overall aim is two-fold, first to improve our basic understanding on MPs by describing the size, morphology and phenotypes of MPs in physiological and pathological conditions, and second to develop reliable methods of EV detection and quantification.

In this initial study, we focused on MPs from platelet-free plasma (PFP) samples of healthy donors. Cryo-Electron Microscopy (EM) was used to reveal the morphology and size of the various types of particulate material present in pure plasma. Gold nanoparticles functionalized with various types of ligands were developed and used for labeling selected sub-populations of MPs, principally MPs exposing phosphatidylserine (PS), MPs derived from platelets and MPs derived from red blood cells, using Annexin-5 (Anx5), anti-CD41 and anti-glycophorin antibodies (Abs), respectively. In parallel, flow cytometry analysis of PFP was performed, focusing essentially on Anx5-binding MPs.

We found that PFP samples contain three main morphological types of MPs, consisting of spherical MPs, tubular-shaped MPs and large cell fragments. Spherical MPs constitute about 75% of all MPs present in PFP, most of them ranging in diameter from 50 to 500 nm. Tubular MPs form about 15% of all MPs, with a length extending over 5 μm, while large cell fragments extend up to 10 μm. Strikingly, we found that, in PFP, the sub-population of MPs that expose PS and bind Anx5 comprises only 25% of all MPs. We found also that about 20% of PFP MPs expose CD41, while most of the large cell fragments derive from red blood cells. Size histograms of the whole MP population and of the sub-populations of PS-positive, CD41-positive and glycophorin-positive MPs were determined, for the first time. In addition,

the influence of treatments like aging, centrifugation and freeze-thawing on MPs' structure and PS-exposure was investigated, showing a direct influence of some of these parameters on PS-exposure.
This study provides novel basic knowledge on plasmatic MPs and will serve as a reference for characterizing MPs in various pathological situations.

AS 15 – New Approaches to Antiplatelet Therapy

AS 15.1

Clinical implications of very low on-treatment platelet reactivity in patients treated with thienopyridines: the poba study (predictor of bleedings with antiplatelet drugs)

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Background: New P2Y12 blockers and platelet monitoring has been proposed to optimize platelet inhibition after acute coronary syndrome (ACS) and improve ischemic outcomes. However, bleeding complications remain the 'Achilles' heel' of antiplatelet therapy and platelet monitoring could be useful to evaluate this risk.
Aims: The present study was designed to define the 'hyper response' to thienopyridine (very low on-treatment platelet reactivity (VLTPR)) as the most predictive threshold value of Platelet reactivity index VASP (PRI VASP) for prediction of non access-site related bleeding events. We also aimed to identify predictors of bleeding and VLTPR in patients treated with thienopyridines.
Methods and Results: One thousand five hundred and forty-two consecutive patients undergoing coronary stenting for ACS were included in the present study (287 on clopidogrel 75 mg, 868 on clopidogrel 150 mg and 387 on prasugrel 10 mg). During 6-month follow-up, 9% of patients ($n = 139$) suffered from non-access site related BARC bleeding complications. These patients were more often women, non-diabetic and had lower PRI VASP values than others ($P < 0.001$). ROC curves analysis (AUC = 0.71, $P < 0.01$) identified a threshold value for VLTPR of PRI VASP $\leq 10\%$ to predict bleeding events with a sensitivity of 17% and a specificity of 97%. While prasugrel was the main predictor of VLTPR in the whole population (OR [95% CI]: 10.2 [3–34.2]; $P < 0.001$), VLTPR was the strongest and independent predictor of bleeding (OR [95% CI]: 4.7 [2.7–8.3]; $P < 0.001$).
Conclusion: The present study identified VLTPR (PRI VASP $\leq 10\%$) as the strongest predictor of bleeding complications in patients treated with thienopyridines. This marker could be useful for tailored therapy and bleeding prevention.

AS 15.2

First ex vivo and *in vivo* assessment of anfibatide, a novel glycoprotein Ib-IV-V complex antagonist, in healthy human volunteers in phase I clinical trial

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Background: Platelet adhesion and aggregation at the site of vascular injury are critical for hemostasis and thrombosis. It has been well accepted that interaction between the GPIb complex and von Willebrand factor (VWF) plays a key role in initiation of platelet adhesion, particularly at high shear. Platelet surface integrin aIIbβ3, through interaction with fibrinogen or other ligands, then mediates platelet aggregation and formation of a hemostatic plug or thrombus. Recently, the indispensable role of the GPIb-VWF interaction in platelet aggregation at extremely high shear (e.g. > 10,000/s; areas of stenosis following arteriosclerosis and/or thrombus growth) has been highlighted. Therefore, both the GPIb complex and aIIbb3 are considered major targets for antithrombotic therapies. Although several inhibitors of aIIbβ3 have been developed for antithrombotic therapies, no drug has been successfully developed to target the GPIb complex even though there are limitations for anti-aIIbβ3 therapies, including severe bleeding in some patients. The GPIb complex is, therefore, an attractive target for anti-thrombotic therapy.
Aims: To assess the effect of anfibatide on platelet function and safety in healthy human volunteers in phase I clinical trial.
Methods: Anfibatide, a 30 kDa polypeptide, was purified from *Agkistrodon acutus* snake venom and analyzed by mass spectrometry. The effects of Anfibatide on human platelet function were studied by *in vitro* platelet aggregometry, *ex vivo* perfusion chamber, and thrombealastography (TEG). The safety and efficacy of Anfibatide on platelet function and coagulation were evaluated in a total of 94 healthy human volunteers in a phase I clinical trial. The review board of Yijishan Hospital approved the study. All subjects provided informed consent.
Results: Anfibatide strongly inhibited ristocetin-induced human platelet aggregation and platelet adhesion, aggregation, and platelet thrombus formation on a collagen coated surface under high shear flow (1500/s). It also inhibited thrombus formation at low shear (300/s) but the efficacy was less than that at high shear conditions. Importantly, Anfibatide effectively dissolved the preformed thrombi when we continuously perfused Anfibatide-treated whole blood through perfusion chambers, demonstrating its potential as an anti-thrombotic therapy. TEG revealed no significant change in coagulation parameters after Anfibatide treatment. In this phase I clinical trial, no serious adverse events, premature discontinuations due to adverse events, or deaths occurred during the study. Results showed that Anfibatide can bind to approximately 95% of GPIb and inhibit up to 90% of ristocetin specific platelet aggregation without significantly prolonging human bleeding time, activated partial thromboplastin time, prothrombin time, or thrombin time. The inhibitory effect was undetectable 4 h after Anfibatide was withdrawn. There was also no spontaneous bleeding and no bleeding from blood collection sites. Anfibatide did not significantly affect platelet count and no anti-Anfibatide antibodies were detected in the subjects, suggesting that Anfibatide is well-tolerated in healthy individuals.
Conclusions: This research represents a comprehensive study of Anfibatide in healthy human subjects. This is the first clinical evidence that Anfibatide exhibits strong anti-platelet effects, excellent reversibility, and no significant bleeding diathesis in healthy human volunteers. Given these promising findings, Anfibatide may represent a novel and safe anti-platelet treatment.

AS 15.3

A network-biology based approach to elucidate determinants of platelet reactivity in aspirin-treated cardiovascular patients

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Background: High platelet reactivity in aspirin-treated cardiovascular patients is a risk factor for recurrence of ischemic event, at least in acute settings. The determinants of platelet reactivity are largely unknown, but the investigation of healthy subjects suggests that they

are genetically determined. Network-biology is a novel and promising approach to identify cellular modulators of complex processes, such as platelet reactivity.
Aims: To identify the molecular mechanisms modulating platelet reactivity in aspirin-treated cardiovascular patients.
Methods: Platelet reactivity was assessed in 110 cardiovascular patients treated with aspirin 100 mg/day by light-transmission aggregometry using several agonists. Patients with extreme phenotypes were further studied by complementary platelet-derived 'omics approaches including quantitative proteomics, transcriptomics and genomics. These datasets were integrated using a network biology approach.
Results: More than 1000 proteins and 5000 mRNAS were quantified and 1.5 million SNPs were identified. The integration of the omics data allowed the construction of a network of 99 nodes (RNA/proteins) and 309 edges, representing platelet activation pathways differentially expressed in both phenotype groups. Genetic variants were then integrated into the network as a way to potentially stratify the patient population. Two network clusters presented a higher density of protein-protein relationships that were also confirmed in the literature. These included genes involved in cytoskeleton dynamics, integrin alpha-IIbIIIa-related aggregation processes and glucose metabolism.
Summary/Conclusion: As it is unlikely that a single causal gene or protein predicts the response to aspirin, a more holistic approach is required. Here we used a network-based strategy that allowed the seamless integration of several 'omics datasets. Using this strategy, we unveiled several candidate pathways modulating platelet reactivity in aspirin-treated cardiovascular patients. Aggregation relying on alpha-IIbIIIa, together with glucose metabolism and cytoskeleton dynamics appear to play a role in the modulation of platelet reactivity in cardiovascular patients under aspirin-treatment. These candidate pathways may delineate new targets for the prevention of ischemic events in cardiovascular patients.

AS 16 – Functional Characterisation of Hereditary Platelet Disorders

AS 16
Genetics, platelet dysfunction and bleeding

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Inherited platelet disorders (IPDs) typically comprise defects in platelet number (thrombocytopenia), volume and/or function. Bleeding due to IPDs typically occurs immediately after injury, primarily in skin, mucous membranes, nose and gastrointestinal tracts. Platelets are easy accessible cells and different assays are available to study platelet function under basal and activated conditions. Defects in platelet morphology, adhesion, aggregation and secretion can result from mutations in platelet-specific genes leading to 'isolated' IPDs or from mutations in widely expressed genes leading to an IPD combined with a broader clinical phenotype. Recently, novel methods in epigenetics, proteomics and functional genetics in zebrafish led to the discovery of novel insights in the complexity of IPDs. I will discuss how we used these techniques to describe the role of DNA methylation in GNAS for regulating platelet function via cAMP, to define changes in lysosomal-like granules with differential cathepsin expression in platelets of Alternating Hemiplegia of Childhood patients and to show a role for Niemann Pick type C1 in megakaryopoiesis and platelet production starting from the observation in the zebrafish to verify thrombocytopenia in NPC1 patients. Though significant progress was made during the last years, the genetic factors that cause IPDs still remain largely unknown. IPDs are characterized by a high degree of genetic heterogeneity and are considered as rare diseases of man. Over the last decade, 274 patients were identified in our laboratory with an IPD of unknown etiology. After exclusion of known genetic causes, 176 patients have an isolated IPD that mainly includes thrombocytopenia (59 cases) or storage pool disease (29). In addition, 98 patients have an IPD combined with bone (26), neurological (60), endocrinological (4) or immune (4) defects. We also studied 6 IPD patients with bleeding problems due to a defect in the extra cellular matrix. By combining a genome-wide exome sequencing approach with knowledge of the platelet defect and extensive studies of the pathophysiology of IPDs, genes and pathways will be discovered that place pointers at new regulators of platelet function and/or formation. This last action will be performed as a collaborative effort of the BRIDGE-BPD consortium (www.bridgestudy.org) for rare bleeding and platelet disorders. Patients are collected by 34 specialized referral centers in Belgium, France, Germany, the Netherlands, the UK and the USA. I will here discuss the six IPD patients with a defect in the extracellular matrix. These patients have a variant of the Ehlers Danlos syndrome (EDS) with spontaneous bleeding problems and platelets with abnormal morphology. Genetics factors have been identified by candidate gene screening (COL1A1) and a pilot exome sequencing project from BRIDGE (novel gene on 15q15). We studied whether their abnormal platelets arose from defects in megakaryopoiesis due to an altered extra cellular matrix. This translational project based on knowledge exchange across different clinical disciplines, is expected to gain insights in molecular regulation of megakaryopoiesis and/or platelet function for also improved diagnosis and treatment of IPDs and to link these findings to other disease aspects.

AS 16.1
Abnormal megakaryocyte development and platelet function in a mouse model of gray platelet syndrome

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Background: Gray platelet syndrome (GPS) is characterized by macrothrombocytopenia, bleeding, splenomegaly and the absence of platelet al. pha granules. The GPS molecular defect has recently been identified by our group (Nat Genet. 2011; 43(8):738–40) and others (Nat Genet. 2011; 43(8):732–4; Nat Genet. 2011; 43(8):735–7) as loss of function mutation of *NBEAL2* (neurobeachin-like 2).
Aims: To study platelet development and function in a mouse model of GPS.
Methods: Deletion of exons 4–11 *of Nbeal2* resulted in viable *Nbeal2*−/− mice. Platelet granule content and morphology were assessed by light, electron and immunofluorescence microscopy (IFM). Platelet protein content was assessed by immunoblotting and IFM, which was also used to observe megakaryocyte (MK) development in bone marrow cultures stained for DNA and structural and granular proteins. Platelet function was assessed by Multiplate analyzer, light transmission aggregometry (LTA) of washed platelets and flow cytometry. Spleen size was determined by weight. Thrombus formation in laser-injured cremaster arterioles was assessed using IFM; bleeding was evaluated by tail tip transection.
Results: Electron microscopy confirmed the absence of alpha granules in *Nbeal2*−/−platelets, and immunoblotting detected an absence of the abundant MK-synthesized alpha granule cargo proteins von Willebrand factor (VWF), thrombospondin-1 and platelet factor 4 (actin and P-selectin were normal). *Nbeal2*−/− MKs cultured from bone marrow showed decreased proplatelet formation, and while P-selectin distribution appeared normal, VWF (normally packaged into alpha granules) was observed to accumulate near the outer membrane, and in some MKs may have been extruded. *Nbeal2*−/− mice exhibited splenomegaly (spleen weight 0.13 +/- 0.03 g compared to WT 0.09 +/- 0.02 g; $n = 8$, $P < 0.005$). Relative to WT, collagen-induced aggregation of *Nbeal2*−/− platelets was reduced by half ($P < 0.001$) in the

Multiplate analyzer. LTA showed similar responses at high agonist concentrations and weaker response of *Nbeal2*−/− platelets to low concentrations, with < 5% aggregation seen with thrombin (0.05 U/mL, WT = 48%), CRP (0.05 μg/mL, WT = 21%) and U46619 (0.2 μM, WT = 76%). Flow cytometry showed CD41 activation (JONA expression) by thrombin and ADP (not collagen) was greatly reduced in *Nbeal2*−/− mice, as were thrombin and collagen (not ADP) induced P-selectin expression; maximal thrombin-induced P-selectin expression in *Nbeal2*−/− mice was 25% of WT. The peak size of laser-induced thrombi were smaller in *Nbeal2*−/− mice ($P < 0.05$). The ratio of P-selectin expression to thrombus size (identified via membrane GPIbβ) increased by less (40 vs. 70 AU, $P < 0.0001$) in thrombi generated in *Nbeal2*−/− mice compared to WT. Time to half maximal ratio (THMR) did not increase and the THMR of αIIb up-regulation was also similar for *Nbeal2*−/− and WT. *Nbeal2*−/− mice showed increased blood loss following tail tip transection (508 ± 162 vs. 43 ± 27 μL for WT).

Summary/Conclusions: *Nbeal2*−/− mice represent the first mammalian model of GPS. Like GPS patients, *Nbeal2*−/− mice exhibit splenomegaly, prolonged bleeding and platelet aggregation abnormalities, and their platelets lack both alpha granules and cargo. Exploiting the experimental advantages of our animal model, we have observed that megakaryopoiesis is abnormal and impaired in *Nbeal2*−/− mice, as is laser-induced thrombus formation. Our results confirm the importance of alpha granule secretion for platelet function *in vivo*.

AS 16.2

Nbeal2-deficient mice reveal a key role of platelet a-granules in arterial thrombosis, thrombo-inflammatory brain infarction and wound healing

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Background: The most abundant platelet organelles, α-granules, contain more than 300 different proteins, which upon release participate in thrombus formation, haemostasis, inflammation and wound healing. However, the exact contribution of the plethora of α-granule proteins to thrombotic and thrombo-inflammatory processes has not been established. The gray platelet syndrome (GPS) is a rare congenital platelet disorder characterised by the lack of α-granules and their contents. Platelets of GPS patients appear gray and enlarged in the light microscope and aggregate poorly upon stimulation with thrombin and collagen. Characteristics of GPS include a macrothrombocytopenia, splenomegaly, myelofibrosis and a mild to moderate bleeding tendency that is occasionally severe. Only recently, mutations in *NBEAL2* (neurobeachin-like 2) have been linked to GPS. NBEAL2 is a large 302 kDa member of the BEACH-WD40 domain protein family. Other members of this family, such as Neurobeachin, Nbeal1, Lrba and Lyst, have been associated with protein trafficking.

Aim: Despite an extensive knowledge of α-granule contents and of the multiple roles for biologically active proteins secreted from platelets, relatively little is known about how they help to maintain haemostasis or of their participation in arterial thrombosis and thrombo-inflammatory processes. We now report the use of *Nbeal2*−/− mice to overcome the limitations of studying haemostasis and platelet pathology in patients with GPS and to investigate for the first time the role of α-granules and their contents *in vivo*.

Methods: Mice lacking the Nbeal2 protein were generated and megakaryocyte (MK) and platelet function and ultrastructure were assessed *in vitro* using biochemical methods combined with confocal and electron microscopy. *In vivo* function of Nbeal2-deficient platelets was studied in models of haemostasis, arterial thrombosis, ischaemic stroke and wound healing.

Results: *Nbeal2*−/− mice are viable and fertile but display the characteristics of human GPS with defective α-granule biogenesis in MKs, which leads to platelets lacking α-granules. Nbeal2-deficiency had no obvious effect on MK-differentiation and proplatelet formation *in vitro* or platelet life span *in vivo*. Nbeal2-deficient platelets showed impaired adhesion, aggregation and coagulant activity under flow ex vivo which translated into severely defective arterial thrombus formation *in vivo* and profound protection from thrombo-inflammatory brain infarction following focal cerebral ischaemia. Furthermore, wound healing experiments showed a reduced myofibroblast differentiation at the site of injury, which was attributed to decreased TGFβ release from Nbeal2-deficient platelets.

Conclusion: This study provides complementary evidence that NBEAL2 is essential for platelet α-granule biogenesis and reveals for the first time that α-granule constituents are critically required not only for haemostasis but also thrombotic and acute thrombo-inflammatory disease states while promoting tissue repair after injury.

AS 16.3

Defective thrombus formation in hermansky pudlak syndrome mice is due to impaired platelet granule exocytosis and reduced protein disulfide isomerase release

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Background: Protein disulfide isomerase (PDI) is secreted from platelets upon cell activation, is captured on platelets and endothelial cells via β3 integrins and is required for thrombus formation.

Aims: To determine the potential role of platelet granules in PDI-mediated thrombus formation we studied a Hermansky Pudlak syndrome 6 (HPS6) mouse model in which platelet dense granules are absent.

Methods: Intravital microscopy using a laser injury model in mouse cremaster muscle arterioles was employed to determine the effect of the HPS6 mutation on platelet deposition, fibrin generation and extracellular PDI release during thrombus formation. Thrombin-induced granule exocytosis *in vitro* was studied using immunoflow cytometry and translocation of P-selectin and Toll Like Receptor 9 (TLR9) and Lysosome Associated Membrane Protein 1 (LAMP1) secretion as markers. Platelet Factor 4 (PF4) secretion was measured by ELISA.

Results: Platelet accumulation and fibrin generation were nearly absent in HPS6$^{-/-}$ mice after laser-induced injury. Extracellular PDI associated with the injured vessel wall was diminished, in the presence or absence of eptifibatide. *In vitro* studies demonstrated that HPS6$^{-/-}$ platelets contain similar levels of PDI compared to wild type platelets, but exocytosis of alpha granules, lysosomes and T-granules, the storage organelles for PDI, from HPS6$^{-/-}$ platelets showed decreased sensitivity to thrombin. At intermediate thrombin concentrations, P-selectin translocation from the alpha granule membrane into the plasma membrane and PF4 secretion from alpha granules were significantly lower in HPS$^{-/-}$ platelets compared to wild type platelets ($P < 0.001$ at 0.05 and 0.1 U/mL thrombin). Similarly, expression of TLR9, a T-granule marker, and LAMP1, a lysosome marker, required higher concentrations of thrombin to obtain comparable release from HPS6$^{-/-}$ platelets and wild type platelets ($P = 0.01$ at 0.05 U/mL thrombin). Release or expression of P-selectin, PF4, TLR9 and LAMP1 was comparable from wild type and HPS$^{-/-}$ platelets at a thrombin concentration of 1 U/mL. These results suggest a relative defect in granule exocytosis in HPS6$^{-/-}$ platelets. Infusion of wild type platelets to approximately 10% of the *in vivo* platelet concentration or infusion of recombinant PDI in HPS6$^{-/-}$ mice rescued both platelet thrombus formation and fibrin generation after laser injury. In contrast, neither infusion of an inactive PDI mutant nor thioredoxin had any effect on thrombus formation in HPS6$^{-/-}$ mice.

Conclusions: These results demonstrate that in HPS6$^{-/-}$ mice in which platelets lack dense granules, the exocytosis of alpha granules, T-granules and lysosomes is defective. At the concentrations of thrombin generated *in vivo* following laser injury, extracellular PDI accumulation is impaired due to either decreased PDI secretion or decreased PDI capture, or both. Defective granule exocytosis, in particular a deficiency in the release of the extracellular PDI necessary for PDI-mediated thrombus formation, is likely responsible for the bleeding disorder observed in Hermansky Pudlak syndrome. In this model, platelet accumulation and fibrin generation can be rescued with exogenous PDI despite the absence of platelet dense granules and their ADP and polyphosphate contents. This is the first example of a model of a human disease characterized by impaired PDI expression leading to a bleeding disorder.

AS 17 – Challenges in Therapeutic Management of VTE

AS 17.1

Risk of bleeding in patients with acute venous thromboembolism treated with rivaroxaban or enoxaparin/VKA and concomitant ASA therapy or NSAIDs: subanalysis from EINSTEIN DVT and PE studies

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Background: Combined anticoagulant and acetylsalicylic acid (ASA) therapy, or to a lesser extent non-steroidal anti-inflammatory drug (NSAID) therapy, is associated with an increased risk of bleeding in patients with atrial fibrillation and arterial vascular disease. The effect of concomitant use of these medications on bleeding in patients with venous thromboembolism is of considerable clinical importance.
Aim: This post hoc analysis assessed the impact of combined anticoagulant and ASA or NSAID therapy on bleeding risk compared with anticoagulant therapy alone in patients with deep vein thrombosis (DVT) and/or pulmonary embolism (PE) who participated in the EINSTEIN DVT and PE studies (The EINSTEIN Investigators. *NEJM* 2010; 363:2499; The EINSTEIN-PE Investigators. *NEJM* 2012; 366:1287).
Methods: ASA therapy was defined as all drugs containing ASA (including acetylsalicylates), while NSAIDs were defined by means of ATC code M01A. Bleeding events that were adjudicated as clinically relevant (major and non-major) were used for this analysis. Antiplatelet therapy other than ASA was used very infrequently and not analysed further.
Event rates per 100 patient-years with or without exposure to ASA or NSAID therapy were computed for the entire at-risk period, and hazard ratios (HR) for use vs. non-use (adjusted for age and creatinine clearance) were calculated using co-medication as a time-dependent covariate.
Results: The safety population comprised 4130 patients who received rivaroxaban and 4116 patients who received enoxaparin/vitamin K antagonist (VKA), of whom 1202 (14.6%) and 1884 (22.8%) patients received concomitant ASA or NSAID therapy, respectively. During ASA-rivaroxaban treatment, clinically relevant bleeding occurred with an event rate of 34.9 per 100 patient-years compared with 16.1 during non-ASA use (HR = 1.79 [95% CI 1.35–2.38]), whereas during ASA-enoxaparin/VKA treatment, an event rate of 39.1 was found, compared with 17.8 when ASA was not used (HR = 1.58 [1.16–2.15]). In patients treated with ASA-rivaroxaban, major bleeding occurred with an event rate of 3.3 per 100 patient-years compared with 1.6 during non-ASA use (HR = 1.49 [0.62–3.57]), whereas during ASA-enoxaparin/VKA treatment an event rate of 6.9 was found, compared with 2.9 during non-use (HR = 1.49 [0.73–3.02]). During NSAID-rivaroxaban treatment, clinically relevant bleeding occurred with an event rate of 37.6 per 100 patient-years compared with 15.8 during non-use (HR = 1.93 [1.47–2.53]), whereas during NSAID-enoxaparin/VKA use, an event rate of 37.3 was found, compared with 17.4 during non-use (HR = 1.68 [1.29–2.21]). During NSAID-rivaroxaban treatment, major bleeding occurred with an event rate of 4.74 per 100 patient-years compared with 1.43 during non-use (HR = 2.60 [1.23–5.48]), whereas during NSAID-enoxaparin/VKA treatment, an event rate of 8.4 was found compared with 2.7 during non-use (HR = 2.32 [1.31–4.12]).
Conclusions: The combination of anticoagulant and NSAID therapy increased the risk of both clinically relevant bleeding and major bleeding by approximately two-fold, whereas for concomitant ASA use, the risk of clinically relevant bleeding was increased by about 1.5-fold without a statistically significant effect on major bleeding alone. The increase in risk of clinically relevant and major bleeding was similar for patients treated with rivaroxaban and those treated with enoxaparin/VKA. DVT and PE patients should avoid taking NSAIDs for prolonged periods without an important clinical indication for NSAID use.

AS 17.2

Vitamin K antagonist treatment patterns and persistence after venous thromboembolism in non-cancer patients: VTE Epidemiology Group (VEG) Study

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Background: Anticoagulation with vitamin K antagonists (VKAs) and low molecular weight heparins (LMWHs) is effective in preventing recurrence of venous thromboembolism (VTE). Guidelines typically recommend 3 months' treatment for events provoked by temporary risk factors, with longer treatment considered for unprovoked VTE. VKA management of VTE, deep vein thrombosis and pulmonary embolism in primary and secondary care is not well documented.
Aims: To assess treatment patterns and persistence with VKA treatment in a large contemporary cohort of unselected patients with non-cancer-related VTE.
Methods: A population-based cohort study, involving two million patients, retrieved data from the subset of general practices in England contributing to the Clinical Practice Research Datalink (CPRD) linked to data from the Hospital Episodes Statistics (HES) and to the Office for National Statistics (ONS). From January 2008 to October 2011 all incident cases of VTE were stratified into provoked and unprovoked non-cancer-related VTE. All VTE cases were verified with an algorithm based on review of medical records, hospital diagnoses, cause of death and anticoagulation therapy.
We identified all patients who initiated VKA use in the first 3 months after an initial VTE and followed them for the entire duration of VKA treatment. The observation of patients with a recurrent VTE and major bleeding was censored at the time of the event. VKA use was defined by prescriptions, repeated international normalized ratio tests, medical codes and electronic search of medical notes. Duration of VKA use was estimated by connecting VKA prescriptions based on a prescription length of 28 days and a 28 days grace period. Persistence with VKA treatment was derived from Kaplan–Meier estimates and expressed as the percentage of those on VKA therapy at 3, 6 and 12 months.
Results: A total of 8504 patients had a first VTE and a follow-up of at least 7 days: 5019 patients with DVT and 3485 patients with PE with or without DVT. The mean age of patients was 63.5 years; 46.4% were male. Of the total, 3130 (36.8%) patients had a provoked non-cancer event and 5374 (63.2%) patients had an unprovoked event.

Of the 5374 patients with unprovoked VTE, 15.4% had no record for anticoagulation use in the 90 days after the venous thromboembolic event and 84.6% of patients received anticoagulation (67.0% VKA alone, 12.2% both LWMH and VKA, and 5.4% LWMH alone). Higher rates of anticoagulation were recorded for PE patients (86.1%) compared with DVT patients (79.3%).

Persistence with VKA therapy in the complete VTE cohort was 77.4% at 3 months, 50.3% at 6 months and 11.4% at 12 months. Treatment persistence for patients with provoked VTE was 76.8%, 49.0% and 10.3% at 3, 6 and 12 months, respectively, and for unprovoked VTE these values were 77.8%, 51.1% and 11.9%, respectively.

Summary/Conclusions: Currently, VKA therapy after provoked VTE is given for longer than recommended. Conversely, after unprovoked VTE, patients receive VKA therapy for shorter durations than recommended. Reasons for the non-adherence to guidelines merit further investigation.

AS 17.3

Elderly patients with venous thromboembolism: cancer or no cancer, a different therapeutic approach?

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Background: Elderly patients are usually excluded from clinical trials assessing anticoagulant therapies in venous thromboembolism (VTE) treatment. However, current guidelines recommend similar anticoagulant therapy for patients with VTE, regardless of their age, even though its efficacy and safety have not yet been assessed in the elderly.

Aims: To assess the rate of recurrent VTE, major bleeding and death in patients older than 75 years who experienced pulmonary embolism (PE) or deep venous thrombosis (DVT) during anticoagulation; to further investigate occurrence of these events in subgroups according to the presence or not of cancer, and type of anticoagulation used.

Methods: We used data from the Registro Informatizado de Enfermedad TromboEmbólica (RIETE) registry, an ongoing international, multicentred, real-life prospective registry of consecutive patients presenting with symptomatic, acute VTE. All patients provided written or oral consent for participation in the registry, in accordance with local ethical committee requirements. Outcome measures includedVTE, PE, DVT, major bleeding, death, presence or not of cancer, and antithrombotic medication.

Results: Up to December 2010, 11,403 patients older than 75 years had been enrolled in RIETE. Of these, 2229 (19.5%) had active cancer. During the course of anticoagulation (mean, 216 ± 440 days), 2.3% of patients developed recurrent PE, 2.2% recurrent DVT, 3.9% had major bleeding, and 12.8% died. Compared with patients with no cancer, patients with cancer were frailer, with more comorbidities; they had a higher risk of VTE recurrence (5.2% vs. 4.4%, $P = 0.04$ [for PE, 3.1% vs. 2.2%, $P = 0.004$; for DVT, 2.2% in both groups]), major bleeding (5.1% vs. 3.6%, $P = 0.001$), and death (28% vs. 9.1%, $P = 0.0001$).

In the group without cancer, patients receiving long-term LMWH experienced significantly more VTE recurrence (5.8% vs. 3.8%, $P = 0.006$), PE recurrence (2.9% vs. 1.9%, $P = 0.005$) and major bleeding (6.0% vs. 2.5%, $P = 0.001$) than those who were receiving VKA.

In the group with cancer, although they were at higher risk for bleeding and recurrent VTE, patients receiving LMWH experienced nonsignificantly less PE recurrence than those receiving VKA (1.8% vs. 2.02%, $P = 0.3$); a similar rate was observed in patients without cancer and receiving VKA (1.9%). In patients receiving long-term LMWH, the rate of major bleeding was similar in patients with and without cancer (6.1% and 6.0%, respectively).

Conclusions: We identified two different populations in elderly patients with VTE. Overall, in patients without cancer, long-term LMWH therapy was harmful in terms of recurrent VTE and major bleeding. However, in patients with cancer, long-term LMWH therapy had a different effect, and kept the risk of PE recurrence within the range of that of patients without cancer, without increasing the risk of bleeding. Further studies are needed to prospectively assess the efficacy and safety of long-term LMWH therapy in the elderly patient.

AS 18 – Prevention of Recurrent VTE

AS 18.1

Assessment of the risk of recurrent venous thrombosis using a genetic risk score comprising five genetic markers

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Background: Previously, we reported on a genetic risk score (GRS) comprising five single nucleotide polymorphisms (SNP) improving the prediction of a first venous thrombosis. The prediction of recurrent venous thrombosis using individual genetic risk predictors has proven to be challenging.

Aim: The aim of this study was to assess whether the 5-SNP GRS is associated with recurrent venous thrombosis.

Methods: Patients with a first venous thrombosis included in the MEGA case-control study between 1999 and 2004, were followed for a recurrent venous thrombosis up to 2009 ($n = 4731$). Included in the current analyses were patients with a first venous thrombosis without a history of malignant disease or oral anticoagulant use during follow-up and with a valid measurement for all five SNPs included in the GRS ($n = 3121$). Consent and ethical approval were obtained. The GRS for each individual was calculated by summing the number of risk-increasing alleles for each of the following five SNPs: rs6025 (F5, factor VLeiden), rs1799963 (F2, 20210 G>A), rs8176719 (ABO), rs2066865 (FGG 10034 C>T) and rs2036914 (F11). The risk of recurrent venous thrombosis associated with the GRS was calculated continuously, i.e. per addition of one risk allele, and after stratification in a low and high risk score (i.e. ≤ 2 risk alleles and ≥ 5 risk alleles) by calculating hazard ratios (HR), adjusted for age and sex. The cumulative incidence of recurrence was calculated after 2 and 4 years of follow-up for individuals with a low GRS and individuals with a high GRS. Analyses were performed in the overall patient group and after stratification into patients with a first idiopathic or provoked event. Idiopathic thrombosis was defined as absence of surgery, trauma, plaster cast, hormone use (oral contraceptives and hormone therapy), or pregnancy within 3 months before the first event, and not within 4 weeks postpartum.

Results: The 5-SNP GRS was positively associated with the risk of recurrent venous thrombosis, i.e. the addition of one risk allele to the GRS increased the risk of a recurrence (HR = 1.2; 95% CI: 1.1–1.3). This risk was similar in patients with an idiopathic or provoked first event. After stratification in a high and low GRS, the risk of recurrence was higher in patients with a high GRS than in patients with a low GRS (Overall HR = 2.0, 95% CI: 1.5–2.6; idiopathic first event: HR = 1.7, 95% CI: 1.1–2.5; provoked first event: HR = 2.2, 95% CI: 1.5–3.2).

The overall 2-year cumulative incidence of recurrence was 6.1% (95% CI: 5.2–6.9). The incidence was clearly higher in patients with a high GRS than in patients with a low GRS (high: 10.3%, 95% CI: 7.2–13.4; low: 4.4%, 95% CI: 3.2–5.6). This difference remained also after 4 years of follow-up (high GRS: 14.9%, 95% CI: 11.3–18.5, low GRS:

8.8%, 95% CI 7.1–10.5). The GRS was predictive of the risk of recurrent thrombosis both in patients with an idiopathic and patients with a provoked first event.
Summary/Conclusion: This 5-SNP GRS was associated with an increased risk of recurrent thrombosis. The GRS can identify patients with a high absolute risk of recurrence.

AS 18.2

Prevention of pulmonary embolism recurrences by retrievable vena cava filter: results of the randomized multicenter trial PREPIC 2

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Background: Vena cava filter insertion in patients with an acute proximal deep vein thrombosis (DVT) with or without pulmonary embolism (PE) is associated with a lower risk of PE at short-term (< 1 month) but an increase risk of DVT at long term (> 6 months) (Decousus H et al. *N Engl J Med* 1998; PREPIC Study Group. *Circulation* 2005). In this way, retrievable filter could be used during the high-risk period of PE recurrences and then removed to avoid the late increase in the risk of DVT recurrences.
Aim: To assess the efficacy and safety of retrievable filter implanted for 3 months in patients with symptomatic PE.
Methods: We conducted a multicenter prospective open randomized study with a blind evaluation that compared retrievable vena cava filter (ALN) maintained for 3 months with no filter insertion in patients receiving anticoagulant therapy for an unprovoked acute symptomatic confirmed PE associated with a thrombosis of the lower limb. Patients were eligible if they were considered at high risk of PE recurrences, i.e. when presenting at least one of the following risk factors: age over 75, right ventricular dysfunction, active cancer, bilateral and/or ilio-caval DVT, cardiorespiratory insufficiency. A systematic retrieval of filter was planned at 3 months in the filter group and both groups were followed-up for three additional months. The primary efficacy outcome was recurrent PE at 3 months. Secondary outcomes were DVT at 3 months, VTE at 6 months, major bleeding, overall mortality at 3 and 6 months and filter complications.
Results: The study recruited 399 patients in 18 centers from August 2006 to July 2012: 200 given filter (filter was really inserted in 193) and 199 given no-filter. All patients received an initial parenteral anticoagulant therapy (heparins 85%, fondaparinux 15%) followed by vitamin K antagonists (89%) or prolonged low molecular weight heparins (11%). Median age was 76 years, 35% had a history of DVT or PE, 97% had a concomitant DVT, 25% had a cancer. Last patient last visit was January 16th, 2013. Recurrence of PE at 3 months occurred in six patients (3.0%) in the filter group compared to three patients (1.5%) in the no-filter group, RR = 2.0 (0.51–7.89). These events included six and two fatal PE respectively. At 3 months, no difference was found on DVT (0.5% in both groups); eight patients in the filter group had major bleeding (4.0%), vs. 10 in the no-filter group (5.0%). At 3 months, 15 deaths (7.5%) occurred in the filter group and 12 (6.0%) in the no-filter group. Filter removal was attempted in 165 patients and successful for 152 (92.1%). Thirteen patients died before retrieval, and retrieval was not attempted for the following reasons: permanent indication of filter or poor clinical status (11 patients), vena cava filter thrombosis (3) or patient's refusal (1). Results at 6 months will be presented at the congress.
Conclusions: In the lack of contra-indication to anticoagulation, these results do not plead for retrieval vena cava filter insertion in patients with an acute PE.

AS 18.3

D-dimer to select patients with a first unprovoked venous thromboembolism (VTE) who have anticoagulants stopped at 3–7 months or have treatment continued indefinitely: a multicentre management study

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Background: Patients with unprovoked venous thromboembolism (VTE) who have a normal D-dimer level 1 month after withdrawing anticoagulant therapy have been reported to have a reduced recurrence risk; however, it remains uncertain if this risk is low enough to justify using D-dimer to decide if anticoagulation is stopped.
Aims and Methods: We did a prospective multicentre study to determine if patients < 75 years with a first unprovoked proximal DVT or PE have a low risk of recurrence (about 5% per year, with an upper 95% CI of no more than 7.0% per year) if they have a negative point-of-care D-dimer test (Clearview-Simplify, Alere; qualitative result [positive or negative]) while on anticoagulants, and again 1 month after stopping therapy (Negative D-dimer group). Patients with a positive D-dimer test remained on, or restarted, anticoagulants and continued them indefinitely (Positive D-dimer group). Patients provided written informed consent, and the study was approved by the research ethics boards of all clinical centres.
Results: Four hundred and ten patients (51 years, SD 14; 44% females) were enrolled after 5.0 months (SD 1.3) of initial anticoagulant therapy and have been followed for 2.0 years (SD 0.9) to date. D-dimer was positive on therapy in 14/410 (3.4%) patients who, therefore, remained on anticoagulants. Of 391 patients with a negative D-dimer on therapy and then had anticoagulants withdrawn, D-dimer converted to positive at 1 month in 57 (15%) patients, and 55 of these restarted anticoagulant therapy. 320/410 (78%) patients were in the Negative D-dimer group and 318 of these remained off anticoagulant therapy. In the Negative D-dimer group, recurrent VTE was: 5.6% per year (95% CI 4.0–7.8; 35 recurrences) in all patients (n = 318); 8.2% per year (95% CI 5.6–11.6; 29 recurrences) in men (n = 179); 3.9% per year (95% CI 1.6–8.1; six recurrences) in women with an initial VTE not associated with estrogens (n = 81); and 0% per year (95% CI 0.0–2.6; 0 recurrences) in women with an initial VTE associated with estrogens (n = 58). The rates of recurrent VTE differed among the three subgroups (P = 0.0014). In the Positive D-dimer group, recurrent VTE and major bleeding were each 2.6% per year (95% CI 0.8–6.2; four recurrent VTE and four major bleeds) in the 69 patients who remained on anticoagulant therapy. Findings will be updated when scheduled follow-up is completed on 30 June 2013.
Conclusion: There was a high risk of recurrent VTE in men with a first unprovoked VTE who had a D-dimer test that remained negative 1 month after stopping therapy; therefore, negative D-dimer testing alone does not justify stopping anticoagulants after 3–7 months in men. However, the risk of recurrent VTE in women with unprovoked VTE who had negative D-dimer testing, including those who had VTE that was not associated with estrogen therapy, appears to be low enough to justify stopping anticoagulants after 3–7 months.

AS 19 – Vascular Disorders

AS 19.1

Risk factors for chronic pulmonary hypertension: VTE Epidemiology Group (VEG) Study

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Background: Chronic pulmonary hypertension (CPH) occurs in around 5% of patients following pulmonary embolism (PE) and

results in significant morbidity, mortality and cost. CPH occurs following index PE and less commonly following an index deep vein thrombosis (DVT), presumably in conjunction with sub-clinical PE. The recognition of risk factors may help to evaluate high risk groups and guide therapy type and duration. To simplify decision making these factors for CPH must be assessed allowing for the presence, quality and absence of anticoagulant therapy.

Aim: To explore potential risk factors for CPH.

Methods: Patient data were retrieved from the subset of general practices in England contributing to the Clinical Practice Research Database (CPRD) that has been linked to data from the Hospital Episodes Statistics (HES) and to the Office for National Statistics (ONS). From January 2001 to October 2011, all VTE cases in the CPRD were verified with an algorithm based on review of medical records and notes, hospital diagnoses, cause of death and anticoagulation therapy. We conducted a nested case-control study in patients with a first VTE. CPH in any time during the entire observational period were defined as cases and the date of CPH as the index day. For each case we selected five non-CPH controls at random matched on the index day and with the same duration of observation as the respective case. The independent association between the potential risk and CPH was derived from conditional multivariate logistic regression models and presented as adjusted odds ratios (ORs) with 95% confidence intervals (CIs). Adjustment included age, gender, socioeconomic risk factors, components of the Charlson index, and the type of antithrombotic therapy (VKA, low molecular weight heparin or antiplatelet therapy).

Results: Of 35,373 patients with first VTE, 29,550 patients survived more than 7 days, had 81,906 person-years of observation, and were at risk of CPH. Over the observational period and after at least 90 days following first VTE 256 cases of CPH were identified.

The OR of CPH was greater in the following risk factors: following PE compared with DVT, OR 3.92 (2.56–6.01), PE with DVT compared with DVT, OR 4.43 (2.19–8.98); and with recurrent PE, OR 2.43 (1.28–4.62) but not with recurrent DVT.

Patients were less likely to develop CPH were males, OR 0.63 (0.42–0.95); as were those with INR with Time in Therapeutic Range (TTR) > 60%, OR 0.22 (0.07–0.66); TTR 40–60%, OR0.33 (0.11–0.99) compared to those with poor control TTR < 40%.

Summary/conclusions: The recognition of risk factors for CPH should facilitate the need for further investigations and the decision for appropriate therapy and extension of anticoagulant therapy in VTE patients.

AS 19.2

Residual vein thrombosis as a strong predictor of recurrent thromboembolism and post-thrombotic syndrome: a prospective cohort study

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Background: Whether residual vein thrombosis (RVT) increases the risk of recurrent venous thromboembolism (VTE) after an episode of deep vein thrombosis (DVT) is controversial. Moreover, it is virtually unknown whether and to what extent RVT increases the risk of the post-thrombotic syndrome (PTS).

Aims: We sought to determine the risk of recurrent VTE, mild and severe PTS over a 3-year follow-up in a wide cohort of patients with proximal DVT who had the ultrasound assessment of the common femoral and the popliteal veins 3 months after the thrombotic episode.

Methods: We followed-up prospectively for up to 3 years 906 consecutive outpatients who referred with an episode of proximal DVT, as shown by compression ultrasonography (CUS), and received conventional anticoagulation. Patients with a recent (< 2 years) episode of DVT involving the same leg were excluded, as were those unavailable for repeating CUS. After 3 months the common femoral and the popliteal veins were scanned in the transverse section with CUS. RVT was accepted in case of a vein diameter > 4 mm under maximum compressibility in at least one spot. Over the 3-year follow-up the development of recurrent VTE, mild and severe PTS (as assessed with the Villalta scale) was independently recorded by three investigators.

Results: RVT was detected in 483 of the 906 patients (53.5%). Patients with RVT were more likely to be males (52.3 vs. 42.3%; $P = 0.038$), to have a previous VTE (15.7 vs. 10.2%; $P = 0.017$) and to have an extensive (both femoral and popliteal) involvement of the vein system (54.9 vs. 28.6%; $P < 0.00001$). After the 3-year follow-up period recurrent VTE developed in 82 patients (17.0%; 32 in the ipsilateral leg) in the group with RVT and in 41 (9.7%; nine in the ipsilateral leg) in that without RVT; overall PTS in 275 (56.9%; severe in 35) and 127 (30.0%; severe in 10), respectively. After adjusting for age, sex, previous VTE, obesity, risk factors for DVT (unprovoked or provoked), location of DVT, presence of clinically symptomatic PE and duration of anticoagulation the HR and its 95% CI for the development of recurrent VTE in patients with RVT as compared with those with earlier vein recanalization was 1.94 (1.32–2.83), and that of PTS 2.27 (1.83–2.81).

Conclusions: RVT, as assessed by CUS 3 months after an episode of proximal DVT, is a powerful and independent predictor of recurrent VTE and PTS in patients with proximal DVT. Males, patients with previous VTE and those with extensive involvement of the deep vein system are more likely to develop RVT.

AS 19.3

Health-related quality of life after catheter-directed thrombolysis for deep vein thrombosis; from the CaVenT study

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Background: Additional catheter-directed thrombolysis (CDT) with alteplase for extensive deep vein thrombosis (DVT) reduces the frequency of the long-term chronic complication post-thrombotic syndrome (PTS) compared to standard treatment. PTS has been shown to be a predictor of reduced quality of life (QOL) following DVT of the lower limb.

Aims: To investigate whether additional CDT improves long-term QOL compared to standard treatment with anticoagulation and compression stockings alone.

Methods: The CaVenT Study was an open, multicenter, randomized, controlled trial. Prior to treatment allocation, written informed consent was obtained. Patients (18–75 years) with high proximal DVT above mid-thigh level and symptoms < 21 days were randomized to receive additional CDT or standard treatment alone. At 6 and 24 months follow-up visits patient reported QOL was obtained using the generic QOL questionnaire EQ-5D and the venous disease-specific QOL questionnaire VEINES-QOL/Sym. PTS was assessed with the Villalta scale. The study protocol was approved by the Regional Committee for Medical and Health Research Ethics and the Norwegian Medicines Agency, and was registered at www.clinicaltrials.gov with the unique trial identifier NCT00251771.

Results: One hundred and eight-nine of 209 patients completed 24 months follow-up. Mean age was 51.5 years (SD 15.8) and 70 (37%) participants were female. Mean duration of symptoms before diagnosis and start of treatment was 6.6 days (SD 4.6). Most baseline demographic and clinical characteristics, including VEINES-QOL/Sym and EQ-5D scores, were fairly equally distributed between the two treatment groups. After 24 months PTS was observed in 41% allocated CDT compared to 56% of control patients ($P < 0.05$). There were no differences between the two treatment arms regarding the

patient reported outcomes EQ-5D index and VEINES-QOL and -Sym scores. Patients who developed PTS reported poorer long-term outcomes in terms of all three scores ($P < 0.001$), and regarding the VEINES scores these differences were also present at 6 months follow-up ($P < 0.001$); all differences were likely to represent clinically meaningful differences.

Summary/Conclusion: Long-term QOL did not differ between patients treated with additional CDT for a high proximal DVT compared to anticoagulation and compression therapy alone. Patients who developed PTS reported poorer generic and disease-specific QOL and more symptoms than patients without PTS. QOL should be included as an outcome measure in clinical studies with patients who are at risk of PTS.

AS 20 – New Developments in Treatment of VTE

AS 20.1

A phase 2 randomized, double-blind, placebo-controlled trial of PRT064445, a novel, universal antidote for direct and indirect factor Xa inhibitors

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Background: Direct factor Xa inhibitors have superior anticoagulant efficacy and/or safety relative to warfarin and low molecular weight heparin but are limited by lack of a specific antidote to reverse anticoagulation during episodes of serious bleeding or before surgery. PRT064445 (PRT) is a modified, human recombinant fXa that is catalytically inactive but retains high-affinity binding to direct fXa inhibitors (fXai) and heparin-antithrombin III complexes. It competes with native fXa for fXai drugs, thus reversing direct and indirect fXai-mediated anticoagulation. In a previous Phase 1 study ($n = 32$), in the presence of PRT in subject plasma samples, ex-vivo thrombin generation and anti-fXa activity of rivaroxaban were reversed in a dose-dependent manner.

Aims: To evaluate dose-response of PRT in reversing the anticoagulant effects of several fXai, pharmacokinetics (PK), and overall safety in up to 144 healthy volunteers.

Methods: This ongoing Phase 2, double-blind, placebo-controlled study is examining the effects of PRT with apixaban, rivaroxaban, enoxaparin, and betrixaban. Each fXai will be studied with up to four different dose cohorts of PRT or placebo in a 6:3 ratio (i.e. up to 36 subjects per fXai). Subjects are treated on Days 1–6 with fXai (to steady state) and then dosed with IV PRT or placebo on Day 6, 3 h after the fXai dose. Pharmacodynamic and safety data are collected through Day 48 and PK through Day 10. A chromogenic assay was used to determine anti-fXa activity.

Results: We are reporting available data from the first PRT dose cohort of nine subjects, who received apixaban 5 mg bid (11 doses), followed by a bolus of PRT (90 mg) or placebo IV. Anti-fXa activity peaked at 3 h after the last dose of apixaban at which time PRT/placebo was administered. Within 2 min, the mean anti-fXa activity decreased by −65% following PRT ($n = 4$) vs. +6% in the placebo group ($n = 2$). At 10, 30, and 60 min post-PRT or placebo, the comparable values were −47% vs. +7%; −29% vs. +11%; and −17% vs. +3%. Similarly, plasma concentrations of *unbound* apixaban decreased immediately after PRT but were unchanged following placebo. Based on a mean apixaban plasma concentration of 474 nM and an estimated PRT plasma concentration of 314 nM, the PRT/apixaban molar ratio was 0.66, indicating that this initial low dose of 90 mg PRT bound approximately two thirds of the apixaban in the plasma. This value is in good agreement with the observed 65% decrease in anti-fXa activity. PRT was temporally associated with a transient reduction in tissue factor pathway inhibitor (TFPI) activity and increase in F1+F2, but no change in D-dimer. There were no thrombotic or allergic-type adverse events, serious adverse events, or deaths. PRT was well tolerated; 5/9 subjects experienced one or more adverse events; all were mild.

Summary/Conclusions: Emerging in vivo data from this trial confirm prior ex-vivo observations and animal models that PRT reverses anticoagulant effects of fXai. PRT is a well-tolerated, and potentially promising, universal antidote for fXai. Additional data with higher doses will be presented.

AS 20.2

No need for a rivaroxaban dose reduction in renally impaired patients with symptomatic venous thromboembolism

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Background: Rivaroxaban is an oral, direct Factor Xa inhibitor. One-third of the rivaroxaban dose is excreted renally as unchanged drug. Consequently, rivaroxaban exposure increases with declining creatinine clearance (CrCl), with area under the curve ratios of 1.44 (95% confidence interval [CI] 1.08–1.91), 1.52 (95% CI 1.15–2.01) and 1.64 (95% CI 1.24–2.17) observed in patients with CrCl 50–79, 30–49 and < 30 mL/min, respectively (Kubitza et al. 2010). Dose-finding studies for deep vein thrombosis (DVT) treatment showed a flat dose-response for recurrent venous thromboembolism (VTE), with no increase in major bleeding for total daily doses of rivaroxaban of 20–60 mg and no increase in major bleeding in patients with CrCl 50–79 mL/min or < 50 mL/min. Therefore, in the EINSTEIN DVT and PE studies, the rivaroxaban dose was not reduced in patients with renal impairment.

Aims: To assess rates of bleeding and recurrent VTE in patients with renal impairment treated with fixed-dose rivaroxaban compared with standard VTE treatment in EINSTEIN DVT and PE.

Methods: Patients with acute symptomatic DVT and/or pulmonary embolism were randomized to receive fixed-dose rivaroxaban (15 mg twice daily for 3 weeks followed by 20 mg once daily) or subcutaneous weight-adjusted enoxaparin followed by a vitamin K antagonist (VKA) (international normalized ratio 2.0–3.0) for 3, 6 or 12 months. Patients with CrCl < 30 mL/min were excluded. Rates of major bleeding and recurrent VTE were assessed. Subgroup analyses were performed for patients with normal renal function (CrCl ≥ 80 mL/min) and mild (CrCl 50–79 mL/min) or moderate (CrCl < 50 mL/min) renal impairment.

Results: A total of 4130 patients received rivaroxaban and 4116 patients received enoxaparin/VKA. Major bleeding occurred in 40 (1.0%, three fatal) and 72 (1.7%, eight fatal) patients in the rivaroxaban and enoxaparin/VKA groups, respectively (hazard ratio [HR] = 0.54; 95% CI 0.37–0.79; $P = 0.002$). In patients with normal renal function, major bleeding occurred in 23/2739 (0.8%) patients receiving rivaroxaban and in 29/2776 (1.0%) patients receiving enoxaparin/VKA (HR = 0.79, 95% CI 0.46–1.37). In patients with mild renal impairment, major bleeding occurred in 14/1024 (1.4%) patients and 30/993 (3.0%) patients, respectively (HR = 0.44, 95% CI 0.24–0.84). In patients with moderate renal impairment, major bleeding was observed in 3/329 (0.9%) patients and 13/320 (4.1%) patients, respectively (HR = 0.21, 95% CI 0.06–0.73). In the rivaroxaban group, the incidence of recurrent VTE increased from 49/2748 (1.8%) patients with normal renal function to 24/1030 (2.3%) and 11/332 (3.3%) patients with mild or moderate renal impairment, respectively. In the enoxaparin/VKA group, the incidence increased similarly from 52/2787 (1.9%) to 30/992 (3.0%) and 11/322 (3.4%) patients, respectively.

Summary/Conclusions: Rivaroxaban 15 mg twice daily for 3 weeks, followed by 20 mg once daily, was not associated with increased major

bleeding in patients with reduced renal function. Compared with enoxaparin/VKA, rivaroxaban was associated with a relative risk reduction of major bleeding of ~55% and ~78% in patients with mild and moderate renal impairment, respectively. The incidence of recurrent VTE increased with declining renal function in both treatment groups (P-value for trend = 0.002). We conclude that a rivaroxaban dose reduction is not indicated in patients with DVT or pulmonary embolism with mild/moderate renal impairment.

AS 20.3

Inhibition of coagulation factor XII provides thromboprotection in extracorporeal circulation without increasing bleeding risk

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Background: Anticoagulation treatment for prevention of thromboembolic diseases is associated with an increase in potentially life-threatening bleeding. Thus, it constitutes a priority to develop novel targets for efficient and safe anticoagulation. Deficiency in coagulation factor XII (FXII) protects mice in models of thromboembolic disease. Despite its critical role in thrombosis, FXII deficiency does not increase bleeding risk in patients.

Aims: Here, we analyzed inhibition of FXII activity in rabbits as a potential novel anticoagulation strategy, devoid of bleeding risk.

Methods and Results: We used phage display to develop a monoclonal antibody directed against activated FXII (anti-FXIIa antibody CSL-3F7). CSL-3F7 selectively interferes with FXII-mediated thrombin formation and clotting in rabbit plasma. CSL-3F7 dose-dependently blocked collagen-induced thrombus formation under venous and arterial flow in rabbit blood. To analyze the efficacy and safety of CSL-3F7 in a clinical setting that requires potent anticoagulation, we set up a model of Extra Corporeal Membrane Oxygenation (ECMO) for rabbits. A single bolus of CSL-3F7 was as efficient as high-dose, heparin in terms of thromboprotection during ECMO and prevented clot formation for up to 6 h. In contrast, bypass systems occluded in < 3 min in buffer-infused animals. Despite its striking thromboprotective efficiency, CSL-3F7 did not increase skin and cuticle bleeding times and had no effect on blood loss as assessed by liver and skin wound injury.

Conclusions: The data establish CSL-3F7, a FXIIa-neutralizing antibody, as a potent thromboprotective agent in extracorporeal circulation and indicate that targeting FXII activity offers a safe strategy for prevention of thrombosis that is not associated with the frequent complications of bleeding seen with currently used anticoagulants.

AS 21 – The Interplay Between Coagulation and Inflammation

AS 21

Tissue factor signaling and inflammation

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The Tissue Factor (TF) pathway is directly linked to physiological repair mechanisms and the host defense against infection. TF is induced by inflammatory mediators in myelo-monocytic cells where danger signals from dying cells or associated with complement activation regulate TF procoagulant activity dependent on protein disulfide isomerase, coupling the initiation of inflammation with local activation of the coagulation cascade. The role of TF is not restricted to the initiation of coagulation and, in addition, signaling of TF protease complexes plays a pivotal role in the regulation of innate immune responses and inflammation. Recent data show that the TF pathway is a critical inducer of the chronic inflammation in obesity. TF adipocyte signaling contributes to the development of obesity, whereas TF expressed by adipose tissue macrophages regulates their inflammatory phenotype. Loss of TF-PAR2 signaling in the hematopoietic compartment of obese mice attenuates adipose tissue inflammation and promotes a switch of adipose tissue macrophages to an anti-inflammatory phenotype typically seen in lean mice. TF forms two distinct signaling complexes. The binary, coagulation inactive TF-VIIa complex interacts with 1 integrins and only cleaves PAR2. The ternary TF-VIIa-Xa complex mediates Xa-dependent activation of PAR1 or PAR2. Xa signaling in this complex is strictly dependent on the endothelial protein C receptor (EPCR). Macrophages found in adipose and tumor tissues express components of these signaling complexes. Further insight into the control of macrophage phenotypes by the TF pathway is expected to emerge from the characterization of regulated expression and interactions of TF signaling components in macrophages during acute and chronic inflammation.

AS 21.1

Formation of neutrophil extracellular traps in skin wounds of mice retards healing

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Background: Wound healing is an important restorative process in response to tissue injury. Failure of effective wound repair in chronic inflammatory conditions can result in ulcers and amputation. Approximately 3–6 million people in the US suffer from non-healing wounds. Inflammation is one of the early phases of wound repair in which neutrophils play a central role. Upon stimulation, neutrophils can release their chromatin and granule proteins, forming neutrophil extracellular traps (NETs) that act as another mechanism of innate immunity. Peptidylarginine deiminase 4 (PAD4) is abundantly expressed in neutrophils and can decondense chromatin by converting arginine on histones to citrulline. PAD4-mediated histone hypercitrullination is a critical step for neutrophils to extrude their chromatin to make NETs. NETs limit the spread of bacteria and may kill them via the locally concentrated antibacterial components. Their non-specific cytotoxicity, however, may also be injurious to host tissue. High concentration of neutrophil elastase, which is also a NET component, can cause excessive degradation of the wound matrix that is unfavorable to healing. Whether NETs are present in wounds and their impact on the healing process has not been explored.

Aims: Our aims were to determine whether NETs are formed during wound healing and to examine whether they facilitate wound repair or delay the process.

Methods: Full thickness wounds were made with sterile 4-mm biopsy punches in the dorsal skin of wild-type (C57BL/6) mice. Wounds were observed for the presence of extracellular DNA. Mice deficient in CD18 ($CD18^{-/-}$) with defective leukocyte recruitment to sites of tissue damage, and wild-type mice depleted of neutrophils using an anti-Ly6G antibody (1A8 clone) were used to examine whether neutrophils were the main producers of such extracellular DNA. $PAD4^{-/-}$ mice whose neutrophils cannot make NETs were used to evaluate the impact of NETs formation in wound healing. The wounds were digitally photographed for 14 days and total wound area was calculated using ImageJ software. Recruitment of neutrophils and re-epithelialization reflected by keratinocyte migration were examined by histology. Citrullination of histone H3 (H3Cit), a marker for NETs formation, and neutrophil marker (Ly6G) were localized in wound sections by fluorescence microscopy and quantified by Western blotting.

Results: Western blot analysis of wounds from wild-type mice showed that H3Cit appeared the first day after wounding and peaked from days 3 to 7. Immunofluorescence imaging revealed the co-localization of H3Cit with $Ly6G^+$ cells in the scab. H3Cit was absent in wounds from $CD18^{-/-}$ or $PAD4^{-/-}$ mice and reduced by ~80% in mice depleted of neutrophils. $PAD4^{-/-}$ mice exhibited accelerated wound repair, re-epithelialization and wound closure. NET-like extracellular DNA structures, seen as blue spikes with hematoxylin, were observed in wounds from wild-type but not $PAD4^{-/-}$ mice.
Summary: Neutrophils are the main producers of extracellular DNA during wound healing and NETs represent a major component of skin wounds. PAD4-dependent NET production has a negative impact on healing, thus inhibition of NET formation may accelerate wound repair.

AS 21.2
Protease-activated receptor 1 contributes to early immune responses in the lung after influenza A infection

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Background: Influenza A (IVA) is a RNA virus that infects the lungs and causes considerable morbidity and mortality. Toll-like receptor-3 (TLR3) signaling plays a central role in controlling IVA replication. TLR3 activation leads to the expression of an antiviral program that includes interferon (IFN). Recruited natural killer (NK) cells kill infected cells. Furthermore, IVA infection was shown to induce local production of different proteases that activate protease-activated receptor-1 (PAR1). Recently, we found that PAR1 deficiency resulted in increased coxsackievirus B3-induced myocarditis. This observation was mediated by a PAR1-dependent increase in TLR3-IFNβ-CXCL10 expression. In contrast to our findings, the Riteau group found that PAR1 stimulation resulted in increased mortality after IVA infection. Two companies have developed anti-PAR1 drugs as novel antiplatelet drugs. However, PAR1 is expressed by many cell types and these drugs may have off-target effects and possibly interfere with early immune responses.
Aim: At present, the role of PAR1 during virus infection of the lung is controversial. Here, we investigated whether PAR-1 plays a role in the innate immune responses to IVA.
Methods: Male $PAR1^{+/+}$ (wild-type, WT) and $PAR1^{-/-}$ mice (age of 10–12 weeks) were infected intranasally with 50 μl of 0.02 hemagglutinating units of IVA H1N1/PR8. IVA genome levels, expression of IFNs and inflammatory mediators were analyzed by qRT-PCR 1, 3 and 7 days post infection (dpi) in lungs of infected WT and $PAR1^{-/-}$ mice. Further, we analyzed the effect of PAR1 stimulation on the TLR3-signaling pathway on bone-marrow derived macrophages (BMDM). Lastly, we analyzed the role of PAR1 for NK cell activity.
Results: $PAR1^{-/-}$ mice exhibited significantly reduced levels of IFNβ in the lung compared to WT mice 1 dpi. ($P < 0.05$). In addition there were reduced levels of the NK cell marker, NK1.1, and five-fold more IVA genomes in the lungs of $PAR1^{-/-}$ mice compared to WT mice 3 dpi ($P < 0.05$). Increased IL1β and TNFα expression was also observed in PAR-1 deficient mice lungs compared to WT mice 3 dpi. However, WT mice exhibited higher levels of IFNγ, CXCL10, IL1β and TNFα in the lungs compared to $PAR1^{-/-}$ mice 7 dpi. TLR3 stimulation experiments with BMDM showed that PAR1 activation enhances TLR3-IFNβ-CXCL10 pathway in a p38 dependent fashion. Furthermore, we found that maximal NK cell activity of a splenocyte population was dependent on the PAR1 expression.
Conclusion: We found that PAR1 contributes to early antiviral immune responses in the lung after IVA infection. An absence of PAR1 was associated with reduced IFNβ and NK cell infiltration in the lung that appears to lead to increased virus replication and inflammation 3 dpi. Our data indicate that PAR1 has a role in early immune responses by inducing early innate immune responses to IVA. We speculate that inhibition of PAR1 might lead to accelerated IVA replication in the early phase of infection.

AS 21.3
Activated protein C glycosylation status dictates protease-activated receptor 1 proteolysis and anti-inflammatory signaling efficacy

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Background: Protein C circulates in plasma as two major glycoforms (α and β), characterized by the presence (α-protein C) or absence (β-protein C) of an N-linked glycan chain at position Asn-329. Upon activation, a recombinant mimic of β-activated protein C (APC^{N329Q}) exhibits significantly improved ability to maintain endothelial cell barrier integrity and limit endothelial cell apoptosis compared to wild type APC. Despite its potential importance in defining endogenous APC anti-inflammatory activity, the molecular basis of enhanced β-APC cytoprotective signaling is currently unknown.
Aims: We sought to determine how enhanced protease-activated receptor 1 (PAR1)-dependent cytoprotective signaling by β-APC is mediated, and investigate whether site-specific glycosylation status is also an important determinant in PAR1-independent APC cell signaling. Furthermore, we examined whether partial glycosylation and associated enhanced PAR1 signaling function was similarly conserved in murine APC.
Methods: A PAR1 cleavage alkaline phosphatase reporter assay was used to assess the rate of PAR1 proteolysis by APC on HEK293T cells co-transfected with endothelial cell protein C receptor (EPCR). APC inhibition of lipopolysaccharide (LPS)-induced pro-inflammatory cytokine secretion from human or murine myeloid cells was assessed by ELISA or by using cytokine-specific reporter cell lines. The role of APC glycosylation in PAR1 proteolytic activity and anti-inflammatory function was determined by enzymatic removal of APC N-linked glycans with PNGase F, and also by utilization of recombinant APC variants rendered incapable of glycan moiety attachment at specific sequons, namely Asn-248 (APC^{N248Q}), Asn-313 (APC^{N313Q}) and Asn-329 (APC^{N329Q}) in human APC, and Asn-330 in murine APC ($mAPC^{N330Q}$).
Results: The rate of EPCR-dependent PAR1 proteolysis by PNGase F-treated APC was more than double that of untreated APC. APC^{N329Q} exhibited comparably enhanced PAR1 proteolysis to that of PNGase F-treated APC, whereas variants APC^{N313Q} and APC^{N248Q} were similar in efficacy to wild type APC. PAR1-dependent inhibition of TNFα and IL-6 secretion from macrophages was significantly more effective in the presence of either PNGase F-treated APC or APC^{N329Q} than with untreated wild type APC. No difference, however, was observed in the ability of untreated, PNGase F-treated, or recombinant site-specific glycan variant APC to mediate PAR1-*independent* inhibition of TNFα secretion from LPS-treated monocytes. Finally, SDS-PAGE analysis revealed that murine APC migrates as a glycosylation-dependent doublet, which, like human APC, mediates significantly increased PAR1-dependent inhibition of TNFα and IL-6 secretion from LPS-stimulated murine macrophages upon pre-incubation with PNGase F. In addition, a recombinant version of murine β-APC ($mAPC^{N330Q}$) exhibited equally improved anti-inflammatory activity to that of PNGase F-treated murine APC in the same assay.
Summary/Conclusions: In this study, we show that the PAR1-dependent anti-inflammatory signaling activity of both human and murine APC is regulated by N-linked glycans at Asn-329 and Asn-330 respectively, which act to impede efficient PAR1 proteolysis and thereby limit cytoprotective signaling. Furthermore, our data imply a key role for the endogenous β-APC glycoform in dictating PAR1-dependent

APC anti-inflammatory activity *in vivo*, and highlights the therapeutic potential of recombinant N-linked glycan sequon-specific APC variants for the treatment of acute inflammatory disease.

AS 22 – Inhibitors in Haemophilia A

AS 22.1

Factor VIII gene (F8) mutation and inhibitor development in non-severe hemophilia A

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Background: The development of neutralizing antibodies (inhibitors) towards factor VIII is a major complication in non-severe hemophilia A, profoundly aggravating the bleeding pattern. Identification of high risk patients is hampered by lack of data on the association between factor VIII gene (*F8*) mutations and the development of inhibitors that take exposure days to therapeutic factor VIII concentrates into account.

Aims: To determine the risk of inhibitor development in patients with non-severe hemophilia A and to analyze the association with *F8* mutation, taking exposure days to therapeutic factor VIII concentrates into account.

Methods: The study population was derived from a source population of 2711 non-severe hemophilia A patients (factor VIII 2–40%), treated in 34 hemophilia treatment centers in Europe and Australia (the INSIGHT consortium). The association between *F8* mutation and inhibitor development was assessed in 1112 patients, only recruited from centers that had genotyped at least 70% of their patients. Inhibitor risk was calculated as Kaplan–Meier incidence with cumulative number of exposure days as time variable. Thus, risk was calculated as the proportion of patients that developed an inhibitor after a certain number of exposure days (e.g. 20 or 50) to therapeutic factor VIII concentrates.

Results: During 44,800 exposure days (median 24 exposure days per patient; Inter Quartile Range (IQR), 7–90), 59 of the 1112 patients developed an inhibitor; cumulative incidence of 5.3% (95% confidence interval (CI), 4.0–6.6) after a median of 28 exposure days (IQR, 12–71). The inhibitor risk at 50 exposure days was 6.7% (95% CI, 4.5–8.9) and at 100 exposure days this risk was further increased to 13.3% (95% CI, 9.6–17.0). Among a total of 221 different *F8* point mutations 19 were associated with inhibitor development. The inhibitor risk was highest for R593C, D2074G, R2159C and W2229C, reaching 19%, 21%, 39% and 42%, respectively, at 50 exposure days.

Conclusion: Among a total of 221 different point mutations, 19 mutations were associated with inhibitor development. Longitudinal analysis revealed that the inhibitor incidence in non-severe hemophilia A patients with certain *F8* mutations approaches the incidence observed in patients with severe hemophilia. These results emphasize the importance of *F8* genotyping in non-severe hemophilia A. New preventive and therapeutic approaches in this patient group are urgently needed.

AS 22.2

Effect of type and intensity of FVIII exposure on inhibitors development in hemophilia A: first results of an individual patient data meta-analytic project

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Background: The role of treatment-related factors in the development of inhibitors in hemophilia A patients is highly debated; in view of the scanty evidence from randomized and large prospective trials, a collaboration between researchers to pool a large amount of individual patient data (IPD) and a rigorous methodological approach to their analysis can maximize the reliability of the obtainable evidence.

Aims: To explore the impact of the source of FVIII concentrate and of the intensity of treatment on inhibitor development in previously untreated patients (PUPs) with hemophilia A using different statistical approaches to investigate the role of possible confounders.

Methods: Data source: prospectively collected IPD on PUPs with moderate-severe hemophilia A treated in hemophilia centers in Germany, Italy, Israel, Egypt. Outcome: Inhibitor formation (Bethesda titer > 0.5 BU). Treatment-related risk factors on study: FVIII source (recombinant [rFVIII] vs. plasma-derived [pdFVIII]) and treatment intensity (high-intensity [initial dose ≥ 150 IU/Kg/week] vs. low-intensity). Other risk factors included as covariates/confounders: family history of inhibitors, severity of hemophilia, gene mutation (null/non-null), age at the first exposure to FVIII, regimen (on-demand/prophylaxis), surgery at first exposure and birth year. Statistical methods: meta-analysis of the pooled cohorts by (i) univariate and multivariate Cox regression, with exposure days (EDs) as time-to-event; (ii) CART analysis; (iii) Cox regression with covariate-adjustment using propensity scores for FVIII source and intensity; (iv) calculating the Average effect of Treatment on Treated (ATT) using different methods of matching/stratification on propensity score. Analyses were stratified by country and/or included country as covariate. Multiple-imputation techniques were used to manage missing data. Sub-group analyses: severe patients and high responding (HR) inhibitors.

Results: 761 PUPs (86% severe) were followed up for over 150 EDs (3–490), 241 (31.7%) of whom treated with rFVIII; among 700 patients with available data on initial weekly dose, 116 (16.6%) received a high-intensity treatment. Twenty-seven percent (208/761) developed inhibitors (172 HR) considering the entire population, 38% among patients treated with rFVIII and 22% among those treated with pdFVIII (unadjusted HR 2.2, 95% CI 1.6–3.0); 53% among those receiving an high-intensity treatment and 23% among those treated less intensively (unadjusted HR 3.6, 95% CI 2.6–4.9). Only treatment intensity, and not FVIII source, held an overall statistically significant effect in multivariate Cox analyses, either directly adjusted for covariates or adjusted for propensity scores, and showed a stable significant ATT across different matching methods. According to a significant interaction effect between the two treatment-related factors, rFVIII was associated to a higher risk of inhibitors vs. pdFVIII only in case of low-intensity treatment. CART analysis assigned hierarchically and

automatically a primary effect to treatment intensity and family history of inhibitors, and a secondary effect to FVIII source. Similar results were found for only severe patients and only HR.
Conclusion: Different statistical approaches to pooled IPD confirmed that a high intensity treatment increased the risk of inhibitor while the effect of recombinant or plasma-derived FVIII sources was partially confounded by patient-related risk factors and dependent on the intensity of the exposure.

AS 22.3
Defective indoleamine 2,3-dioxygenase induction is associated with inhibitor development in severe hemophilia A patients carrying F8 gene null mutations

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Background: The occurrence of neutralizing antibodies (inhibitors) to FVIII is a major obstacle to successfully treating hemophilia A patients. Mutations resulting in the absence (or severe truncation) of FVIII protein are associated with the highest risk of inhibitor formation in that they prevent a patient's immune system from initiating central tolerance to FVIII. However, the occurrence of inhibitors only in a minority of those patients on replacement therapy suggests that effective regulatory mechanisms of peripheral tolerance operate extrathymically in most patients harboring FVIII-reactive lymphocytes. A recently elucidated mechanism whereby antigen-presenting cells (APCs) promote and maintain peripheral tolerance involves immunosuppressive tryptophan catabolism by indoleamine 2,3-dioxygenase (IDO). When activated in APCs, the immunoregulatory enzyme IDO changes the antigen-presenting profile of those cells from immunogenic to tolerogenic, contributing to immune homeostasis under a variety of pathophysiological conditions.
Aims: To study the possible role of peripheral tolerance to FVIII, we investigated IDO induction, expression, and activity in severe hemophilia A patients for any possible relationship with inhibitor development.
Methods: We enrolled 45 severe hemophilia A patients who had developed high-titer inhibitors (> 5 BU/mL) and 45 age-matched inhibitor-negative patients (consistently inhibitor-free after at least 150 ED). All subjects in this study, whether inhibitor-positive or inhibitor-negative, carried F8 gene mutations expected to cause complete absence of functional FVIII protein, namely large deletions, nonsense mutations, or intron 1 and 22 inversions, which are all thought to prevent central tolerance development to FVIII. PBMCs were isolated by standard technique. IDO competence was assessed by evaluation of IDO protein and mRNA induction, while IDO activity was analyzed by HPLC measurement of l-kynurenine production (the first breakdown product of tryptophan catabolism by IDO). Informed consent was obtained before enrollment, according to a protocol approved by the ethical committees of the individual participating Centers, in accordance with the Declaration of Helsinki.
Results: Significant differences in IDO induction were found between inhibitor-positive and inhibitor-negative patients by: (i) immunoblot analysis of protein expression (densitometry units; inhibitor-positive patients, 8.9 ± 7.3; inhibitor-free patients 20.5 ± 9.2; $P < 0.0001$); (ii) RT-PCR for IDO mRNA (mRNA fold change; inhibitor-positive patients 1.65 ± 0.54; inhibitor-free patients 3.35 ± 1.6; $P < 0.0001$); and by (iii) kynurenine production (μm/L; inhibitor-positive patients 0.90 ± 0.53; inhibitor-free patients 1.84 ± 0.94; $P < 0.0001$). Overall, 32 out of the 45 inhibitor-free patients showed upregulated IDO expression and function in response to stimulation, whereas 16 of the 45 inhibitor-positive patients were capable of up-regulating IDO (OR 4.45; 95% CI 1.83–10.8, $P = 0.001$).
Conclusions: Our results support the relevance of peripheral tolerance mechanisms in the development of FVIII inhibitors. In particular, our data show that defective IDO induction is associated with inhibitor development in severe haemophilia A patients. Further details on peripheral tolerance in haemophilia A patients could help develop an effective strategy to predict or even prevent (or eradicate) inhibitors through mechanisms of tolerance acquired in the periphery, whether in prophylaxis – to reduce the incidence rate of inhibitors – or in ITI to increase its likelihood to succeed.

AS 23 – What's New in Fibrinolysis?

AS 23.1
PAI-1 is a critical determinant of senescence and survival in klotho mice, a murine model of accelerated aging

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Background: Advanced age contributes to the development of frailty and disease in humans, but the fundamental mechanisms that drive aging are incompletely understood. Cellular senescence correlates with the synthesis of several secreted factors, collectively termed the Senescence-Messaging Secretome (SMS), including insulin-like growth factor binding proteins (IGFBPs), interleukins (ILs), TGF-b, and plasminogen activator inhibitor-1 (PAI-1). The *klotho* gene functions as an aging-suppressor gene; thus klotho-deficient (*kl*/*kl*) mice develop an accelerated aging-like phenotype characterized by a truncated lifespan (64 ± 26 days), infertility, ectopic calcification, and emphysema. Since PAI-1 is markedly elevated in *kl*/*kl* mice and is a critical determinant of replicative senescence *in vitro*, we hypothesized that PAI-1 is essential for development of the phenotype in *kl*/*kl* mice.
Aims: We aimed to investigate the effects of PAI-1 deficiency (partial or complete) on the phenotypic abnormalities exhibited by *klotho* mice.
Methods: Heterozygous *klotho* and PAI-1 knockout mice were bred to generate partial (*kl*/*klpai-1*+/−) or complete (*kl*/*klpai-1*−/−) PAI-1 deficiency in *klotho* mice. Lifespan, body weight and physical activity level of these mice were monitored. Plasma levels of SMS factors, FGF23 and Vitamin D_3 were measured by ELISA methods. Senescence in kidneys was assessed by measuring expression levels of $p16^{Ink4a}$ by qRTPCR and immunostaining. Telomere length in various tissues was quantitatively determined using a qRTPCR method. Emphysema phenotype was characterized by histological evaluation of lung tissues and measuring the partial oxygen pressure in arterial blood. The von Kossa stain was performed on kidney and heart tissues to study ectopic calcification.
Results: Partial (*kl*/*klpai-1*+/−) or complete (*kl*/*klpai-1*−/−) PAI-1 deficiency increased the average lifespan of *kl*/*kl* mice by 4.0-fold to 257 ± 171 and 275 ± 127 days, respectively ($P = 0.0001$). The median survival was 58 days for *kl*/*kl*, 163 days for *kl*/*klpai-1*+/− and 246 days for *kl*/*klpai-1*−/− mice. Furthermore, both *kl*/*klpai-1*−/− and *kl*/*klpai-1*+/− mice exhibited increased body weight, normal fertility, and were protected against emphysematous changes and ectopic calcification developed by *kl*/*kl* mice. While plasma levels of SMS factors IGFBP-3 and IL-6 were significantly elevated in *kl*/*kl* mice, PAI-1 deficiency normalized these factors in *kl*/*klpai-1*+/− and *kl*/*klpai-1*−/− mice. Similarly, *kl*/*kl* mice had shortened telomere length in a number of tissues including liver, aorta, and skin, whereas in *kl*/*klpai-1*+/− and *kl*/*klpai-1*−/− mice, telomere length in these tissues was preserved. Moreover, the renal expression of the senescence biomarker $p16^{Ink4a}$ was 3.1-fold higher in *kl*/*kl* and reduced by 80% in *kl*/*klpai-1*−/− mice. Finally, *kl*/*klpai-1*+/− and *kl*/*klpai-1*−/− mice had significantly

decreased plasma levels of FGF23 (99%) and vitamin D3 (76%), which are biochemical hallmarks of klotho deficiency.
Conclusion: This is the first demonstration that modulating the activity of PAI-1, a key component of the SMS, can prolong survival and prevent the development of senescence in a mouse model of accelerated aging. These findings indicate that PAI-1 is a critical determinant of senescence *in vivo* and that novel therapies targeting PAI-1 or other components of the SMS may prevent senescence and aging-related pathologic changes in humans.

AS 23.2

Hyperfibrinolytic states induce blood-brain barrier disruption by a plasmin-bradykininn dependent mechanism.

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Background: Hyperfibrinolysis is a pathological condition occurring in various clinical disorders and treatments, such as trauma, liver cirrhosis or administration of fibrinolytics. It is characterized by an exacerbated activity of endogenous clot-bursting proteases, including tissue-type plasminogen activator (tPA). Apart from increased bleeding tendency, the pathophysiological implications of hyperfibrinolysis remain largely unknown.
Aims: To develop an experimental model of hyperfibrinolysis and to study its effects on the intact blood-brain barrier (BBB).
Methods: In order to induce sustained high plasmatic levels of tPA, in C57BL6J mice, we performed hydrodynamic transfection of a cDNA encoding for tPA with specific expression in the liver. Subsequently, we assessed the effects of tPA overexpression on BBB permeability and cerebrovascular inflammation by Near Infrared Fluorescence, immunohistology, quantitative PCR and molecular magnetic resonance imaging (MRI).
Results: 48 h after hepatic transfection, plasmatic overexpression of tPA induced BBB leakage. We did not find difference between control and tPA transfected animals in inflammation markers: Iba-1 positive cells, positive vessels overexpressing vascular endothelial adhesion molecule 1 (VCAM-1), mRNA levels of: interleukine-1 beta (IL-1B), tumor necrosis factor (TNF). Deletion of the finger domain (to prevent its interaction with the low density lipoprotein related receptor, LRP) or mutation of the K2 domain of tPA (to prevent its interaction with the N-Methyl-D-Aspartate receptor, NMDA-R) did not influence post-transfection BBB permeability. In contrast, concomitant administration of tranexamic acid, epsilon-aminocaproic acid or aprotinin completely prevented BBB leakage, suggesting a plasmin-dependent mechanism. This effect was NMDA-R, protease activated receptor-1 and matrix-metalloproteinases independent. In fact, we demonstrated that tPA-induced BBB leakage was dependent on bradykinin and the subsequent activation of endothelial B2 receptors, because Icatibant (a clinically available B2 receptor antagonist) completely prevented the BBB disruption. This suggests that plasmin triggers bradykinin generation, effect that provokes BBB leakage.
Conclusion: Circulating plasminogen activators induce BBB leakage in a plasmin, bradykinin and B2 receptor activation-dependent manner. Icatibant appears as a promising therapy to prevent the deleterious effects of fibrinolytics on the BBB.

AS 23.3

The interplay of DNA, histones and neutrophil leukocytes with plasmin dependent lysis of plasma clots

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Background: Neutrophil extracellular traps (NETs) are networks of DNA and associated proteins produced by nucleosome release from activated neutrophils in response to inflammatory stimuli. Our recent studies have shown that DNA and/or histones modify the structure of fibrin clots and their resistance to mechanical and enzymatic destruction.
Aim: The present study addresses the effect of certain NET components (DNA and histones) on clot lysis expanding our previous observations from purified fibrin to more physiologically relevant conditions in a more complex system including plasma clots and human neutrophil leukocytes.
Methods: Neutrophil leukocytes were isolated from the buffy coat of human blood and following activation with phorbol myristic acid (PMA) or N-formyl-methionine-leucine-phenylalanine (fMLP) the cells were incorporated in fibrin clots. Scanning electron microscopy (SEM) was used to evaluate the structure of cell-fibrin clots. Penetration of a yellow fluorescent protein-fusion variant of tissue plasminogen activator (tPA-YFP) in plasma clots supplemented with Alexa-labeled fibrinogen was monitored by laser scanning confocal microscopy (LSM) as an indicator of tPA-induced lysis. Plasmin-catalyzed lysis of fibrin and plasma clots was followed with time lapse photoscanning of the transparent fluid/opaque clot boundary in 0.4 mm high channels of microslides (IBIDI[TRADEMARK]). Turbidimetric assay was applied as an additional tool for studying the effects of NET components on plasma clot lysis. Residual plasmin activity measured on a synthetic chromogenic plasmin substrate (Spectrozyme-PL) was used to monitor the time course of plasmin inactivation by defibrinogenated plasma and pure α_2-plasmin inhibitor.
Results: SEM evidenced that PMA and fMLP-activated neutrophils produced a meshwork of fine fibers (diameter in the range of 10 nm) tangled in the fibrin scaffold composed of fibers about 10-fold thicker than the NET filaments. In the functional fibrinolytic assays this meshwork was modelled with the addition of DNA and histones to clotting blood plasma. According to LSM studies, addition of DNA and histones at 0.05–0.2 mg/mL inhibited penetration of tPA in plasma clots: the average distance for penetration over 30 min decreased by 28% in line with changes in the generated plasmin activity detected with Spectrozyme-PL. If lysis of clots prepared in microslides was initiated with plasmin applied to their surface, DNA and/or histones had negligible effect on the resolution of pure fibrin, whereas in plasma clots DNA slowed down the movement of the lysis front by 50% and histones partially reversed this effect. DNA enhanced the inhibition of plasmin by defibrinogenated plasma (plasmin activity was 29% and 37% lower after 10 and 40 s of inactivation, respectively, compared to the DNA-free assay), but the interaction of plasmin with isolated α_2-plasmin inhibitor was not significantly affected by DNA.
Conclusion: NET constituents hamper both tPA- and plasmin-induced fibrinolysis on the surface of plasma clots through effects on plasminogen activation and plasmin inactivation. Expanding previous work with purified components, the present data provide further support for NET-dependent modulation of fibrinolysis relevant to deep vein thrombosis in inflammatory diseases or clots formed on atherosclerotic plaques with necrotic debris.

AS 24 – Pleiotropic Effects of Fibrinogen

AS 24

Fibrinogen and β-amyloid association alters thrombosis and fibrinolysis: a possible contributing factor to Alzheimer's disease

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The Tissue Factor (TF) pathway is directly linked to physiological repair mechanisms and the host defense against infection. TF is induced by inflammatory mediators in myelo-monocytic cells where danger signals from dying cells or associated with complement activation regulate TF procoagulant activity dependent on protein disulfide isomerase, coupling the initiation of inflammation with local activation of the coagulation cascade. The role of TF is not restricted to the initiation of coagulation and, in addition, signaling of TF protease complexes plays a pivotal role in the regulation of innate immune responses and inflammation. Recent data show that the TF pathway is a critical inducer of the chronic inflammation in obesity. TF adipocyte signaling contributes to the development of obesity, whereas TF expressed by adipose tissue macrophages regulates their inflammatory phenotype. Loss of TF-PAR2 signaling in the hematopoietic compartment of obese mice attenuates adipose tissue inflammation and promotes a switch of adipose tissue macrophages to an anti-inflammatory phenotype typically seen in lean mice. TF forms two distinct signaling complexes. The binary, coagulation inactive TF-VIIa complex interacts with 1 integrins and only cleaves PAR2. The ternary TF-VIIa-Xa complex mediates Xa-dependent activation of PAR1 or PAR2. Xa signaling in this complex is strictly dependent on the endothelial protein C receptor (EPCR). Macrophages found in adipose and tumor tissues express components of these signaling complexes. Further insight into the control of macrophage phenotypes by the TF pathway is expected to emerge from the characterization of regulated expression and interactions of TF signaling components in macrophages during acute and chronic inflammation.

Alzheimer's disease (AD) is a neurodegenerative disorder in which vascular pathology plays an important role. Since the -amyloid peptide (A) is a critical factor in this disease, we examined its relationship to fibrin clot formation in AD. In vitro and in vivo experiments showed that fibrin clots formed in the presence of A are structurally abnormal and resistant to degradation. Fibrin(ogen) was observed in blood vessels positive for amyloid in mouse and human AD samples, and intravital brain imaging of clot formation and dissolution revealed abnormal thrombosis and fibrinolysis in AD mice. Moreover, depletion of fibrinogen lessened cerebral amyloid angiopathy pathology and reduced cognitive impairment in AD mice. These experiments suggest that one important contribution of A to AD is via its effects on fibrin clots, implicating fibrin(ogen) as a potential critical factor in this disease.

AS 24.1

Fibrinogen promotes obesity and high fat diet-associated diseases through a mechanism linked to its aMβ2 binding motif

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Obesity is a worldwide epidemic that predisposes individuals to numerous comorbidities including atherosclerosis, hypertension, type II diabetes, and non-alcoholic fatty liver disease (NAFLD). Systemic inflammation has been proposed as a contributing factor in virtually all of the obesity-associated diseases. Notably, obesity is further characterized by enhanced coagulation activity including extravascular fibrin deposition in multiple organ systems. Fibrin(ogen) has been shown to promote pro-inflammatory activity through engagement of the leukocyte integrin receptor $\alpha_M\beta_2$ (Mac-1). To determine if fibrin (ogen)/$\alpha_M\beta_2$ interactions promote the development of obesity-associated pathologies, we fed wild-type mice and mice expressing a mutant form of fibrinogen that retains normal clotting function, but lacks the leukocyte integrin receptor $\alpha_M\beta_2$ binding motif (i.e. Fib$\gamma^{390-396A}$ mice) a high fat diet (HFD) for up to 20 weeks. Intriguingly, Fib$\gamma^{390-396A}$ mice gained significantly less weight following HFD challenge relative to wild-type animals. Differences in adipose tissue mass, but not lean tissue mass, as determined by EchoMRI analysis, directly correlated with differential weight gain in HFD fed wild-type mice compared to Fib$\gamma^{390-396A}$ mice. In agreement with analysis of body composition, the weights of multiple white adipose tissue depots, as well as brown adipose tissue, were reduced in Fib$\gamma^{390-396A}$ mice at the end of the 20-week challenge period. The genotype-dependent difference in body and adipose tissue weight could not be accounted for by differential consumption of HFD. In concert with significant changes in body weight, Fib$\gamma^{390-396A}$ mice displayed significantly lower blood fasting glucose levels when analysed at 12, 14 and 18 weeks on HFD. Fib$\gamma^{390-396A}$ mice were also found to be profoundly resistant to HFD-induced NAFLD development. Specifically, Fib$\gamma^{390-396A}$ mice exhibited significantly reduced histological evidence of hepatic steatosis and inflammation, lower hepatic triglyceride levels, and reduced hepatic mRNA levels of pro-inflammatory mediators known to exacerbate NAFLD (i.e. TNFα, MCP-1). These data suggest fibrin(ogen) influences the pathogenesis of obesity-driven disease sequelae by controlling both local inflammatory processes and by an unanticipated, and as yet uncharacterized, mechanism linking fibrin(ogen) to HFD-induced increase in adiposity.

AS 24.2

Plasma fibronectin supports hemostasis, controls the diameter of fibrin fibers, and regulates thrombosis

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Background: Plasma fibronectin (pFn) has long been suspected to play a role in thrombosis/hemostasis, but this has remained controversial. We previously demonstrated that pFn supports thrombosis in pFn-conditional deficient mice (PNAS, 2003). Interestingly, depletion of pFn in fibrinogen (Fg)/VWF−/− mice resulted in enhanced platelet aggregation and thrombus formation in these mice, revealing a functional switch of pFn in the absence of Fg or VWF (Blood, 2009). However, the mechanism controlling this switch is unknown. Furthermore, the hemostatic function of pFn is unclear.

Methods: In addition to triple deficient mice (Fg/VWF/pFn−/−, TKO), we established Fg/pFn−/− and VWF/pFn−/− mice by crossing pFn conditional knockout mice with VWF−/− or Fg−/− mice. Injection of polyI-polyC into Cre+ mice resulted in depletion of pFn in plasma and platelets by over 98% and 80%, respectively. In contrast, there was no depletion of pFn when polyI-polyC was injected into Cre- littermate controls. To avoid contamination with Fg, pFn was purified from Fg−/− mice.

Results: TKO mice had significantly higher mortality (23.1% vs. 9.1%) and longer tail bleeding time than their pFn+ Fg/VWF−/− litter-

mates. Autopsy revealed severe subcutaneous or abdominal bleeding. pFn depletion in Fg−/− mice also increased mortality from 5.5% to 25.6% and prolonged bleeding times; the latter effect was reversed with pFn infusion. Using intravital microscopy, we observed rapid deposition of fluorescently-labeled pFn at sites of vessel injury in wild-type, Fg−/−, Fg/VWF−/−, and β3 integrin−/− mice. pFn deposition preceded significant platelet accumulation, suggesting that pFn is an efficient and rapid hemostatic factor.

Based on thromboelastography, fibrin clots formed in whole blood or platelet-poor plasma from pFn+ mice were significantly stronger than those formed in blood or plasma from pFn−/− mice. Inferior vena cava occlusion after $FeCl_3$ injury was significantly delayed in pFn−/− mice. Fluorescently-labeled pFn was actively recruited into the fibrin network in mouse and human plasma. Under scanning electron microscopy, physiological levels of pFn doubled the diameter of fibrin fibers compared with those formed in pFn−/− plasma, suggesting that pFn contributes to lateral aggregation of fibrin protofibrils.

Interestingly, pFn inhibited platelet aggregation when fibrin was absent. In Fg−/− mice, pFn depletion enhanced aggregation of gel-filtered platelets in response to thrombin and TRAP. By contrast, in VWF−/− mice (with Fg), pFn depletion attenuated the aggregation of gel-filtered platelets in response to thrombin but enhanced TRAP-induced aggregation (which cannot convert Fg to fibrin). Using *ex vivo* perfusion chamber and intravital microscopy, pFn inhibited thrombus formation in Fg−/− mice. Thus, pFn plays a dual role in platelet aggregation based on the presence of fibrin.

Conclusion: pFn contributes to the survival of Fg−/− mice and supports hemostasis via both fibrin-independent and dependent pathways. pFn doubles the diameter of fibrin fibers and enhances the mechanical strength of the clot. In the presence of Fg, pFn augments platelet aggregation, likely because of fibrin-pFn complex formation. By contrast, non-fibrin-linked pFn (at the top of thrombi where thrombin is diluted) inhibits platelet aggregation and prevents excessive thrombosis. Therefore, pFn is likely a crucial supportive factor in hemostasis and a key regulator in thrombosis.

AS 24.3

Inhibition of thrombin-mediated factor V activation as a novel anticoagulant mechanism of fibrinogen ?'

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Background: Besides its well-known procoagulant function as a precursor of fibrin, fibrinogen also plays a less understood anticoagulant role by binding to thrombin and thereby modulating its activity. The alternatively spliced fibrinogen γ' chain exists as a heterodimer with the main fibrinogen γA chain as γA/γ' fibrinogen. Both γA/γA and γA/γ' fibrinogens bind with low affinity to thrombin exosite I, whereas exclusively γA/γ' fibrinogen binds to thrombin exosite II. The binding is mediated through the anionic carboxyl-terminal end of the fibrinogen γ' chain, which contains two sulfotyrosine and seven acidic residues. This high-affinity interaction has been reported to down-regulate some of the procoagulant activities of thrombin, such as factor VIII (FVIII) activation and platelet aggregation.

Aim: To investigate the thrombin-mediated anticoagulant effect of fibrinogen and particularly of fibrinogen γ' in plasma.

Methods: Thrombin generation was measured by Calibrated Automated Thrombography in whole and defibrinated plasma and in plasma supplemented with synthetic peptides containing the unique amino acid sequence of the fibrinogen γ' carboxyl-terminal end and various modifications of the two tyrosine residues at positions 418 and 422. The peptides were used as a model for the fibrinogen γ' chain domain that binds to thrombin exosite II. The effect of the doubly sulfated peptide on thrombin-mediated factor V (FV) activation was studied in model systems and in plasma.

Results: Total fibrinogen prolonged the lag time of thrombin generation when coagulation was triggered with low tissue factor concentrations, but this anticoagulant effect was lost at higher tissue factor concentrations. The fibrinogen γ' peptides prolonged the lag time and also decreased the peak height of thrombin generation determined at low tissue factor. Tyrosine sulfation, rather than phosphorylation, considerably enhanced the effect of the peptides. The doubly sulfated peptide was the most potent in a dose-dependent manner, whereas a scrambled control peptide was ineffective. Interestingly, the effects of the doubly sulfated peptide on thrombin generation persisted in the presence of an anti-FVIII antibody, a condition where the feedback loops of thrombin on FVIII and factor XI do not contribute to thrombin generation and FV is the only relevant substrate of thrombin. This suggested that the fibrinogen γ' peptide may inhibit thrombin-mediated activation of FV. This was subsequently confirmed in model systems and in plasma, where the fibrinogen γ' peptide strongly reduced the rate of FV activation by thrombin.

Conclusions: Total fibrinogen and the fibrinogen γ' carboxyl-terminal peptide have an overall anticoagulant effect on thrombin generation determined at low procoagulant stimuli. Inhibition of thrombin-mediated FV activation by the fibrinogen γ' peptide is a novel aspect of the anticoagulant activity of fibrinogen γ'.

AS 25 – Contact Activation 2.0

AS 25.1

Role of cell-free DNA in sepsis pathophysiology: novel studies in murine models of sepsis

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Background: Sepsis is characterized by a dysregulated host response to microbial infection of the blood resulting in the systemic activation of coagulation and inflammation. We have recently observed that high levels of cell-free DNA (cfDNA) released in the circulation is a powerful prognostic marker in severe sepsis patients. However, the potential pathogenic effects of cfDNA itself is unknown.

Aims: Our objective is to investigate the role of cfDNA in experimental sepsis, its association with the inflammatory and procoagulant responses, as well as to explore the potential therapeutic effects of degrading cfDNA.

Methods: Healthy C57Bl/6 male mice were subjected to cecal ligation and puncture (CLP), a sepsis-inducing procedure involving ligation of the cecum distal to the ileocecal valve and two punctures of the ligated cecum (100% at 24 h) or sham surgery (no ligation or puncture). Blood was collected from 0 to 10 h post-surgery and plasma levels of cfDNA and IL-6 were quantified. Additionally, mice subjected to CLP were injected with DNase (0.20 μg/g) and blood and organs were collected 2 h post-injection to quantify plasma levels of cfDNA and IL-6 and to examine organ histology. This was repeated in a lipopolysaccharide (LPS, 0.04 μg/g) model of inflammation.

Results: Baseline levels of cfDNA in healthy mice were 1.1 ± 0.2 μg/mL. At 6 h post-CLP, cfDNA levels increased more than two-fold compared to non-septic mice subjected to sham surgery (5.5 ± 0.4 vs. 2.4 ± 0.2 μg/mL; $P < 0.01$) and remained elevated for 10 h post-CLP. Increases in levels of IL-6 in septic mice accompanied the rapid, time-dependent elevations in cfDNA. Levels of cfDNA and IL-6 in sham-operated, non-septic mice did not change significantly over the 10 h period. Injection of DNase in septic mice resulted in suppressed levels of cfDNA (3.75 ± 0.6 μg/mL DNase-treated vs. 6.35 ± 0.2 μg/mL untreated; $P < 0.01$) comparable to cfDNA levels of nonseptic mice as well as reduced levels of IL-6. In histological studies, photomicro-

graphs of hematoxylin and eosin stained organ sections showed decreased leukocyte infiltration, red blood cell congestion, and microvascular clotting in the lungs and kidneys of septic mice treated with DNase. Similar to observations made in the CLP model, LPS-challenged mice had significant elevations in cfDNA compared to healthy controls (6.24 ± 0.3 vs. 2.1 ± 0.2 μg/mL respectively; $P < 0.01$) which were also suppressed following DNase injection (4.72 ± 0.1 μg/mL; $P < 0.01$). The suppressed levels of cfDNA in DNase-treated septic mice correlated with reductions in IL-6 and improved organ pathology compared to untreated septic mice, suggesting a potential association between cfDNA and inflammatory pathways activated in sepsis.

Conclusions: These studies demonstrate that rapid elevations in cfDNA accompany an early proinflammatory response in experimental sepsis, supporting the importance of early intervention (i.e. administration of resuscitation fluids and antibiotics within 1 h of diagnosis). Furthermore, modifying cfDNA in septic and LPS-challenged mice via treatment with DNase not only causes a reduction in cfDNA levels but may also suppress the inflammatory response and improve organ pathology. These findings suggest that early therapeutic interventions which modulate levels of cfDNA may improve clinical outcome of septic patients.

AS 25.2

Inhibition of factor XII activation reduces experimental thrombus formation in baboons

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Background: Factor XI (FXI) activation by activated factor XII (FXIIa) contributes significantly to vascular occlusion in murine thrombosis models. However, the importance of this reaction to thrombosis in primates, including humans, has yet to be established. Indeed, while low FXI levels are associated with reduced risks for arterial and venous thrombosis in humans, a similar relationship between factor XII (FXII) and thrombosis has not been conclusively observed, in contrast to experimental data in mice.

Aims: We sought to identify a role for FXII in primate thrombosis using a novel monoclonal antibody (15H8) that binds to the fibronectin type I domain of FXII and inhibits FXII activation on polyanionic surfaces.

Methods: The hybridoma clone secreting the monoclonal IgG 15H8, which prevents arterial thrombosis in FXII-deficient mice reconstituted with human FXII, was generated by immunizing FXII-deficient mice with human FXII using standard techniques. The antithrombotic effects of 15H8 were evaluated in an established baboon thrombosis model by comparing platelet and fibrin deposition within a thrombogenic device (TD) in 15H8- and vehicle-treated animals. The TD was placed for 1 h in a chronic femoral arteriovenous shunt. The TD consisted of a proximal collagen-coated vascular graft segment to initiate thrombogenesis (4 mm i.d.) and an expansion chamber (9 mm i.d.) 20 mm distal to the graft. The proximal graft segment is intended to model the flow rates of large arteries, while the thrombus chamber creates shear rates more typical of the venous circulation. Impairment of hemostasis was evaluated by template bleeding times and bleeding volumes.

Results: Saturating concentrations of 15H8 in normal human plasma prolonged the aPTT 1.9-fold, while baboon aPTTs were prolonged up to 3.6-fold. An intravenous bolus of 15H8 (6 mg/kg) prolonged the aPTT in baboons by 1.7-fold ($n = 4$) within 10 min, corresponding to a > 99% inhibition of circulating FXII procoagulant activity. The effect lasted for > 24 h. An hour following 15H8 infusion, a $49 \pm 8\%$ reduction in platelet accumulation was observed in the expansion chamber compared to vehicle-treated controls ($P < 0.02$, $n = 4$–9), while fibrin accumulation was reduced by $95 \pm 1\%$. Interestingly, while platelet deposition was not affected within the collagen-coated segment of the TD that models arterial-type thrombosis, fibrin deposition was reduced by $70 \pm 5\%$ ($P < 0.05$) compared with controls. Bleeding times and bleeding volumes were not affected by 15H8 treatment.

Summary/Conclusion: Inhibition of FXII activation produces an antithrombotic effect in our baboon model. 15H8 appears to be more effective at limiting thrombosis on collagen-coated grafts at shear rates typically seen in the venous circulation than under arterial shear. Previously, we showed that inhibition of factor IX activation by activated FXI significantly reduces collagen- or tissue factor-initiated platelet deposition under arterial shear rates in this model. Given this, the present data suggest that activation of FXI by enzymes other than FXII (e.g. thrombin) may be more important for arterial-type thrombus formation. In summary, our data further delineate the role of FXII in thrombosis and hemostasis. Moreover, the results support the hypothesis that pharmacological inhibition of FXII activation may provide safe thromboprophylaxis.

AS 25.3

Blood-borne and wall-derived DNA as a kinetically significant contact pathway activator: DNase inhibits thrombin formation *in vitro* and *in vivo*

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Given recent findings of reduced thrombus stability in the Factor XII knockout mouse and the discoveries of platelet-released polyphosphate and neutrophil-released extracellular traps (NETs), we investigated the source and potency of various contact activators. We report that addition of recombinant DNase (rDNase) dramatically delayed thrombin production in recalcified citrated PFP, PRP, and whole blood. Using very low levels of corn trypsin inhibitor (5 μg/mL) added to human whole blood, rDNase (1 mg/mL) delayed thrombin production, while RNase (1 mg/mL) or alkaline phosphatase (0.1 mg/mL) had no effect, with similar outcomes in treated recalcified citrated PRP (no neutrophils observed in PRP wells at 20× magnification). Direct visualization of annexinV-positive platelets revealed the exposure with time of Sytox-Green (a cell impermeable DNA dye) staining nodules as well as detection of ~5 μ long thin strands linked to platelets or in solution, a size consistent with the size of mitochondrial DNA (mtDNA). Platelet mtDNA was elevated (by qPCR) in plasma obtained from convulxin-stimulated PRP and purified platelet mtDNA caused a modest acceleration of thrombin production in recalcified PFP, PRP, and whole blood. This is new evidence for platelet-derived, blood-borne mtDNA as a contact activator following platelet activation. In the arteriole laser injury model using wildtype mouse, rDNase delayed thrombin production (detected with an anti CD41-linked thrombin sensor), delayed platelet accumulation, resulted in less platelet accumulation, and enhanced clot instability. The rDNase caused a 75% reduction ($P < 0.05$, $n = 8$) of wall localized Sytox staining at the injury site and 25% reduction ($P < 0.05$, $n = 11$) of the faint and punctate staining of the thrombus mass. We conclude wall exposed DNA is a major activator of the contact pathway during laser injury, with staining evidence for the existence of clot-derived DNA that was too small to originate from neutrophils. While DNA has been recognized as a contact activator, this evidence provides new insights into its source, locality, and role in the laser injury model *in vivo* as well as its role in the clotting of blood *in vitro*. Understanding the most proximal triggers of contact activation during arterial thrombosis may be particularly relevant to the development of safe anticoagulants with reduced bleeding risk.

AS 26 – Assays for Haemostatic Drugs

AS 26.1

Evaluation of whole blood clotting activity of recombinant factor VIII Fc fusion protein by ROTEM analysis in a multi-center Phase 3 clinical trial

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Background: The recombinant coagulation factor VIII Fc (rFVIIIFc) fusion protein consists of a single molecule of human factor VIII covalently linked to the dimeric Fc domain of human immunoglobulin G1 (IgG_1). The rFVIIIFc utilizes the FcRn receptor-mediated immunoglobulin cycling pathway to extend its plasma half-life. In a global, multi-center, phase 3 clinical study (A-LONG), rFVIIIFc demonstrated effective prevention and control of bleeding episodes in 1–2× weekly prophylactic dosing intervals.

Aims: To evaluate the whole blood clotting potential of rFVIIIFc in comparison to a currently marketed rFVIII product by *ex vivo* rotation thromboelastometry (ROTEM) of post-infusion samples.

Methods: Thirteen clinical sites (5 US, 5 EU, 1 IL, 1 AU, and 1 JP) performed ROTEM analysis as part of the pharmacokinetic evaluation of 44 subjects treated with rFVIIIFc. All participating sites were trained on a standardized ROTEM procedure and used the same lots of custom reagents, supplies and assay controls. Citrated whole blood samples were rested at ambient temperature for 30–45 min before initiating clot formation by NATEM (recalcification), INTEM (1:300 dilution of the ellagic acid in-tem reagent) or EXTEM (1:10,000 dilution of the recombinant TF ex-tem reagent). Four key ROTEM parameters (clot time [CT], clot formation time [CFT], α-angle, and maximum clot firmness [MCF]) were evaluated relative to FVIII activity determined by the one-stage clotting assay at a central laboratory. Informed consent was obtained from all participating subjects and the study was approved by local site medical ethics committees.

Results: ROTEM results from 16 subjects that had complete pharmacokinetics (PK) profiles for rFVIII and rFVIIIFc, followed by a repeat rFVIIIFc PK profile approximately 3 months later, were included in this report. The 77 rFVIII and 143 rFVIIIFc samples showed a dose-dependent response for CT, CFT and α-angle through the entire range of measurable FVIII activity (0.5% to > 100%). MCF was largely unaffected by the amount of FVIII. Despite significant inter-subject differences in the ROTEM profiles, the mean whole blood clotting activity over time profiles for rFVIIIFc compared to rFVIII closely mirrored the PK extension observed for plasma rFVIIIFc activity. Whole blood clotting activity was, on average, comparable for rFVIII and rFVIIIFc at the first post-dosing time point, while the ROTEM activity seen for Advate 48 h after dosing was comparable to the activity of rFVIIIFc at 72 h. Regression analysis of the integrated ROTEM data vs. FVIII activity showed parallel and comparable ROTEM responses for rFVIIIFc and rFVIII at equivalent FVIII activities.

Summary/Conclusions: *Ex vivo* ROTEM analysis in 16 subjects demonstrated prolonged improvements in clot formation consistent with the reduced rate of plasma clearance observed for rFVIIIFc compared to rFVIII. Equivalent whole blood coagulation potential was demonstrated for rFVIIIFc and rFVIII in a physiologically relevant setting. The comparable ROTEM response for both products throughout the assay range confirms our previous biochemical assessment of rFVIIIFc demonstrating full coagulation potential of the rFVIII fusion protein.

AS 26.2

Activated factor VII: antithrombin complex plasma concentration is an independent predictor of total and cardiovascular mortality in patients with angiographically demonstrated coronary artery disease

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Background: Plasma concentration of activated factor VII (FVIIa)-antithrombin (AT) complex has recently been proposed as a marker of intravascular exposure of tissue factor (TF) and, thus, of activation of the extrinsic pathway of coagulation cascade. However, the results of studies investigating the potential relationship between FVIIa-AT complex and coronary artery disease (CAD) are only preliminary so far.

Aims: The aim of this study was to investigate the predictive value of FVIIa-AT complex plasma concentration for total and cardiovascular mortality in the setting of secondary prevention of CAD.

Methods: Within the framework of the Verona Heart Study (VHS), a regional survey aimed to search for new risk factors for cardiovascular disease, we selected a cohort of 510 patients with angiographically proven CAD (mean age 61.8 ± 10.6 years; females 23.7%), who were not taking anticoagulant drugs at time of enrolment and for whom plasma citrate samples for FVIIa-AT complex assay were available. These patients were prospectively followed for a median period of 64 months. Plasma concentration of FVIIa-AT complex was determined by ELISA.

Results: The majority of patients had severe CAD and received surgical or endovascular coronary revascularization during the period 1999–2006 (81.8%). Eight patients had peri-operative deaths and were excluded from subsequent analyses. During follow-up, 105 (20.9%) subjects died, with 68 (13.5%) events attributed to cardiovascular causes. CAD patients who died had a significantly higher plasma concentration of FVIIa-AT complex than those survived ($P = 0.002$). Stratifying the study population on the basis of the FVIIa-AT complex quartiles, we noted an evident increase of both total and cardiovascular mortality for the subjects in the two upper quartiles. CAD patients with plasma concentration of FVIIa-AT higher than the median value (79 pM) had an increase in both total and cardiovascular mortality than those with lower values (26.7% vs. 15.0%, $P = 0.001$ by Log Rank test, and 17.3% vs. 9.7%, $P = 0.006$ by Log Rank test, respectively).

After adjustment for the other predictors of mortality at univariate analysis (i.e. sex, age, number of coronary vessels involved, MI history, hypertension, diabetes, BMI, renal function, and hs-CRP concentration), elevated FVIIa-AT complex (≥ 79 pM) significantly predicted both total and cardiovascular mortality (HR for total and cardiovascular mortality: 2.24 (1.35–3.70) and 1.92 (1.02–3.61), respectively). Such associations remained significant also after adjustment for left ventricular ejection fraction (HR for total and cardiovascular mortality: 1.91 (1.21–3.03) and 1.86 (1.03–3.34), respectively), as well as for the main cardiovascular therapies at discharge, like beta-blockers, ACE-inhibitors, statins, and antiplatelet/anticoagulant drugs (HR for total and cardiovascular mortality: 1.67 (1.08–2.59) and 1.77 (1.01–3.11), respectively).

Conclusions: In this study high plasma concentrations of FVIIa-AT complex were an independent predictor of both total and cardiovascular mortality in patients with angiographically demonstrated CAD, speculatively reflecting a prothrombotic diathesis due to TF-related activation of coagulation cascade. Thus, FVIIa-AT may be a useful prognostic marker in the setting of secondary prevention of CAD.

AS 26.3

Evaluation of a recombinant inactive antithrombin to reverse fondaparinux anticoagulant activity by measuring thrombin generation: an asset compared to other non specific hemostatic agents

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Background: Fondaparinux, a synthetic pentasaccharide mediating its anticoagulant activity through antithrombin (AT) activation, is now widely used for preventive and curative treatment of thrombosis. This drug exhibits advantageous pharmacokinetic and safety profiles but no antidote is still available to reverse fondaparinux overdosing and limit associated blood losses. However, non-specific procoagulant products such as recombinant factor VIIa (rFVIIa) or activated prothrombin complex concentrates (aPCCs), have been tested but with limited evidence of efficacy. To secure the clinical use of fondaparinux, we have already designed a recombinant inactive AT (iAT) that retains its pentasaccharide binding capacity. Such an iAT traps fondaparinux in an inactive complex, and might thus act as a potent reversal agent in situations of fondaparinux overdosing.

Aims: Inactive AT efficiently reverses fondaparinux *in-vitro* and *in-vivo* when assayed by chromogenic anti-factor Xa activity. The goal of this study was to compare the asset of iAT as a specific antidote for fondaparinux, as compared to nonspecific hemostatic agents (rFVIIa and aPCC).

Methods: The impact on coagulation of iAT, rFVIIa and aPCC was evaluated by thrombin generation assay in platelet-poor plasma (PPP) overloaded or not with a supratherapeutic dose of fondaparinux.

Results: The anticoagulant effect of a high dose of fondaparinux (3 mg/L) used to mimic overdosing was evidenced by perturbation of the main thrombogram parameters, in particular by a longer lag time (7.3 ± 0.4 min vs. 2.9 ± 0.3), and both decreased endogenous thrombin potential (ETP) (275 ± 76 nM.min vs. 1741 ± 282) and thrombin peak height (20 ± 5 nM vs. 294 ± 37). As previously shown, addition of increasing rFVIIa concentrations (0.3–2.4 μg/mL) in fondaparinux-overloaded plasma restored a normal lag time though non dose-dependently, while other thrombogram parameters were unchanged. In contrast, aPCC (from 0.5 tio 4 U/mL) shortened the lag time and slightly increased the ETP, which nevertheless remained 2.4-fold lower than the ETP measured in plasma without fondaparinux. Under the same conditions, iAT dose-dependently shortened the lag time, increased the ETP and the thrombin peak height. For example, the estimated ETP in the presence of iAT at concentrations of 0.075, 0.15, 0.3, 0.6, and 1.2 g/L in fondaparinux overloaded plasma were 397 ± 85, 550 ± 77, 900 ± 101, 1331 ± 454, and 1360 ± 380 nM.min, respectively. Strikingly, the same concentrations of iAT in fondaparinux-free plasma had no impact on the thrombin generation curves, whereas rFVIIa only significantly shortened the lag time and aPCC (1 U/mL) both shortened the lag time and greatly increased the ETP by 178%.

Conclusions: This study confirms the potent effect of our recombinant iAT as a specific reversal agent to fondaparinux in a thrombin generation assay. Indeed, iAT normalized thrombin generation curves and parameters measured in fondaparinux-overdosed plasma, Compared to non-specific hemostatic agents, our iAT appears much more efficient to reverse fondaparinux anticoagulant activity and overall could be considered much safer since it appears devoid of any anticoagulant or procoagulant activity by itself.

AS 27 – Platelet Response to Injury

AS 27.1

A talin mutant that disrupts talin-integrin binding in platelets decelerates aIIbβ3 activation without pathological bleeding

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Background: Tight regulation of integrin affinity is a critical component of thrombosis and hemostasis. The final step of integrin inside-out activation is the binding of the cytoskeletal protein talin to two distinct sites within the integrin β cytoplasmic tail. First, binding of talin to an integrin NPLY sequence provides most of the binding energy. Second, a weaker interaction with the membrane-proximal region (MPR) of the integrin is required for talin-mediated integrin activation. Selective deletion of talin1 from platelets and megakaryocytes ($Tln1^{fl/fl}$Pf4-Cre+) completely prevents agonist-induced activation of α2β1 and αIIbβ3 integrins, impairs thrombus formation and results in profound defects in hemostasis. Interestingly, expression of a talin1 mutant (L325R) that lacks the ability to bind the MPR, but can still bind the NPLY sequence, phenocopies the integrin activation and hemostatic defects observed in mice with talin-deficient platelets. In transfected cells, a talin1 mutant (W359A) with a markedly impaired ability to bind the NPLY region of the β3-integrin tail did not rescue the talin1 knockdown phenotype.

Aim: In this study we sought to analyze the effects of a platelet-specific talin1 mutation (W359A) on thrombosis and hemostasis.

Methods: Homozygous knock-in of talin1(W359A) is embryonic lethal in mice. Thus, $Tln1^{W359A/wt}$ mice were crossed with $Tln1^{fl/fl}$PF4-Cre+ mice to generate $Tln1^{W359A/fl}$PF4-Cre+ ($Tln^{W359A/ko}$) and $Tln1^{wt/fl}$ PF4-Cre+ ($Tln^{wt/ko}$) control mice. aIIbb3 integrin activation and granule secretion of mouse platelets was monitored by flow cytometry and standard aggregometry. Adhesion under static conditions was determined by measuring spreading of ADP-stimulated platelets on fibrinogen-coated slides. For adhesion studies under flow conditions murine whole blood was perfused over fibrillar collagen type I. Thrombosis *in vivo* was studied by applying 5% $FeCl_3$ to the carotid artery for 3 min and by monitoring the flow rate with an ultrasound flow probe. Hemostasis was determined both by tail-bleed and gastrointestinal-bleed assays.

Results: Expression of talin1(W359A) in platelets partially rescued talin1 deficiency. Compared to $Tln^{wt/ko}$ controls, agonist-induced αIIbβ3 activation was reduced by ~50% and both granule release and spreading on fibrinogen were only moderately impaired in $Tln^{W359A/ko}$ platelets. However, kinetic studies demonstrated decelerated αIIbβ3 activation in $Tln^{W359A/ko}$ platelets, which resulted in delayed aggregation under static conditions and reduced thrombus size at low shear rates. Interestingly, adhesion of $Tln^{W359A/ko}$ platelets to collagen at high venous and arterial shear rates was not significantly better than that of talin-deficient cells and $Tln^{W359A/ko}$ mice were completely protected from $FeCl_3$-induced carotid artery occlusion. However, we reproducibly observed a distinct thin layer of platelets adhering to the carotid artery 20 min after injury. Lastly, in contrast to platelet-specific talin-knockout or $Tln^{L325R/ko}$ mice, $Tln^{W359A/ko}$ mice showed no detectable gastrointestinal bleeding and only modestly increased tail-bleeding times compared to littermate controls.

Conclusions: Our studies demonstrate that the talin1(W359A) mutation decelerates, but does not abolish, talin-dependent integrin activation in platelets. Importantly, we also show that the slower kinetics of talin-integrin binding may have beneficial anti-thrombotic effects without increasing the risk of bleeding.

AS 27.2

Tetraspanin Tspan18 regulates GPVI-induced platelet activation by interacting with the store-operated Ca2+ entry channel Orai1

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Background: Intracellular Ca^{2+} elevation following platelet activation regulates shape change, granule secretion, integrin activation and phosphatidylserine exposure. Together these events promote platelet adhesion, aggregation and the coagulation cascade at sites of vascular injury. A major means of Ca^{2+} elevation is across the plasma membrane through Orai1 store-operated Ca^{2+} entry channels. Orai1 is particularly important for platelet activation by the collagen receptor GPVI. Mice lacking platelet Orai1 or GPVI are protected against arterial thrombosis and ischaemic stroke, and both proteins are potential anti-thrombotic drug targets. The tetraspanins are a superfamily of 33 transmembrane proteins in humans, of which at least 10 are platelet-expressed, including the previously unstudied Tspan18. Tetraspanins are emerging as important regulators of the trafficking and/or clustering of certain associated receptors. We have previously identified GPVI as tetraspanin-associated, but how tetraspanins regulate GPVI signaling is unknown.

Aims: The aim of this study was to identify tetraspanins that regulate the GPVI signaling pathway, and to subsequently characterize the previously unstudied tetraspanin Tspan18 using knockout mice.

Methods: A panel of tetraspanins was co-expressed with GPVI in a cell line model to determine which could regulate GPVI signaling. The readout was activation of the Ca^{2+}-responsive nuclear factor of activated T cells (NFAT) transcription factor. Cells deficient for various intracellular signaling proteins were used to characterize the mechanism by which the identified tetraspanin, Tspan18, regulated the GPVI signaling pathway. Platelet function was then assessed in the Tspan18 knockout mouse. To address the mechanism of Tspan18 function, its interaction with Orai1 was assessed biochemically.

Results: Expression of the previously uncharacterized tetraspanin Tspan18 in a cell line model promoted GPVI-induced NFAT activation. Moreover, Tspan18 expression in the absence of GPVI was sufficient to activate NFAT, raising the possibility of an effect on Ca^{2+}/NFAT downstream of GPVI. Other platelet tetraspanins did not have this capacity, suggesting a unique function for Tspan18. The active site of Tspan18 mapped to a 61 amino acid portion of the main extracellular region, which is known to mediate specific protein-protein interactions within the tetraspanin superfamily. Tspan18-induced NFAT activation did not require signaling proteins upstream of store-operated Ca^{2+} entry, but did require functional Orai1 channels. The Tspan18 knockout mouse exhibited defective haemostasis, since five-fold more blood was lost than wild-type mice in a tail bleeding assay. Tspan18-deficient platelets were defective in aggregation and secretion in response to the GPVI-specific agonist collagen-related peptide, although the aggregation defect could be overcome at high concentration of agonist. Surface levels of GPVI and other major glycoproteins were normal, as were platelet number and size. Platelet aggregation to thrombin was unaffected by the absence of Tspan18. These observations are similar to those previously reported for mice with Orai1-deficient platelets. Consistent with a role for Tspan18 in regulating Orai1, Tspan18 co-immunoprecipitated with Orai1 but other platelet tetraspanins did not.

Summary/Conclusions: We have characterized a new platelet tetraspanin Tspan18 that positively regulates GPVI signalling via interaction with the store-operated Ca^{2+} entry channel Orai1. The Tspan18-Orai1 complex is a potential anti-platelet drug target for the prevention and treatment of thrombosis.

AS 27.3

Integrin alpha6beta1 is the main receptor for vascular laminins and plays a role in platelet adhesion, activation and arterial thrombosis

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Background: Laminins are major components of endothelial and smooth muscle cells basement membranes, well located to interact with platelets upon vascular injury. Laminin-111 ($\alpha_1\beta_1\gamma_1$) is known to support platelet adhesion but is absent from most blood vessels, which contain isoforms with the α_2, α_4 or α_5 chain. The importance of vascular laminins in platelet functions remains unknown.

Aims: The aim of this study was to evaluate (i) the ability of a series of vascular laminin isoforms to support platelet adhesion and activation under hemodynamic conditions, and (ii) the significance of these interactions in hemostasis and thrombosis.

Methods: Glass microcapillaries coated with recombinant human vascular laminins were perfused with hirudinated blood using a syringe pump and platelet adhesion was monitored by differential interference contrast microscopy. Activation of adherent platelets was evaluated by observing morphological changes and cytosolic Ca^{2+} increases. Concerning the second objective, we generated knock-out mice bearing a platelet-specific deletion of the major laminin receptor, and measured their tail transection bleeding time and thrombotic response upon mechanical-, laser- and chemical-induced arterial injury.

Results: We found that laminin-411 ($\alpha_4\beta_1\gamma_1$), laminin-511 ($\alpha_5\beta_1\gamma_1$) and laminin-521 ($\alpha_5\beta_2\gamma_1$), but not laminin-211 ($\alpha_2\beta_1\gamma_1$), allow efficient human platelet adhesion across a wide range of arterial wall shear rates up to 5000/s. In addition, platelets adhering to laminin-411, laminin-511 and laminin-521 became activated, as indicated by their shape change with filopodia extension and cytosolic Ca^{2+} oscillations. Use of blocking antibodies revealed that platelet/laminin interactions were critically dependent on integrin $\alpha_6\beta_1$. Therefore, to address their *in vivo* significance, we developed a platelet-specific knock-out of integrin α_6 ($\alpha_6^{-/-}$). As expected, integrin $\alpha_6\beta_1$ was not critical for platelet activation in response to a series of agonists (ADP, TxA_2 mimetic, thrombin, PAR-4 agonist peptide, collagen, collagen-related peptide), as indicated by normal aggregation, binding of soluble fibrinogen and P-selectin exposure in $\alpha_6^{-/-}$platelets. In contrast, adhesion of these platelets to vascular laminins under flow was abrogated, whereas it remained unchanged on other subendothelial proteins such as von Willebrand factor, collagen, fibrinogen and fibronectin. Interestingly, compared to their wild-type littermates, $\alpha_6^{-/-}$ mice exhibited a marked inhibition of 69 ± 10% ($n = 6$, $P < 0.05$) in thrombosis induced by endothelium denudation of the common carotid with a guide wire. Following compression of the abdominal aorta with forceps, thrombus formation was likewise reduced by 56 ± 12% ($n = 5$, $P < 0.01$). A more pronounced decrease in thrombosis of 82 ± 3% ($n = 12$, $P < 0.01$) was observed in a model of moderate laser injury removing the endothelial cell layer of mesenteric arterioles. In contrast, $\alpha_6^{-/-}$ mice did not exhibit any protection after a deeper laser lesion exposing the media and adventitia, and following $FeCl_3$ injury of the common carotid artery. The tail bleeding time and volume of blood lost remained unchanged in $\alpha_6^{-/-}$ mice, suggesting normal hemostasis.

Conclusion: This study reveals an unsuspected important contribution of laminins to platelet adhesion and aggregation upon arterial injury depending on the type and severity of lesion. From a clinical point of view, targeting their main receptor, integrin $\alpha_6\beta_1$, could represent an alternative antithrombotic strategy with a potentially low bleeding risk.

AS 28 – Thrombocytopenia

AS 28.1

Impact of an avoid-heparin program on the incidence, clinical consequences and resource use associated with heparin-induced thrombocytopenia (HIT)

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Background: Heparin-induced thrombocytopenia (HIT) is a serious complication of heparin, occurring in up to 5% of patients exposed to unfractionated heparin (UFH). In comparison, the risk of HIT among patients exposed to low molecular weight heparin (LMWH) is substantially lower.

Aim: To examine the impact of a hospital-wide HIT reduction strategy, through limiting exposure to UFH and replacing UFH with LMWH, on the incidence of HIT and its consequences.

Methods: A comprehensive 'Avoid-Heparin Program' was implemented at a large university hospital in 2006 as a specific attempt to reduce the burden of HIT and improve patient safety. This involved replacing intravenous and subcutaneous (SC) UFH with SC LMWH for prophylactic and therapeutic indications, and removing UFH from arterial and central venous lines. After 2006, UFH was limited to intraoperative use in cardiovascular surgery and hemodialysis. All cases of suspected HIT from 2003 to 2011 were adjudicated using explicit criteria for negative and positive HIT enzyme-linked immunosorbent assay (ELISA), HIT and HIT with thrombosis (HITT). Outcomes in the pre-intervention phase (2003–2005) were compared to those occurring in the Avoid-Heparin phase (2007–2011). Data from 2006 was not included since this was the program implementation year. There were no changes to the investigation of HIT over the study period.

Results: The annual number of suspected HIT cases decreased 34%, from 85 per 10,000 admissions in the pre-intervention phase to 56 per 10,000 admissions in the Avoid-Heparin phase ($P < 0.001$). The annual number of patients with a positive HIT ELISA decreased 59% from 17 to 7 per 10,000 admissions ($P < 0.001$); adjudicated HIT decreased 73% from 11 to 3 per 10,000 admissions ($P < 0.001$); and HITT decreased 80% from 5 to 1 per 10,000 admissions ($P < 0.001$). Patients with HIT in the pre-intervention phase more frequently developed HITT (43%) than patients with HIT in the Avoid-Heparin phase (20%, $P = 0.044$). The proportion of patients with HIT exposed to LMWH alone was significantly greater in the Avoid-Heparin phase (44%) than in the pre-intervention phase (8%, $P < 0.001$). However, the use of LMWH increased more than six-fold from 2003 to 2011 and the annual rate of LMWH-associated HIT remained constant (at 1/10,000 admissions per year, $P = 1.000$). The hospital expenditure on HIT-safe anticoagulants also decreased 41% in the Avoid-Heparin phase. Patients with HIT in the pre-intervention ($n = 53$) and Avoid-Heparin phases ($n = 25$) had similar characteristics including age, sex, admitting service, duration of heparin exposure and length of stay. We did not identify any other factors that could account for the observed reduction in HIT and HITT.

Summary/Conclusion: Implementation of an 'Avoid-Heparin Program' led to a dramatic decrease in the burden of HIT (suspected HIT, diagnosed HIT and HITT) and in the costs of HIT care. To our knowledge, this is the first study demonstrating the success of a hospital-wide HIT prevention strategy. Our findings suggest a feasible heparin avoidance intervention can improve hospital patient outcomes.

AS 28.2

Platelet-monocyte interactions enhance procoagulant activity in heparin induced thrombocytopenia

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Background: Heparin-induced thrombocytopenia (HIT) is a well recognized complication of heparin therapy. The most feared complication of HIT is the development of thrombosis. HIT is caused by the formation of antibodies against heparin bound to platelet factor 4 (PF4) and the PF4/heparin/antibody (HIT) complexes cause platelet activation via the FcgRIIa receptor. We have previously demonstrated that HIT complexes induce monocyte tissue factor (TF) via the FcgRI receptor.

Aim: The objective of this study was to characterize the role of platelet-monocyte interactions in the HIT complex mediated procoagulant response.

Methods: Peripheral blood mononuclear cells (PBMCs, 1×10^6 per condition) and platelet rich plasma (PRP, 25×10^6 platelets per condition) were isolated from citrated blood donated by healthy volunteers. PBMCs and/or PRP aliquots were incubated with PF4 (10 μg/mL), heparin (1 U/mL), and the PF4-heparin specific antibody KKO (100 μg/mL) at 37°C between 30 min and 6 h based on the experiment. Procoagulant activity (PCA, pg/mL) was measured using a one-stage clotting assay.

Results: Incubation of PBMCs with PRP for 6 h resulted in an increase in PCA compared to PBMCs alone (114 ± 34 vs. 6 ± 1 pg/mL; $n = 4$, $P = 0.02$). Incubation of PBMCs with PF4, heparin and KKO (HIT complex) resulted in significantly increased PCA compared to control ($224 +/- 63$ vs. $6 +/- 1$ pg/mL; $n = 4$, $P = 0.014$). Pre-incubation of PRP with the HIT complex for 30 min followed by incubation with PBMCs did not lead to significant increase in PCA compared to incubation of PBMCs and PRP without the HIT complex (120 ± 50 vs. 114 ± 34 pg/mL; $n = 3$). Combining PBMCs with PRP and incubating them with the HIT complex for 6 h resulted in a further increase in PCA (1114 ± 411 pg/mL; $n = 4$) compared to HIT complex and PBMCs alone. In preliminary experiments, pre-incubation of PRP with the anti-FcgRII antibody IV.3 before combining with PBMCs and incubating for 6 h decreased the PCA by 70% (1704 vs. 504 pg/mL). The addition of a P-Selectin blocking antibody in the PRP+PBMC+ HIT complex experiment did not decrease the PCA compared to PRP+PBMC+ HIT (1704 vs. 2015 pg/mL).

Conclusion: HIT complexes are known to cause activation of both monocytes and platelets. Interestingly, activation of platelets by the HIT complex and subsequent incubation with PBMCs did not cause significant increase in PCA. This suggests that platelet activation in itself doesn't lead to monocyte activation in HIT. On the other hand, when PBMCs and platelets were combined together prior to exposure, the HIT complex caused marked increase in PCA, much greater than the effect of both HIT complexes and LPS on PBMCs. The effect of platelets on the PCA was significantly decreased by blocking FcgRIIa but not by blocking P-Selectin. This suggests that platelet activation enhances the activation of coagulation caused by HIT complexes and that this platelet-monocyte interaction in HIT is not mediated by P-Selectin.

AS 28.3

A mutation in ANKRD18A is associated with a severe congenital thrombocytopenia

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Background: Inherited thrombocytopenias are a group of heterogeneous disorders which are associated with bleeding of all types of

severity depending both on the reduction in platelet count and whether there is co-existing altered platelet function. The condition can be associated with abnormal bleeding ranging from severe bleeding diatheses recognised early in life to mild bleeding which can remain undetected even to advanced adulthood. Here we describe two cousins from a consanguineous family with severe thrombocytopenia and serious clinical bleeding including intracerebral haemorrhage.

Aims: In this study, we used a whole exome-sequencing approach to elucidate the genetic basis of a severe form of congenital thrombocytopenia in two cousins from a consanguineous family.

Methods: We studied a consanguineous family that included two cousins with severe thrombocytopenia. The proband was aged 3 years with a platelet count of 3×10^9/L when first entered into the study. His affected cousin was aged 7 years when recruited into the study and has a baseline platelet count of 15–20 $\times$ 10^9/L. To identify the causative mutation in this family we sequenced the whole exome of the two affected individuals with the SureSelect human AllExon 50 Mb kit (Agilent Technologies) and sequencing on the HiSeq 2000 (Illumina) with 100 bp paired-end reads. To examine whether the mutation within *ANKRD18A* led to hypofunction, we first measured the abundance of *ANKRD18A* mRNA by real time quantitative RT-PCR (qRT-PCR) in the two patients and compared to healthy controls. To analyse expression of *ANKRD18A,* quantification of gene expression was performed using the ABI Prism 7900HT Sequence Detection System. To investigate platelet function in the two patients we initially measured the levels of the surface glycoproteins CD42b (GPIbα), CD41 (αIIb) and GPVI by flow cytometry. Furthermore PRP was stimulated with ADP, collagen-related peptide (CRP) and PAR-1 peptide and the expression of P-selectin and binding of fluorescent fibrinogen was assessed by flow cytometry on an Accuri C6 flow cytometer.

Results: Whole exome sequencing performed in the two patients identified a homozygous in frame deletion mutation (c.2401_2403delGAA) in the ankyrin repeat domain 18A gene (*ANKRD18A*). The mutation removed a glutamic acid residue at position 801 of a coiled-coil domain of the protein (p.Glu801del). RT-PCR analysis in the two affected individuals revealed loss of *ANKRD18A* mRNA expression compared to control samples demonstrating an unstable mutant transcript. In addition to a severely low platelet count, platelet function testing demonstrated a marked impairment of platelet activation by a range of agonists.

Summary/Conclusions: In summary, these findings identify a novel gene that underlies thrombocytopenia and defines the genetic basis of the severe thrombocytopenia in this family. *ANKRD18A* belongs to the same family of proteins that has recently been discovered to be a major cause of a dominant form of thrombocytopenia (THC2). This study led to the identification of mutations in *ANKRD26 gene*, encoding ankyrin repeat domain 26. Therefore the discovery of *ANKRD18A* mutations further implicates ankyrin repeat domain containing genes in the global regulation of platelet formation and possibly platelet function.

AS 29 – Genetics in Coagulation

AS 29
Anticoagulant pathways: genotype-phenotype relationships

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The protein C pathway is a pivotal anticoagulant mechanism which is responsible for the proteolytic inactivation of coagulation factors Va (FVa) and VIIIa (FVIIIa), the essential cofactors of the prothrombinase and tenase complexes, respectively. Several genetic and acquired risk factors for venous thromboembolism (VTE), such as the deficiencies of protein C and protein S, the FV Arg506 Gln (FV Leiden) and prothrombin G20210A mutations, elevated FVIII levels, pregnancy and oral contraceptive use, directly or indirectly hamper the inactivation of FVa and/or FVIIIa by activated protein C (APC). Not surprisingly, a poor anticoagulant response of plasma to APC (APC resistance) is the most common risk factor for VTE. In our laboratory, we use a thrombin generation-based assay to determine plasma APC resistance. In this assay, thrombin generation is measured at ~10 pM tissue factor in the absence and presence of added APC, and the ratio between the endogenous thrombin potentials (ETP) determined in the presence and absence of APC is taken as a measure of plasma APC resistance. Thrombin generation is a global assay that probes all three phases of coagulation (initiation, propagation and termination) and reflects the overall tendency of a plasma sample to clot. Several retrospective and prospective studies have shown a correlation between the ETP or peak height of thrombin generation, especially if determined in the presence of APC, and VTE risk, making thrombin generation a promising intermediate phenotype to discover new (genetic) determinants of hypercoagulability and possibly VTE. In this lecture I will illustrate this concept with practical examples. In particular, I will show that: 1) thrombin generation determined in the absence and presence of APC varies widely among different FV Leiden carriers and is a marker of VTE risk; 2) some of this inter-individual variability can be explained by common genetic variation in haemostasis-related genes; and 3) the genetic dissection of thrombin generation parameters in patient populations may help identify new candidate genes that contribute to VTE risk.

AS 29.1
Fibrinogen ?′ increases the sensitivity to activated protein C in normal and FV Leiden plasma

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Background: Activated protein C (APC) inhibits coagulation by proteolytically inactivating activated factor V (FVa) and activated factor FVIII (FVIIIa). An insufficient response of plasma to APC (APC resistance), which is often due to the FV Leiden mutation, is the most common risk factor for venous thrombosis (VT). In a recent study of 188 FV Leiden carriers we observed an unexpected relationship between carriership of the fibrinogen γ gene (*FGG*) haplotype 2 (H2) and thrombin generation determined at high tissue factor in the presence of APC. The endogenous thrombin potential (ETP_{+APC}) was 397 ± 100 nM.min in *FGG* H2 homozygotes, 338 ± 91 nM.min in heterozygotes and 230 ± 62 nM.min in non-carriers ($P < 0.001$). *FGG* H2, a known risk factor for VT, is associated with reduced levels of the alternatively spliced fibrinogen γ' chain. The latter binds and inhibits thrombin *via* a high-affinity interaction with thrombin exosite II. Occupation of exosite II by the highly anionic carboxyl-terminal peptide of the fibrinogen γ' chain has been shown to interfere with the ability of thrombin to activate both FVIII and (Omarova et al., this congress) FV.

Aims: To investigate the effect of total fibrinogen and fibrinogen γ' on plasma APC resistance.

Methods: Thrombin generation was measured by Calibrated Automated Thrombography in the absence and presence of exogenous APC in: (i) whole and defibrinated plasma, (ii) congenitally fibrinogen-deficient plasma reconstituted with the purified fibrinogen isoforms (γAγA or γAγ'), and (iii) pooled plasma from normal individuals or heterozygous FV Leiden carriers in the presence of a synthetic fibrinogen γ' carboxyl-terminal peptide. The ratio of the ETPs determined in the presence and absence of APC was taken as a measure of plasma APC resistance.

Results: Whole plasma was less APC-resistant than defibrinated plasma, suggesting that (total) fibrinogen improves the response of plasma to APC. Reconstitution experiments in fibrinogen-deficient

plasma indicated that this phenomenon is mainly attributable to the γA/γ' fibrinogen isoform, with γAγA fibrinogen having only a minor effect. The synthetic fibrinogen γ' carboxyl-terminal peptide (added to plasma at a final concentration of 250 μM) considerably increased the APC sensitivity not only of normal pooled plasma, but also of pooled plasma from FV Leiden heterozygotes. These effects were specific, as a scrambled control peptide was ineffective. Moreover, the APC-sensitizing action of the fibrinogen γ' peptide was not abolished by the addition of an anti-FVIII antibody, indicating that the effect of fibrinogen γ' on the APC response is not (exclusively) mediated by FVIII, but probably also by FV.

Conclusions: Total fibrinogen and especially fibrinogen γA/γ' increase the APC sensitivity of normal and FV Leiden plasma. This APC-sensitizing effect can be reproduced by the carboxyl-terminal peptide of the fibrinogen γ' chain, suggesting that the underlying mechanism is due to the inhibition of thrombin-mediated activation of FV and FVIII, rather than to a stimulation of the APC-mediated inactivation of FVa and FVIIIa. The fibrinogen γ' peptide might form the basis for pharmacological interventions to counteract plasma APC resistance.

AS 29.2

The functional effect of pleotropic mutations on Antithrombin

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Background: Antithrombin is a serine protease inhibitor with a key role in hemostasis and its deficiency significantly increases the risk of thrombosis. Two types of antithrombin deficiency have been described: type I with no mutant antithrombin in plasma, and type II, with a plasma variant that has reduced anticoagulant activity. This group of deficiencies is sub-classified according to the localization of the mutation and its functional consequence: II-a, and II-b affects the heparin-binding site and the reactive center loop, respectively. The type II-c or pleiotropic deficiencies include mutations located in C-sheet that impair the heparin affinity and the inhibitory activity by not well defined mechanisms. Recently, we described an abnormal glycosylation of a pleiotropic mutation (K241E) that explained the impaired heparin affinity.

Aim: To evaluate potential effects of different natural pleiotropic mutations on the glycosylation of antithrombin and their functional effects.

Methods: We selected pleiotropic mutations identified in patients with antithrombin deficiency located in each one of the strands of the C-sheet: K241E, M251I, M315K, F402L and P429L. Recombinant mutants produced in HEK-EBNA cells in a β-antithrombin context (S137A) were purified by heparin affinity chromatography and anion exchange. Then, we evaluated differences on size in SDS gel under reducing conditions, and the glycan alteration by treatment with *N*-glycosidase F. We also determined heparin affinity by intrinsic fluorescence. Finally, functional assays with FXa and thrombin in presence or absence of heparin were done.

Results: All pleiotropic mutants presented two isoforms that were purified by anion exchange due to their different isoelectric points, except for M315K, which only produced one isoform. Treatment with *N*-glycosidase F verified that these differences are caused by distinct glycosylation. For every mutant the glycoform with less electrophoretic mobility (V1) had reduced heparin affinity, which for the M251I mutant reached six-fold lower affinity than the recombinant β wild-type antithrombin. Moreover, these V1 glycoforms also had severely affected the reactivity with target proteases (FXa and FIIa) in presence and absence of heparin. In contrast, the V2 glycoform showed the same electrophoretic mobility and similar heparin affinity that recombinant β wild-type antithrombin. Moreover, this V2 glycoform also had an impaired inhibitory activity but it was partially compensated by activation with heparin.

Conclusions: Our study shows a common glyosylation defect for every tested natural pleiotropic mutation affecting C-sheet, apart from M315K, probably because the localization of this residue, close to the reactive center, might not affect the normal interaction with glycosyl transferases involved in the maturation of the *N*-glycan, that seems to allow other mutations. However, mutants with two glycoforms showed a proportion of protein undegoing an abnormal glycosylation. In these cases, V1 with high glycan content had reduced heparin affinity and a significantly impaired anticoagulant function. In contrast, V2 had normal glycosylation and heparin affinity. Thus, although the inhibitory activity of V2 glycoform is impaired as a consequence of the mutation, the activation with heparin restores partially the inhibitory capacity.

AS 29.3

Genetic variation in the annexin V gene and the risk for pregnancy related venous thrombosis

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Background: Annexin A5 is a natural anticoagulant mainly expressed by placental trophoblasts. It is assumed to have thrombomodulary functions as it shields phospholipid layers from coagulation complexes. There is evidence that anti-Annexin A5 antibodies can interfere with the Annexin A5 crystallization on the phospholipid layers and thereby promote pregnancy losses and/or thrombosis in the anti-phospholipd antibody syndrome. Recently, it has been demonstrated that the M2 haplotype within the Annexin A5 gene (*ANXA5*) promotor could be responsible for a diminished transcriptional activity. In the general population, some studies have shown that the M2 haplotype is associated with recurrent fetal loss, pregnancy-related hypertensive disorders as well as venous thrombosis (VT).

In developed countries, VT is a leading cause of maternal morbidity and death during pregnancy and puerperium, and occurs in approximately 1/1000 pregnancies. The mechanism behind pregnancy related VT is considered multicausal, but risk factors for ante- and postnatal VT differ, indicating different pathophysiological mechanisms.

Aim: To investigate if the M1 or M2 haplotypes or other genetic variations in *ANXA5* was associated with pregnancy related VT.

Methods: We investigated women in the Norwegian VIP study which is a population based case-control study of VT in pregnancy or within 3 months post partum (cases) and women without pregnancy related VT (controls). Informed consent was obtained from all women and the study was approved by the regional medical ethics committee. The M1 and M2 haplotypes were analysed by direct sequencing of a PCR-amplified 491 bp fragment of the *ANXA5* promotor. In addition, tag SNPs were selected for *ANXA5* from the CEU population of the Hapmap project with an r2 of 0.80 and minimum allele frequency of 0.10. DNA samples from 313 cases and 353 controls were investigated. Odds ratios were calculated for each haplotype with the wild type as the reference and for each tag SNP with the most common genotype as reference. Antenatal and postnatal VT were analysed separately.

Results: Women homozygous for the M2 haplotype had an odds ratio of 2.8 (95% confidence interval 0.83–9.2) for antenatal VT. For the other haplotypes the odds ratios for both antenatal and postnatal VT were close to 1.

The tag SNP rs17384348 had an odds ratio for antenatal VT of 1.5 (95% confidence interval 0.8–2.7). The other tag SNPs had odds ratios close to 1 for both antenatal and postnatal VT.

Conclusion: All in all, neither the M1/M2 haplotypes nor the tag SNPs in *ANXA5* were convincingly associated with pregnancy related VT.

AS 30 – Thrombus Resolution and Stroke

AS 30

The effects of DNA and histones on fibrin clot structure-function and on the regulation of fibrinolysis

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Background and Aims: Neutrophil extracellular traps (NETs) are networks of DNA, histones and other proteins, released by neutrophils and are recognised as mediators between immunity, inflammation and haemostasis. They have been shown to promote clotting and form a scaffold with fibrin within clots which increases the likelihood of unwanted thrombosis. However, little is known of the effects of DNA and histones on the mechanical stability of fibrin and resistance to fibrinolysis, the subjects of the present study.
Methods: An array of biophysical techniques and binding studies were applied to investigate the effects of DNA and histones on fibrin structure and stability. These included electron microscopy (EM) and confocal microscopy (CM), rheology, isothermal titration calorimetry (ITC), small angle X-ray scattering (SAXS) and thromboelastography. Fibrinolysis assays, with tissue plasminogen activator (tPA) and plasminogen, in a variety of formats were included to understand the kinetics of plasminogen activation and of fibrin breakdown.
Results: DNA, histones or DNA+histones could have strong and sometimes opposing effects on fibrin structure and stability to mechanical stress. This may be important physiologically as we found surgically removed arterial thrombi stained positively for DNA and histones either separately or in complex. Rheology studies showed DNA added to fibrin produced weak, floppy clots, whereas histones or DNA+histones produced firmer clots requiring more shear stress to be disassembled. EM studies showed histones and DNA+histones in clots resulted in significantly thicker fibrin fibres. Fibrinolysis was also affected by DNA and histones. Setting tPA activity in the absence of DNA or histones to 100%, fibrinolytic activity of tPA with 70 μg/mL DNA was 85.4 ± 6.6% and 146.8 ± 8.2% with 50 μg/mL histones. These results were in agreement with experiments tracking the progress of a fluorescent tPA chimera (tPA-GFP) through red AlexaFluor fluorescently-conjugated fibrin by CM. The rate of movement of the lysis front in clots with no additions was set to 100% and was 50 ± 3% in the presence of DNA and was 161 ± 3% in the presence of histones or DNA+histones. However, important differences were seen in studies of gross changes in clot structure during lysis. Time lapse photography showed dramatically slower clot lysis (~3 times longer) in the presence of DNA+histones. ITC data indicated that DNA and histones both bind to large FDP suggesting a mechanism whereby DNA-histones bind to FDP to maintain clot structure during lysis, significantly delaying complete dissolution until FDP are reduced to smaller fragments which bind to DNA-histones only weakly. DNase mixed with tPA reverses this delay. Heparin was able to break down fibrin-DNA-histone complexes, as evidenced in SAXS studies, also enhancing clot lysis rates.
Conclusions: DNA and histones affect fibrin structure and properties, plasminogen activation and fibrinolysis in a number of distinct ways. However, profound effects were observed with DNA+histones which modify clot structure and markedly delay clot lysis. DNA and histones released by neutrophils or other apoptotic cells found in the pro-inflammatory environment of atherosclerotic plaques may regulate fibrinolysis *in vivo*. New therapeutic strategies including DNase or non-anticoagulant heparins added to plasminogen activators may improve thrombolysis.

AS 30.1

A new effective strategy for thrombolysis in ischemic stroke: low-dose tPA in combination with MMP-10

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Background: Early reperfusion using tissue-type plasminogen activator (tPA) is the only therapeutic strategy to treat focal cerebral ischemia with proven efficacy in stroke patients. Intracerebral haemorrhage is one of the major complications of t-PA administration. t-PA has also been linked to neurotoxicity. Therefore, there is an unmet need for new strategies to improve tPA-induced thrombolysis.
Aim: Based on our previous data showing a profibrinolytic effect of matrix metalloproteinase-10 (MMP-10) in ischemic stroke model (Orbe et al., Circulation 2011), we wanted to determine if combined therapy of tPA and MMP-10 improves the beneficial effect of tPA.
Methods: We used a murine ischemic stroke model induced by the local injection of thrombin into the middle cerebral artery, to analyze the effect of different tPA doses (1–10 mg/Kg) or tPA combined with recombinant MMP-10 (6.5 μg/Kg) on the infarct size. *In vitro* cultures were also performed to determine the effect of tPA+MMP-10 on endothelial cells (permeability assay) and neurons (excitotoxicity assay).
Results: We observed that 10 mg/Kg of tPA plus MMP-10 significantly reduced the infarct size in the thromboembolic ischemic stroke model compared with tPA alone. Moreover, a 10-fold reduction in tPA dose in the presence of MMP-10 produced the same effect on infarct size than full-dose of tPA+MMP-10 ($P < 0.05$) without increasing haemorrhage. *In vitro* data showed that MMP-10 reduced the tPA endothelial permeability and improved neuronal excitotoxicity of tPA.
Conclusions: These data suggest that the combination tPA/MMP-10 could be a new strategy for thrombolysis in ischemic stroke, allowing significant reduction of the tPA dose and similar decrease in infarct size.

AS 30.2

Kinase activity of TRPM7 enhances PLC? Two mediated calcium responses in platelets and plays an important role in arterial thrombosis and stroke

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Background: Transient receptor potential melastatin-like 7 channel (TRPM7) has been identified as a constitutively active divalent cation selective channel which regulates intracellular Mg^{2+} concentration in vertebrates. In addition, the C-terminus of TRPM7 is covalently linked to an α-type Ser/Thr kinase domain which is able to phosphorylate its own residues and several substrates including phospholipase C (PLC) γ2. Interestingly, the activation mechanism and the physiological role of the kinase domain still remain elusive. TRPM7 has been shown to play an important role in the pathogenesis of ischaemic stroke and its expression in brain tissue was found to be upregulated under ischaemic conditions, whereas knock-down of TRPM7 resulted in enhanced viability of neurons under hypoxia. TRPM7 seems to exhibit an ambivalent role in cation homeostasis; hence under normal physiological conditions Mg^{2+} influx through TRPM7 can support cell survival, while under pathophysiological conditions the channel or its kinase domain might enhance Ca^{2+} influx leading to cell death. TRPM7 function in platelet physiology and thrombosis has not been investigated so far.
Aims: We hypothesized that the kinase domain of TRPM7 and its signalling mechanism might regulate Ca^{2+} mobilization and Ca^{2+} influx during platelet activation and thrombus growth.

Methods: To identify the physiological importance of the TRPM7 kinase *in vivo*, a *Trpm7ΔKIN* mouse line was generated by introducing a point mutation in the *Trpm7* gene, resulting in an amino acid substitution at position 1646 from lysine to arginine. The K1646R mutation inhibits Mg^{2+}-ATP binding in the catalytic site of the kinase domain, leading to the complete blockade of TRPM7 kinase activity. *Trpm7ΔKIN* platelets were analyzed using a wide range of *in vitro* and *in vivo* platelet functional assays.
Results: We report the first experimental evidence that *Trpm7ΔKIN* mice display a marked signalling defect in platelets. Inositol triphosphate (IP_3) production, Ca^{2+} mobilization and Ca^{2+} influx were severely impaired upon activation of the immunoreceptor tyrosine based activation motif (ITAM)-coupled collagen receptor, GPVI. These results indicated that the kinase activity of TRPM7 is essential to enhance ITAM-PLCγ2-mediated Ca^{2+} responses. Impaired TRPM7 signalling also led to altered integrin activation, degranulation and aggregation responses to GPVI agonists *in vitro* and impaired thrombus formation under flow *ex vivo*. In line with these findings, *in vivo* experiments showed that *Trpm7ΔKIN* mice were significantly protected from arterial thrombosis and ischaemic brain infarction.
Conclusions: These results establish the physiological role of TRPM7 kinase as a key modulator of Ca^{2+} store release and SOCE in platelets, and highlight the role of this new signalling mechanism in the pathogenesis of thrombosis and ischaemic stroke.

AS 31 – Coagulation and Complement

AS 31
Coagulation and complement

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The blood coagulation system and the complement system are connected in many ways. Coagulation proteases, such as thrombin, but also factor Xa, IXa and XIa, can directly activate complement factors, although the biological and clinical relevance of this action *in vivo* is not clear. Factor XIIa is known to activate complement factor I, leading to the initiation of the classical complement pathway. Conversely, C1 inhibitor does not only block complement factor I but is also the most important inhibitor of factor XIIa and kallikrein. In patients with C1-inhibitor deficiency (leading to hereditary angio-edema) attacks of angio-edema are associated with minor activation of coagulation but have major impact on fibrinolysis. An interesting role of thrombin activatable fibrinolysis inhibitor (TAFI) in complement regulation has been established. TAFI is activated upon binding of thrombin to the endothelial surface receptor thrombomodulin and acts as an important inhibitor of C3a and C5a, thereby rendering another important connection between coagulation and inflammation. Lastly, C4b binding protein serves a binding protein for free protein S, interfering with the regulation of coagulation by physiological anticoagulants.

AS 31.1
Human megakaryocytes and platelets contain complement C3 and C5 and release it upon interaction with *Escherichia coli*

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Background: Platelets link coagulation, inflammation and infection. Following exposure to bacteria and a complement dependent activation, platelets can contribute to defense against pathogens. It is known that complement proteins, especially C3, are important for protection from endotoxin shock, and individuals with C3 deficiency are more susceptible to bacterial infections. In sepsis, C3 activation, leading to high C3b levels, is involved in the development of septic shock. In haemolytic uremic syndrome with Shiga toxin producing *E. coli* infection, platelet microparticles carrying high levels of surface bound C3 and C9 are found. Currently, it is assumed that the complement proteins are bound to platelets from plasma.
Aim: We studied whether complement proteins on platelets are only derived from plasma or are synthesized in megakaryocytes and stored in platelets intracellularly. We hypothesized that platelets contain complement proteins and, upon contact with gram-negative bacteria, platelets release complement factors and their activation products to support defense against the bacteria.
Methods: Mature megakaryocytes (MK) generated from cord blood CD34+ cells and platelets obtained from platelet concentrates were exposed to heat killed (HK) *E. coli* 018: K1 in the absence of plasma or other sources of complement proteins. Platelets and MKs were tested for complement C3 and C5 by Western blot and immunocytochemistry. The release of complement proteins was evaluated by ELISA, the presence of C3 and C5 mRNA was analyzed by real time PCR (RT-PCR). The effect of platelets on viability of *E. coli* 018: K1 (pathogenic) and *E. coli* K12 C600 (non-pathogenic) was tested following incubation with platelets before growing the bacteria on culture plates.
Results: RT-PCR analyses showed the presence of C3 and C5 mRNA in MKs and platelets. Proteins migrating at 110, 100, 75, 64, 45 and 38 kD were detectable in platelet concentrates by Western blot with C3 antibody. Exposure of platelets and MK to *E. coli* 018: K1 lead to secretion of C3a and C5a, and ELISA results revealed a 10-fold increased release of C3a and a 100-fold increase of C5a compared to platelets incubated with buffer. Higher expression of C3 after contact with bacteria was also found by confocal microscopy. Activated C3 released from platelets was immediately bound to bacteria. Growth of *E. coli* K12 C600 was completely abolished following platelet contact, while the growth of *E. coli* 018: K1 was not affected. Increased levels of C3a or C5a were not induced by *E. coli* 055:B5 LPS.
Conclusion: Our data indicate that complement C3 and C5 mRNA and protein are present in megakaryocytes and platelets. Contact of washed platelets with *E. coli* leads to activation of C3 and C5 and generation of C3a, C3b, C3d and C5a. The lacking effect of LPS indicates that the activation of complement in platelets is not primarily mediated via TLR4. Already 30 min contact between platelets and bacteria seems to be sufficient to inhibit the growth of *E. coli* K12 C600. The underlying mechanisms have yet to be elucidated and the relevance for the pathogenicity of *E. coli* needs to be evaluated.

AS 31.2
Polyphosphate suppresses complement activation

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Background: In response to vascular injury, coagulation and complement are simultaneously activated to limit blood loss and restrict pathogen invasion, respectively. Although it is reasonable to consider that complement and coagulation are co-regulated, it is only recently that the molecular mechanisms have started to be delineated.
Initiation of coagulation is achieved by exposure of blood to tissue factor or an anionic surface, resulting in a proteolytic cascade that leads to generation of thrombin and a fibrin clot. Complement activation is initiated via the classical, lectin or alternative pathways, whereupon the pathways merge, ultimately leading to cleavage of C5 to yield C5a and C5b. C5b complexes with C6 (C5b,6), which initiates the terminal pathway by sequentially binding to C7, C8 and multiple C9 molecules.

This resultant C5b-9, the membrane attack complex (MAC), becomes integrated into the membrane of the cell that is targeted for destruction.

Recent studies have shown that polyphosphate (polyP), anionic strings of phosphate ions that are released from granules of activated platelets, has important procoagulant properties. With increasing evidence of links between complement and coagulation, we sought to evaluate whether polyP also modulates complement activation.

Aim: To evaluate the effect of polyP on complement activation.

Methods: Binding of polyP to complement components was evaluated by electrophoretic mobility shift assays. Complement-mediated hemolytic activity was quantified by measuring lysis of rabbit or chicken erythrocytes. The terminal pathway was initiated with C5b,6 in the presence of C7, C8 and C9 or human serum. Different polymer lengths and concentrations of polyP were added prior to initiation of complement. As controls, polyP polymers were digested with calf intestinal alkaline phosphatase (CIAP) into monomers (P_1). Concentrations of polyP were expressed as monomeric phosphate equivalents.

Results: We determined that polyP binds to alterative pathway components factor H and factor B, and to C5, C5b,6 and C6 of the terminal pathway. Total hemolytic activity was dampened by increasing concentrations of long-chained polyP ($P_{>1000}$) as compared with P_1. Long-, medium- and short-chain polyP ($P_{>1000}$, $P_{40–160}$, and $P_{<30}$) interfered with terminal pathway activation in a concentration-dependent manner, but the $P_{>1000}$ and $P_{40–160}$ were more effective, i.e., the concentration of $P_{>1000}$ or $P_{40–160}$ to achieve half-maximal inhibition (IC_{50}) was ~10 μM with total inhibition at ~200 μM. In contrast, an IC_{50} with $P_{<30}$ could not be achieved even with 5 mM polyP. The ability of polyP to inhibit terminal pathway activation was absent once the C5b-7 complex formed, suggesting that polyP binding to C5b,6 may alter its ability to form a fully functional MAC. Pretreatment of polyP with CIAP restored hemolytic activity.

Summary/Conclusions: In addition to interacting with factors that regulate coagulation, polyP binds to several proteins in the complement system, including those that initiate and propagate the terminal pathway. All polyP preparations tested inhibited hemolytic activity in terminal pathway assays with $P_{>1000}$ being the most effective. The physiological relevance of polyP in response to injury requires further study, but our findings are consistent with an important role in protecting host cells from complement-mediated damage.

AS 31.3

The lectin like domain of Thrombomodulin is involved in the defense against pyelonephritis

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Background: Urinary tract infections (UTI), frequently caused by *E. coli*, are among the most common bacterial infections, affecting 50% of women worldwide at one point of their life and causing considerable paediatric problems. Among UTI acute pyelonephritis forms a substantial health issue and can lead to end stage renal failure.

Thrombomodulin (TM) is a cell surface-expressed protein, mainly expressed on vascular endothelium but also on some epithelium such as urothelium. TM is part of a natural anticoagulation pathway that limits the action thrombin. Binding of thrombin to TM inhibits the thrombin-mediated conversion of fibrinogen into fibrin. Furthermore the thrombin-TM complex activates the protein C which has anticoagulant, anti-inflammatory and cytoprotective activities. The TM protein consists of several domains of which the lectin and EGF-like domains are crucial for TM structure and function.

Aim: In the current study we wanted to investigate the role played by thrombomodulin in a model of acute pyelonefritis.

Methods: We used two TM transgenic mouse strains: TMpro/pro mice, which bear a mutation in the EGF-like domain making them unable to activate protein C, and TMled/led mice which lack the lectin-like domain of TM which is critical for its anti-inflammatory and cytoprotective properties. These mice were subjected to pyelonephritis model by intravesical inoculation of $1x10^9$ CFU of *E. coli* 1677. Mice were sacrified after 24 and 48 h after inoculation. Kidneys and the bladder were harvested and homogenized in sterile saline for determination of bacterial outgrowth on blood agar plates. Circulating numbers of leukocytes were counted using a haemocytometer.

Results: TMpro/pro mice did not show any differences in renal and bladder bacterial loads, nor in blood leukocytes compared to WT mice.

In contrast, TMled/led mice showed more severe disease than WT mice. TMled/led mice showed significantly more bacterial outgrowth in bladder homogenates at 24 and 48 h after inoculation compared to WT mice ($P < 0.01$ and $P < 0.05$ respectively). Also, TMled/led mice had significantly more bacteria in kidney homogenates at 48 h after inoculation ($P < 0.05$) and showed elevated blood leukocyte counts at 24 h after inoculation than WT mice ($P < 0.05$).

Conclusion: We conclude that the lectin-like domain of thrombomodulin is critically involved in host defense against *E. coli* induced acute pyelonephritis.

AS 32 – Treatment of Von Willebrand Disease

AS 32.1

Long-term correction of von Willebrand disease via Sleeping Beauty transposon-mediated gene therapy

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Background: Von Willebrand disease (VWD) is the most common inherited bleeding disorder. Severe VWD (type 3) is characterized by complete absence of the hemostatic protein von Willebrand factor (VWF). Current treatment of severe VWD is limited to the administration of exogenous VWF concentrates derived from plasma. Given the relatively short half-life of VWF (8–12 h), only a short-term therapeutic effect can be achieved, often requiring multiple subsequent treatments. Gene therapy for this monogenic bleeding disorder is an interesting treatment alternative with long-term therapeutic potential. We previously showed that the liver is an attractive target for ectopic expression of physiologically active VWF.

Aim: The aim of this study was to achieve long-term correction of murine severe VWD via non-viral gene therapy.

Methods: We constructed two Sleeping Beauty transposon vectors containing full-length murine VWF under the control of either the ubiquitous CAG promoter or the hepatocyte-specific alpha-1 antitrypsin promoter. Each transposon, together with a plasmid encoding the optimized Sleeping Beauty transposase SB100x, was directed to the liver of VWF-deficient mice via hydrodynamic gene delivery. At regular intervals after gene delivery, we measured VWF antigen levels in plasma. Correction of the bleeding diathesis was investigated using a tail-clipping bleeding assay.

Results: Use of the transposon containing the CAG promoter resulted in a sustained long-term (> 1 year) expression of VWF in VWF-deficient mice, although VWF levels were low (< 5% of normal wild-type level). Integration of the murine VWF cDNA into the mouse liver genome was confirmed using splinkerette PCR. Higher levels of sustained VWF expression were observed using the transposon with the more potent alpha-1 antitrypsin promoter. Three months after gene delivery, plasma levels remained 75.8 ± 17.6% ($n = 5$) of wild-type values in male mice while VWF expression was lost within 3 weeks in mice that received the VWF transposon vector but no transposase plasmid. At this timepoint, tail-clipping bleeding times were corrected in treated male mice. This phenotypic correction was confirmed by the observation that the blood loss volume was

significantly lower in treated mice when compared to VWF-deficient control mice. Female animals had significantly lower levels of VWF after treatment, resulting in less blood loss volume but not in correction of bleeding time.

Conclusion: We herewith present the first proof-of-concept for long-term treatment of VWD by non-viral gene therapy. Ongoing follow-up studies will establish how long phenotypic correction can be maintained.

AS 32.2

Determinants, frequency, types, and management of bleedings in severe VWD type 3: final results of the retro/prospective analyses in 86/52 Italian patients

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Background: VWD3 is the most rare (1–5 cases/million) and severe form of VWD because is due to virtually complete deficiency of the von Willebrand factor (VWF). VWD3 is of major interest because of severe clinical presentation, need for replacement therapy with VWF/FVIII concentrates, risk of occurrence of anti-VWF inhibitors in a few cases. Even though VWD3 has been known since its original description in 1926, detailed information on the determinants of bleeding are not available so far.

Aims and design of the study: To evaluate possible predictors of bleedings requiring VWF/FVIII concentrates in VWD3 patients, data were collected by Members of the Italian Association of Hemophilia Centers (AICE) using standard protocols. 6/20 Italian Centres could also follow-up for 1 year patients with confirmed VWD3 diagnosis.

Methods: Inclusion criteria were VWF:Ag < 3 U/dL with FVIII:C < 20 U/dL and autosomal-recessive inheritance. Bleeding score (BS) was calculated at enrolment. Gene mutations were searched for in all available DNA.

Results: In the retrospective analysis, among cases originally reported, only 86/114 (75.4%) patients met the inclusion criteria and were characterized by the following demographic, clinical-lab parameters [median(range)]: gender (M/F) = 42/44; age = 38 (6–71); BS = 14 (3–35); FVIII:C = 3 (1–20); anti-VWF-inhibitors = four from three families. Molecular diagnosis was available in 54/86 (59.5%) cases with the following gene defects(pt-n): large-deletion (4); small-deletions-insertions (14); nonsense (9); splice-site (8); missense mutations (19). Mucosal bleedings (64%) were more frequent than hematomas-hemarthrosis (24%). In the retrospective study 77/86 (89.5%) VWD3 had been already exposed to VWF/FVIII concentrates. In the prospective observational study, 52/86 (60.5%) VWD3 were followed-up by 6/20 Centres and 27/52 (88%) were treated in a year for 72 spontaneous bleeding events. BS of VWD3 bleeders (16 [5–35]) was significantly ($P = 0.01$) higher than that (10 [3–22]) of non-bleeders; BS > 10 (HR = 6.8, CI = 3.8–12.3) and FVIII < 5 U/dL (HR = 4.1, CI = 2.4–7) were associated with high risk of bleeding. VWD3 patients with BS < 10 and FVIII > 15 had very few bleeding events. On the other hand, BS > 10 could identify VWD3 patients with higher frequency and severity of events requiring intensive therapy (8100 [3500–25,400] VWF:RCo IU/event) with VWF/FVIII concentrates. Patients with anti-VWF inhibitors were off therapy during this follow-up.

Discussion and Conclusions: these retro/prospective studies show for the first time that also VWD3 can be very heterogeneous. BS and FVIII levels are good predictors of bleeding. An international and multicenter project (3WINTERS-IPS) has been recently organized (2011–2016) to collect more information about clinical and molecular markers associated with increased risk of bleeding and of anti-VWF inhibitors in a larger cohort of at least 250 patients with VWD3.

AS 32.3

High rate of postpartum hemorrhage in women with von Willebrand disease or carriership of hemophilia, despite specialized care

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Background: In the general Dutch population the incidence of severe postpartum hemorrhage (PPH) is 5%. It is likely that this incidence is higher in women with bleeding disorders, as the increase of coagulation factors during pregnancy, such as von Willebrand factor and factor VIII, may be insufficient for adequate hemostasis. It is known that pregnancy and delivery are associated with a high risk of bleeding in women with bleeding disorders. Therefore, pregnant women with bleeding disorders require specialized peripartum care to prevent PPH. It is generally accepted that coagulation factors < 0.50 IU/mL are an indication for prophylactic replacement therapy with factor concentrate or desmopressin during delivery.

Aims: to study the incidence of PPH in women with von Willebrand disease (VWD) and hemophilia carriers managed in Hemophilia Treatment Centers (HTC).

Methods: We included pregnancies in women with VWD or hemophilia carriers treated in two Dutch HTCs between 2002 and 2011. We collected information about pregnancy outcome, i.e. blood loss during or after delivery, need for red blood cell transfusion, and mode of delivery. Primary PPH was defined as blood loss ≥ 500 mL, severe PPH as ≥ 1000 mL within 24 h postpartum. Analyses were performed separately for all pregnancies and for first pregnancies only. As results did not essentially differ, only the former are presented. The study was not subject to the Medical Research Involving Human Subjects Act and was approved by the Medical Ethics Committee.

Results: We included 201 pregnancies in 141 women. Mean age at time of delivery was 31 ± 4.7 years. Median parity was 1 (10–90th percentile: 0–2). Sixty-eight women had VWD (58 type 1, 10 type 2) and 73 women were hemophilia carriers (60 hemophilia A, 13 hemophilia B). Seventy-nine percent (158/201) of deliveries were vaginal and 21% (42/201) were caesarean sections (11% elective, 10% emergency). Of 180 pregnancies data on the amount of milliliters blood loss during or after delivery were available. Thirty-six percent (65/180) of deliveries were complicated by primary PPH, 10% (18/180) by severe PPH. No difference in PPH incidence was seen between women with VWD and hemophilia carriers. Coagulation factor levels were measured at 25–40 weeks of gestation. In 26 pregnancies these levels were < 0.50 IU/mL and consequently prophylactic replacement therapy was given. Despite replacement therapy, 50% (13/26) of deliveries in these patients still resulted in PPH, 15% (4/26) in severe PPH. These PPH incidences were higher compared to deliveries in which no prophylactic replacement therapy was given according to the guidelines, i.e. 34% (52/154) for all PPH and 9% (14/154) for severe PPH. Compared to pregnancies with factor level ≥ 1.00 IU/mL at 25–40 weeks of gestation (before correction with factor concentrate), pregnancies with factor level < 0.50 IU/mL had a seven-fold increased risk of severe PPH.

Summary/Conclusion: The incidence of severe PPH is high in women with VWD and in carriers of hemophilia, despite specialized care in a HTC. The high rate of PPH in pregnancies managed with prophylactic factor replacement therapy indicates that current management of deliveries in women with bleeding disorders needs to be improved.

AS 33 – Major Bleeding

AS 33
Bleeding and thrombosis in patients with liver disease

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Patients with chronic or acute liver diseases frequently acquire a complex disorder of hemostasis secondary to their disease. These hemostatic changes include thrombocytopenia and platelet function defects, decreased levels of pro- and anticoagulant proteins, and alterations in plasma levels of the fibrinolytic system. These changes have long been believed to result in a hypocoagulable status. Recent laboratory studies have shown that the prohemostatic defects in patients with liver diseases are, at least in part, compensated for. Specifically, the thrombocytopenia is compensated for by substantially elevated levels of von Willebrand factor, decreased levels of procoagulants are balanced by decreased levels of anticoagulants, and defects in profibrinolytic systems are balanced by changes in the regulatory mechanisms of the fibrinolytic system. The hemostatic system in patients with liver disease thus appears to be in a 'rebalanced' state due to concomitant changes in pro- and antihemostatic pathways. This new hemostatic balance, however, is unstable and can easily tip towards either a hypo- or a hypercoagulable status. Careful analysis of the clinical phenotype of these patients supports the theory of a 'rebalanced' hemostatic system. Patients with liver diseases do not have an overt bleeding diathesis, as many patients can undergo major surgical procedures such as liver transplant surgery without the requirement for transfusion or prohemostatic therapy. In addition, both bleeding complications as well as thrombotic episodes frequently occur in patients with liver diseases. Nevertheless, the most important bleeding complication in patients with chronic liver disease (i.e. variceal bleeding) is unrelated to defective hemostasis, but rather depends more on local vascular abnormalities and portal hypertension leading to increased vascular pressure. Management strategies of bleeding complications that are related to defective hemostasis are still incompletely defined, and no laboratory tests to predict bleeding complications in these patients is available. Despite laboratory indications of a hypocoagulable status such as prolongations in the PT and APTT, patients with liver disease are not protected against venous thrombosis. In addition, thrombosis of the portal vein is common in patients with advanced chronic liver disease, and results in clinical deterioration. Prevention and treatment of thrombosis of patients with liver diseases is complicated by multiple factors including (i) a (perceived) bleeding risk, (ii) Uncertainty of dosing regimens or drug targets (e.g. the target INR of a patient that requires vitamin K antagonists but already has a prolonged INR as a result of the liver disease is unclear), (iii) issues with monitoring tests (assessment of the INR or of anti-Xa levels is inaccurate in patients with liver diseases), (iv) The anticoagulant potency of some of the available drugs appear different in patients with liver disease compared to individuals with intact liver function. Clinical studies on safety, efficacy, and monitoring of both pro- and antihemostatic strategies in patients with liver disease are thus urgently required.

AS 33.1
Comparison of three different prothrombin complex concentrates efficacy, in reversal of anti-vitamin K effect in patients under oral anticoagulant treatment: an *in vitro* study

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Background: Intra-cranial and major haemorrhages in patients undergoing anticoagulant treatment are life-threatening events that require rapid reversal of anticoagulation with target INR < 1.5.

Aims: To compare '*in vitro*' efficacy of three different prothrombin complex concentrates (PCC) in reversing INR and in restoring thrombin generation and ROTEM® profiles in anticoagulated blood or plasma samples. Two of them, 'PCC-A' and 'PCC-B' were four factors containing PCC. The third, 'PCC-C', was a three factors containing PCC.

Methods: Primarily, we tested three different concentrations of PCC (0.5, 1 and 1.5 U/mL of plasma) to detect the lower dose sufficient to restore a normal INR. The lower dose was found to be 0.5 U/mL. In a second phase *n*. 60 anticoagulated blood samples, both whole blood and plasma, were collected: (i) *n*. 20 with INR 1.5–2; (ii) *n*. 20 with INR 2–3.5; (iii) *n*. 20 with INR > 3.5. In each plasma sample, coagulation factors concentrations, INR, PTT and thrombin generation were measured at baseline (t_0) and after the addition of 0.5 U/mL of each of the three different PCC (t_1). Finally, ROTEM® profiles were evaluated in whole blood samples at t_0 and t_1.

Results: The four factors PCC (PCC-A and PCC-B) were able to restore a normal INR value independently from the INR at baseline. On the contrary, PCC-C restored an INR value < 1.5 only when the INR t_0 value was 1.5–2. Moreover, when INR value at baseline was 2–3.5 the mean INR value at t_1 was 1.64 (range 1.4–2.5), and when INR value at baseline was > 3.5 the mean INR corrected value was 1.88 (range 1.7–2.3). PCC-A determined a significantly ($P < 0.0001$, in both cases) lower factor IX activity (FIX act) at t_1 (increase vs. basal of 96.4%, range 40–155.4) than PCC-C (170%, range 101–273.6%) and PCC-B (164.3%, 106.6–270.6). Differently from PCC-A and PCC-B, PCC-C did not restore Factor VII activity. PCC-C completely restored thrombin generation profile in all degrees of anticoagulation (mean Peak increase vs. basal 453.1%, 96.7–1250.6) with performance comparable to PCC-B (483.4, 152.1–1433.6). PCC-A failed to restore normal thrombin generation even at lower levels of anticoagulation (INR < 2). In particular, with PCC-A was observed only a small increase of Peak compared to basal condition (38.5%, −82.3 to 495.6). No significant differences were observed as for ROTEM profiles among the three PCC tested.

Conclusions: INR is rapidly corrected by low doses of PCC-A and PCC-B independently of basal INR while PCC-C gives only a partial INR correction at higher degrees of anticoagulation. This is most likely due to the lack of FVII in PCC-C. TG is completely restored by low doses of PPC-B and PCC-C while PCC-A has a paradoxical behavior evidenced by a prolongation of PTT and CT-INTEM and absent TG restoration *in vitro*.

On the basis of TG profiles, it can be observed that PCC-B and PCC-C are good tools to rapidly reverse anticoagulation. Data concerning PCC-A need to be carefully evaluated and correlated with the real *in vivo* efficacy.

AS 33.2
Liver cirrhosis is associated with hypercoagulability, decreased clot strength and normal fibrinolysis

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Background: In patients with cirrhosis substantial changes in the haemostatic system occur, making them prone not only to bleeding but also to thrombotic events. Conventional coagulation tests support the theory of a combined hypocoagulable and hyperfibrinolytic state. However, recent studies postulate that in cirrhosis pro-haemostatic changes compensate these antihaemostatic changes, thus 'rebalancing' the coagulation system.

Aim: To gain better insights in the coagulation and fibrinolytic capacity in patients with increasing severity of liver cirrhosis we performed the thrombin generation assay (TGA) combined with tPA-induced fibrinolysis by rotational thromboelastometry ('tPA-ROTEM').

Methods: Blood (citrated, 0.105 M) was obtained from 71 patients with all-cause liver cirrhosis. Cirrhosis severity was assessed by means of the Child Pugh (CP) score (CP-A $n = 52$, CP-B $n = 14$, CP-C $n = 5$). A panel of coagulation parameters was assessed. TG was determined by means of the calibrated automated thrombogram in platelet poor plasma using a 1 pM Tissue Factor trigger in the absence or presence of thrombomodulin (parameters: lag time [LT], peak height [PH] and endogenous thrombin potential [ETP]). tPA-ROTEM was determined in recalcified citrated whole blood triggered with either 35 pM TF or 35 pM TF and 125 ng/mL tissue plasminogen activator (tPA). The following parameters of clot formation and clot lysis were analyzed: coagulation time (CT), α-angle, clot formation time (CFT), maximal clot firmness (MCF), lysis onset time (LOT), lysis time (LT) and fibrinolysis velocity (FV). Statistical analysis of potential differences between CP-groups was performed using the Kruskal-Wallis test.

Results: With increasing CP-score the classic coagulation tests (PT, aPTT) showed a significant prolongation while the coagulation parameters antithrombin, protein C and fibrinogen showed, as expected, a significant decrease. In the TGA we found a significant decrease of the median LT with increasing CP-score (CP-A: 6.83 min vs. CP-C: 4.00 min, $P < 0.05$). Despite relatively high normalized ETP and PH values, no significant differences between the CP-groups were observed with ETP being: CP-A 146%, CP-B 167% and CP-C 174%, and PH: CP-A 200.5%, CP-B 265.6%, CP-C 261.7%. To evaluate the protein C pathway, TM was added to the TGA resulting in a significantly lesser reduction of the ETP in the CP-C group in comparison to the CP-A group (7.2% vs. 24%, resp. $P < 0.05$). Parameters that reflect stable clot formation in the tPA-ROTEM showed significant alterations with increasing disease severity. In the measurements without added tPA there was a decrease of the α-angle ($P < 0.05$), increase of the CFT ($P < 0.05$), decrease in the MCF ($P < 0.05$), None of the tPA-ROTEM parameters of clot degradation (LOT, LT and FV) showed significant differences between the different CP-groups (data not shown).

Conclusion: Thrombin generation demonstrated a hypercoagulable profile in patients with liver cirrhosis. This hypercoagulability is most likely due to the reduced levels of the anticoagulant factors antithrombin and protein C. tPA-ROTEM analysis however, showed diminished clot strength with increasing cirrhosis severity, resulting in a less stable clot. Fibrinolysis on the other hand was comparable between the different CP-groups. Taken together our data therefore do not support the theory of a combined hypocoagulable and hyperfibrinolytic state.

AS 33.3

Prolonged shock phase in a pig model with multiple injuries is associated with a decreased onset of tPA induced fibrinolysis

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Background: Current concepts of trauma induced coagulopathy indicate that prolonged shock leads to an acivation of the protein-C pathway with anticoagulation and induce hyperfibinolysis in severley injured patients. However, large animal models investigating this mechanisms are largely missing.

Aims: In a pilot pig study with multiple injury we elaborated the effects of a prolonged shock phase on coagulation parameters as compared to non-shock animals receiving volume resuscitation.

Methods: After local ethical approval trauma was induced in 10 anaesthetized pigs. Multiple injury was inflicted by the fracture of both femurs and a lung contusion using a captive bolt gun followed by blunt liver injury (Injury Severity Score: 27). Following a shock phase of 30 min animals received either no volume (group: NV, $n = 5$) or Ringer's solution (group: RS, $n = 5$) to maintain a mean arterial pressure of 55–60 mmHg. Thromboelastometry (ROTEM), including a modified method for fibrinolysis ('tPA-ROTEM' with parameters lysis onset time (LOT) and Lysis Index after 30 min (LI.30)), and thrombin generation (TG) were monitored for 4 h and blood loss was measured. Statistical analysis was performed using ANOVA with Tukey *post hoc*. A $P < 0.05$ value was considered as statistical significant.

Results: Severity of inury was confirmed both by macroscopical analysis and a significant drop in cardiac output (CO) (baseline: 4.3 ± 0.6 L/min; 30 min post injury: 1.3 ± 0.14 L/min). Fluid resuscitation led to an increase of CO (3.1 ± 0.8 L/min) whereas in non-resuscitated animals CO remained low (1.6 ± 0.2 L/min). Due to haemorrhagic shock lactate levels (baseline: 1.7 ± 0.2 mM) significantly increased over time in NV animals 8.3 ± 2.9 mM and remained stable in RS animals (1.7 ± 0.6 mM). After injury, tPA-ROTEM induced fibrinolysis (both groups, baseline: LOT: 355 ± 66 s; LI.30: 4.4 ± 0.1%) was significantly reduced in the NV group (240 min post inury: LOT: 982 ± 74 s; LI.30: 81 ± 9.7%). In contrast, tPA induced fibrinolysis in RS animals remained constant (240 min post injury: LOT: 399 ± 37 s; LI.30: 10 ± 4.4%). TEM analysis showed a decrease of clot formation over time (both groups, baseline: 79 ± 0.5 mm) with significantly reduced values in the RS group (67 ± 4 mm; NV group: 72 ± 2 mm, $P < 0.05$). Plasma thrombin generation without exogenous trigger and in the presence of a tissue factor inhibitor was enhanced upon infliction of trauma. The endogenous thrombin potential (ETP) increased from 92 ± 65 nM.min at baseline to 203 ± 86 nM.min at 30 min after trauma. This increase in contact activated coagulation continued upto 4 h after trauma (ETP: 182 ± 54 nM.min) and attenuated at 6 h after trauma (ETP: 80 ± 60 nM.min).

Conclusions: Severe haemorrhagic shock over 4 h with elevated shock parameters following multiple injury induced a contact activation mediated hypercoagulable state, without alterations in global haemostasis (ROTEM) or signs of hyperfibrinolysis. In contrast, omitted volume resuscitation led to an enhancement of clot stability. This observation conflicts current ideas of shock induced hyperfibrinolysis and protein-C activated systemic anticoagulation, which may be explained by lower protein-C levels in pigs as compared to humans.

AS 34 – Venous Thrombosis and Cancer

AS 34.1

The value of CT-scanning for detection of occult cancer in patients with unprovoked venous thromboembolism. The D'Acquapendente study

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Background: Patients with unprovoked venous thromboembolism (VTE) may harbor occult cancer. Literature shows that the risk of malignancy after unprovoked VTE ranges from 2% to 12%. Cancer may be identified by baseline screening (history, physical, lab-testing, chest X-ray) in up to 4% of these patients; nonetheless, overt malignancy may occur in up to 10% within 2 years in those with normal findings.

Aims: To assess if an extensive diagnostic strategy based on CT-scan yields a higher cancer detection rate than a non-standardized approach based on physicians' preferences, we embarked in a prospective multicenter randomized open-label clinical trial in patients with a first episode of unprovoked VTE and a normal baseline screening.

Methods: Consecutive patients at their first episode of symptomatic unprovoked VTE were eligible for inclusion. VTE was deemed unprovoked when occurring in patients without known malignancy, trauma, recent surgical procedures/immobilization, family history of VTE, thrombophilia, estrogen use, pregnancy/childbirth. Patients with a history of VTE, younger than 18 years, pregnant, unable to attend follow-up visits and those who underwent torso CT-scan within 3 months of presentation, were excluded. Only patients with a normal baseline screening, who undersigned informed consent, were randomized. Patients in the CT-group were scheduled for mandatory thoracic, abdominal and pelvic CT-scan plus hemoccult. Patients in the CCP-group underwent any diagnostic testing at the discretion of the attending physician, except for CT as first-line imaging. Patients with a normal work-up were followed-up for up to 2 years. The main endpoint of the study was the difference in the cancer detection rate between the two strategies. Secondary endpoints were the sensitivity of the diagnostic strategies, using the incidence of cancer during follow-up as a reference; and the cancer-related mortality.

Results: Between September 2006 and May 2008, 242 patients had a first diagnosis of unprovoked VTE, and 205 (84.4%) were included. Malignancy was identified in 10/205 (4.9%) by baseline screening; thus 195 patients were randomized. Cancer was diagnosed in 10/98 (10.2%) patients of the CT-group, and in 8/97 (8.2%) of the CCP-group, yielding a 2% absolute difference of cancer detection-rate (95% CI −7.2 to 11.1%). During follow-up, cancer developed in 2 (2.2%) patients of the CT-group and in 2 (2.2%) of the CCP-group. Therefore, the sensitivity of the CT strategy was 83.3% (95% CI 55.2–95.3%), while that of the CCP strategy was 80% (95% CI 49.0–94.3%). Overall, 7 (7.1%) patients of the CT-group and 11 (11.3%) of the CCP-group died, with 2 and 4, respectively, as a result of cancer; yielding a −2.1% absolute difference of cancer-related mortality (95% CI −8.0 to 3.8%, OR 0.48, 95% CI 0.04–3.48; $P = 0.665$ by 2-tailed Log-rank test).

Conclusions: A CT-based screening approach yields an early cancer detection rate comparable to a non-standardized approach based on physicians' preferences, and does not impact on overall and cancer-related mortality. Noteworthy, most (6/8, 75%) cancers in the CCP-group were identified by ultrasonography, which may represent a similarly effective non-invasive alternative to CT for cancer screening in patients with unprovoked VTE.

AS 34.2

A risk scoring model for cancer-associated venous thromboembolism is predictive for overall survival in patients with cancer

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Background: Patients with cancer are at high risk of venous thromboembolism (VTE), a complication associated with increased risk of mortality. A simple and multi-factorial risk scoring model was developed by Khorana et al. (*Blood* 2008; 111:4902–7) to improve prediction of VTE in patients receiving chemotherapy. This risk scoring model was confirmed by several groups to reliably stratify also broader populations of cancer patients according to their VTE risk and identify those patients at the highest risk. We hypothesized that cancer patients who are at risk of VTE may also have higher mortality rates and poor prognosis of their disease.

Aim: The aim of the present study was to evaluate whether the risk scoring model for cancer-associated VTE developed by *Khorana* et al. is able to predict mortality and overall survival in cancer patients.

Methods: We assessed the 'Khorana-Score' in a prospective and observational cohort study of patients with newly diagnosed cancer or progressive disease after remission that were included in the Vienna Cancer and Thrombosis (CATS), an ongoing study initiated in year 2003. The primary endpoint of the study was occurrence of objectively confirmed VTE and the secondary was death within a maximum follow-up period of 2 years. According to the Khorana-Score and our validation of this risk model (Ay et al. *Blood* 2010; 116(24):5377–82) assignment of points for five parameters was done: site of cancer (2 points for very high-risk site, 1 point for high-risk site), platelet count of 350×10^9/L or more, hemoglobin < 10 g/dL, leukocyte count more than 11×10^9/L, and body mass index of 35 kg/m^2 or more (1 point each). Cox-regression and Kaplan–Meier analyses were applied to investigate the association of risk scores with risk of mortality and overall survival.

Results: We included 1544 patients with different types of cancer (45% women, median age: 62 [25–75th percentile: 52–68] years). During a median observation time of 563 days, death was reported in 649 (42%) patients. The Khorana-Score was significantly associated with risk of mortality (hazard ratio [HR] per 1 score increase: 1.56 [95% confidence interval: 1.45–1.68], $P < 0.001$). In multivariable Cox-regression analysis including age, sex and VTE, compared to patients with a score 0 ($n = 466$) the HR for risk of mortality was 1.61 ([1.28–2.02], $P < 0.001$) in those with score 1 ($n = 489$), 2.80 ([2.24–3.51], $P < 0.001$) in score 2 ($n = 405$) and 4.01 ([3.09–5.21], $P < 0.001$) in those with score ≥ 3 ($n = 184$). The overall survival probabilities for patients with score 0, 1, 2 and ≥ 3 were 87%, 78%, 65% and 58% after 1 year and 67%, 55%, 38% and 27% after 2 years, respectively (Log-rank test: $P < 0.001$).

Summary/Conclusion: The Khorana-Score had been externally validated in several studies to predict risk of developing VTE. In our current analysis, we were able to demonstrate that this simple risk scoring model predicts also risk of mortality and overall survival in patients with cancer, independently of VTE occurrence. This may support clinical decision strategies with regard to thromboprophylaxis and anticancer treatments.

AS 34.3

Mechanistic insights into zymogen protein C protection against cancer progression

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Background: Cancer is frequently associated with activation of coagulation, and a procoagulant state facilitates tumor metastasis. Recent studies have suggested that the activated protein C (aPC) pathway plays a role in modulating tumor metastasis. Notably, we have uncovered that the inactive precursor, zymogen PC (zyPC), exhibits a robust protective effect against metastasis.

Aim: The aim of this study was therefore to explore mechanisms through which zyPC prevent metastatic cancer progression in a murine cancer model.

Methods: Liver directed adeno-associated viral vectors (AAV) were utilized to achieve a wide range of sustained expression of wildtype (WT) or mutant murine zyPCs. Mice expressing stable levels of zyPCs after AAV administration and control mice receiving PBS were injected intravenously with 2.5×10^5 murine melanoma B16F10 cells. After 3 weeks the numbers of pulmonary tumors were determined.

Results: Expression of zyPC-WT in normal C57BL/6s decreased the rates of metastasis in a dose-dependent manner compared to controls ($P < 0.01$). These effects were noted even in mice with only a two-fold increase in zyPC levels. Conversely, when PC-deficient mice (residual 3%) were administered B16F10s without zyPC-expression, they did not survive 3 weeks, while their normal littermates did. These data demonstrate the protective role of zyPC in tumor progression. We then tested modified zyPCs to identify the critical functions responsible for our observations. zyPC-R15Q is unable to dock to the thrombin-thrombomodulin complex and cannot be converted to aPC. Compared to PBS controls, mice expressing zyPC-R15Q still showed a decrease

in the number of tumor foci ($P < 0.001$) similar to the zyPC-WT ($P = 0.28$). Additionally, mice expressing zyPC-S195A, which has a mutation in its serine protease active site, also showed a significant decrease in the number of tumor foci compared to PBS controls ($P < 0.05$). As with the R15Q, mutating the S195 did not affect the ability of zyPC to protect against metastasis ($P = 0.22$). These data suggest that zyPC protection is not dependent upon local activation of zyPC to aPC and aPC's anticoagulant function. Next, we tested the importance of the main PC cellular receptors in our model. Binding to endothelial protein C receptor (EPCR) enhances activation of PC. zyPC-expressing mice that received anti-EPCR antibody that blocks PC binding still had a significant reduction in tumor rates compared to PBS controls ($P < 0.01$). Moreover, mice expressing zyPC had similar levels of protection whether they received the anti-EPCR antibody or an isotype control ($P = 0.31$). EPCR binding not only increases activation of PC, it also mediates the cytoprotective effect by clustering with and facilitating the activation of the signaling protease-activated receptor (PAR) 1. PAR1 −/− mice expressing zyPC had reduced rates of metastasis compared to PAR1 −/− PBS controls ($P < 0.01$). The zyPC protection in PAR1 null mice was comparable to that in PAR1+/− littermate controls ($P = 0.619$). PAR4 is an alternative PAR found on murine platelets. PAR4 −/− mice were also protected by zyPC expression ($P < 0.01$) similar to their +/− littermate controls ($P = 0.14$).

Conclusion: Collectively, these findings suggest a distinct mechanism by which zyPC modulates tumor progression independent of anticoagulant function, EPCR, PAR1 or PAR4.

AS 35 – Clot Structure

AS 35
Determinants of clot stability

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Fibrinogen (340 kDa) circulates at 2–5 mg/mL (~6–15 μM) as a hexameric homodimer consisting of two sets each of three polypeptide chains (A , B , γ). Following vascular disruption or dysfunction, biochemical and biophysical cross-talk between extravascular and intravascular cells, plasma procoagulant and anticoagulant proteins, and blood flow promote generation of thrombin, which proteolytically cleaves N-terminal peptides from fibrinogen monomers. Fibrin, the polymerized, insoluble product generated by assembly of fibrin monomers into fibers, exhibits remarkable extensibility and elasticity and is essential for the formation and stability of both hemostatic and thrombotic clots. Clinical and epidemiological studies have consistently correlated abnormal fibrin clot characteristics with disease; coarse, open fibrin networks that are susceptible to fibrinolysis are associated with bleeding, whereas dense fibrin networks that are resistant to fibrinolysis are associated with thrombosis. Major determinants of fibrin network characteristics (fiber thickness, branching, density, and susceptibility to lysis) include the concentrations of fibrinogen and thrombin present during fibrin formation. At a constant thrombin concentration, increasing fibrinogen concentrations produce denser, highly-branched fibrin networks. Accordingly, in both humans and mice, reduced fibrinogen levels are associated with bleeding; whereas, elevated fibrinogen in congenital hyperfibrinogenemia, pregnancy, and inflammation is associated with both arterial and venous thrombosis. Similarly, at a constant fibrinogen concentration, low thrombin concentrations produce coarse, un-branched networks of thick fibrin fibers; whereas, high thrombin concentrations produce dense, highly-branched networks of thin fibers. Consequently, therapeutic approaches to modify thrombin generation or activity though the use of hemostatic agents in bleeding patients (e.g. patients with hemophilia) or anticoagulants in patients at risk for thrombosis have important implications for fibrin formation and quality, and therefore, clot stability. Platelets mediate clot stability by both supporting procoagulant activity and inducing integrin-mediated clot retraction, which consolidates clots and protects against fibrinolysis. Additional determinants of fibrin network properties include the levels of alternatively-spliced or post-translationally-modified fibrinogen, polyphosphates, and fibrin(ogen)-binding proteins (e.g. factor XIII). These elements both alter fibrin formation, and modify fibrin's susceptibility to fibrinolysis. Finally, the pattern of blood flow during fibrin formation profoundly affects fibrin structure. Fibrin networks formed under static conditions exhibit an isotropic fiber distribution; whereas, networks formed under flow are anisotropic, with fibers aligned with flow vectors. Anisotropy of fibers within a clot is expected to modulate the clot's interactions with cells and fibrinolytic enzymes. Growing appreciation of the complexity of these interactions increasingly warrants the use of assay systems that incorporate multiple aspects of clot formation, including *in situ* thrombin generation in a plasma milieu that incorporates physiologically-relevant fluid flow.

AS 35.1
Fibrinogen a-chain and ?-chain play specific roles in fibrin clot formation and structure

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Background: Factor XIII (FXIII) plays a major role in stabilising fibrin clots by cross-linking fibrinogen (Fbg) γ- and α-chains. To date, few studies have investigated the individual roles of α- and γ-chains in fibrin clot formation and structure. Investigation of the role of α-chain in clot stiffness at individual fibres (Helms et al., 2012) and whole clot (Standeven et al., 2007) levels, have shown that α-chain cross-linking partly increases stiffness of individual fibrin fibres and of the whole fibrin clot respectively.

Aims: The aims of the present study were to investigate the effects of α-chain and γ-chain cross-linking on fibrin clot formation and structure.

Methods: FbgWT and Fbg3X (γQ398N/Q399N/K406R, eliminates all γ-chain cross-linking) were produced as previously described (Standeven et al., 2007). All experiments were performed with the following: 0.5 mg/mL fibrinogen, 0.1 U/mL thrombin, 5 mM $CaCl_2$. Fibrin formation and lysis were studied by turbidity (± 0.24 μM plasminogen and 100 pM tPA). Fibrin structure was studied by confocal (+1% AlexaFluor488 fibrinogen), and electron microscopy. Microscale fibrin viscoelasticity was analysed by magnetic tweezers. FXIII-A_2B_2 (3.7 μg/mL) was added for cross-linking.

Results: Turbidity experiments showed that in the absence of FXIII FbgWT clots had a 1.33-fold higher maximal absorbance compared to Fbg3X, indicating that Fbg3X formed denser clots with thinner fibres than FbgWT. FXIII induced an increase in maximum absorbance (FbgWT: 1.21-fold; Fbg3X: 1.18-fold) over a prolonged period of time for both fibrinogens (WT: 2.76-fold; Fbg3X: 2.13-fold). Lysis experiments showed similar lysis rates for both fibrinogens (FbgWT: -2.30×10^{-4} U/s; Fbg3X: -2.32×10^{-4} U/s), which were reduced by FXIII (FbgWT: -1.97×10^{-4} U/s; Fbg3X: (-1.96×10^{-4} U/s). Confocal microscopy showed that Fbg3X formed a denser clot (64 fibres/200 μm) with thinner fibres compared to FbgWT (40 fibres/200 μm). Fibres in clots formed with FbgWT (2.5 min) appeared quicker than Fbg3X (3.0 min) in the absence of FXIII. Both fibres appearance times were reduced in the presence of FXIII (FbgWT: 2.0 min; Fbg3X: 2.5 min). Electron microscopy showed that without FXIII, both fibrinogens formed curly fibres. In the presence of FXIII, fibres appeared straighter for both FbgWT and Fbg3X. Magnetic tweezers showed a similar clot stiffness (storage modulus) between the two fibrinogens in the absence of FXIII, but the loss modulus was significantly increased by two-fold in clots formed with Fbg3X compared to FbgWT, indicating that the former are more likely to deform. When FXIII was added, clot stiffness was significantly increased for clots formed by FbgWT (1.3-fold), but not Fbg3X; and clot non-elastic

deformation was significantly decreased in clots formed by FbgWT (1.5-fold), but not Fbg3X.
Summary/Conclusion: In this study, we found that fibrinogen α-chain cross-linking plays a role in a time-dependent increase in fibre thickness. Furthermore, α-chain cross-linking decreases lysis rate and straightens fibres. Our findings also show that fibrinogen γ-chain cross-linking influences fibre density, increases clot stiffness and decreases clot elasticity. These data extend our knowledge regarding the role of α-chain and γ-chain cross-linking in fibrin fibre morphology and fibrin micro-elastic properties.

AS 35.2
Multiscality of the structure and mechanics of fibrin clots

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Background: Blood clots form from three-dimensional mesh of the protein fibrin. These fibrin networks consist of branched fibers, are highly extensible and can be stretched up to four times its original length. Such extraordinary mechanical properties are important in resisting blood shear flow *in vivo* and render fibrin clots one of the most resilient naturally-occurring biopolymer networks.
Aims: Although recent works have started to explore the elastic properties of fibrin fibers and networks, the link connecting the mechanics and the underlying structure of fibrin is still missing. In particular, we seek to unravel how the remarkable mechanics of the fibrin clots derive from the self-assembled structures at the fiber and molecular levels.
Methods: We combine mechanical measurement using rheology and quantitative structural characterization using confocal and electron microscopy, as well as light and X-ray scattering, to study the physical and structural origins of fibrin clotting and extensibility at multiple length scales.
Results: Like many other biopolymer networks, fibrin clots stiffen nonlinearly with stress. Interestingly, however, we find that the rheological response shows multiple plateau-stiffening steps depending on protein concentration, a behavior unique to fibrin. Comparison with theoretical predictions reveals that the stiffening regimes correspond to different deformation modes at fiber and network level. Moreover, the presence of Factor XIII is found to alter the polymerization kinetics, time-dependent response, and network stiffness in the linear regime, but not beyond. Although the network microstructure is not significantly affected, the coupling between fibrils can vary with both fibrin and Factor XIII concentrations, and this manifests macroscopically in the ability of the clots to withstand mechanical strain and stress.
Summary/Conclusions: By dissecting the relation between structure and mechanics of fibrin clots at different length scales, we find that structural hierarchy plays a major role in determining the integrity and behavior of clots. A better understanding of the structure-mechanics interconnection can not only shed important light on the molecular origins of fibrin behavior and regulation in thrombosis, but also provide guidance in designing haemostatic materials, particularly in surgical settings and tissue engineering.

AS 35.3
Dissolution of tPA-resistant thrombi in occlusive intracranial thrombosis

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Background: Large artery intracranial occlusive disease is now considered as the most common subtype of ischemic stroke. Unfortunately currently available fibrinolytic treatments, such as tissue-type plasminogen activator (tPA), glycoprotein (Gp) IIb/IIIa inhibitors and anticoagulants, are poorly efficient to promote reperfusion following *in situ* intracranial thrombosis (ICT).
Aims: In the present study, our aims were to determine the most efficient pharmacological strategy to restore vessel patency after ICT and thus to further understand the molecular mechanisms involved in such type of thrombus formation.
Methods: We developed an original model of ischemic stroke in mice, by inducing occlusive thrombosis of the middle cerebral artery using topical application of $FeCl_3$. In this model, cerebral blood flow was continuously monitored using laser Doppler flowmetry and the arterial status was assessed post-treatment using magnetic resonance angiography. To dissect the mechanisms driving occlusive thrombosis, we used different experimental approaches including transgenic mice, pharmacological inhibitors of platelet receptors, recombinant proteins, F(ab) from monoclonal antibodies and two-photon microscopy.
Results: We demonstrated that the thrombi resulting from occlusive thrombosis are resistant to fibrinolytics (such as rtPA), GpIIb/IIIa inhibitors and anticoagulants. In contrast, administration of inhibitors of the GpIba-von Willebrand Factor (VWF) axis resulted in efficient thrombus dissolution and dramatically improved stroke outcome (68% reduction of the ischemic lesion size), without inducing bleeding complications. From a mechanistic point of view, we demonstrated that during the last steps of thrombus formation, the very-high pathological shear rate in the narrowing arterial lumen promotes GpIba-VWF-dependent platelet cross-linking. Our study provides evidence that this unique mechanism of platelet aggregation is critical to achieve arterial lumen closure. Accordingly, inhibitors of the GpIba-VWF axis induce specific dissolution of the last-formed platelet aggregates and restore vessel patency after occlusive thrombosis.
Conclusion: GpIba-VWF interaction drives arterial lumen closure during occlusive thrombosis. Therefore, inhibition of the GpIba-VWF axis represents a new therapeutic strategy to restore vessel patency after stroke or myocardial infarction.

AS 36 – Structure-Function of Factor V

AS 36
Molecular Basis of Factor V Procofactor Activation

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Most coagulation factors circulate in blood as inactive precursors and must transition to an active form to participate in blood clotting. Despite its significance, mechanisms that underpin these molecular and structural conversions have proven difficult to pinpoint especially for coagulation factor V (FV). Factor V circulates in blood as an inactive procofactor and cannot participate to any significant degree in its macromolecular enzyme complex. Following proteolytic removal of a central B-domain activity is generated indicating that the conversion of the procofactor to FVa must result in structural changes that impart cofactor function. A key focus of my laboratory is to decipher these molecular processes and provide new biophysical and structural insight into how FV is preserved as an inactive procofactor. This molecular process undoubtedly plays critical regulatory roles, evolved to maintain normal hemostasis since FVa has a tremendous influence on thrombin generation. Over the past few years we have shown that proteolysis of FV functions to dismantle and release autoinhibitory sequences within the B-domain termed the procofactor regulatory region. Understanding how this evolutionary conserved region functions, where it binds, how it disengages from FV following activation, and how this region is regulated by a variety of physiological ligands are major research areas in the laboratory.

AS 36.1

Alternatively spliced factor V isoform in the east Texas bleeding disorder inhibits coagulation by binding and increasing tissue factor pathway inhibitor alpha (TFPIa) plasma levels

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Background: The autosomal dominantly inherited East Texas bleeding disorder (OMIM 605913) has been linked to the coagulation FV gene (*F5*) and all affected individuals carry an A2440G variant in exon 13 encoding the large B domain activation fragment. The disease is moderately severe and all affected family members have prolonged prothrombin time (PT) and/or activated partial thromboplastin time (APTT). However, they have normal concentrations of all coagulation factors, including FV.

Aim: The aim was to elucidate the pathogenic molecular mechanisms resulting in the inherited bleeding phenotype.

Methods: The family spans over 4 generations and has 22 known affected members. All participants gave written informed consent. The Institutional Review Board and the Center for Protection of Human Subjects at the University of Texas HealthScience Center at Houston approved this study (MS-02-157). Total blood RNA was reverse transcribed using *F5* gene exon 13 specific primers and subjected to real-time PCR. The FV-Short splice variant was targeted using exon 13 specific reverse and forward primers. FV- and TFPI- isoforms in plasma were tested with western blotting using mono- and polyclonal antibodies against FV and TFPI. Thrombin Generation of plasma, in presence of tissue factor and negatively charged phospholipid membranes, was monitored with fluorogenic substrate. Different isoforms of FV and TFPI in patient plasma were immune depleted using various FV- and TFPI- antibodies. FV- and TFPI- isoforms were quantified with several ELISAs using domain-specific monoclonal antibodies.

Results: We now demonstrate that A2440G causes up-regulation of an alternative spliced form of the FV transcript that results in an in-frame deletion of 702 amino acids of the large activation fragment of FV, the B domain. The novel ~250 kDa FV isoform (FV-Short), which can be fully activated to FVa by thrombin, is present in all A2440G carriers' plasma (n = 16), but is also present at much lower concentrations in non-carriers' plasma (n = 20). FV-Short inhibits coagulation through an indirect mechanism involving tissue factor pathway inhibitor alpha (TFPIα). In affected family members, FV-Short forms a complex with TFPIα. As a result, the TFPIα plasma concentration in affected individuals is increased approximately 10-fold when compared to unaffected individuals' plasma, suggesting that the TFPIα-FV-Short complexes are retained in circulation. The TFPIα-FV-Short complexes are demonstrated to efficiently inhibit thrombin generation of both intrinsic and extrinsic coagulation pathways.

Conclusions: The A2440G FV gene mutation results in up-regulation of a previous unidentified, alternatively spliced transcript of FV, which yields a FV-Short protein (Δ702 amino acids residues) that binds and retains TFPIα in the circulation. The FV-Short-TFPIα complex acts to inhibit activation and propagation of coagulation, leading to the East Texas bleeding disorder. This is the first autosomal dominant bleeding disorder due to a gain of function mutation in the *F5* gene. The identification of TFPIa as the active inhibitory compound in the affected family members opens possibilities for future therapeutic intervention in the affected family members as TFPI inhibitors are presently under development.

AS 36.2

Site-specific glycan trimming in megakaryocyte-endocytosed factor V

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Background: Coagulation factor V exists as two phenotypically- and functionally-distinct pools in whole blood: 75–80% is found in the plasma, while the remaining 20–25% is stored within α-granules of platelets, and appears to be the more physiologically-relevant pool. As platelet-derived factor V originates *via* megakaryocyte endocytosis of plasma-derived factor V, we hypothesize that the unique functional characteristics expressed by platelet-derived factor V are a result of its processing within megakaryocytes subsequent to its endocytosis. This processing includes glycosylation, as Thr^{402} is glycosylated in platelet-derived, but not plasma-derived factor V, suggesting that endocytosed factor V is intracellularly transported to the Golgi network for O-glycosylation. In addition, since glycans account for 13% to 25% of the molecular mass of factor V, and glycosylation is known to affect several plasma-derived factor V/Va activities, we hypothesize further that glycosylation differences between the plasma- vs. platelet-derived pools contribute to their functional differences.

Aims: Our initial goal was to characterize potential N-linked glycosylation sites in the heavy and light chains of plasma-derived factor Va. Subsequent to the identification of those sites, as well as analyses of their composition and determination of any microheterogeneity of the attached glycan chains, our next goal was to determine any donor-dependent differences between the platelet- and plasma-derived factor Va pools.

Methods: Electrospray ionization-liquid chromatography mass spectrometry was performed on proteolytically-derived peptides from purified heavy and light chains of both plasma- and platelet-derived factor Va. Confocal microscopy was used to determine the co-distribution of endocytosed factor V with the Golgi-specific enzyme, α-mannosidase.

Results: Of the nine potential N-linked glycosylation sites within the factor Va heavy chain, two glycans were confirmed (Asn269 and Asn432) and characterized, an additional three were identified (Asn23, Asn27 and Asn439) but only one was characterized (Asn439). Three sites were not glycosylated (Asn354, Asn526, and Asn639) and a last site remains unidentified. Regarding the factor Va light chain, the glycan on Asn2181 was reconfirmed and glycan chains were identified and characterized on the two potential sites remaining (Asn1675, Asn1982). While glycan chains composed of either high mannose carbohydrates or sialic acid-containing complex carbohydrates were observed in factor V purified from plasma pooled from seven donors, little, if any, heterogeneity was apparent at each individual glycosylation site, suggesting that the composition of glycans is relatively consistent between individuals. When individual donor's platelets/plasma were analyzed, again little heterogeneity was observed with one exception. Whereas plasma-derived factor Va contained high-mannose glycans with either 6, 7, 8, or 9 mannose residues at Asn1982, platelet-derived factor Va glycans at the same site had only 4, 5, or 6 mannose residues. This trimming process was site-specific, as neither the high-mannose glycan at Asn269, nor the complex glycans at Asn432, Asn439, Asn1675, and Asn2181 incurred substantial modification from what is observed in plasma-derived factor Va. Subsequent, confocal microscopy studies demonstrated the co-distribution of megakaryocyte-endocytosed factor V with Golgi-specific, α-mannosidase.

Summary/Conclusions: These combined data suggest that factor V endocytosed by megakaryocytes undergoes retrograde transport to the Golgi network where site-specific glycan trimming occurs.

AS 36.3

Functional characterization of a structural element unique to venom factor V from the Australian common brown snake Pseudonaja textilis

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Background: The venom of the Australian snake *Pseudonaja textilis* (pt) contains a robust prothrombin activator homologous to the human coagulation factors V (FV) and Xa (FXa). To effectively escape hemostatic regulation, this venom-derived prothrombinase-like complex has been subject to remarkable gain-of-function adaptations. We previously revealed that the constitutively active FV subunit (pt-FV) not only bypasses the requirement for a membrane surface to achieve high affinity FXa binding, but is also functionally resistant to activated protein C (APC).

Aim: Here we examined the functional linkage between these distinct procoagulant properties and a disulfide bond covalently connecting the A2-A3 domains This structural element is nonconserved throughout vertebrate evolution and exclusive to pt-FV.

Methods: To assess its functional implications in human FV, we introduced the disulfide bond into constitutively active human FV-810 (Pro811-Gy1491 deleted) by substituting the appropriate residues to Cys. In a second variant, we additionally exchanged human A3 region Tyr1756-His1778 for the homologous snake region Phe994-Pro1016, which we hypothesize to promote disulfide linkage in pt-FV based on its unique sequence. Alternatively, we eliminated the disulfide bond in pt-FV by substituting the Cys residues involved for Ser. The recombinant FV variants were stably expressed in BHK cells and purified to homogeneity using ion-exchange chromatography.

Results: All purified FV variants demonstrated full cofactor activity in a PT-based clotting assay. SDS-PAGE analysis prior to and following thrombin-activation demonstrated that in both human FV variants approximately 50% of the molecules had successfully incorporated a covalently linked heavy and light chain. This suggests that formation of the disulfide bond is impaired in human FV. Furthermore, introduction of the *Pseudonaja textilis* A3 sequence into human FV does not promote disulfide linkage under the conditions employed. Using a purified prothrombinase assay, no gain-of-function was observed since the membrane-dependent and -independent cofactor function of the variants was identical to FV-810. Incubation of all human FV variants with physiological concentrations of APC (10 nM) resulted in full proteolysis and loss of cofactor activity. As such, human FV function was not significantly improved following partial introduction of the disulfide bond. In pt-FV, full elimination of the disulfide link was confirmed by SDS-PAGE analysis. However, we observed no loss in cofactor activity of this variant, irrespective of the availability of anionic membranes. Furthermore, pt-FV lacking the disulfide bond was completely active following incubation with high concentrations of APC, even though the heavy chain was fully proteolyzed. This strengthens the assumption that noncovalent interactions stabilize fragments generated by APC.

Conclusion: Taken together, these data indicate that the disulfide bond exclusively found in venom-derived pt-FV is not a functional requirement for its powerful procoagulant properties. Accordingly, transferring this structural element to human FV did not alter cofactor function nor any of the other protein characteristics studied. This concurs with the three-dimensional coordination of the A3 region replaced in human FV, which is a solvent-exposed loop and may therefore tolerate structural rearrangement. Whether its venom counterpart imposes conformational constraints essential to other aspects of the FV life cycle remains to be determined.

AS 36.4

Regulatory sequence 1000 to 1008 of human coagulation factor V maintains the procofactor in a quiescent state

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Background: Human factor V (fV) circulates in blood as a single chain inactive procofactor with a M_r 330,000 consisting of multiple domains (A1-A2-B-A3-C1-C2) at a concentration of 20 nM. The sequential cleavage of fV by thrombin at Arg^{709}, Arg^{1018}, and Arg^{1545} is physiologically necessary to remove the heavily glycosylated B-domain, and produce factor Va (fVa) which is composed of the light and heavy chains. fVa binds to factor Xa (fXa) on a phospholipid membrane in the presence of divalent metal ions to form the prothrombinase complex. A comparative sequence analysis of fV among mammals revealed that amino acid sequence 1000–1008 of the B domain contains seven conserved basic amino acids and could play a major part in inhibiting the interaction of fVa with fXa.

Aim: To investigate the functional importance of the basic amino acid region 1000–1008 within fV.

Methods: We constructed three mutant recombinant fV molecules as follows: fV^{Q3} all activation sites (R709/R1018/R1545) were mutated to glutamine, $fV^{\Delta B9}$ had region 1000–1008 deleted and $fV^{\Delta B9/Q3}$ was a combination of both mutations. All recombinant molecules were expressed as full-length derivatives in mammalian COS-7 cells and were purified to homogeneity. The molecules were assessed for clotting activity using fV-deficient plasma and for cofactor activity in the presence of fXa and saturating concentrations of recombinant fV molecules prior and following incubation with thrombin. The ability of the molecules to be activated was investigated and visualized through Western blotting techniques.

Results: Kinetic analyses revealed that fV^{Q3} was severely impaired in its interaction with fXa before and after incubation with thrombin. In contrast, $fV^{\Delta B9}$ and $fV^{\Delta B9/Q3}$ had similar affinity for fXa when compared to wild type (fVa^{WT}) before and after activation by thrombin. Two-stage clotting assays revealed that while fV^{Q3} was devoid in clotting, $fV^{\Delta B9/Q3}$ had clotting activity comparable to fVa^{WT}. Under similar experimental conditions prothrombinase assembled with recombinant $fV^{\Delta B9/Q3}$ had a K_m that was comparable to the K_m of prothrombinase assembled with fVa^{WT} while the k_{cat} was decreased by ~20-fold. Increasing the concentration of $fV^{\Delta B9/Q3}$ within the mixture resulted in a 57-fold increase in the K_m and a four-fold increase in the k_{cat} of prothrombinase. Western blotting of the reaction mixture during the process of the experiment confirmed that $fV^{\Delta B9/Q3}$ remained intact during the course of the assay whereas $fV^{\Delta B9}$ became activated.

Conclusions: Our data demonstrate that amino acid sequence ^{1000}KTRKKKKEK^{1008} controls spontaneous binding of fV to fXa. Overall the data reveal that the amino acid stretch 1000–1008 and B region of the procofactor keep the molecule in a quiescent state by both covering the fXa binding domain and by impeding the productive interaction of prothrombin with the COOH-terminus of the heavy chain of fVa. Our data demonstrate that the clotting activity of $fV^{\Delta B9/Q3}$, which is a measure of its physiological *in vivo* activity, was indistinguishable from the clotting activities of fVa^{WT} or fVa^{PLASMA}. Thus, it becomes obvious that even a small mutation within the 1000–1008 region of the B domain could have profound and devastating clinical implications for normal hemostasis.

AS 37 – Signal Transduction

AS 37.1

Role of Class I PI3K a and β in platelet activation and functions: new potential antithrombotic targets?

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During platelet activation, class I phosphoinositide 3-kinases (PI3Ks) generate lipid second messengers (D3-phosphoinositides) which are important players in the organisation and the regulation of intracellular signalling processes. Using a selective inhibitor of class I PI3Kβ, TGX221, it has been suggested that targeting this isoforme could be a potentially interesting antithrombotic strategy (Jackson et al. *Nat Med*. 2005). Due to the lack of tools, the role of α isoform of class I PI3K has remained unclear in platelet activation. However, recent studies using pharmacological inhibitors suggest that both PI3Kα and β are required, in a non redundant way, for full platelet activation by collagen (Gilio et al. *J. Biol. Chem*. 2009).

To analyse the respective role of PI3Kα and β during platelet activation and thrombosis, we have generated two mice lines expressing an inactive form of these isoforms selectively in the megakaryocyte lineage (*PF4*-Cre/p110$\alpha^{flox/flox}$ and *PF4*-Cre/p110$\beta^{flox/flox}$).

Using the *PF4*-Cre/p110$\beta^{flox/flox}$ mouse model, we demonstrated that PI3Kβ plays a critical role downstream of GPVI and $\alpha_{IIb}\beta_3$ integrin. This isoform also contributes to platelet activation induced by GPCR. *Ex vivo* and *in vivo* approaches show that PI3Kβ is not mandatory for primary haemostasis but regulates thrombus growth and stability mainly under high shear rates. *PF4*-Cre/p110$\beta^{flox/flox}$ mice are protected against occlusive thrombus formation in the $FeCl_3$ carotid injury model. The mice line presenting conditional invalidation of PI3Kα shows that this isoforme is partially implicated in signalling downstream of GPVI but not downstream of GPCR. *Ex vivo* and *in vivo* experiments reveal a role for this PI3K in thrombus growth but not in thrombus stability or the control of bleeding time.

Altogether, these results reveal different role for PI3Kα and β during platelet activation and thrombus formation *in vitro* and *in vivo*. PI3Kβ appears as an interesting antithrombotic target since inhibition of this isoform protects mice from occlusive thrombus without increasing the hemorrhagic risk.

AS 37.2

The Epac1-Rap1 pathway regulates Weibel-Palade body exocytosis from endothelial cells through the activation of Rac1 via PREX-1.

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Background: Vascular endothelial cells provide a dynamic interface between circulating blood and underlying tissues that is critically involved in maintaining vascular integrity and homeostasis. A significant number of haemostatic components and inflammatory mediators originate from endothelial cell-specific, cigar-shaped organelles called Weibel-Palade bodies (WPBs). WPBs function as storage vesicles for von Willebrand factor (VWF), a multimeric adhesive glycoprotein crucial for platelet plug formation, the leukocyte receptor P-selectin and a number of bioactive compounds that include the chemoattractants IL-8 and eotaxin-3. WPBs release their content following stimulation with agonists increasing intracellular Ca^{2+}, like thrombin, or agonists increasing intracellular levels of cAMP, such as epinephrine. The physiological importance of the cAMP-mediated pathway is illustrated by the rise in VWF levels in patients with von Willebrand's disease and mild haemophilia A following administration of the vasopressin analogue desmopressin (DDAVP).

Aim: Previously, we have shown that the exchange protein activated by cAMP, Epac1 and its substrate, the small GTPase Rap1 are involved in cAMP-mediated release of WPBs. In this study, we explored whether Rac1 and other potential downstream-effectors of Rap1 are involved in cAMP-mediated WPB release.

Methods: Studies were performed in primary human umbilical vein endothelial cells. Activation of the small GTP binding protein Rac1 was monitored by its ability to bind to the CRIB domain of the serine/threonine kinase PAK1. Downstream-effectors of active Rap1 were identified by a proteomic screen using a GST-fusion of the Ras binding domain of RalGDS. Functional involvement of candidate proteins in WPB release was determined by RNAi mediated knockdown of gene expression.

Results: We identified the PI3K-dependent Rac exchange factor 1 (PREX-1) using a pulldown for putative downstream targets of activated Rap1 in endothelial cells. We subsequently explored whether Rac1 is activated in response to agonists that induce WPB release. Incubation of endothelial cells with epinephrine and forskolin but also the Epac1-specific cAMP-analogue Me-cAMP-AM and protein kinase A (PKA)-specific Bnz-cAMP-AM results in the activation of the small GTPase Rac1. Epinephrine and forskolin-induced Rac1 activation is partially abolished in Epac1 depleted cells. Additional treatment with the PKA inhibitor (PKI) completely abolishes the activation of Rac1. Depletion of Rac1 employing RNAi resulted in reduced epinephrine-induced VWF secretion. Also the Rac1 inhibitor EH $t_1$864 reduced both Rac1 activation and epinephrine-induced WPB release. These findings show that Rac1 modulates WPB release. RNAi-mediated downregulation of PREX-1 resulted in a reduced activation of Rac1 in response to Me-cAMP-AM. After silencing of PREX-1, endothelial cells required higher concentrations of epinephrine to induce VWF secretion. Also, the PI3K inhibitor Ly294002 reduced epinephrine-induced WPB release further implicating PREX-1 in WPB release.

Summary/Conclusions: Taken together our findings suggest that Epac1-Rap1-mediated WPB release proceeds through activation of Rac1 by PREX-1, thereby regulating release of haemostatic, inflammatory and angiogenic components from WPBs.

AS 37.3

Endoplasmic reticulum stress in diabetic nephropathy is mechanistically linked to coagulation protease-activated protein C signaling

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Background/Aim: Diabetic nephropathy (DN) is a multifactorial disease associated with substantial changes in the haemostatic system. A hallmark of diabetes induced haemostatic dysfunction is impaired thrombomodulin (TM) dependent-protein C (PC) activation. Impaired PC activation triggers glomerular podocyte and endothelial cell dysfunction, thus promoting DN. The intracellular mechanism through which loss of TM and PC activation contributes to DN is not known. Here we show that the haemostatic mediator activated PC (aPC) regulates cellular homeostasis by inhibiting hyperglycemia induced endoplasmic reticulum (ER)-stress in DN.

Methods: Hyperglycaemia was induced in wild-type (wt) mice or mice with altered activity of the TM-PC system (loss of function secondary to impaired PC activation [$TM^{Pro/LacZ}$] or gain of function with higher plasma levels of aPC [APC^{high}]). Subsets of diabetic mice were treated with the chemical chaperone tauroursodeoxycholic acid (TUDCA) to inhibit ER-stress. After 26 weeks of persistent hyperglycaemia

markers of DN were determined and tissue samples were isolated for *ex vivo* analysis. Supplementary *in vitro* assays were performed in podocytes and endothelial cells.
Results: Persistent hyperglycaemia in wt mice caused severe ER-stress and DN. *Ex vivo* analysis of transcription factors regulating the ER-stress response showed an increase of the ER-stress markers C/EBP homologous protein (CHOP) and activating transcription factor 6 (ATF6), while nuclear translocation of the highly conserved transcription factor X-box binding protein-1 (XBP1) was reduced in DN. These changes were aggravated in diabetic $TM^{Pro/LacZ}$ mice which has impaired protein C activation. Conversely, in a mouse model with constitutively higher aPC levels (APC^{high} mice) nuclear levels of XBP1 were normalized and expression of ATF6 and CHOP was reduced despite persistent hyperglycaemia. Restoring aPC levels or deletion of CHOP reverses the pathological ER-stress alterations in diabetic $TM^{Pro/LacZ}$ mice. In addition, podocyte specific inducible expression of active form of ATF6 aggravated DN. Pharmacological inhibition of ER-stress by using TUDCA normalized nuclear levels of XBP1, inhibited CHOP/ATF6 expression, and protected against DN in diabetic wt and $TM^{Pro/LacZ}$ mice. *In vitro* hyperglycaemia inhibited nuclear translocation of XBP1 in endothelial cells and podocytes, the two cellular components of glomerular filtration barrier. In agreement with *in vivo* observations, hyperglycaemia induced impaired nuclear translocation of XBP1 resulted in sustained activation of ER-stress markers CHOP/ATF6. Likewise, knock down of XBP1 in podocytes and endothelial cells aggravated ER-stress and hyperglycaemia induced cellular apoptosis. Activated PC directly promotes the nuclear translocation of XBP1 in these cells, which is required to inhibit hyperglycaemia induced ER-stress. Deletion of XBP1 in podocytes or endothelial cells abolished the cytoprotective effect of aPC. Furthermore, aPC regulates the interaction of PI3K regulatory subunit p85α with XBP1 which is known to mediate its nuclear translocation and diminished hyperglycaemia induced ER-stress.
Conclusion: These studies demonstrate that hyperglycemia induced ER-stress is causally linked to DN and establish a novel mechanistic link between haemostatic system and ER function in regulating cellular homeostasis in chronic kidney disease.

AS 37.4
Junctional adhesion molecule-A suppresses platelet integrin aIIbβ3 signaling by recruiting Csk to the integrin-c-Src complex

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Background: Dysregulation of the endogenous negative regulators present within the platelet may aid the thrombotic complications seen in various diseases. How endogenous negative regulators suppress platelet functions is not well understood. We have recently shown that junctional adhesion molecule-A (JAM-A), a member of the CTX (Cortical Thymocyte marker of the Xenopus) family is expressed on platelets. Using gene-targeted Jam-A null mice, we showed that *Jam-A* knockout mice show a prothrombotic phenotype, as assessed by significantly ($P < 0.00001$) shortened tail bleeding time, decreased carotid vessel occlusion time, ($P < 0.002$) and significantly increased susceptibility to pulmonary thromboembolism ($P < 0.008$). Platelet functional studies revealed that Jam-A null platelets hyperaggregate to physiological agonists. Furthermore, integrin inside-out signaling events were not affected, although outside-in signaling, such as platelet spreading and clot retraction, were significantly enhanced ($P < 0.0001$) in Jam-A null platelets compared to wild-type (WT).
Aim: To determine the molecular mechanism through which JAM-A suppresses platelet function.
Methods: We used gene-targeted Jam-A null mouse platelets. Wild-type (WT) mouse platelets were used as controls. Co-immunoprecipitation studies were used to evaluate association. Site-directed mutagenesis and GST pull-down assays were used to assess interaction.
Results: We found that outside-in signaling events such as Y^{773} phosphorylation of integrin b_3 subunit and Y^{418} phosphorylation of c-Src were significantly enhanced in Jam-A null platelets. Interestingly, we also found the absence of inhibitory Y^{529} phosphorylation of c-Src in resting Jam-A null platelets compared to WT, suggesting that C-terminal Src kinase (Csk), which is responsible for phosphorylation of Y^{529} may be absent from the integrin-Src complex. When analyzed for the presence of Csk, we found that in WT platelets, Csk, was abundantly present in the integrin $a_{IIb}b_3$ immunoprecipitate, but was completely absent in the integrin immunoprecipitate of the Jam-A null platelet lysates, suggesting that JAM-A may be responsible for the recruitment of Csk to the integrin complex in resting platelets. We also found that JAM-A associates with integrin $a_{IIb}b_3$ in resting platelets, which dissociates upon platelet activation. Concomitantly, Csk was also found to associate with JAM-A in unactivated platelets and dissociates upon platelet activation. Since Csk contains an SH2 domain, it is possible that Csk binds phosphotyrosine residue on its binding partner. In order to understand how Csk binds to JAM-A, we determined if JAM-A is tyrosine phosphorylated. We found that JAM-A is tyrosine phosphorylated in resting platelets and is rapidly dephosphorylated upon platelet activation. This tyrosine phosphorylation appears to be necessary for the association of Csk since Y^{280}-F mutant of JAM-A failed to associate with Csk, but not the integrin. In addition, recombinant SH2-domain of Csk efficiently pulled down endogenous tyrosine-phosphorylated JAM-A.
Summary: The results presented here strongly suggest that tyrosine-phosphorylated JAM-A is an endogenous negative inhibitor of integrin signaling and thus thrombosis. JAM-A recruits Csk to the integrin-c-Src complex, where Csk negatively regulates c-Src activation and thus suppresses the initiation of thrombus growth by attenuating outside-in signaling. Upon agonist stimulation, JAM-A is dephosphorylated on the tyrosine, allowing the dissociation of Csk from the integrin complex and thus facilitating outside-in signaling.

AS 38 – Blood Coagulation Tests

AS 38.1
Endothelial cell-based fluorogenic thrombin generation assay for the evaluation of the protein C anticoagulant system

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Background: *In vivo* thrombin generation (TG) is regulated by cooperation between the vascular endothelium and the pro- and anticoagulant systems in blood, such as the thrombin/endothelial cell (EC)-dependent activation of the protein C anticoagulant pathway that ultimately leads to inactivation of factors Va and VIIIa. *In vitro*, TG is assessed most commonly in platelet-poor plasma by a fluorogenic substrate-based thrombin generation assay (TGA) in microtiter plates. While this assay can accurately measure the kinetics of TG in plasma (including, Peak thrombin [PT], and endogenous thrombin potential [ETP]), it does not assess the influence of the EC-dependent protein C pathway on TG. As a result, the assay has a limitation in the assessment of the hypercoagulable patient.
Aim: In the present study, by introducing a surrogate endothelium to the TGA, we aim to enable activated protein C-induced inactivation of factors Va and VIIIa in the assay system.
Methods: Wells of flat-bottomed microtiter plates were coated with 4×10^4 quiescent EA.hy926 endothelial-like cells which consistently express thrombomodulin (TM) and the endothelial protein C receptor (EPCR). The concentration of active TM associated with EA.hy926 in the assay well (determined by a chromogenic assay) was ~0.5 nM. Tissue factor (TF)-initiated thrombin generation was evaluated in normal pooled plasma (NP), and in protein C-deficient (PCd), protein S-deficient (PSd), and heterozygous factor V Leiden (fVL) plasmas, in the presence or absence of EC.

Results: Thrombin generation in NP was inhibited in the presence of EC as evidenced by a 56% reduction in PT and 39% reduction in ETP. However, in PCd, PSd and fVL plasmas, endothelial-induced suppression of TG was blunted. Specifically, only relatively small reductions were observed in PT (31% in PCd, 13% in PSd, 23% in fVL) and ETP (11% in PCd, 7% in PSd, and 18% in fVL). Furthermore, the blunted inhibition of TG observed in PCd, PSd and fVL plasmas, was proportionally reversed when these plasmas were mixed with different percentages of NP or by the addition of protein C or S to PCd or PSd plasmas. In separate experiments in the absence of EC, we noted that while similar results were obtained in the presence of the soluble form of thrombomodulin, the concentration required (30 nM) was ~60 times higher than that offered by the EC monolayer. This suggests that protein C activation occurs more efficiently in the presence of EA.hy926 cells. The contribution of EC-derived tissue factor pathway inhibitor (TFPI), to the endothelial cell-induced inhibition of TG was ~15%.

Summary: By introducing an EC monolayer to the TGA, and measuring TG kinetics in the presence or absence of cells, we have adapted the assay to assess the contribution of the protein C anticoagulant system to TG in a physiologically relevant manner. This novel approach not only enables the functions of the endothelial-dependent PC pathway by expressing TM and EPCR, but also may provide other endothelial components relevant to TG (such asTFPI). This approach to TG assessment may therefore have both research and clinical applicability.

AS 38.2

Defining time in therapeutic range for clinicians: Frequency of dose changes and INR testing as surrogate markers for adequate vitamin K antagonist management

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Background: The RELY trial has shown that dabigatran (150 mg BID) is superior to vitamin K antagonist (VKA) for stroke prevention in patients with atrial fibriallation. The quality of VKA management was reported using the time in therapeutic range (TTR). A subgroup analysis of the RELY trials has shown that dabigatran is no longer superior to VKA in patients with TTR over 65%. Therefore, numerous national drug coverage agencies and private insurance companies have decided to only financially cover new anticoagulants if patients have TTR $\leq$ 65%. The calculation of the TTR is difficult and not readily available in daily clinical practice. Clinicians need to have a simple and convenient way to identify patients on VKA with TTR $\leq$ 65% who would benefit from coverage for these new anticoagulants.

Aim: We sought to predict a TTR $\leq$ 65% using a surrogate method of measurement (Number of changes in VKA dosing and INR testing) that can be used by primary care physicians.

Method: A cross-sectional study including patients on VKA actively being followed for anticoagulation management using the DAWN software was conducted. Consecutive patients who had received a minimum of 9 months of VKA therapy with a target INR between 2.0 and 3.0 were enrolled into the study. The TTR for each patient was calculated using the linear interpolation of the Rosendaal method. The number of dose changes and the number of INR tests done over the last 6 months were recorded.

Results: A total of 1381 were included in the analyses. The median age was 63 ($\pm$ IQR 52–75) and the median duration of anticoagulation was 1287 days ($\pm$ IQR 764–2268), most patients were anticoagulated for secondary prevention of venous thromboembolism (83.4%) or for atrial fibrillation (11.9%). The mean TTR was 81% ($\pm$ IQR 70–90). Both the number of dose changes and the number of INR tests were independent predictors of TTR (Spearman's correlation coefficient: 0.62 and 0.58 [$P < 0.001$]). Patients were stratified according to their mean TTR (TTR $\leq$ 65% [$n = 238$] and TTR > 65% [$n = 1142$]). In patients with TTR > 65%, the median number of dose changes and the number of INR test done within the previous 6 months were 1 (IQR 0–3) and 7 (IQR 5–9) respectively. In patients with TTR $\leq$ 65%, the 6-month median number of dose changes was 5 (IQR 3–7) and the number of INR tests done was 12 (IQR 9–15). The combination of $\geq$ 3 doses changes and/or $\geq$ 9 INR testing done within 6 months had a sensitivity of 87% (95% CI: 82–90%), specificity of 63% (95% CI: 60–65%) to identify patients with TTR < 65%. The NPV was 96% (95% CI: 94–97%).

Conclusion: Both the number of dose changes and the number of INR tests can be used as surrogate markers of TTR. Therefore, these surrogate markers may offer a simple and clinically relevant way for clinicians to identify which patients on VKA would benefit from coverage for the new anticoagulants.

AS 38.3

Prothrombin fragment 1+2 in urine; a new way of analysing blood coagulation activity

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Background: D-dimer may be used as a pretest to exclude deep vein thrombosis (DVT) and thus reduce the number of patients requiring imaging evaluations. A test based on spot urine could be more simple in several clinical situations. Prothrombin fragment 1+2 (F1+2) is excreted in the urine where it can be measured with an enzyme-linked immuno-sorbent assay. Several studies show an association between F1+2 levels in urine (uF1+2) and postoperative DVT.

Aims: We wanted to assess if levels of uF1+2 were associated with radiological confirmed DVT in symptomatic non-selected patients.

Methods: Patients aged $\geq$ 18 years with suspected DVT were included over a 2.5 year period. The study was conducted in accordance with the Declaration of Helsinki and approved by the regional Ethics Committee. Before the radiological procedure a urine sample (10 mL) was collected. Imaging was conducted with B-mode compression ultrasound and supplemented with venography when inconclusive. A commercial available ELISA kit (Enzygnost F1+2, Monoclonal) and a BEP 2000® analyzer (both Dade Behring, Marburg, Germany) were used for measuring uF1+2 levels. All patients were followed up for at least 3 months to detect new venous or arterial thromboembolic events or other conditions causing thrombin activity. Data analyses were conducted using SPSS version 19 (IBM, Armonk, New York, USA). Statistics were given as percent and number of patients or median with 25–75 percentile as appropriate. The non-parametric Mann-Whitney U test was used to assess differences in uF1+2 levels. The results were considered statistically significant if the two-sided P-value was $\leq$ 0.05.

Results: Median age of the 534 (260 men and 274 women) included patients was 60 (range 18–93) years and 108 (20.2%) patients had imaging verified DVT. Venography was performed in 52 (9.7%) patients due to inconclusive ultrasound. Median levels of uF1+2 was 28.1 pM and the DVT positive patients had statistically significant higher levels of uF1+2 than those without DVT (56.8 vs. 24.3 pM, $P < 0.001$).

Summary/Conclusions: We found that the urine levels of F1+2 was statistically significantly higher in patients with radiologically confirmed DVT compared to those without DVT. This indicates that F1+2 in urine can be used to determine increased procoagulant activity in patients with suspected DVT.

AS 38.4

Real-time dynamic measurement of hemostasis and fibrinolysis and detection of hemostatic and prothrombotic blood disorders by T2 magnetic resonance

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Background: There is no single currently available test to rapidly identify and assess impaired hemostasis, hypercoagulable states and fibrinolysis. This is in part because of difficulties in measuring integrated reactions in whole blood with one platform. T2 magnetic resonance (T2MR) is a new approach that allows for monitoring these processes in real time by measuring relaxation times of the hydrogen nuclei within the sample. The time-resolved signals generated with this method provide dynamic information about the physical states of complex samples, such as changes that occur in the blood during hemostasis.

Aims: Apply a novel detection method, T2MR, to monitor the dynamics of clot formation and fibrinolysis across a range of hemostatic conditions.

Methods: We used a small, portable instrument to measure changes in T2MR signal that continuously report on the dynamically changing microscopic environment of water during coagulation and clot contraction in whole blood. In these foundational studies, we measured 34 μL blood samples from consented normal adult donors continuously over 20 min using T2MR. We tested clotting of recalcified citrated whole blood initiated by the addition of thrombin or kaolin. To assess the sensitivity of this platform to platelet activity, we measured samples activated with ADP or arachidonic acid in the presence and absence of 2-methylthioadenosine 5′-monophosphate (2-MeSAMP) or aspirin. Results were confirmed by standard platelet aggregometry. Fibrinolysis in whole blood was induced by addition of different concentrations of tissue plasminogen activator (tPA) before, during or after clot formation.

Results: At normal platelet counts (150,000–300,000/μL) and normal hematocrit (HCT) (38–48%), the T2MR signals resolved into two peaks corresponding to: (i) water trapped within a retracted clot and (ii) water in the surrounding liquid, i.e. serum. Platelet counts < 50,000/μL or addition of aspirin or 2-MeSAMP eliminated the signal peak originating from clot contraction. Higher platelet counts (300,000–1,300,000/μL) and high thrombin concentrations (2 U/mL) generated a third, even more highly ordered peak. The third peak was confirmed to correspond to a tightly packed fibrin clot enriched in red blood cells (RBCs) with D2O exchange experiments and scanning electron microscopy. The signals also revealed the procoagulant influence of RBCs on clot formation/contraction over a wide range of HCTs (20–73%). Addition of tPA coincident with thrombin generated diverse species of fibrin/fibrin degradation products reflected in the dynamics of clot contraction and dissolution. Addition of tPA after clot contraction revealed heterogeneity in the susceptibility of clots formed under normal and procoagulant conditions to lysis.

Summary/Conclusions: These studies show that the dynamics and properties of clot formation and lysis across a range of hemostatic conditions can be characterized with T2MR measurements. This approach may be applicable to the study, diagnosis, and management of a spectrum of disorders of hemostasis, thrombosis and fibrinolysis. Additional studies will be needed to develop a more complete understanding of the biochemical events measured by T2MR and to more fully explore its clinical utility.

AS 39 – Regulation of Platelet Function

AS 39.1

Src-like adapter proteins (SLAPs) are critical negative regulators of GPVI/ITAM-signalling in arterial thrombosis and ischaemic stroke

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Background: The central activating platelet collagen receptor *glycoprotein* (GP) VI has been established as an attractive potential target for antithrombotic therapy as its blockade or antibody-induced deficiency provided powerful protection from experimental arterial thrombosis and acute ischaemic stroke without affecting platelet haemostatic functions. GPVI signals through an *immunoreceptor tyrosine-based activation motif* (ITAM) in a similar manner to the *T*- and *B-cell antigen receptors* (TCR, BCR).

The *Src-like adapter protein* (SLAP) and the closely related SLAP2 constitute a family of haematopoietic adapter proteins that are involved in the regulation of TCR and BCR surface expression levels and signalling via interaction with the phosphorylated ITAMs of their signalling complexes. However, the function of SLAP and SLAP2 in platelets, and in particular their effects on GPVI, are unknown.

Aims: The aim of this study was to investigate the role of SLAP and SLAP2 in platelet biology.

Methods: Mice deficient in SLAP and SLAP2 were generated and platelet function was analysed by flow cytometry, aggregometry, flow adhesion assays and biochemical methods. In addition, SLAP and SLAP2 were expressed in a cell line model system to study GPVI/ITAM signalling *in vitro*. The role of SLAP and SLAP2 *in vivo* was assessed in models of arterial thrombosis and ischaemic stroke.

Results: *Slap*/*Slap2*″**◆**/″**◆** mouse platelets displayed markedly increased integrin activation, granule release, aggregation and procoagulant activity upon stimulation with GPVI/ITAM″**◆**specific agonists. In contrast, activation responses to soluble agonists operating via G protein-coupled receptors were normal. The negative regulatory function of both adapters was confirmed in a heterologous system where overexpression of either SLAP or SLAP2 could almost completely inhibit GPVI signalling in response to collagen. Furthermore, SLAP/SLAP2-deficiency was associated with enhanced and sustained tyrosine phosphorylation of different proteins involved in the GPVI/ITAM″**◆**signalling cascade, including FcR γ-chain, Syk, LAT and PLCγ2. This effect was not based on the elevated GPVI expression levels observed in *Slap*/*Slap2*″**◆**/″**◆** platelets as it was fully preserved in *Slap*/*Slap2*″**◆**/″**◆**/*Gp6*+/− mice which exhibited reduced GPVI expression levels compared to the wild-type. *In vivo*, *Slap*/*Slap2*″**◆**/″**◆** mice displayed faster occlusive thrombus formation in the $FeCl_3$-injured carotid artery and a dramatically worsened outcome in a model of ischaemic stroke. This was evident by increased infarct volume and neurologic deficits in *Slap*/*Slap2*″**◆**/″**◆** mice compared to the wild-type 24 h after 30 min of transient middle cerebral artery occlusion.

Summary/Conclusion: Our results establish SLAP and SLAP2 as critical inhibitors of platelet GPVI/ITAM signalling, which has major pathophysiological significance in the setting of arterial thrombus formation and ischaemic stroke.

AS 39.2

Connexin40 regulates platelet function and thrombosis

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Background: Connexins are membrane proteins that assemble into connexons or hemichannels on the plasma membrane to facilitate the transport of small signalling molecules (up to ~1000 Da) between the interior and exterior of isolated cells. Upon docking, hemichannels form gap junctions between adjacent cells allowing direct intercellular communication. The presence of multiple connexins is recently reported on platelets, small circulating blood cells that maintain haemostasis and their inappropriate activation leads to thrombosis, a trigger for heart attack and strokes. The mechanistic phenomenon on the functions of various connexins in regulating platelet function is yet to be explored.
Aims: This study was performed to determine if connexin40 (Cx40) has a role in the regulation of platelet function and to establish whether Cx37 and Cx40 are able to function independently in these cells.
Methods: Human platelets were tested in the presence or absence of a selective peptide inhibitor of Cx40, ^{40}Gap27 on various functional assays such as aggregation, fibrinogen binding and granule secretion. Plasma clot retraction was also tested in the presence of ^{40}Gap27. To assess the effects of Cx40 deletion, platelets from *Cx40−/−* mice were analysed in comparison with those from *Cx40+/+* mice using aggregation, fibrinogen binding, granule secretion and clot retraction assays. To explore, if Cx40 function independently from Cx37, the platelets from *Cx40−/−* were analysed in the presence and absence of ^{37,43}Gap27 and similarly platelets from *Cx37−/−*were analysed using ^{40}Gap27.
Results: The Cx40 selective inhibitor, ^{40}Gap27 inhibited CRP-XL- (a GPVI selective agonist) and thrombin-induced human platelet aggregation, fibrinogen binding and granule secretion in concentration-dependent manner. Inhibition of Cx40 also resulted in reduced clot retraction, a process driven by outside-in signalling by integrin αIIbβ3 on the platelet surface. Deletion of Cx40 in mice also resulted in retarded platelet function and clot retraction. The ability of ^{40}Gap27 to inhibit the function of *Cx37−/−* (but not *Cx40−/−*) platelets, and conversely ^{37,43}Gap27 to inhibit *Cx40−/−* (but not *Cx37−/−*) platelets, suggests that Cx40 and Cx37 are able to function independently in platelets.
Conclusions: This study indicates that multiple members of the connexin family of proteins, beyond the previously characterised Cx37, are likely to be of importance in the regulation of platelet function, haemostasis and thrombosis.

AS 39.3

CalDAG-GEFI deficiency protects mice from FcγRIIa-mediated thrombotic thrombocytopenia induced by CD40L and β2GPI immune complexes

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Introduction: Platelet activation via Fcγ receptor IIa (FcγRIIa) is implicated in the pathogenesis of thrombotic complications triggered by immune complexes (ICs) resulting from autoimmune disease or the administration of therapeutic antibodies. We previously showed that ICs of antigen and antibodies targeting CD40 ligand (CD40L) or β2 Glycoprotein I (β2GPI) induce thrombocytopenia and thrombosis in mice transgenic for human FcγRIIa (hFcR) but not wild-type control mice (which lack FcγRIIa). *In vitro*, platelet aggregation downstream of FcγRIIa was dependent on signaling by the calcium-sensing guanine nucleotide exchange factor, CalDAG-GEFI, and the Gi-coupled receptor for ADP, P2Y12.
Aim: Here we studied the signaling response required for IC-mediated thrombocytopenia and thrombosis (ITT) in mice. Specifically, we evaluated ITT in hFcR transgenic mice deficient in CalDAG-GEFI and/or treated with the P2Y12 inhibitor clopidogrel bisulfate.
Methods: Pre-formed anti-CD40L ICs (M90+rCD40L) or anti-β2GPI (β2pAb+β2GPI) ICs were injected intravenously into hFcR/CalDAG-GEFI$^{+/+}$ or hFcR/CalDAG-GEFI$^{-/-}$ mice with or without clopidogrel pre-treatment. Inhibition of platelet P2Y12 by clopidogrel was confirmed by flow cytometry ex vivo. Animals were observed for symptoms of shock for 30 min, during which time core body temperature was monitored. Platelet counts were obtained before and 30 min after IC injection. Lungs were harvested at 30 min for assessment of thrombosis by histology or by near-infrared imaging (mice pretreated with Alexa-800-labeled anti-P-selectin antibody).
Results: CD40L and β2GPI ICs both rapidly induced severe symptoms of shock and marked reduction in body temperature in hFcRmice along with profound thrombotic thrombocytopenia. hFcR/CalDAG-GEFI$^{-/-}$ mice were almost completely protected from CD40L IC-induced thrombocytopenia/thrombosis and shock. Clopidogrel had a small effect on IC-induced ITT in hFcR and hFcR/CalDAG-GEFI$^{-/-}$ mice. CalDAG-GEFI deficiency also conferred marked protection from β2GPI IC-induced ITT. However, significant thrombocytopenia/thrombosis and hypothermia was still observed in clopidogrel treated hFcR/CalDAG-GEFI$^{-/-}$ mice, suggesting that β2GPI ICs can trigger platelet activation in the absence of these major signaling pathways.
Summary/Conclusions: Our studies demonstrate that clinically relevant ICs cause platelet FcγRIIa-dependent thrombocytopenia and thrombosis in hFcR mice. We further show that signaling by CalDAG-GEFI, but not by P2Y12, is critical for IC-induced platelet activation *in vitro* and *in vivo*. Thus, CalDAG-GEFI may be a promising target for the intervention of IC-associated, FcγRIIa-mediated thrombotic conditions.

AS 39.4

Platelet ITAM signaling is critical for maintenance of vascular integrity during inflammation

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Background: Blood platelets, long recognized for their importance in primary hemostasis, have recently been identified as critical regulators of vascular integrity during inflammation and cancer. Interestingly, the contribution of platelets to the maintenance of vascular integrity in inflammation seems to be independent of their ability to form a clot.
Aim: To identify signaling molecules critical for the contribution of platelets to vascular integrity in inflammation.
Methods: We here describe a novel method for the generation of mice with platelet-specific signaling defects, which is based on the adoptive transfer of platelets into thrombocytopenic (TP) mice. Depletion of circulating platelets was achieved in transgenic mice with antibodies that recognize hIL-4R, a heterologous antigen expressed on circulating platelets in these animals. Platelets from various knockout mice or inhibitor-treated wild-type (WT) platelets were transfused into TP mice and tested for their ability to support vascular integrity in a reverse passive Arthus reaction (rpA) model and LPS-induced inflammation in the lung. Hemoglobin (Hb) content in skin tissue and bronchoalveolar lavage (BAL) was used to quantify hemorrhage at sites of inflamamtion.
Results: Inflammation-induced hemorrhage was significantly increased in TP mice compared to controls. Vascular integrity at sites of

inflammation was not impaired in TP mice transfused with 8×10^8 WT platelets before challenge. Unexpectedly, platelets defective in GPCR signaling, a crucial component of platelet plug formation and hemostasis, were indistinguishable from WT platelets in their ability to support vascular integrity in both models of inflammation. In contrast, genetic deletion of *Clec2* or inhibition of GPVI (inhibitory antibody JAQ1), the only ITAM receptors expressed on mouse platelets, significantly reduced the ability of platelets to prevent inflammation-induced hemorrhage. Moreover, transfusion of JAQ1-treated *Clec2−/−* platelets or platelets lacking SLP-76, an adapter protein critical to ITAM signaling, into TP mice had no significant effect on vascular integrity during inflammation.

Summary/Conclusions: By generating mice with multiple platelet-specific signaling defects, we demonstrate that vascular integrity in inflammation depends on both ITAM receptors, GPVI and CLEC2, as well as the adapter protein SLP-76. Our studies highlight potential complications associated with novel antiplatelet drugs targeting the ITAM signaling pathway, which may lead to bleeding at sites of inflammation.

AS 40 – Thrombotic Micro-Angiopathies

AS 40.1

A novel CD46 gene mutation in a patient with normal ADAMTS13 activity and microangiopathy

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Background: The differential diagnosis and etiologic classification of the two main forms of thrombotic microangiopathy, thrombotic thrombocytopenic purpura (TTP) and atypical hemolytic uremic syndrome (HUS), remain challenging. TTP is usually characterized by the severe deficiency of the von Willebrand factor (VWF) cleaving protease, ADAMTS13. Atypical HUS is characterized by hyperactivation of the alternative complement-system. The prevalence of severe ADAMTS13 deficiency in TTP is particularly high in patients with idiopathic disease and absence of renal involvement, reaching 75–80% in some cohorts. In a small fraction of patients with a diagnosis of idiopathic TTP and absent (or minor) renal involvement, ADAMTS13 activity may be only slightly reduced or normal at acute disease presentation. The pathophysiological mechanisms underlying microvascular thrombosis in these patients are unknown.

Aims: To investigate etiology in a 27-year-old woman with a diagnosis of TTP with minor renal involvement and normal ADAMTS13 activity at presentation.

Methods: The patient was referred to the Milan TTP registry (URL: www.ttpdatabase.org) with a diagnosis of TTP (thrombocytopenia, microangiopathic hemolytic anemia and no overt renal failure). Blood samples were collected both at acute episode before starting any transfusional therapy and during remission. We measured ADAMTS13 antigen, activity, VWF- and complement system-related plasmatic parameters. Mutations in six complement-system genes (*CFH, CFI, CD46, CFB, THBD* and *C3*) were searched for by PCR and Sanger sequencing. The protein coding area of the genome (i.e. the exome) was sequenced by target-capture and sequencing on Illumina HiSeq 2000. The role of a novel mutation of the *CD46* gene was studied by expression of MCP on leucocytes by fluorescence-activated cell sorter (FACS).

Results: A 27-year-old woman with thrombocytopenia (platelet counts: 14×10^9/L; normal values [n.v.]: 150–450 $\times 10^9$/L), microangiopathic hemolytic anemia (hemoglobin: 8.9 g/dL; n.v.: 11.5–16 g/dL), and slightly increased serum creatinine (117 μM; n.v.: 53–106 μM), was diagnosed as TTP and was treated with plasma-exchange. Remission was achieved after 7 daily exchange procedures. Large multimers of von Willebrand factor were depleted and complement system was activated during acute disease, while ADAMTS13 activity was normal. Sequencing of six complement system genes revealed a novel splice-site mutation of *CD46*. Exome sequencing revealed 12 nonsynonymous changes in 11 genes encoding proteins implicated in the pathophysiology of thrombotic microangiopathies, including the novel *CD46* mutation. The mutation was predicted to create an alternative splice site within the open reading frame of exon 4, resulting in the in frame deletion of six aminoacids of the encoded complement membrane-cofactor. Studies on mRNA isolated from the patient and from family members carrying the mutation showed the presence of abnormal transcripts bearing the deletion. Low surface expression of MCP on PBMC was confirmed by FACS.

Summary/Conclusions: This study shows that complement system gene mutations may be implicated in the occurrence of TTP with normal ADAMTS13 at presentation, even when there are little signs of renal involvement.

AS 40.2

Complement activation and cytokine response in TTP

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Background: Thrombotic Thrombocytopenic Purpura (TTP) is a microangiopathy associated with organ dysfunction and a high level of morbidity and mortality. Complement dysregulation and subsequent overactivation of complement effector proteins (including the anaphylatoxins C3a and C5a) is established in atypical Haemolytic Uraemic Syndrome (aHUS), and responds clinically to C5 inhibition. However, the role of complement in TTP is unknown.

Aims: We carried out a prospective study investigating the role of complement anaphylatoxins C3a and C5a in patients with TTP during acute episodes, and in remission, and compared these with normal controls. For both acute and remission groups we also investigated the cytokine response (Th1/Th2/Th17- involving IL-2, IL-4, IL-6, IL-10, TNF, IFN-γ and IL-17a) and association with disease state.

Methods: EDTA plasma was obtained from 21 patients (12F, 9M) with acute TTP, ADAMTS13 activity < 5% and positive anti-ADAMTS13 IgG antibodies (median IgG 59%, range 8–180%). Also, EDTA Plasma from 50 patients (34F, 16M) previously treated for acute TTP, now in clinical remission (normal ADAMTS13 activity and a platelet count > 150×10^9/L). Paired EDTA plasma was obtained from 12 patients (6F, 6M) for whom both acute and remission samples were available. Measurement of complement anaphylatoxins C3a (NR 32.5–56.1 ng/mL) and C5a (NR 1.7–13.6 ng/mL) was performed using BD™ Human Anaphylatoxin Kit, (BD Biosciences, San Jose, CA, USA) using a Cytometric Bead Array (CBA) method. Serum was obtained from acute and remission patients, enabling the measurement of cytokines, using BD™ Human Th1/Th2/Th17 Kit, (BD Biosciences, San Jose, CA, USA) also incorporating the CBA method.

Results: In acute TTP, median C3a and C5a were significantly elevated compared to patients in remission, C3a 66 vs. 38.5 ng/mL ($P < 0.001$) and C5a 17 vs. 10 ng/mL ($P < 0.001$), respectively. For the 12 patients with paired acute and remission samples, median C3a levels were significantly higher during the acute episode than in remission, C3a 45.5 vs. 35.8 ng/mL ($P = 0.041$); there was a non-significant difference in C5a levels 15.2 vs. 10.6 ng/mL ($P = 0.084$). Compared to controls, remission median C5a levels were significantly higher 10 vs. 5.81 ng/mL ($P = 0.001$); however remission C3a levels were lower than controls 38.5 vs. 43.7 ng/mL ($P = 0.046$). Within the acute TTP group, there was no significant difference in C3a or C5a levels for patients

requiring ITU admission vs. those not requiring ITU care or patients with or without neurological features at presentation. Both median IL-6 and IL-10 levels were significantly higher in the acute vs. remission groups, IL-6: 8 vs. 2 pg/mL ($P = 0.002$), IL-10: 6 vs. 2 pg/mL ($P < 0.001$). C3a levels were strongly correlated with both anti-ADAMTS13 IgG ($r_s = 0.731$, $P = 0.002$) and IL-10 ($r_s = 0.627$, $P = 0.012$) levels.

Summary/Conclusion: These results suggest anaphylatoxin levels in TTP vary between acute and remission cases, and highlight the possibility that the complement response seen acutely may relate to both the degree of anti-ADAMTS13 IgG antibody level and cytokine (IL-10) levels. Complement inhibition at presentation in the acute phase may have therapeutic potential.

AS 40.3

Plasmin cleavage of von Willebrand factor; a physiological and therapeutic bypass for ADAMTS13 deficiency and thrombotic microangiopathy

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Background: ADAMTS13 controls Von Willebrand Factor (VWF) multimer size through continuous proteolysis. This prevents spontaneous platelet agglutination on ultra-large VWF multimers and formation of pathological microthrombi. ADAMTS13 deficiency is often caused by inhibiting autoantibodies and can result in microangiopathy. This is treated by plasmapheresis, which is inefficient and costly. Enigmatically, a complete congenital or persistent acquired ADAMTS13 deficiency does not lead to continuous thrombotic disease.

Hypothesis: Plasmin, the key enzyme of the fibrinolytic system, can serve as a physiological backup enzyme for the degradation of unfolded VWF multimers in absence of ADAMTS13. We base this hypothesis on the following evidence: 1) Plasmin can cleave VWF under purified conditions 2) Plasmin activity is reported in ADAMTS13 deficient persons. 3) uPA triggers plasmin activation on activated endothelial cells, but is redundant for the clearance of fibrin.

Results: Plasminogen activation, either induced by uPA/uPAR or streptokinase, leads to rapid degradation of endothelial-cell tethered strings of platelet-decorated VWF. In the presence of plasmin, ristocetin-induced platelet agglutinates form normally, whereafter they are fully degraded. In contrast, plasmin acivity does not reduce aggregate formation on collagen surfaces at arterial shear rates. These findings indicate that platelet-VWF complexes are preferred substrates for plasmin degradation. Plasminogen binds to immobilized VWF in a lysine-dependent manner through multiple binding sites, one of which resides in the A1 domain. Patients with acute TTP episodes have elevated levels of PAP-complexes, indicating plasminogen activation.

Conclusions: We propose that during reduced or absent activity of ADAMTS13, plasmin serves as a natural backup for the degradation of pathologically large VWF multimers. As streptokinase is able to degrade platelet-decorated VWF strings on endothelial cells, as well as platelet VWF-agglutinates, induction of plasmin activity may have therapeutic value in the treatment of VWF-mediated microangiopathies. Especially when neutralizing autoantibodies cause ADAMTS13 deficiency, this bypassing strategy may prove useful.

AS 40.4

The phenotype of ADAMTS13 mutation 4143_4144insA – a study of 11 homozygous cases in Norway

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Background: Patients with congenital thrombotic thrombocytopenic purpura (TTP) suffer recurrent acute attacks of microangiopathic haemolytic anaemia and thrombocytopenia due to an inherited severe deficiency of von Willebrand factor cleaving metalloprotease ADAMTS13. However, time of onset and severity of the disease, in terms of number of acute attacks, end-organ damage, and dependency on regular plasma infusions vary widely among patients. Effort has been put down to determine to what extent different genotypes correspond to different clinical phenotypes. The fact that more than 100 different mutations have been reported in congenital TTP, most of them in compound heterozygous state, represents an obstacle in this work. In Norway, we have a particularly high prevalence of patients homozygous for the common mutation 4143_4144insA.

Aim: To study the clinical phenotype of Norwegian patients homozygous for mutation 4143_4144insA and compare it with previously published cases with the same mutation.

Methods: The study combined retrospective review of participating patients' medical records, and literature searches. Ethical review board approval and patients' informed consents were obtained.

Results: There were five (46%) females, and six males from eleven different families of non-consanguineous marriages. Median age at inclusion in the study was 30 years (range 5–63). Seven patients (64%) had symptoms of TTP in the neonatal period, although diagnosed years later. Two patients (18%) had disease onset during childhood, and two (18%) in adulthood. Mean and median age at first TTP attack requiring plasma therapy was 25 and 22 years, respectively (range 3–52). Five patients (46%) received regular prophylactic plasma infusions, and two (18%) only on special occasions. The average amount of plasma required per month was 4 units (range 2–6). Two patients (18%) had five or less TTP attacks, seven patients (64%) had between six and nine, and two patients (18%) had ten or more documented acute attacks. Three patients had complications in pregnancy related to TTP, one had a mild behavioural developmental disorder, one had chronic renal insufficiency, one had neurological sequelae, and six had no complications. Only two patients were correctly diagnosed with TTP at their first presentation of disease. Other diagnoses were haemolytic-uremic syndrome (3), Evans syndrome (2), immune thrombocytopenic purpura (1), disseminated intravascular coagulation (1), glomerulonephritis (1), hepatitis (1), and pregnancy related haemolytic crisis (1). Twenty-four reported cases with 4143_4144insA mutation were identified in the literature, eleven of which were homozygous (from nine families). There were limited clinical data on ten patients, and no strict definitions on disease severity.

Summary: Overall, patients homozygous for *ADAMTS13* mutation 4143_4144insA tend to have a relatively mild phenotype, despite symptoms since early childhood in most. However, we cannot rule out that we in this retrospective analysis have selected milder cases that survive long enough to get a molecular diagnosis. Misdiagnosis was usual at first presentation. Due to lacking definitions on disease severity it was difficult to compare our material with previously published cases. We suggest evidence of end-organ damage and dependency on prophylactic plasma infusions may be used to assess disease severity in congenital TTP.

AS 41 – Natural Anticoagulants

AS 41.1

Protease-activated receptor 3 (PAR3) tethered-ligand peptides derived from non-canonical cleavage at Arg41 by activated protein C provide vascular barrier protective effects *in vitro* and *in vivo*

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Background: Activated protein C (APC) conveys cytoprotective effects on cells and in various murine disease models that generally require the endothelial protein C receptor (EPCR) and protease-activated receptor 1 (PAR1). In contrast, thrombin signaling via PAR1 is proinflammatory, thus it is unclear how PAR1 can mediate both cytoprotective APC signaling as well as proinflammatory thrombin signaling. Neuro- and nephro-protective activities of APC that required PAR3 suggested involvement of PAR3. We found that cleavage of PAR3 by APC is EPCR dependent and proportional to the expression of EPCR on cells. Remarkably, APC and thrombin cleaved the *N*-terminal domain of PAR3 at different sites, with proteolysis of PAR3 by thrombin at K38 and cleavage by APC at R41.
Aim: We hypothesize that the specific cleavage site of PAR3 by APC creates a non-canonical tethered-ligand that, in contrast to the canonical PAR3 tethered-ligand generated by thrombin, will convey APC-like cytoprotective activities and improve vascular barrier function *in vitro* and *in vivo*.
Methods: PAR3 peptides of different length, either starting at T39 (P3K) or G42 (P3R) were tested for vascular barrier function *in vitro* on EA.hy926 endothelial cells. Vascular permeability *in vivo* was determined in immune-competent hairless mice by analyzing VEGF-induced Evans blue leakage in the skin and quantification by infrared fluorescent imagery.
Results: Induction of endothelial barrier disruptive effects by thrombin requires PAR1 but blocking antibodies against PAR3 revealed PAR3 contributions, indicating that PAR3 could modulate endothelial barrier function. PAR3 tethered-ligand peptides (13- and 24-mer P3R peptides) derived from APC cleavage at Arg41, but not 6-mer P3R peptides, prevented endothelial barrier disruptive effects induced by either thrombin or histones similar to APC. In contrast, P3K peptides (either 6-, 13- or 24-mer) did not prevent disruptive effect by thrombin or histones. Since PAR3 cannot signal on its own, but was shown to modulate PAR1 signaling, assays were performed in spontaneously leaky endothelial cells that do not rely on PAR1 to induce permeability. P3R peptides decreased spontaneous leakage but the barrier protective effects of P3R were abolished by a PAR1 antagonist (SCH79797), indicating that the barrier protective effects of non-canonical PAR3 tethered-ligand peptides required PAR1. *In vivo*, intravenous injection of 24-mer P3R peptides 60 min before injection of VEGF decreased vascular leakage by 38% ($P < 0.05$, $n = 8$ mice). In contrast, P3K had no effect and neither P3R nor P3K peptides affected vascular leakage in the absence of VEGF. Remarkably, injection of P3R peptides 5 min before VEGF was not vascular protective, indicating that P3R-mediated vascular protection is not an acute event.
Conclusions: Collectively, these results demonstrate that peptides (13-mer or longer) derived from PAR3 cleavage by APC at R41 act as APC-mimetics, reflecting the broad vascular barrier protective effects of APC *in vitro* and *in vivo*. The protective effect required PAR1, suggesting that the P3R peptides may act as allosteric modulators of PAR1 signaling. We propose a novel biochemical mechanism for APC-induced cell signaling via PAR3 and the generation of a novel non-canonical *N*-terminal tethered ligand that initiates APC-mimetic vascular protective signaling.

AS 41.2

Direct inhibition of factor VIIa by TFPI and TFPI constructs

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Background: TFPI is a multi-Kunitz domain protease inhibitor that down-regulates the extrinsic coagulation pathway by inhibiting FXa and FVIIa. TFPI inhibits FXa via a slow-tight binding mechanism and inhibits TF-FVIIa in a direct FXa-independent manner (formation of a TFPI-TF-FVIIa complex) and FXa-dependent manner (formation of a quaternary TFPI-FXa-TF-FVIIa complex).
Aims: To investigate the role of the three Kunitz domains (KD) of TFPI in FVIIa inhibition using full length TFPI (TFPI-fl) and truncated TFPI constructs.
Methods: Recombinant full length TFPI (TFPI-fl) and TFPI constructs (KD1, KD1-KD2, TFPI1-150) were purified from bacterial expression systems. Inhibition of FVIIa with/without relipidated tissue factor (TF) or soluble TF (sTF) by TFPI-fl/TFPI constructs was quantified with a FVIIa-specific chromogenic substrate.
Results: TFPI-fl inhibited TF-FVIIa via a monophasic reaction that was rather slow at low TFPI-fl concentrations ($t_{½}$~5 min at 2 nM TFPI) and had a K_i of 4.6 nM. In the presence of sTF and without TF, TFPI-fl was a poor FVIIa inhibitor with K_i values of 122 and 1118 nM, respectively. TFPI constructs without KD3-*C*-terminus (TFPI1-150 and KD1-KD2) were 7–10-fold less effective than TFPI-fl in inhibiting TF-FVIIa and sTF-FVIIa. Compared to KD1-KD2, KD1 was a poor TF-FVIIa inhibitor (K_i = 434 nM). Protein S, which is a co-factor of TFPI-fl in FXa inhibition, also stimulated TF-FVIIa inhibition by TFPI-fl and decreased K_i ~7-fold (K_i = 0.7 nM). Protein S required phospholipids and the KD3-*C*-terminus of TFPI to express TFPI cofactor activity. In presence of FXa, a tight quaternary TF-FVIIa-TFPI-FXa complex was formed with TFPI-fl, TFPI1-150 and KD1-KD2 with K_i values < 0.15, 0.5 and 0.8 nM, respectively. Phospholipids and the Gla-domain of FXa were required for quaternary complex formation.
Summary and Conclusions: Our study shows that TFPI-fl is a direct TF-FVIIa inhibitor with protein S as co-factor, which decreases the Ki value close to the plasma TFPI-fl concentration (0.25–0.5 nM). Truncated forms of TFPI were much less active than TFPI-fl indicating that the KD3-*C*-terminus significantly contributes to direct inhibition of FVIIa by TFPI. Although it is generally accepted that KD1 binds to and inhibits FVIIa, KD1-KD2 was much more effective in FVIIa inhibition than KD1. This indicates that the KD2 domain of TFPI, which actually targets FXa, also contributes to FVIIa inhibition. Phospholipids and TF significantly enhance direct TF-FVIIa inhibition by TFPI-fl. In presence of FXa, TFPI-fl and truncated TFPI constructs efficiently formed a quaternary complex indicating that the KD3-*C*-terminus is not a prerequisite for quaternary complex formation.
Considering the TFPI-fl concentration in plasma (0.25–0.5 nM), one may question whether direct inhibition of TF-FVIIa by TFPI in the absence of FXa but in the presence of phospholipids and protein S (Ki~0.7 nM) is physiologically important. In this respect it should be emphasized that at the site of a growing thrombus the local TFPI concentration may be significantly higher due to release of TFPI from platelets. At such conditions, direct inhibition of TF-FVIIa by TFPI may contribute to the down-regulation of thrombin formation.

AS 41.3

Protective effects of non-anticoagulant activated protein C variant (D36A/L38D/A39V) in a murine model of ischaemic stroke

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Background: Ischaemic stroke is caused by occlusive thrombi in the cerebral vasculature that lead to ischaemia/tissue damage. Patients treated within 4.5 h of onset of symptoms can receive tissue-plasminogen activator (tPA) as thrombolytic therapy, which however has some major limitations including blood brain barrier disruption and increased risk of bleeding. Treatments that prevent/limit such deleterious side-effects could be of clinical importance. Activated protein C (APC) regulates thrombin generation through the protein S-dependent inactivation of FVa and FVIIIa. APC also exhibits cytoprotective cell signalling mediated through activation of protease-activated receptor 1. In murine models of stroke, APC in conjunction with tPA can limit the deleterious effects of tPA, however, APC in this setting can further elevate the risk of bleeding. Thus, APC variants with reduced anticoagulant, but normal cytoprotective function represent potential therapeutic agents.

Aims: Three APC variants with reduced anticoagulant, but normal cytoprotective function have been engineered; 1) APC KKK191-193AAA/R229A-R230A – APC(5A) 2) APC D222C-R237C – APC (Ca-ins), and 3) APC D36A/L38D/A39V – APC(36–39) – the latter which abolishes the ability of APC to be enhanced by its cofactor, protein S. We aimed to compare the relative anticoagulant functions and rates of inactivation by protein C inhibitor (PCI) of these three APC variants, and to explore the therapeutic benefit of the APC(36–39) in a murine model of ischemic stroke.

Methods: Human wild type (wt) and variant protein C, and their murine counterparts, were expressed, purified, activated and quantified. The relative anticoagulant functions of these variants were assessed using thrombin generation assays (TGA). The rate of inactivation of human variants by PCI was assayed using the chromogenic substrate S2366. Using a murine middle cerebral artery occlusion (MCAo) model, we measured neurological scores, cerebral infarction, oedema and cerebral haemoglobin 24 h post-MCAo and the influence of administration of t-PA with and without co-administration of either murine wtAPC or APC(36–39).

Results: Human and murine wt protein C, protein C(36–39) were expressed and secreted normally. The protein C(Ca-ins) variants were secreted partly as disulphide-linked dimers. The protein C(5A) variants were secreted with lower efficiency. TGA suggested reductions in anticoagulant function of ~50-fold for APC(36–39), ~25-fold for APC (Ca-ins) and ~17-fold for APC(5A). In PCI inactivation assays, wtAPC, APC(36–39) and APC(Ca-ins) were all inhibited similarly ($t_{½}$ – 33 to 39 mins). APC(5A) was inactivated ~9-fold faster ($t_{½}$ – 4 mins). Using the murine MCAo ischaemia/reperfusion injury model, APC (36–39) in combination with tPA improved neurological scores, cerebral infarct area and oedema ratio as well as, or better than, wt APC. APC(36–39) alone also significantly reduced cerebral haemoglobin (a measurement of bleeding) induced by administration of tPA, whereas wt APC was unable to significantly improve cerebral bleeding.

Summary/Conclusions: APC(36–39) is a non-anticoagulant form of APC that is expressed and activated normally and inactivated by PCI with similar kinetics to wtAPC. APC(36–39) in conjunction with tPA can ameliorate ischaemia/reperfusion injury in mouse brains and reduce the deleterious effects of tPA. These attributes suggest that APC(36–39) is a potential therapeutic agent with possible advantages over other non-anticoagulant APC variants.

AS 41.4

Identification of a novel pathway for encryption of the endothelial protein C receptor (EPCR) by TNFa that results in loss of activated protein C (APC) binding and induction of cellular APC resistance

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Activated protein C (APC) provides antithrombotic and cytoprotective beneficial effects that require the endothelial protein C receptor (EPCR). EPCR is a CD1-like endothelial receptor with a phospholipid embedded in its hydrophobic groove that is required for (A)PC binding. According to the current paradigm, cytokine-induced EPCR shedding is responsible for EPCR inactivation on cells. However, upon incubation of EA.hy926 endothelial cells or blood outgrowth endothelial cells (BOECs) with TNFα, a disproportional loss of APC binding compared to EPCR surface expression was observed, inconsistent with EPCR shedding. Using a novel on-cell Western approach, we found that TNFα decreased EPCR-dependent APC binding on EA.hy926 cells to 25% of normal, whereas surface EPCR remained unchanged. In contrast, induction of EPCR shedding with PMA decreased APC binding and released abundant soluble (s)EPCR, which was not observed with TNFα. Accordingly, inhibition of EPCR shedding prevented PMA but not TNFα-induced loss of APC binding to cells. Thus, shedding was not responsible for loss of EPCR-dependent APC binding induced by TNFα on endothelial cells. Another proposed mechanism for EPCR inactivation involved cleavage of the fatty-acid chain at the sn-2 position of the lipid embedded in the hydrophobic groove of EPCR by phospholipase A_2 (PLA_2). However, inhibitors of PLA_2 did not restore loss of APC binding to cells induced by TNFα. In contrast, inhibitors of PLA_2 did block the loss of EPCR-dependent APC binding to endothelial cells induced by serum derived from TNFα-incubated whole blood. Thus, lipid editing by PLA_2 is important for EPCR inactivation on cells but was not involved in TNFα-induced loss of APC binding to endothelial cells. No *N*- or *C*-terminal proteolysis of EPCR in cells treated with TNFα was observed. However, APC binding to purified EPCR derived from TNFα-treated EA.hy926 cells was defective, whereas EPCR purified from non-treated cells showed normal APC binding. Next, two distinct nodes involved in TNFα signaling, NFκB and p38 MAPK were targeted. Interestingly, inhibition of NFκB completely normalized APC binding to TNFα-treated cells while inhibition of p38 had no effect. Insights gleaned from lipid editing in CD1 molecules suggest the involvement of clathrin-mediated mechanisms. An inhibitor of clathrin-mediated transport prevented TNFα-induced EPCR encryption but not NFκB-dependent ICAM1 expression induced by TNFα, indicating that the inhibitor did not interfere with general NFκB-dependent TNFα signaling. In contrast, an inhibitor of dynamin-mediated transport prevented both TNFα-induced EPCR encryption and ICAM1 expression. Remarkably, neither inhibitors inhibited TNFα-induced EPCR internalization, suggesting that the clathrin inhibitor may have affected endocytic sorting pathways rather than internalization of EPCR.

In summary, our results conceptualize a novel EPCR encryption mechanism that is responsible for TNFα-induced loss of EPCR-dependent binding of APC to endothelial cells. The EPCR encryption mechanism seemed partially homologous to lipid editing in CD1 molecules, and involved NFκB and clathrin-mediated effects. Insight into the molecular mechanisms for EPCR encryption may help to protect EPCR function on cells, improve efficacy of EPCR-dependent cytoprotective effects on cells and prevent inflammation-induced cellular APC resistance.

AS 42 – Diagnosis of Deep Vein Thrombosis and Pulmonary Embolism

AS 42.1

Age-adjusted D-Dimer for venous thromboembolism exclusion in the elderly: a systematic review and meta-analysis

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Background: In combination with clinical probability assessment, a negative D-Dimer test allows to safely rule out the diagnosis of venous thromboembolism (VTE) in approximately one-third of patients, without any imaging test. D-Dimer levels increase with age. As a result, the proportion of patients in whom VTE could be ruled out on the basis of negative D-Dimer decreases with age. After 80 years of age, only 5% of patients will benefit from D-Dimer test. Recently, an age-adjusted cut-off was derived (age-adjusted cut-off = patient's age × 10, in μg/L), that might rule out VTE in a higher proportion than the conventional 500 μg/L cut-off without compromising diagnostic exclusion safety. Several retrospective external validation studies were published, but the limited number of patients included in these studies didn't allow to draw firm conclusions.

Aims: To assess the usefulness and safety of the age-adjusted cut-off for VTE exclusion in elderly patients.

Methods: A systematic literature search was conducted to identify potential studies on Medline and Embase, as well as on proceedings of recent American Society of Hematology and International Society of Thrombosis and Hemostasis meetings. From each retrieved study, we extracted the total number of patients, the number of patients with a low/intermediate or unlikely clinical probability of DVT or PE, the number of patients with negative D-Dimer test using the conventional and the age-adjusted cut-off, respectively, and the number of false-negative tests. We computed the pool proportions and 95% confidence intervals using inverse variance-weighted proportions, with a focus on the proportion of false-negative patients in those with a D-Dimer level above the conventional but below the age-adjusted cut-off. Subgroup analyses were performed on patients aged > 80 years.

Results: We identified 12 studies, gathering 22 537 patients with suspected VTE. Overall, 40% (95% CI 34–46%) of low/intermediate or unlikely probability of VTE had a negative D-Dimer at the conventional cut-off, with a false-negative rate of 0.5% (0.2–0.9%). Using the age-adjusted cut-off, 45% (39–51%) would have had a negative D-Dimer, with a false-negative rate of 0.7% (0.4–1.19%). Among the 1399 patients aged > 80 years, only 13% (9–18%) had a negative D-Dimer at the conventional cut-off, but this proportion increased to 33% (26–40%) when using the age-adjusted cut-off. The false-negative rates for the conventional and the age-adjusted cut-off were 0.3% (0.1–0.6%) and 2.3% (1.1–4.1%), respectively. The false-negative rate among the 240 patients aged > 80 years with a D-Dimer level > 500 μg/L but below their age-adjusted cut-off was 3.3% (95% CI 1.5–5.9%).

Summary/Conclusions: Age-adjusted cut-off allows to significantly increase the proportion of elderly patients in whom VTE could be excluded on the basis of D-Dimer test in combination with clinical probability assessment. However, the false-negative rate appears to be higher and might raise concerns about the safety of this strategy in elderly patients. The results of an ongoing prospective management outcome study are needed to formally assess the safety of the age-adjusted D-Dimer cut-off.

AS 42.2

Safety and feasibility of a diagnostic algorithm combining clinical probability, D-dimer and ultrasonography in suspected upper extremity deep vein thrombosis: a prospective management study

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Background: Traditionally, the focus of the diagnosis of venous thromboembolism (VTE) is on deep vein thrombosis (DVT) of the leg and pulmonary embolism. Until recently, upper extremity DVT (UEDVT) was regarded as an uncommon presentation of VTE; however, the more widespread use of central venous catheters has caused a significant increase in its incidence. Therefore, effective and safe diagnostic strategies are needed.

Aims: This diagnostic management study assessed the safety and feasibility of a new diagnostic algorithm in patients with clinically suspected UEDVT.

Methods: In- and outpatients with suspected UEDVT were recruited from January 2010 until July 2012 in 16 hospitals in Europe and the United States, after approval of the protocol by the institutional review boards. Main exclusion criteria were previous UEDVT and the use of therapeutic doses of anticoagulants. Informed consent was obtained from all participants. The algorithm consisted of the sequential application of the Constans' clinical decision score[1] (score), D-dimer testing and compression ultrasonography. Patients were first categorized as UEDVT likely or unlikely by the score. In patients with an unlikely score and a normal D-dimer, UEDVT was considered excluded and no further testing was done. All other patients underwent ultrasonography, which first assessed the presence of UEDVT and then superficial vein thrombosis (SVT). The primary outcome was the 3-month incidence of symptomatic UEDVT and pulmonary embolism in patients with a diagnostic work-up excluding both UEDVT and SVT. To confirm an acceptable failure rate of excluding UEDVT (upper 95% confidence interval below 3%), approximately 400 patients needed to be included.

Results: The study population comprised of 406 consecutive patients with suspected UEDVT. The algorithm was feasible and could be completed in 96%. Of the 406 patients, 203 had an unlikely probability score and D-dimer was measured, except in three cases. In 87 patients (22%) an unlikely score was combined with a normal D-dimer, and therefore UEDVT was excluded. All these patients had an uneventful 3 month follow up. The remaining 113 patients with an unlikely and 203 patients with a likely probability score underwent ultrasonography. Ultrasonography was repeated if indicated according to protocol; seven times (1.7%) because of an indeterminate ultrasonography result and in 45 of the 51 patients (13%) with the combination of a likely score, abnormal D-dimer and normal ultrasonography. To summarize, of the 406 included patients, 103 patients had UEDVT (25%), 55 had SVT (14%) and in 249 patients the algorithm excluded UEDVT and SVT. Of these, one patient developed UEDVT during follow-up, hence, an overall failure rate of 0.40% (95% CI: 0.0–2.2%).

Summary/Conclusions: A new diagnostic algorithm which combines a clinical decision score, D-dimer and ultrasonography can safely and effectively exclude venous thrombosis of the upper extremity. This approach is attractive as it is simple, quick and non-invasive, and very similar to the well established algorithm for suspected DVT of the leg which could facilitate its implementation in clinical practice.

[1]Constans et al, Thromb Haemost 2008.

AS 42.3

Combined pretest probability assessment for acute coronary syndrome and pulmonary embolism in emergency department patients with chest pain and shortness of breath

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Background: In the United States, large registries show that most emergency department patients with chest pain and shortness of breath undergo at least one diagnostic test for both acute coronary syndrome (ACS) and pulmonary embolism (PE). Most of these patients have neither PE nor ACS. We hypothesize that quantitative pretest probability (PTP) can reduce unnecessary cost and radiation exposure in emergency department (ED) patients with symptoms of acute coronary syndrome (ACS) and pulmonary embolism (PE). In this study, we examine the scope of the problem and potential role for quantitative pretest probability to reduce testing and radiation exposure.

Methods: Study design was a prospective, four center, non-interventional study of diagnostic accuracy. Patients were adults with non-diagnostic electrocardiograms and no obvious diagnosis. The clinician in charge of care provided inputs into a web-based computer program that provides quantitative pretest probability estimates for both ACS and PE using the method of attribute matching. Patients were followed for 90 days. The sample size was estimated with the assumption that 25% of patients would have PTP estimates for both ACS and PE below the predefined test threshold (< 2.5% for both), and that the outcome rate of ACS and PE would be at or near zero in this very low risk group, and that $n = 850$ would narrow the top limit 95% CI for the actual outcome rate of ACS or PE to < 2%.

Results: We enrolled 851 patients and excluded 11 because of screen failures. 840 patients had complete data. 23/840 (3%) had ACS and 15 (2%) had PE and 60 (7%) had another significant cardiopulmonary diagnosis within 90 days. Diagnosis was delayed in four patients with ACS and one with PE and all five had PTPs > 2.5%. Both the ACS and PE PTP estimates were below the predefined test threshold of 2.5% in 227 patients (27%, 95% CI 24–30%). None of these 227 very low risk patients had ACS or PE diagnosed within 90 days (0%, 0–1.6%) Of these with very low PTPs, 7 (3%, 1.5–6.2%) had another serious cardiopulmonary diagnosis, including four with pneumonia, two with pneumothorax, one with heart failure and none with aortic dissection. Only the patient with heart failure had a delay in diagnosis. The median chest radiation exposure per patient in the very low risk group was 0.06 mSv (1st–3rd quartiles 0.06–1.53) including 46 (21%, 95% CI: 16–26%) receiving > 5 mSv radiation to the chest as part of their evaluation which yielded no significant diagnosis. The median cost of care in this very low risk group was $653/patient ($454-$1863, $US).

Conclusion: These data show the safety and large opportunity for combined quantitative PTP for ACS and PE to reduce unnecessary testing and radiation exposure in ED patients with chest pain and shortness of breath.

AS 42.4

Validity of the primary care rule for risk stratification in patients with suspected DVT in auckland new zealand

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Background: The majority of patients who present with symptoms of deep vein thrombosis (DVT) do not have the disease. Clinical decision rules aim to identify those who require diagnostic ultrasound while safely excluding those who do not. The "Wells Rule"has been widely validated in the secondary care setting however its performance in primary care appears to be less accurate. The Primary Care Rule was reported in the Netherlands to be safe and accurate in the general practice environment. The rule includes points based on clinical risk factors and a whole blood (WB) point of care (POC) D-dimer which reduces the waiting time for test results and contributes significantly to the clinical probability score.

Aims: To validate the Primary Care Rule in Auckland, New Zealand using the same POC D-dimer (*Clearview Simplify*) and reduce unnecessary community ultrasonography through use of a decision rule.

Methods: A prospective study of consecutive patients with suspected DVT presenting to one of 100 primary care medical centres within the Auckland metro region (Pathway group). Each patient was assessed using the primary care rule incorporating POC WB D-dimer and followed up at 3 months. Staff received training in the POC methodology. Subsequently a separate comparative analysis of the POC D-dimer was undertaken in 200 consecutive patients in whom risk scores were initially evaluated using a laboratory quantitative D-dimer (Innovance) to compare the performance of the POC D-Dimer when undertaken by laboratory staff. Where required, whole leg ultrasonography (USS) was used to confirm or exclude DVT in both groups.

Results: Pathway group: DVT was identified in 17% (5/29) of the POC D-dimer negative (low risk) group. Sensitivity was 0.61 (95% confidence interval (CI) 0.32–0.84), specificity was 0.27 (CI: 0.18–0.37). Negative Predictive Value (NPV) was 0.82. DVT was confirmed in 11% (8/72) of the POC D-dimer positive (high risk) group. All five of the DVTs in the low risk group were identified early in the 3 month follow up period due to ongoing clinical suspicion.

Comparative analysis: There were concerns over the Pathway performance so 200 subsequent patients were evaluated for possible DVT using the laboratory D-Dimer. Parallel POC D-dimers were negative in 132 of these patients and in this group DVT was confirmed (positive laboratory D-dimer and ultrasound) in 2% (3/132). For the POC D-dimer performed in the laboratory, sensitivity was 0.8 (CI 0.51–0.94), specificity 0.69 (CI: 0.62–0.7), NPV of 0.97. DVT was confirmed in 18% (12/68) with positive POC D-dimer.

Conclusions: The POC D-dimer performed to the manufacturers' specifications when tested by experienced laboratory staff. Although training was given to clinical staff at each medical centre, sensitivity was poor in their hands and the Primary Care Rule did not adequately distinguish high and low risk patients. The pathway validation was stopped due to the high DVT incidence in patients stratified as low risk. The primary objective of reducing the number of USS was not met in the first 100 patients with adequate safety. The choice of clinical decision rule in primary care requires further study.

AS 43 – Non-Inherited Risk Factors for Venous Thrombosis

AS 43.1

Impaired glucose metabolism, assessed by HbA1c, and future risk of venous thromboembolism – The Tromsø study

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Background: Experimental studies have suggested that hyperglycemia facilitate thrombosis development, but previous cohort studies on the impact of diabetes mellitus (DM) on risk of venous thromboembolism (VTE) have reported diverging results. Glycated hemoglobin (HbA1c), a marker of average plasma glucose during the last 8–12 weeks, is associated with future risk of cardiovascular disease (CVD) and all-cause mortality. However, no study has so far investigated the impact of HbA1c on risk of VTE.

Aims: The purpose of the present study was to examine the association between impaired glucose metabolism, assessed by HbA1c, and future risk of VTE in a prospective cohort recruited from a general population.

Methods: HbA1c was measured in 16 156 unique subjects, aged 25–87 years, who participated in one or more surveys of the Tromsø study, starting in 1994–95 (Tromsø 4; 1994–95, Tromsø 5; 2001–2, and Tromsø 6; 2007–8). Incident events of VTE were recorded to the end of follow-up, December 31, 2010. Cox's proportional hazard regression models were used to estimate crude and multivariable-adjusted hazard ratios with 95% confidence intervals. The analyses were adjusted for age, sex, body mass index, smoking, physical activity and CVD. HbA1c was analyzed in predefined categories (< 5.7%; normal, 5.7–6.4%; impaired glucose metabolism (IGM), and ≥ 6.5%; DM) and as a continuous variable. Moreover, to minimize regression dilution effects, Cox-regression was performed in subjects with at least two recordings of HbA1c during follow-up, allowing for time-dependency of the exposure variable and important co-variates. The study was approved by the regional committee for research ethics, and all participants gave their informed written consent to participate.

Results: There were 333 validated first VTE events (2.91 per 1000 person years), of which 137 were unprovoked, during a mean of 7.1 years of follow-up. HbA1c was not associated with future risk of VTE in analysis treating HbA1c as a continuous variable, or in categorized analyses. The risk of VTE increased by 5% per 1 SD (0.7%) increase in HbA1c (Multivariable-adjusted HR 1.05; 95% CI 0.97–1.14), and subjects with HbA1c ≥ 6.5% had 27% increased risk compared to those with HbA1c below 5.7% (Multivariable-adjusted HR 1.27; 95% CI 0.72–2.26). There was no significant trend for increased risk of VTE across categories of HbA1c ($P = 0.27$). Only minor changes in risk estimates were observed in time-dependent analyses.

Conclusion: In our study, levels of HbA1c were not associated with future risk of VTE in multivariable analysis. Our findings suggest that impaired glucose metabolism does not play an important role in the pathogenesis of VTE.

AS 43.2

Insulin resistance is not independently associated with unprovoked venous thromboembolism: results from the EDITH case-control study

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Background: Shared risk factors help explain the association between venous thromboembolism (VTE) and atherothrombosis. Insulin resistance is an independent risk factor for the development of metabolic syndrome and diabetes, and thus, leads to an increased risk of cardiovascular diseases. The homeostasis model assessment of insulin resistance (HOMA-IR) is an accurate and precise method to measure insulin resistance. To date, the potential association between insulin resistance and VTE has been poorly evaluated.

Aims: To assess the association between insulin resistance and VTE in the EDITH hospital-based case-control study.

Methods: Between May 2000 and December 2004, 677 patients with unprovoked VTE and their age and sex-matched controls were included. Fasting glycemia and insulinemia were measured and insulin resistance was estimated with the HOMA-IR equation (glucose [mM] × insulin [mUI/L]/22.5). Conditional logistic regression taking into account the matching factors was carried out to estimate odds ratios (OR) and 95% confidence intervals (CI) for VTE risk associated with HOMA-IR index in non-diabetic patients. The association between HOMA-IR and VTE was determined in a quintile-based analysis.

The protocol was approved by our hospital's Ethics Committee. After complete description of the study to the subjects, written informed consent was obtained from all patients.

Results: A total of 590 non-diabetic cases (median age 73.0 years, 255 men) and 581 non-diabetic controls (median age 72.0 years, 247 men) were analyzed. There was a trend for a higher median level of HOMA-IR index in cases than in controls (1.21 [interquartile range 0.84–2.10] vs. 1.19 [interquartile range 0.72–2.02], $P = 0.08$). The unadjusted analysis showed an increased risk of unprovoked VTE associated with increasing HOMA-IR (OR 1.53; 95% CI 1.00–2.34 for the highest quintile of HOMA-IR compared with the first quintile). Body mass index and fibrates use were also associated with unprovoked VTE in the unadjusted analysis (OR 1.07; 95% CI 1.05–1.10 per each standard deviation increase and OR 2.08; 95% CI 1.34–3.23 respectively), whereas aspirin use was associated with a decreased risk of VTE (OR 0.58; 95% CI 0.42–0.80), and statins use with a non-significant decreased risk of VTE (OR 0.72; 95% 95% CI 0.47–1.08). Adjustment for lipid lowering drugs and antiplatelets agents use (potential markers of insulin resistance) slightly modified the association between increasing HOMA-IR and VTE (OR 1.51; 95% CI 0.97–2.34 for the highest quintile of HOMA-IR compared with the first quintile). When body mass index was added in the adjusted model, HOMA-IR was no longer associated with VTE (OR 1.08; 95% CI 0.67–1.73 for the highest quintile of HOMA-IR compared with the first quintile).

Conclusions: Our study showed that insulin resistance assessed by the HOMA-IR index was not an independent risk factor for VTE. Our results highlight the key role of body mass index in the association between traditional cardiovascular risk factors and VTE. Changing lifestyle in order to lose weight is likely to be beneficial for overweight and obese patients after a VTE event.

AS 43.3

Lower leg cast immobilisation and risk of venous thrombosis: results from the MEGA study

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Background: Guidelines and clinical practice vary considerably with respect to antithrombotic treatment during plaster cast immobilisation of the lower leg. Due to the heterogeneity of the evidence on the risk of venous thrombosis (VT) in patients with lower leg cast immobilisation, the exact risk remains unclear and it is uncertain whether the benefits of thromboprophylaxis outweigh the risks.

***Aims*:** To estimate the risk of venous thrombosis after cast immobilisation of the lower leg and to identify high risk groups by taking genetic and other acquired risk factors as well as the indication for immobilisation into account.

Methods: We used data from a large population based case-control study (MEGA-study) into the aetiology of venous thrombosis (4420 cases, 6151 controls, consent and ethical approval obtained). Odds ratios (OR) with 95% confidence intervals (CI95) were calculated as a measure of the relative risk and adjusted for age, sex, body mass index and regular exercise. We used varying exposure time risk windows for reasons of statistical precision. Analyses of joint effect all have those with none of the risk factors in the analysis as reference group. Absolute risks were estimated from the ORs, assuming an incidence of VT of 1–2 per 1000 person-years in the general population.

Results: Patients with cast immobilisation of the lower leg (134 cases, 23 controls) had an eight-fold increased risk of venous thrombosis in the subsequent year (OR 8.3 [CI95; 5.3–12.9]). Most thromboses were seen in the first 3 months after cast application, leading to a 56-fold increased risk (OR 56.3 [CI95; 17.9–177]) in this period. This corresponds to an incidence of VT of 1.4–2.8% over 3 months. Considering events during 1 year after cast application showed that traumatic indications (fractures, tendon and ligament ruptures and contusions) led to a higher risk of VT (OR 12.7 [CI95; 6.6–24.6]) than non-traumatic indications (overuse injuries, plantar fasciitis and joint complaints): OR 7.6 (CI95; 0.9–66.4). An even more pronounced risk was found for the combination of genetic and other acquired risk factors. The combination of a lower leg plaster cast and oral contraception use led to an OR of 18.2 (CI95; 6.2–53.4); with obesity (BMI > 30 kg/m^2) to an OR of 17.2 (CI95; 5.4–55.2); with carriership of the factor V Leiden mutation, prothrombin G20210A mutation or a non-O blood type to an OR of 23.0 (CI95; 11.5–46.0) (all during the 1 year following the cast application). No information was available on thromboprophylaxis, while a proportion of the patients most likely did receive this. However, this implies that our estimate most likely represents an underestimation of the true risk.

Conclusion: Cast immobilisation of the lower leg is associated with a high risk of venous thrombosis, particularly in the first 3 months after application. Patients with lower leg cast immobilisation in combination with well known genetic and other acquired risk factors are high-risk groups. Prophylactic treatment can be individualised and optimised when the indication of cast immobilisation and the presence of genetic and other acquired risk factors are taken into account.

AS 43.4

Acute infection, immobility and venous thromboembolism: an analysis from the RIETE Registry

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Background: The strong association between immobility and venous thromboembolism (VTE) is well established. Acute infection and sepsis are frequent cause of immobility, and several risk assessment models include them among the items to be considered for VTE prevention. However available real-world evidence for an association among acute infection, immobility and VTE is limited, as well as detailed information on the role of specific types of infection.

Aims: Aim of the present analysis was to evaluate clinical characteristics of VTE patients with acute infection leading to immobility, in general and according to type of infection.

Methods: The RIETE registry is an ongoing, worldwide, prospective cohort of consecutive patients presenting with symptomatic deep vein thrombosis (DVT) or pulmonary embolism (PE) confirmed by objective tests, and followed-up for at least 3 months. Patients' characteristics were analyzed by means of the χ^2 test or the Student's t test as appropriate. A P value < 0.05 was considered to be statistically significant.

Results: The overall population of patients included in the RIETE registry at January 2013 (n = 44 898) was considered for this analysis. Acute infection leading to immobility was reported in 889 patients (2%), being isolated DVT diagnosed in 47.8% and PE in 52.2% of patients. At 3-month follow-up, recurrent VTE occurred in 2.4% of patients, major bleeding in 2.7% and overall mortality in 15.0% (2.0% VTE-related). Among different types of infection, pneumonia was present in 23.1%, other respiratory infections in 29.1%, urinary tract infections in 10.7%, and sepsis in 5.2% of cases. Patients with respiratory infections/pneumonia had more frequently PE as initial presentation of VTE if compared with other types of infection (61.6% vs. 41.9%, P < 0.001). By comparing patients with pneumonia and those with other respiratory infections, the latter had more frequent concomitant chronic lung disease (36% vs. 28%, P < 0.05), while no significant differences were noted neither for other risk factors (heart failure, cancer, previous VTE), nor for duration of immobility. Significantly more patients with pneumonia had received thromboprophylaxis prior to VTE (51.7%, vs. 30.5% in patients with other respiratory infections, P < 0.001).

Summary/Conclusions: Acute infection, the associated inflammation and their clinical consequences may increase the risk of VTE due to one or more of the precipitants proposed by Virchow, and namely venous stasis. Data from the very large RIETE registry allowed us to better characterize real-world patients with acute infection leading to immobility, and developing VTE. Respiratory infections accounted for the majority of cases, and they more frequently had PE at presentation. One potential strength of our study is that, being VTE events objectively confirmed, we avoided possible diagnostic misclassification of PE in case of respiratory infection. Of interest, patients with pneumonia had a high percentage of ineffective prophylaxis; future clinical trials might evaluate the opportunity of a more systematic and aggressive approach in these patients.

AS 44 – Inflammation and Coagulation Axis

AS 44.1

Akt2 plays a critical role in regulating alphaMbeta2 integrin function and heterotypic platelet-neutrophil interactions during vascular inflammation.

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Background: Heterotypic interaction of activated platelets and neutrophils on the activated endothelium plays an important role in mediating pathological thrombosis and inflammation. Heterotypic cell-cell aggregation results from the interaction of specific receptors-counter receptors which are well identified. However, the regulatory mechanisms of receptor interactions between platelets and neutrophils in thrombo-inflammatory disease remain unknown.
Aims: To investigate the role of Akt in regulating heterotypic neutrophil-platelet aggregation in vascular inflammation.
Methods and results: Using real-time intravital microscopy of Akt knockout (KO) mice, we have demonstrated that Akt2, but not Akt1 and Akt3, plays a critical role in neutrophil adhesion to the activated endothelium during TNF-alpha-induced vascular inflammation. Further, heterotypic platelet-neutrophil interaction on the activated endothelium was markedly inhibited in Akt2 KO mice. Studies with chimeric mice generated from bone marrow transplants on wild-type (WT) and Akt2 KO mice revealed that hematopoietic cell but not endothelial cell Akt2 regulates neutrophil adhesion and platelet-neutrophil interaction during vascular inflammation. Using *in vitro* reconstituted systems in which platelets and/or neutrophils were treated with an Akt2 specific inhibitor or both cells were isolated from WT and Akt KO mice, we observed that both platelet and neutrophil Akt2 play a central role in platelet-neutrophil aggregation under shear conditions. Akt1, Akt2, or Akt3 KO platelets showed a partial defect of P-selectin exposure by 30–40% of WT platelets during thrombin activation, whereas surface expression of P-selectin glycoprotein ligand-1 – a counter receptor for P-selectin – was similar on WT and Akt KO neutrophils. Notably, surface expression of alphaMbeta2 integrin is enhanced on WT and Akt1 KO but not Akt2 KO neutrophils upon fMLF stimulation. Consistently, human neutrophils pre-treated with an Akt2 specific inhibitor significantly reduced cell adhesion to surfaces coated with fibrinogen or intercellular adhesion molecule-1 (ICAM-1) and diminished activation of alphaMbeta2 integrin – a key receptor for stable interaction of neutrophils with platelets and endothelial cells. Further, neutrophils lacking Akt2 exhibited decreased adhesion to ICAM-1, and the adhesion was further inhibited by a combination with a blocking anti-beta2 but not anti-alphaM antibody, suggesting that Akt2 regulates alphaMbeta2 integrin-mediated neutrophil adhesion. We observed that compared to WT neutrophils, Akt2 KO neutrophils show decreased complex formation of SNARE proteins which are important for exocytosis-mediated alphaMbeta2 translocation during neutrophil activation.
Conclusion: These results indicate that neutrophil Akt2 is a key regulator for the membrane translocation, activation, and adhesiveness of alphaMbeta2 integrin, thereby orchestrating heterotypic platelet-neutrophil interaction in thrombo-inflammatory disease.

AS 44.2

Shedding of protease activated receptor 1 (PAR1) by *Streptococcus pyogenes* pyrogenic exotoxin B (SpeB) – a novel mechanism to evade host immune system

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Background: Protease activated receptor (PAR)-1 allows cells to sense proteolytic activity and mediates pleiotropic actions affecting coagulation/hemostasis endothelial barrier function and inflammation. Few bacterial proteases have been shown to blunt PAR1 signaling through *N*-terminal shedding. *Streptococcus pyogenes*, a major human pathogen leading to superficial and life threatening infections such as necrotizing fasciitis and streptococcal toxic shock toxin syndrome, expresses a multitude of virulence factors including efficient proteases. We hypothesized that streptococcal proteases cleave PAR1 and contribute to *streptococcal* virulence.
Aims: We analyzed whether streptococcal proteases cleave PAR, what the neo *N*-termini generated are and whether cleavage of PAR1 by streptococcal protease causes biological effects.
Methods: *S. pyogenes* M1T1was grown overnight in mammalian tissue culture medium and diluted supernatant were incubated with endothelial cells natively expressing PAR1. *Streptococcal* proteases were identified by zymography, protease deficient mutant strains and recombinant *streptococcal* proteases served as controls. Removal of *N*-terminal PAR1 antigen on natively expressing EA.hy926 endothelial cells was quantified using anti-PAR1 recognizing epitopes around R41, R 46 and D53 by cell surface ELISA. Alkaline phosphatase tagged PAR1 cleavage reporter constructs carrying mutants and deletions were transiently overexpressed in 293T embryonic kidney cells to localize cleavage sites. Complementary, soluble peptides corresponding to PAR1's *N*-terminus were incubated with streptococcal proteases and cleavage products were identified by *N*-terminal sequencing. To test how streptococcal proteases affect endothelial cells, direct induction of ERK1/2 phosphorylation and desentization of thrombin mediated ERK1/2 induction was analyzed. As biological read out we tested whether streptococcal proteases modify platelet aggregation.
Results: In natively endothelial expressed PAR1 proteases of *streptococcal* supernatants removed *N*-terminal R41 and R46 but not D53 epitopes. Consistent with cleavage between R46 and D53 in overexpressed PAR1 reporter constructs carrying either alanine substitutes or deletions, the cleavage site could be mapped at or close to L44. *N*-terminal sequencing of a peptide corresponding to PAR1's *N*-terminus revealed a L44 cleavage product upon incubation with streptococcal supernatants. Reporter constructs of PAR2, 3 and 4 were not cleaved by streptococcal supernatants. Zymography and cysteine protease inhibitor E64 identify *streptococcal* pyrogenic exotoxin B (SpeB), a cysteine protease expressed in stationary growth phase, as candidate protease. This was further supported by low nanomolar concentrations of recombinant SpeB efficiently cleaving PAR1 at L44 and failure of SpeB deficient streptococcus in cleaving PAR1. SpeB via cleavage of PAR1 prevented thrombin mediated ERK 1/2 phosphorylation in endothelial cells and blunted platelet aggregation.
Conclusion: We provide novel evidence that PAR1 is a target of *streptococcal* SpeB, a major *streptotoccal* virulence factor. Cleavage at the novel L44 abolishes responsiveness of endothelial cells as well as aggregation of platelets which are important for bacterial entrapping and pathogen clearance.

AS 44.3

CXCL7 (platelet basic protein) and CXCL4 (platelet factor 4) contribute to the pathogenesis of acute lung injury

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Background: Acute lung injury (ALI) is characterized by abnormally enhanced coagulation, microvascular barrier dysfunction and tissue necrosis. Platelets are known as primary effector cells of hemostasis and thrombosis, but a growing body of evidence also supports an important role for platelets in inflammatory response, including leukocyte recruitment. Activated platelets release chemokines and other proteins stored in their alpha-granules, including its two most abundant proteins, CXCL4 and CXCL7. CXCL4 role in thrombosis, atherosclerosis and megakaryopoiesis, and in the pathogenesis of heparin-induced thrombocytopenia has been well established in part due to studies using mouse (m) CXCL4 knockout (mCXCL4-KO) and mice overexpressing human (h) CXCL4 specifically in platelets ($hCXCL4^+$). CXCL7 belongs to the same family of chemokines as CXCL4, but has an *N*-terminal Glu-Leu-Arg (ELR) motif after *N*-terminal truncation to the 70 amino acid neutrophil activating peptide-2 (CXCL7-NAP-2). This ELR motif is key for CXCL7-NAP-2's binding to the chemokine receptor CXCR2, which mediates leukocyte (PMN) migration.
Aim: Since migration of PMNs into the lungs is a crucial element in lung injury, the aim of this study was to establish the role of platelet-released CXCL7 in acid-induced mouse model of ALI. We first determined if mouse (m) PBP can be processed to yield biologically active degradation product that bind CXCR2 and induces PMN chemotaxis and then studied over and under CXCL7-expressing mice in a model of lung injury. Similar studies were done for CXCL4.
Methods: Purified recombinant (r) mPBP and mNAP-2 was cleaved by cathepsin G and products were evaluated by mass spectrometry and Western blot. Created by us, CXCL7 knockout (mCXCL7-KO) and overexpressing human (h) CXCL7 specifically in platelets ($hCXCL7^+$) mice were used in a mouse model of severe lung injury and evaluated by *ALI score*, presence of neutrophils and protein in bronchoalveolar lavage fluid (BALF), and vascular permeability measured by extravasation of FITC-dextran from systemic circulation into the lung.
Results: We examined full-length recombinant mPBP and native mPBP released from mouse platelets and found that both are *N*-terminally processed by cathepsin G to yield a biologically active mNAP-2 version that like its human counterpart, increased PMN chemotaxis.
We found that mCXCL7-KO mice are protected from severe lung injury. In contrast, $hCXCL7^+$/mCXCL7-KO mice develop lung injury comparable to WT animals. We have also found that $hCXCL4^+$ mice had more severe injury as compared to mCXCL4-deficient mice.
Summary/Conclusions: Both platelet-released CXCL7 and CXCL4 contribute to ALI severity. As CXCL4 is not a ligand for CXCR2, the mechanism of CXCL4 effect may be different, possibly involving lipoprotein receptor-related protein-1, which has been implicated to help maintain the integrity of the capillary-alveolar barrier. These studies show a clear mechanism by which platelets may influence the degree of ALI and provide important new insights into the pathogenesis of ALI. They may also lead to novel therapeutic strategies to modify the severity of this challenging disorder.

AS 44.4

The P2X1 receptor plays a key role in LPS-induced lethal endotoxemia

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Background: The $P2X_1$ receptor, an ATP-gated cation channel, is expressed in platelets and in cells of the immune system (neutrophils, macrophages) which all contribute to acute systemic inflammation. The $P2X_1$ receptor has been reported to play a role in neutrophils migration.
Aims: Our aim was to evaluate the role of the $P2X_1$ receptor in LPS-induced lethal endotoxemia.
Methods: Wild-type (WT) mice and mice deficient for the $P2X_1$ receptor ($P2X_1^{-/-}$ mice) were studied in an experimental model of lethal endotoxemia, induced by intraperitoneal injection of lipopolysaccharide (LPS, 10 mg/kg). Mortality was recorded over 5 days. Blood samples were collected to measure cytokines production and lung histology was examined. The migration capacity of neutrophils was evaluated in the dorsal air pouch mouse model of inflammation.
Results: Within 5 days after LPS injection, 70% of WT mice died whereas only 15% of $P2X_1^{-/-}$ mice died (***$P = 0.0003$, $n = 20$). Histopathologic alterations associated with endotoxemia, such as lung injury with alveolar wall thickening and airspace collapse, were much less pronounced in $P2X_1^{-/-}$ mice as compared to WT mice. Neutrophil accumulation into the lung, assessed by immunostaining of lung sections with an anti-neutrophil antibody, was reduced in $P2X_1^{-/-}$ mice ($429 \times 10^3 \pm 41 \times 10^3$ pixels) as compared to WT mice ($660 \times 10^3 \pm 57 \times 10^3$ pixels, **$P = 0.0017$), indicating reduced inflammation. Inflammatory cytokines (TNFα, IL6) and chemokines (MIP1α, MIP1β, RANTES) reached significantly lower levels in the plasma of LPS-challenged $P2X_1^{-/-}$ mice as compared to the WT. In addition, the neutrophil recruitment was significantly reduced in the dorsal skin air pouch of LPS injected $P2X_1^{-/-}$ mice as compared to the WT, confirming *in vivo* the role of the $P2X_1$ receptor in neutrophil migration. As endotoxemia is accompanied by intravascular coagulation, thrombin-antithrombin (TAT) complexes were measured and found to reach lower levels in LPS-challenged $P2X_1^{-/-}$ mice (29.4 ± 3.2 ng/mL) as compared to WT mice (57.9 ± 5.2 ng/mL, $n = 5$, **$P = 0.0016$). Accordingly, platelet sequestration in the lung microvasculature, as evaluated by immunostaining of lung sections with an anti-platelet antibody, was reduced in $P2X_1^{-/-}$ mice ($125 \times 10^3 \pm 25 \times 10^3$ pixels) as compared to WT mice ($292 \times 10^3 \pm 33 \times 10^3$ pixels, ***$P = 0.0002$).
Conclusion: Our data indicate an important role for the $P2X_1$ receptor in LPS-induced lethal endotoxemia through a major contribution of migrating neutrophils. The respective contributions of the other blood cells or tissues which express the $P2X_1$ receptor remain to be delineated. In any case, selective antagonists of the $P2X_1$ receptor might be promising pharmacological means to limit the damages caused by acute systemic endotoxemia.

AS 45 – Innovative Treatments of Haemophilia A and B

AS 45

Alternative strategies for haemophilia treatment

Mast A

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Current therapy for hemophilia A and B is replacement of the missing coagulation factor VIII (fVIII) or IX (fIX). This requires i.v. infusions several times per week to prevent spontaneous bleeding in patients with severe disease and carries risk for development of inhibitory anti-

bodies that block activity of the transfused clotting factor. Alternative non-i.v. therapies administered less frequently would be of great benefit for patients, many of whom are very young. One strategy that has shown promise in several animal models is to block the function of Tissue Factor Pathway Inhibitor (TFPI), an anticoagulant protein present within plasma and platelets and on the endothelium surface. TFPI exerts anticoagulant activity by inhibiting tissue factor/fVIIa (TF-fVIIa) and fXa. Under physiological conditions TFPI "forces"TF-initiated coagulation to proceed through activation of fIX. Therapeutically blocking TFPI inhibitory activity, or "inhibiting the inhibitor", allows for TF-initiated coagulation to proceed directly through activation of fX, bypassing the need for fVIII or fIX. TFPI is a multivalent Kunitz-type serine protease inhibitor with two primary alternatively spliced isoforms, TFPIα and TFPIβ. These isoforms both inhibit TF-fVIIa and fXa but differ in their *C*-terminal domains, where TFPIα has a third Kunitz domain and basic *C*-terminal region; and TFPIβ has a stretch of amino acids encoding a GPI-anchor attachment site. Thus, TFPIα is a soluble protein and TFPIβ is bound to the cell surface. Several recent advances defining the cellular expression and potentially unique functional activities of TFPIα and TFPIβ are important for understanding how pharmaceuticals targeted to different regions of TFPI may alter hemostasis in hemophilia patients. A key finding is that TFPIα and TFPIβ are distinctly expressed within the vasculature. TFPIα is the only isoform within platelets. TFPIα and TFPIβ are made by endothelial cells, but have distinct cellular expression with TFPIβ on the cell surface and TFPIα within an intracellular granule. Their distinct expression patterns suggest that TFPIα and TFPIβ have unique anticoagulant functions, which we have sought to define using cellular and murine model systems. Our current working hypotheses are that TFPIβ serves primarily to dampen the procoagulant potential of intravascular TF expressed in inflammatory conditions, while TFPIα released from platelets at the site of vascular injury actively regulates clot development and hemostasis. In this regard, mice lacking hematopoietic TFPI, which is primarily within platelets, have increased intravascular thrombus volume following large vessel injury. Interestingly, this is due to increased platelet accumulation within the clot rather than increased fibrin formation. Studies of fVIII deficient mice demonstrated that infusion of anti-TFPI antibody continued to decrease tail vein bleeding at concentrations beyond that necessary to totally inhibit plasma TFPI suggesting that platelet TFPIα released at the injury site promoted bleeding in this model. Consistent with notion, tail vein bleeding fVIII deficient mice lacking hematopoietic TFPI was significantly decreased compared to mice with hematopoietic TFPI, implicating platelet TFPIα as a primary physiological regulator of bleeding in hemophilia, which could be specifically targeted to restore hemostasis in hemophilia patients.

AS 45.1

A new class of coagulation factor VIII molecules that achieved four-fold longer half-life than recombinant FVIII in hemophilia A mice

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Background: The circulating half-life of coagulation factor VIII (FVIII) determines the frequency of injections required to maintain protective plasma levels of FVIII activity. Numerous approaches to extending half-life have been investigated, and molecules in late-stage development have achieved an approximately 1.6-fold half-life increase relative to currently marketed rFVIII. In order to achieve further improvement, the association between FVIII and von Willebrand factor (VWF) must be addressed. VWF couples the clearance rate of FVIII to its own due to the formation of a high affinity non-covalent complex between the two molecules in circulation. Engineering of FVIII molecules, disassociated with endogenous VWF, but containing elements sufficient to confer greater protection than that provided by VWF constitutes a promising strategy for further extending the half-life of FVIII molecules.

Aim: To increase the half-life of FVIII molecules by combining two approaches: 1) Fuse FVIII with the D'D3 region of VWF to preserve normal FVIII conformation but block its interaction with endogenous VWF; and 2) Insert XTEN at multiple locations within FVIII sequence to extend its circulating half-life.

Methods: The new class of FVIII molecules was designed to contain two polypeptides; one that consists of a single chain B-domain deleted (BDD) FVIII with XTEN inserted at one or more locations within the FVIII sequence, and one that is composed of the D'D3 region of VWF. Each polypeptide was also recombinantly fused to the Fc region of IgG1 to enable the D'D3 region to be correctly aligned to bind the FVIII moiety. The resulting FVIII variants were expressed in HEK 293 cells by transient transfection, and purified from the conditioned media. FVIII activity was evaluated by FVIII chromogenic assay and the pharmacokinetic properties were assessed in both FVIII knockout (HemA) and FVIII/VWF double knock-out (DKO) mice.

Results: Incorporating XTEN and D'D3 region of VWF into rFVIII led to the uncoupling of the clearance of the fusion proteins from endogenous VWF while extending their circulating half-life. FVIII in this fusion configuration is completely shielded from interacting with VWF, as measured by biolayer interferometry (Octet) analysis. Consistent with this, their pharmacokinetic profiles in HemA and DKO mice were found to be identical, indicating that their clearance rate in mice was effectively disconnected from VWF. Optimization of XTEN length and the locations for inserting XTEN identified a subset of the proteins that have exceeded the VWF limitation (16 h), reaching a circulating half-life of up to 30 h in HemA mice representing a four-fold improvement over BDD-FVIII. Importantly, these proteins maintained their functionality, as judged by FVIII chromogenic assay.

Conclusion: The VWF dependency has set a fundamental limitation for half-life of therapeutic FVIII. Uncoupling FVIII from VWF clearance pathways while extending half-life by the fusion of D'D3 region of VWF and XTEN has generated a novel FVIII molecule with a four-fold half-life extension. This is the first report of an engineered FVIII that has exceeded the half-life limitation observed through industry-wide efforts in development of long-lasting FVIII, representing a potentially significant advancement in prophylactic treatment of hemophilia A.

AS 45.2

IPSC-based strategy to correct the bleeding phenotype in haemophilia A

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Background: Hemophilia A (HA) is a X-linked bleeding disorder caused by mutations in the coagulation factor VIII (FVIII) gene. Existing therapy includes recombinant or plasma-derived FVIII infusion. Reprogramming of genetically corrected somatic cells can be used to generate high amount of autologous, disease-free induced Pluripotent Stem Cells (iPSCs), which can be differentiated into progenitor cells relevant for gene and cell therapy applications. In hemophilic patients, to harvest fibroblasts from skin biopsies is risky; thus, we utilized patient-derived peripheral blood cells as an easy-to-access source of cells to reprogram.

Aim: Development of a novel strategy for HA treatment, generating human B-domain-deleted FVIII (hBDD-FVIII)-corrected patient-specific iPSCs and differentiating them into functional endothelial cells (ECs), secreting FVIII in hemophilic mice after transplantation.

Methods: Mononuclear cells (MNC) were isolated from healthy and hemophilic donors by ficoll gradient. Cells were corrected by LV-hBDDFVIII transduction and reprogrammed with a cre-excisable polycistronic LV carrying OCT4, SOX2 and KLF4. MNC-derived iPSCs were characterized by Alkaline Phosphatase (AP), immunofluorescence stainings and RT-PCR of specific stem cell markers, telomers length and demethylation of Oct4 promoter. The IPSCs *in vitro* differentiation potential was analyzed by RT-PCR of three germ layers markers in embryoid bodies (EBs) and by adipogenic, osteogenic and condrogenic differentiation. IPSCs were differentiated in ECs using EB medium containing 50 ng/mL of hVEGF. Cells were evaluated for morphology, expression of endothelial markers and GFP-expression when transduced with LV carrying GFP under the control of endothelial-specific promoters: Flk-1 and Tie2. Two millions Flk1-GFP+ cells were transplanted by portal vein injection in MCT-treated NOD-SCID HA mice. Mice were killed 96 h later and cell engraftment analyzed by GFP-staining of liver sections.

Results: Reprogrammed MNC gave rise to iPSCs in about 45 days. iPSCs displayed embryonic stem cells (ESC)-like morphology: colonies were compact, uniform and with defined borders when grown on feeders. iPSCs were positive for AP staining and expressed specific stem cell markers at RNA and protein level, showing activation of the endogenous reprogramming factors. Moreover, iPSCs differentiated in osteogenic, condrogenic and adipose tissues. EB expressed markers of the three germ layers: alpha-fetoprotein, brachiury and nestin. Nevertheless, iPSCs showed demethylation at CpG islands at the core of Oct4 promoter, epigenetic marker of complete reprogramming to a pluripotent state. Additionally, telomeres length of original and reprogrammed cells did not show changes indicating telomerase reactivation. Furthermore, RT-PCR on HA-iPSCs showed the expression of hBDD-FVIII, confirming genetic correction of HA-MNC by LV transduction.

Importantly, iPSCs differentiated into ECs, acquiring a typical endothelial-like morphology with an increased expression of ECs markers, such as CD31, KDR, vWF and FVIII. Then, iPSC-derived ECs were transduced with LV-expressing GFP under the control of Flk-1 and Tie2. Flk-1-GFP+ cells were transplanted in NOD-SCID HA mice. GFP+ cells were detected in liver sections up to 1 week post transplantation.

Conclusion: Overall, these data will be instrumental to assess the engraftment, the proliferation and the levels of FVIII expression from differentiated, gene corrected and reprogramming factor free iPSCs to confirm the suitability of this approach for HA gene-cell-therapy.

AS 45.3

Platelet-specific expression of FIX induced by lentiviral gene delivery to hematopoietic stem cells restores hemostasis and induces immune tolerance in hemophilia B mice

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Background: Gene therapy is an attractive approach for hemophilia treatment, however, immune responses to the transgene product or viral vector can occur during such approaches.

Objective: To develop a novel gene therapy approach that will not only provide therapeutic protein FIX, but also induce FIX-specific immune tolerance for hemophilia B.

Methods: Platelet- FIX (2bF9) expression, driven by the platelet-specific αIIb promoter, was introduced by lentivirus-mediated hematopoietic stem cell (HSC) transduction followed by syngeneic transplantation. Recipients were analyzed by PCR, flow cytometry, and FIX assays to assess 2bF9 expression and then challenged with recombinant human FIX (rhF9) to investigate whether immune tolerance was induced after platelet gene therapy.

Results: The 2bF9 transgene was detected by PCR in all transduced recipients. Flow cytometry showed that there were 20.8 ± 12.1% (n = 7) and 14.8 ± 10.7% (n = 6) 2bF9-transduced platelets respectively in the recipients preconditioned with 1100 cGy or 660 cGy. The antigen levels of FIX were 2.89 ± 1.75 mU/10^8 platelets (n = 9) in the recipients preconditioned with 1100 cGy and 1.87 ± 1.30 mU/10^8 platelets (n = 7) in the 660 cGy group. There was a small amount of FIX detected in recipient plasma with the average levels of 2.22 mU/mL in the 1100 cGy group and 1.44 mU/mL in the 660 cGy group. To analyze the distribution of the FIX between platelets and plasma, we normalized FIX levels to total whole blood FIX content. The results demonstrated that 90–95% of whole blood FIX was stored in platelets. The tail clip survival test demonstrated that 15 of 16 mice that received 2bF9-transduced HSCs survived the tail clipping, while only two of 10 FIX^{null} control mice survived under the same challenge. Nine months after transplantation, sequential transplantation was performed on some of the primary recipients. Platelet-FIX expression in the secondary recipients was sustained, leading to phenotypic correction and confirming that long-term engrafting HSCs were successfully transduced with 2bF9 LV. Notably, none of the transduced recipients developed anti-FIX antibodies after platelet gene therapy. To investigate whether immune tolerance was induced in 2bF9-transduced recipients, we challenged the recipients with rhF9 in the presence of adjuvant. Only one of nine 2bF9-transduced recipients developed a low titer of inhibitory antibodies (1.6 BU/mL) as measured by a modified Bethesda assay. In contrast, all of the FIX^{null} controls developed inhibitors ranging from 17 to 37 BU/mL after the same challenge (n = 5). To ensure that the immune system was not defective in the 2bF9-transduced recipients and that the tolerance induction is FIX antigen-specific, we further challenged the animals with ovalbumin (OVA) absorbed on Alum. Both the 2bF9-transduced and FIX^{null} control mice developed high-titer anti-OVA antibodies. The levels of anti-OVA antibodies in the 2bF9-transduced recipients were not significantly different from FIX^{null} mice after the OVA immunization, confirming that tolerance induction in 2bF9 LV-transduced mice is FIX-specific.

Conclusion: Our data suggest that platelet gene therapy can not only provide sustained phenotypic correction, but also induce immune tolerance in hemophilia B mice, indicating that this approach may be a promising strategy for gene therapy of hemophilia B in humans.

AS 45.4

A novel mutation that alters an ubiquitination site on adeno-associated virus serotype (AAV) – 8 capsid improves hepatic coagulation factor IX expression *in vivo*

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Background: Recombinant adeno-associated virus vectors based on serotype (AAV)-8 have shown significant promise for hepatic gene therapy of hemophilia B. However, in a recent clinical trial, patients who received the highest dose (2×10^{12} vg/kg) of the self-complementary AAV8 vector developed transaminitis and capsid specific T cells that was attenuated by glucocorticoid therapy. To circumvent this vector dose dependent immunotoxicity, it is important to develop better AAV8 vectors that provide enhanced gene expression at significantly less vector doses.

Aims: We hypothesized that AAV8 vectors during intracellular trafficking, are targeted for destruction in the cytoplasm by the host-cellular kinase/ubiquitination/proteasomal degradation machinery and modification of specific serine/threonine kinase or ubiquitination targets on AAV8 capsid may improve its transduction efficiency.

Methods/Results: To test this, point mutations at specific serine (S)/threonine (T) à alanine (A) or lysine (K) àarginine (R) residues were generated on AAV8 capsid. Using a reporter transgene construct

(p.CB-EGFP), we have identified that one of the mutant vector (K137R), had increased liver directed gene expression (46-fold) and higher vector DNA copy numbers (22-fold) when compared to WT-AAV8 administered animals. The K137R-AAV8 vector also showed significantly decreased ubiquitination of the viral capsid as measured by immunoblotting experiments. Furthermore, the K137R vector demonstrated reduced activation of markers of innate immune response (IL-6, IL-12, TNF- α, Kupffer cells and toll like receptor-9) or decreased (two-fold) levels of cross-neutralizing antibody formation compared to animals that received WT-AAV8 vector. To further study the utility of the novel AAV8-K137R mutant in therapeutic gene transfer, we delivered human coagulation factor IX (h.FIX) under the control of liver specific promoters (LP1 or hAAT) at two different doses ($2.5 \times 10e^{10}$ and $1 \times 10e^{11}$ vgs per mouse) in 8–12 weeks old male C57BL/6 mice. Mice were bled retro-orbitally 2, 4 and 8 weeks post vector administration and the antigenic activity (hFIX:Ag) was measured (Asserachrom, Stago). The circulating levels of h.FIX:Ag were higher in all the K137R-AAV8 treated groups as compared to the WT-AAV8 treated groups either at 2 weeks (62% vs. 37% for hAAT constructs and 47% vs. 21% for LP1 constructs) 4 weeks (78% vs. 56% for hAAT constructs and 64% vs. 30% for LP1 constructs) or 8 weeks (90% vs. 74% for hAAT constructs and 77% vs. 31% for LP1 constructs) post hepatic gene transfer.

Conclusions: These studies demonstrate the feasibility of the use of this novel AAV8 vector for potential gene therapy of hemophilia B.

AS 46 – Tissue Factor, Cancer and Thrombosis

AS 46

Tissue factor in brain cancer

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Brain tumours emerge and progress within a unique tissue microenvironment, which they modulate in ways that depend on the underlying oncogenic defects. The highly specialized brain vasculature that responds to, and actively participates in the malignant process in ways that are still poorly understood. In this regard glioblastoma multiforme (GBM), an incurable, infiltrative, high grade astrocytic tumour is exhibits an extensive and diagnostically important vascular involvement, including hyperproliferaitve endothelial cells, presence of myeloid cells, intravascular microthrombosis coupled with occlusion and hypoxia as well as high risk for peripheral venous thromboembolism (VTE). While pathomechanisms involved in these changes remain to be fully elucidated, recent studies revealed that GBM cells express several coagulation proteins including ectopic factor VII, tissue factor (TF) and several effectors of the coagulation-related signalling circuitry, notably protease activated receptors 1 and 2 (PAR-1/2). This expression is driven, at least to some extent, by overexpression (amplification) in GBM cells of the epidermal growth factor receptor (EGFR) and its constitutively active mutant (EGFRvIII). Oncogenic EGFR stimulates vesiculation of GBM cells, including emission of TF-containing extracellular vesicles (EVs, microparticles) and EVs containing EGFRvIII (oncosomes). Mesenchymal transition of EGFR-expressing cancer cells also leads to a shift in TF emission resulting in shedding of procoagulant exosomes. The link between EGFR and TF is observed not only in engineered GBM cell lines but also in the clinical material. Thus, our analysis of coagulation-related transcripts (coagulome) of GBM specimens reveals a close correlation between levels of EGFR and TF in the classical subtype of GBM. In contrast, molecular GBM subtypes not known to be driven by oncogenic EGFR (mesenchymal, proneural, neural) exhibit lesser or no TF upregulation. Indeed several coagulation related genes are expressed (often ectopically) in GBM, medulloblastoma (MB) in a manner that corresponds to molecular subtypes of these (and other) malignances. TF plays different roles at different stages of GBM progression in that growth of advanced experimental tumours can be partially attenuated by suppression of TF activity in cancer cells. Notably, TF-negative indolent human GBM cells fail to initiate tumour growth and remain viable but dormant in mice for nearly a year. Expression of TF in these cells leads to cessation of dormancy and the onset of tumour growth after 4–6 week long latency, a process related to the onset of angiogenesis and inflammatory infiltration. Thus, TF involvement in the pathogenesis of brain tumours is notable but also complex, and extends beyond thrombosis. The diagnostic and therapeutic implications of this pathway in brain tumours may depend on the type, stage of the disease as well as the configuration of oncogenic mutations and molecular subtype.

AS 46.1

Neuronal cell tissue factor contributes to microvascular thrombosis in the brain after injury

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Tissue factor (TF), the major cellular initiator of the coagulation cascades, is highly expressed within the central nervous system (CNS). Astrocytes are the main source of TF in the brain. Astrocyte endfeet form the glia limitans interna which surrounds the microvasculature within the brain. Therefore, it has been proposed that astrocyte TF forms the hemostatic envelope within the brain vasculature. However, this hypothesis has never been tested and the role of TF in the brain remains largely unknown. To study the role of TF in the brain we generated mice lacking TF gene in all neuronal cells, including astrocytes, by crossing $TF^{flox/flox}$ mice with mice expressing Cre recombinase under control of a nestin promoter ($TF^{flox/flox}$/nestin Cre). These mice were viable and were born at the expected frequency from crosses between $TF^{flox/flox}$ and $TF^{flox/+}$/nestin Cre mice. First, we determined the level of TF expression in the brains of $TF^{flox/flox}$/nestin Cre mice and $TF^{flox/flox}$ control littermates. We observed a 99.5% reduction of TF mRNA expression in in the brain of $TF^{flox/flox}$/nestin Cre ($n = 4$) mice compared to $TF^{flox/flox}$ ($n = 4$) mice ($P < 0.0001$). Consistent with the mRNA data, total procoagulant activity of the brain tissue extract was reduced by 98.7% ($P < 0.01$). Mice lacking TF in the CNS exhibited normal survival without any evidence of spontaneous bleeding. Next, we determined the effect of TF deficiency in all neuronal cells on microvascular thrombosis using a photoactivation model. After craniotomy, visualization of individual vessels and thrombosis was performed using a 40× water-immersion objective attached to an Axioskop 2 FS Plus research microscope. 5% FITC dextran (10 mg/kg) was injected intravenously and allowed to circulate for 10 min before photoactivation (Gavins FN et al, Blood 2011, PMID:21304105). Thrombus formation, assessed as time for complete cessation of microvascular blood flow after light/dye injury, was quantified using intravital fluorescence microscopy in WT ($n = 6$), $TF^{flox/flox}$ ($n = 8$) $TF^{flox/flox}$/nestin Cre ($n = 9$) male mice within arterioles, venules and capillaries. The average diameter of these three vessel types was the same in all groups of mice. We hypothesized that deletion of TF from all neuronal cell would significantly affect thrombosis within the capillaries, because of direct contact of astrocyte endfeet with the basal membrane under the endothelium. Consistent with this notion, a significant prolongation in flow-cessation time was observed in capillaries of $TF^{flox/flox}$/nestin Cre mice (11.8 ± 1.6 min) compared with flow-cessation time recorded for $TF^{flox/flox}$ (8.5 ± 0.9 min, $P = 0.02$) and WT mice (7.52 ± 0.7, $P = 0.04$). Interestingly, deletion of TF from all neuronal cells had no effect on the flow-cessation time in arterioles or venules. Our results indicate that TF expression by astrocytes plays an important role in brain hemostasis at the capillary level, and likely

contributes to microvascular brain thrombosis upon damage to this vascular bed. Studies investigating the other roles of neuronal TF in the pathophysiology of the CNS are currently ongoing in our laboratories.

AS 46.2

Transient phosphorylation of the cytoplasmic domain of tissue factor at serine 253 promotes its interaction with filamin-A and is prerequisite for tissue factor release into microparticles

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Background: The release of tissue factor (TF) into cell-derived microparticles (MPs) is positively regulated by the phosphorylation of serine 253 within the cytoplasmic domain of TF. However, the molecular basis for this phosphorylation-dependent release of TF into MPs is unknown. The cytoskeletal protein filamin-A has been shown to directly bind to the cytoplasmic domain of TF. Moreover, this interaction is enhanced by the phosphorylation of serine residues 253 and 258 within the cytoplasmic domain of TF.
Aims: This study examined the role of filamin-A in the release of TF into MPs and examined the importance of the phosphorylation of serine 253 for the interaction of TF with filamin-A prior to MP release.
Methods: Human dermal blood endothelial cells (HDBEC) and human coronary artery endothelial cells (HCAEC) were transfected with pCMV-XL5-TF to express wild-type TF. Alternatively, cells were transfected with mutant forms of this construct in order to express TF with alanine or aspartate substitutions of residue 253 in order to prevent phosphorylation or mimic phosphorylation of this residue, respectively. MDA-MB-231 cells were used as a cell line that constitutively expresses TF. MP release was induced by activating protease-activated receptor 2 (PAR2) with an agonist peptide or factor Xa. MPs were then isolated from the culture media by ultracentrifugation. The interaction between TF and filamin-A was examined using a proximity ligation assay and co-immunoprecipitation. The expression of filamin-A was suppressed using filamin-A specific siRNA. The TF antigen content of the MPs was measured using a TF-ELISA and confirmed by western blot analysis. TF activity of the MPs was determined using a thrombin-generation assay. Total numbers of released MPs and size distributions were determined using nanoparticle tracking analysis.
Results: The interaction between TF and filamin-A was transient and peaked in HDBEC at 45 min post-activation of PAR2, in HCAEC at 60 min post-activation, and in MDA-MB-231 cells at 10 min post-activation. Two *C*-terminal fragments of filamin-A of 35 and 40 kDa were shown to co-immunoprecipitate with TF. HDBEC transfected with a mutant form of TF with alanine substitution of serine 253 resulted in reduced interactions between TF and filamin-A compared to wild-type TF. Mutation of serine 253 to aspartate resulted in increased interactions between TF and filamin-A, even in the absence of PAR2 activation. Furthermore, the interaction between aspartate substituted-TF with filamin-A was also transient, indicating that decreases in interactions observed at later time-points is not the result of serine 253 de-phosphorylation. siRNA-mediated suppression of filamin-A expression resulted in reduced levels of TF in the released MPs, but had no effect on the expression of TF. In addition, knockdown of filamin-A expression did not significantly alter the total number or size distribution of the released MPs.
Conclusions: These data show that filamin-A interacts with TF prior to MP release and the presence of filamin-A is required for the release of TF into MPs. This study therefore provides an insight into the mechanism by which the phosphorylation of serine 253 within the cytoplasmic domain of TF promotes the release of TF-positive MPs.

AS 46.3

Alternatively spliced Tissue Factor fuels breast cancer growth by binding to a non-canonical site on beta1 integrins

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Full-length tissue factor (flTF), the coagulation initiator, is overexpressed in breast cancer (BrCa) and induces coagulation-independent tumor angiogenesis in murine orthotopic breast cancer models though activation of PAR2. Nevertheless, associations between flTF expression and clinical outcome remain controversial. Interestingly, TF can be alternatively spliced yielding the non-coagulant soluble protein alternatively spliced tissue factor (asTF), but it is currently unknown whether asTF is expressed in BrCa and/or impacts BrCa progression.
To study the impact of TF isoform expression on tumor progression, breast tumor specimens from 574 patients were stained with flTF and asTF specific antibodies and percentages of positive cells were correlated with clinical parameters. Using this approach, we found for the first time that asTF, but not flTF, strongly associates with both tumor size and grade. To study this mechanistically, we generated MCF7-based breast cancer cells selectively expressing flTF, asTF or no TF protein. asTF expression, but not flTF expression, in these cells showed strong proliferative effects, compared to control cells. These proliferative effects were dependent on asTF binding to a non-canonical site on b1 integrins. Furthermore, asTF expression in these cells promoted oncogenic gene expression, anchorage-independent growth and strongly upregulated tumor expansion in a orthotopic luminal BrCa model. Furthermore, we characterized *in vivo*-selected MDA-MB-231 and found that these more aggressive MDA-MB-231-mfp cells expressed similar amounts of flTF, but more asTF, compared to MDA-MB-231. Increased proliferation rates of these cells compared to MDA-MB-231 were again dependent on asTF expression and b1 integrin. *In vivo*, asTF-blockade using specific antibodies resulted in diminished tumor growth and proliferation, but not diminished angiogenesis.
We propose that asTF plays a critical role in BrCa progression acting as an autocrine factor fueling tumor growth, while flTF impacts tumor angiogenesis. Targeting asTF either alone, or in combination with flTF/PAR2 blockade, may comprise a novel therapeutic strategy in BrCa that stems tumor growth, yet does not impair normal hemostasis.

AS 46.4

Alternatively spliced tissue factor contributes to tumor spread and activation of coagulation in pancreatic ductal adenocarcinoma

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Background: Alternatively spliced Tissue Factor (asTF) is a minimally coagulant TF form that promotes neovascularization and monocyte recruitment through integrin mediated mechanisms. While it has been shown that asTF is expressed in some pancreatic ductal adenocarcinoma (PDAC) cell lines and increased asTF expression potentiates PDAC growth in a subcutaneous model, the expression of asTF in human PDAC lesions and its potential role in the growth and/or spread of PDAC in a physiologically relevant setting are yet to be ascertained.

Aims: To assess the expression of asTF protein in bona fide human adenocarcinoma tissue; to evaluate the effect(s) of asTF overexpression in PDAC cells in an orthotopic model; to investigate the changes in the procoagulant potential of PDAC cells overexpressing asTF.
Methods: Tumor microarrays (PDAC, breast, urothelial, ovarian, and prostatic carcinomas) were evaluated for asTF expression and monocyte infiltration ($n = 9$). Primary PDAC tumors and regional lymph node metastases were then analyzed for asTF expression. PDAC cell lines expressing various levels of asTF were implanted in the pancreases of nude mice and subsequent in-vivo imaging studies were performed. PDAC cells were treated with recombinant asTF and the resultant changes in gene expression were assessed using microarrays. TF activity was measured in PDAC cell lines Capan-1, Pt45P1, and asTF overexpressing cells Pt45P1/asTF+ as well as the corresponding microparticles (MP) using fXa generation-based chromogenic assay; full-length TF (flTF) and phosphatidylserine (PS) levels were assessed using flow cytometry.
Results: asTF was detected in lesional and stromal compartments in all five studied types of carcinoma; PDAC tissue contained significantly higher levels of $CD68^+$ monocytes ($P < 0.05$). Analysis of 29 specimens of human PDAC revealed detectable asTF in > 90% of lesions with a range of staining intensities (low – 15%, moderate – 70%, high – 15%). Intense staining of asTF in perineurally invasive cancer glands and pancreatic intraductal neoplasia lesions was identified. Staining intensity for asTF in PDAC lesions positively correlated with the degree of monocyte infiltration. In nude mice, Pt45P1/asTF+ cells exhibited the most aggressive growth with metastases to distal lymph nodes, which stained positive for asTF. PDAC cells stimulated with recombinant asTF exhibited upregulation of genes involved in EGFR binding, epithelial branch elongation/development, and epithelial-mesenchymal signaling. Pt45P1/asTF+ cells and MP derived from these cells displayed higher TF activity compared to Pt45P1 cells and Pt45P1 derived MP ($P < 0.05$). While there were no significant differences between the levels of PS detectable on the surface of these two cell lines and/or cell derived MP, and the total MP levels remained unchanged, MP produced by Pt45P1/asTF+ cells carried more flTF compared to MP produced by Pt45P1 cells.
Conclusions: Our findings demonstrate that asTF is expressed in bona fide PDAC lesions and lymph node metastases, and potentiates PDAC spread *in vivo*. asTF elicits global changes in gene expression likely involved in tumor progression and metastatic dissemination, and it also enhances the procoagulant potential of PDAC cells and cell derived MP. Thus, asTF may comprise a novel therapeutic target to treat PDAC.

AS 47 Late Breaking Abstracts: New Insights

AS 47.1

Antidote for new oral anticoagulants: mechanism of action and binding specificity of PER977

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The new oral anticoagulants (NOACs) offer significant advantages over the heparins and warfarin therapies with regards to route of administration, drug interactions and predictability of bioactivity. Currently NOACs lack a specific reversal agent and concern over the need for rapid reversal for emergency procedures, serious bleeding or potential over dosage is heightened. As such we rationally designed, synthesized, and characterized a synthetic small molecule anticoagulant antidote (PER977).
In silico modeling data predicted the locations of non-covalent hydrogen bonding between PER977 and NOACs and heparins. *In vitro* dynamic light scattering (DLS) data reversal data correlate the *in silico* predicted non-covalent binding specificity of PER977 directly to NOACs and heparins (e.g. enoxaparin). Dynamic light scattering (DLS) of mixtures of PER977 and enoxaparin evidence the formation of molecular complexes formed at 1:1 and increasing in size at 10:1 ratios, indicating a strong physical, non-covalent association between PER977 and enoxaparin that accounts for the enoxaparin reversal activity of PER977.
Pre-clinical *in vivo* anticoagulant (rat-tail transection bleeding) assays demonstrate full reversal of all NOACs, with edoxaban requiring the lowest dose for full reversal of edoxaban by PER977. Importantly, PER977 exhibits no binding to any human plasma coagulation factors or albumin. Thromboelastography (TEG) measurements also demonstrate that PER977 returns edoxaban anti-coagulated rats to their naïve coagulation state. TEG reaction time (TEG-R) measurements demonstrate a statistically significant decrease ($P < 0.05$) back to normal TEG-R levels in edoxaban anticoagulated rats administered PER977 within 30 min of administration, as compared to rats receiving edoxaban followed by a saline sham.
In vivo and *in vitro* toxicology and safety studies have been completed and a first-in-human clinical trial to demonstrate safety and efficacy in healthy human volunteers with PER977 and edoxaban will follow. PER977 has also shown no pro-coagulant properties as measured in human blood. In conclusion, PER977 directly and specifically binds NOACs and heparins reversing their anticoagulant properties.

AS 47.2

A case of thrombotic thrombocytopenic purpura and two novel mutations on complement system genes: a new etiology of disease?

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Background: The differential diagnosis between thrombocytopenic purpura (TTP) and atypical hemolytic uremic syndrome (HUS), is challenging due to the overlapping clinical signs and symptoms. Severe deficiency of ADAMTS13 (A Disintegrin And Metalloprotease with ThromboSpondin 1 repeats) is a well-recognized cause of TTP, whereas HUS is caused either by enterohemorrhagic *E. coli* infection (typical HUS) or by mutations in genes encoding complement system proteins (atypical HUS). As the therapeutic approach to the different forms of TMA diverges, it becomes increasingly important to establish the underlying etiology. We report a case that redefines the boundaries between TTP and aHUS, which may have major future consequences for the treatment of patients with TTP.
Aims: To investigate etiology in a TTP patient without renal involvement and normal ADAMTS13 activity at presentation.
Methods: ADAMTS13 antigen (by ELISA), activity (by fluorescence resonance energy transfer), VWF:Ag, multimers and complement system-related plasmatic parameters were measured. Mutational analysis in six complement-regulator genes (*CFH, CFI, CD46, CFB,* thrombomodulin (*THBD*) and *C3* were performed by PCR and Sanger sequencing. The role of a new mutation on *CFI* was studied by the concentration of complement factor I (*CFI*) in plasma with an ELISA. *THBD* variation was investigated by stable *in vitro* expression studies in HEK293 cells followed by functional characterization of TAFIa production in the presence of thrombin in both wild type (WT) and mutant (p.P368L) THBDs.

Results: A 49-year-old woman with fever, abdominal pain, vomiting and confusion, platelet counts of 46×10^9/L; and microangiopathic hemolytic anemia (hemoglobin: 8.3 g/dL, negative Coombs test) was diagnosed with idiopathic TTP. There was no renal impairment (creatinine 52.8 μM; normal values: 53–106 μM), urinary sediment was normal and 24-h proteinuria was negative. Remission was achieved after six daily plasma exchange procedures. ADAMTS13 activity and antigen were normal, but there was an increase in VWF antigen, with the presence of ultra-large VWF multimers. The alternative complement system pathway was activated, as documented by very low residual activity of AP50 (5%, normal value 30–113). Sequencing revealed the presence of two novel missense mutations in *CFI* (c.805G>A, p.G269S) and in *THBD* (c.1103C>T, p.P368L), for both of which she was heterozygous. Plasmatic complement factor I concentration was normal 58 mg/L (normal value: 40–80 mg/L). The mutant THBD (p.P368L) recombinant form was less effective than wild-type in the generation of TAFIa (50% reduction vs WT).

Summary/conclusions: We report a classical case of TTP, without renal impairment but with normal ADAMTS13 activity, who has two mutations in complement regulation genes CFI (c.805G>A, p.G269S) and in THBD (c.1103C>T, p.P368L). *In vitro* expression studies showed a reduced generation of TAFIa caused by p.P368L mutation on *THBD* leading probably to endothelial activation by increasing circulating anaphylatoxins (C3a, C4a). This alteration associated with an impaired complement regulation caused by p.G269S mutation on *CFI* (increased C3 and C5 cleavage to C3a/C3b and C5a/C5b, thus creating a positive feedback loop of inflammation) may be a hitherto unknown pathophysiologic mechanism in patients affected with TTP and might open a new therapeutic horizon in these patients.

AS 47.3

Complement is not activated in nonhuman primates during development of hemolytic uremic syndrome and thrombotic microangiopathy induced by *E. coli* Shiga toxins

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Background: Enterohemorrhagic *Escherichia coli* (EHEC) produce ribosome inactivating Shiga toxins (Stx1, Stx2) responsible for endothelial injury, development of hemolytic uremic syndrome (HUS) and acute kidney injury (AKI). Some patients show evidence of complement activation during EHEC infection, raising the possibility of therapeutic targeting of complement for relief. *In vitro* and murine Stx2 + LPS studies indicate that Stxs induce activation of the alternative complement pathway causing glomerular injury and microvascular thrombosis. However, these models do not recapitulate Stx-induced HUS, but the coagulopathy of disseminated intravascular coagulation (DIC). Our non-human primate (*Papio*) models of endotoxin-free Stx challenge exhibit full spectrum HUS including thrombocytopenia, hemolytic anemia, and AKI with glomerular thrombotic microangiopathy.

Aim: We examined whether complement was activated in Stx-challenged non-human primates during the development of HUS and AKI. In addition, we quantified D-dimer as a marker for fibrinolysis and the damage associated molecular patterns (DAMPs) HMGB1 and histone as markers of cell injury.

Methods: Plasma terminal complement complex (TCC), D-dimer, plasma/urine HMGB1 and histones were measured by ELISA in timed samples from juvenile baboons challenged i.v. with 100 ng/kg Stx1 or 50 ng/kg Stx2. Plasma from septic, bacteremic baboons with DIC after i.v. challenge with sub-lethal 5E + 09 CFU/kg *E. coli* B7 O86a:K61or lethal 3E + 09 CFU/kg attenuated *Bacillus anthracis* were used as positive controls.

Results: There were no significant increases in TCC levels in animals challenged with Stx1 ($n = 6$) or Stx2 ($n = 5$) in timed plasma samples from T0 to euthanasia at 49.5–128 h post-challenge. TCC levels in bacteremic animals with DIC peaked ≤ 48 h at 399.15 mAU/mL (O86a:K61) and 2204.99 mAU/mL (*B. anthracis*). D dimer consistently increased from 4 h, peaking at 48 h (Stx1, 736.89 ± 175.44 ng/mL) or 96 h (Stx2, 1009.75 ± 192.95 ng/mL). Plasma and urine HMGB1 was detectable by 8–24 h, rising earlier and higher after Stx1 compared with Stx2. Histones increased, but were variable between animals.

Conclusions: Complement is not activated in this pre-clinical HUS model, despite clear coagulopathy and cellular injury. This suggests complement activation is not required for the development of thrombotic microangiopathy and HUS induced by EHEC Shiga-like toxins. This was unexpected given the close links between coagulation, complement and endothelial injury. Instead, other bacterial and/or host factors must contribute. Complement is an important defense mechanism and benefits or risks of therapeutic inhibition should be studied further for this infection.

AS 47.4

The interaction between complement factor H and von Willebrand factor increases factor H cofactor activity and regulates von Willebrand factor prothrombotic status

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Background: Vascular endothelial cells are an important interface between primary hemostasis, coagulation and complement activation. EC synthesize both the clotting-initiator von Willebrand factor (VWF) and the complement regulator factor H (CFH). VWF is stored in EC Weibel-Palade bodies (WPB), but the intracellular location of CFH is not well defined.

Aim: Here, we address the question of the localization of CFH within endothelial cells and of the interaction of CFH with VWF.

Methods: The localization of CFH and VWF within human umbilical endothelial cells (HUVEC) was assessed by fluorescent microscopy. The binding of CFH to VWF was analyzed by SPR, ELISA and immunoprecipitation. The functional consequences of the interaction were probed by a cofactor test of CFH for cleavage of C3b by factor I, by VWF:RCo and by ADAMTS13-mediated VWF proteolysis.

Results: CFH co-localized with VWF in the WPB of HUVEC. Moreover, FH bound directly to VWF in real time and under static conditions with a K_D of 200 ± 25 nM. CFH-VWF complexes were isolated from normal human plasma. VWF bound to the C-terminal fragment of CFH. Besides, CFH bound to the D'D3 fragment of VWF, an interaction that was inhibited by FVIII. The binding of VWF with CFH enhanced CFH cofactor activity toward factor I as well as VWF-mediated platelet aggregation, whereas it reduced the proteolysis of VWF by its specific protease ADAMTS13.

Conclusion: The simultaneous secretion of VWF and CFH by activated EC may promote adhesion of platelets to sites of endothelial injury to assure wound healing, simultaneously dampening the pro-inflammatory effect of complement in order to limit bystander tissue damage.

AS 47.5

Thrombin binds to human ceruloplasmin and proteolytically hinders its antioxidant activity

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Background: Human ceruloplasmin (CP) is a component of alpha-2 globulin fraction of blood plasma, structurally homologous to coagulation factors V and VIII. CP is a member of the multi-copper oxidase family and plays a key role in iron metabolism. Purified CP preparations contain the intact protein form (132 kDa) and, depending on the pathophysiological state of the donor and storage time of the sample, variable amounts of nicked species produced by some yet unidentified protease. In addition to its involvement in iron metabolism, intact CP (the 132 kDa form) efficiently inhibits the peroxidase and chlorinating activity of leukocyte myeloperoxidase (MPO), thus functioning as an effective antioxidant in inflammatory diseases characterized by activation of leukocytes and release of MPO (sepsis and rheumatoid arthritis). Strikingly, the MPO inhibitory function of CP (but not its oxidase activity) is hindered in the nicked species (Sokolov et al., 2008), thus suggesting that the antioxidant activity and tissue protective effects of CP might be regulated proteolytically. Human thrombin is the final effector protease in the coagulation cascade and plays a pivotal role at the interface of coagulation, inflammation and cell proliferation. Interestingly, the concentrations of both thrombin and CP are significantly increased at inflammatory sites, such as the synovial fluid of patients with rheumatoid arthritis.

Aims: (i) Verify that thrombin cleaves CP *in vivo*; (ii) quantify the affinity of CP for thrombin; (iii) mapping the binding sites in thrombin-CP interaction.

Methods: CP was purified from human plasma by ion exchange and heparin affinity chromatography. Recombinant wild-type and mutant thrombins were expressed in *E. coli* and *in vitro* refolded. CP-thrombin interaction was studied by fluorescence and surface plasmon resonance (SPR). Cleavage sites in CP were identified by SDS-PAGE, Western-blotting, and Edman sequencing. Samples of synovial fluid from 24 patients were tested for CP, thrombin and MPO concentrations.

Results: SPR analyses were performed either by injecting the inactive thrombin mutant S195A over the chip-bound CP and, oppositely, by injecting CP over thrombin-bound sensor chip. In both cases a similar affinity was measured, with $K_d = 3.1 \pm 0.5$ mM and $K_d = 2.0 \pm 0.7$ mM. A comparable value was obtained from fluorescence binding measurements. Displacement experiments, carried out with specific binders of thrombin exosite-1 (hirugen and HD1 aptamer) or exosite-2 (fibrinogen γ′-peptide and HD22 aptamer) and with thrombin mutants (Arg73Ala and Arg101Ala), having one of the two exosites selectively compromised, indicate that both exosites are involved in CP binding. After treating purified, intact CP with thrombin, the fragmentation pattern of CP in SDS-PAGE was identical to that of the nicked CP species found in human plasma. The major fragmentation sites correspond to Lys887-Val888 and Arg481-Ser482 peptide bonds, both located in highly flexible loops in the 3D structure of CP. Cleavage is abolished in the presence of hirudin, a potent and selective thrombin inhibitor. Notably, proteolytic degradation of CP was more pronounced in samples of synovial fluids with higher titres of thrombin and increased MPO activity.

Conclusions: Thrombin cleaves CP and hinders the inhibitory effect that intact CP exerts on MPO function in inflammatory diseases.

AS 47.6

A novel gene identified at an erythrocyte quantitative trait locus has a profound effect on thrombocyte formation in zebrafish

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Background: The bone marrow residing precursor cells of platelets and red cells share a common progenitor, named the megakaryocyte-erythroid progenitor. Therefore important regulatory genes that regulate events in myeloid maturation may exert an effect on the formation of both red cells and platelets.

Aims: As part of an immunogenomics study, we sought to resolve the underlying genetic basis of the clinically relevant red cell blood group Vel.

Methods: The Vel group was discovered 60 years ago but efforts to identify the underlying sequence variant have resisted repeated attempts. We sequenced by targeted pull-down the exome of five individuals lacking the Vel group. Variants observed in 1% or more samples were removed by using the results of the 1000 and the UK10K Genomes projects. A single candidate gene was identified and by computational biology and gene knockdown in zebrafish its function in the formation of red cells and thrombocytes was elucidated.

Results: We identified the putative 78 amino-acid transmembrane protein Smim1 as the carrier of the high incidence Vel blood group, most commonly through the homozygous presence of a low frequency 17 nucleotide frameshift deletion. RNA-sequencing blood cell progenitors and precursors revealed that *SMIM1* transcription is restricted to the myeloid lineage. A common expression-quantitative trait locus (eQTL) at rs1175550 with a minor allele frequency of 0.21, contributed to variable expression of the *SMIM1* transcript ($P = 10^{-250}$). This SNP also pinpointed *SMIM1* as the causal gene underlying a genome-wide association signal at $P = 8.6 \times 10^{-15}$ for the mean haemoglobin concentration of red blood cells. Knock down of the zebrafish ortholog *smim1* caused a mild reduction of the number of red cells and a complete abrogation of thrombocytes.

Conclusions: We have discovered the previously hypothetical gene *SMIM1*, encoding one of a novel class of small integral membrane proteins on myeloid cells and carrying the Vel group. Genome-wide typing of nearly 100,000 individuals showed that the SNP rs1175550 that regulates the *SMIM1* transcript level is causally correlated with the haemoglobin concentration of red cells. This association observation was corroborated by knock-down in zebrafish which showed that *smim1* not only regulates red cell formation but also thrombocytogenesis. Our discovery adds to an expanding list of novel genes that regulate red cells and thrombocyte formation in a model system, for further evaluation in humans.

FS 01 – Antithrombotics and Pregnancy

FS 01.1

The Thrombophilia in Pregnancy Prophylaxis Study (TIPPS): a multi-national randomized trial of dalteparin vs. no dalteparin to prevent pregnancy complications in pregnant thrombophilic women

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Background: Placental thrombosis is a common pathophysiologic feature of placenta mediated pregnancy complications (pre-eclampsia (PET), birth of a small for gestional age child (SGA), pregnancy loss and/or placental abruption). Thrombophilias are common and are purported to increase the risk of placenta mediated pregnancy complications and pregnancy-associated venous thromboembolism (VTE).
Aim: We hypothesized that dalteparin would reduce placenta mediated pregnancy complications and venous thromboembolism (VTE) in pregnant thrombophilic women at increased risk of these complications.
Methods: In a multi-national 19 center study, we randomized consenting pregnant women with laboratory confirmed thrombophilia at increased risk of placenta mediated pregnancy complications or VTE to open label dalteparin (5000 units SC daily until 20 weeks gestation and then 5000 units twice daily until at least 37 weeks gestation) or no dalteparin in a 1:1 allocation. Women were excluded if they had an indication for anticoagulation, were ≥ 20 weeks gestation, geographically inaccessible for follow-up or had contraindications to dalteparin. Post-partum dalteparin 5000 units/day was administered to all participants. The primary composite outcome was independently adjudicated placenta mediated pregnancy complications (severe or early onset pre-eclampsia, birth of a small for gestational age child (< 10th percentile) and/or pregnancy loss) and/or major VTE up to 6 weeks post-partum.
Results: We present preliminary data analysis on 289 of 292 recruited women (three deliver in 03/2013). Final analysis on all participants will be presented in July. Between 02/2000 and 09/2012, we recruited 292 thrombophilic women at increased risk of placenta mediated pregnancy complications (46 with prior PET, 42 with prior SGA < 10th percentile, 43 with three or more pregnancy losses < 10 weeks, 25 with two or more losses > 10 weeks, 58 with one or more losses > 16 weeks) or increased risk of venous thrombosis (86 with a family history of VTE in a first degree relative and 35 with prior VTE). Mean maternal age was 31.8 years and mean gestational age at enrolment was 12.0 weeks. There were three post-randomization exclusions, leaving 144 assigned to dalteparin and 142 assigned to no dalteparin for this preliminary analysis. No women were lost to follow-up or withdrew consent but 26 women crossed over from their allocated group (12 from dalteparin to no dalteparin and 14 from no dalteparin to dalteparin). In intention to treat analysis, dalteparin did not reduce the incidence of the primary composite outcome (25/144, 17.4% [95% CI:11.2–23.6%]) compared with the no dalteparin group (27/142, 19.0%, (95% CI: 12.6–25.5%). In on treatment analysis, the results were similar (dalteparin 28/146, 19.2% (95% CI:12.8–25.6%) vs. no dalteparin 24/140, 17.1% [95% CI:10.9–23.4%]). In secondary outcome analysis, major bleeding was no different in the dalteparin (2/144, 1.4% [95% CI:0–3.3%]) compared with the no dalteparin group (2/142, 1.4% [95% CI: 0–3.4%]).
Summary/Conclusions: In a large randomized trial, ante-partum prophylactic dalteparin in thrombophilic women at increased risk of placenta mediated pregnancy complications and/or venous thrombosis did not reduce the incidence of these complications.

FS 01.2

RhuFVIIa reduces the rate of interventional second line therapies in severe primary postpartum haemorrhages resistant to uterotonics: a multicenter, randomised, open controlled trial

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Background: Postpartum haemorrhage (PPH) is the most common cause of obstetric haemorrhage. The early introduction of uterotonic agents is recommended as a first-line therapy, before specific interventional second-line therapies (uterine compression sutures, vascular ligations, embolisation, and hysterectomy). Recombinant human activated factor VII (RhuFVIIa, Novoseven®, Novonordisk A/S Baagsvaerd, Denmark) has been empirically administered as a last-ditch attempt to prevent hysterectomy but no randomised controlled trial (RCT) is currently available.
Aims: to conduct an open RCT testing a single rhuFVIIa infusion vs. standard care in women with PPH still ongoing after first-line therapies applied accordingly to French national guidelines, i.e. refractory to uterotonic agents.
Methods: This trial was conducted between February 2007 and November 2010 at eight University hospitals sites (Nimes, Montpellier, Nice, Saint- Etienne, Clamart, Paris-Cochin, Lille and Geneva) in two countries (France and Switzerland). After oxytocin then sulprostone failure, the patients underwent block randomisation according to site to receive an early single intravenous dose of rhuFVIIa 60 μg/kg (intervention arm), or no early rhuFVIIa (standard care arm). The sample size calculation was obtained to detect an absolute reduction of 30% in specific second line therapies. The clinical criteria which triggered the inclusion procedure was failure to control the bleeding 1 h after the onset/start of the sulprostone infusion. The primary efficacy outcome was the need of second line therapies. The primary safety outcome was a composite of any thrombotic events during the 5 days after rhuFVIIa infusion and death.
Results: Eighty-four parturient were enrolled. A second-line therapy was needed in 39 women from the standard care arm (92.9%) and in 22 women from the intervention arm (52.4%), RR 0.56 (0.42–0.77), $P < 0.0001$, mean number of women to avoid one-second-line therapy: 2.6. A significant decrease in uterine artery embolisation needs, alone ($P = 0.0082$) or associated with other second-line procedures ($P = 0.008$), was evidenced. The rate of final haemostatic hysterectomies was 19% in the standard care arm, with a trend for lower needs in the study drug group (7%, NNT 8.4, $P = 0.10$). After categorisation of the patients according to the mode of delivery, we observed similar rates of the primary efficacy outcome in both delivery categories. Safety concerns only occurred in the intervention arm: one ovarian vein thrombosis case (day 2) and one late pulmonary embolism case (PE; 5 days after a C-section delivery for placental abruption and stillbirth).
Summary/Conclusions: This first RCT on rhuFVIIa use in women with a severe PPH (refractory to uterotonics) evidenced an absolute 40.5% decrease in the need of interventional second-line therapies and a 2.4% risk of late PE. In severe PPH settings, thromboprophylaxis modalities being defined, future studies will have to investigate the saving potential of rhuFVIIa on end-line haemostatic hysterectomies.

FS 01.3

Polymorphisms in the Annexin A5 promoter, placental Annexin A5 gene expression and risk of preeclampsia

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Background: Annexin A5 (ANXA5) is an anticoagulant protein in the placenta. Reduced ANXA5 protein expression may induce placenta-mediated pregnancy complications such as recurrent miscarriage and preeclampsia. The M2 haplotype, encompassing four SNPs (rs112782763, rs28717001, rs28651243 and rs113588187) in the ANXA5 gene, is reported to be associated with recurrent miscarriage and with reduced ANXA5 promoter activity (N. Bogdanova et al., Human Molecular Genetics, 2007 vol. 16). Whether the placental expression of ANXA5 mRNA is reduced in the presence of this haplotype is unknown. Previous studies report an association between pregnancy outcome and the maternal ANXA5 genotype. We hypothesize that a stronger association with the neonatal than the maternal genotype is more likely, as placental tissue is of fetal origin and the placenta is a prerequisite to develop pregnancy complications as preeclampsia.
Aims: The aim of this study was to evaluate whether maternal or neonatal SNPs or haplotypes in the ANXA5 gene are associated with reduced placental ANXA5 mRNA expression.
Methods: The ANXA5 promoter region was evaluated by direct sequencing of maternal and umbilical cord blood DNA from a cohort of 287 women who delivered between 2005 and 2009 (45 with pre-eclampsia), which was obtained with informed consent. ANXA5 gene expression in placental tissue was determined by RT-qPCR and normalized to PSMD4 expression. Association between SNPs and placental expression of ANXA5 was evaluated in Haploview (X^2 test) and in SPSS (ANOVA and student's t-test). Mean ANXA5 gene expression in preeclamptic vs. normotensive women was compared (student's t-test).
Results: We identified seven SNPs, including the four SNPs that encompass the previously described M2 haplotype. We did not detect any association between placental ANXA5 mRNA levels and M2 haplotype in mother ($P = 0.72$) or neonate ($P = 0.66$) but did observe that placental ANXA5 expression is significantly increased in the presence of the minor allele of one SNP (c.-390C>T, rs62319820). This association is more prominent in umbilical cord DNA samples ($P = 0.002$) than in maternal DNA samples ($P = 0.007$) homozygous for the T allele. A mean of 13.2 ANXA5 normalized copy numbers was measured (range 0.40–27.12). Mean ANXA5 gene expression did not differ between preeclamptic and normotensive pregnancies ($P = 0.85$) and no SNP in maternal or neonatal genotype was associated with preeclampsia.
Conclusion: The presence of c.-390C>T is associated with increased placental ANXA5 mRNA expression. Whether ANXA5 protein levels are correlated to genotype in a similar fashion is yet to be determined. No SNP was associated with preeclampsia. In our population, the previously described M2 haplotype is not associated with reduced placental ANXA5 mRNA levels.

FS 02 – Thrombosis and Haemostasis in Children

FS 02

New approaches to unravelling the mysteries of clotting in children

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One can argue that clotting in children is a different pathophysiological entity to clotting in adults based on the observed age related differences in the coagulation system, the vastly different incidence of thrombosis in children compared to adults, and the epidemiology of disease presentation in patients with inherited thrombophilia. There is also increasing evidence that the differences in plasma proteins alter the interactions of anticoagulant drugs, significantly affecting efficacy and safety. However as yet we poorly understand the differences. While many differences have been recently described, the practical implications for clinical care of patients and robust strategies to improve patient outcomes remain to be elucidated. A variety of approaches are required to help unravel these mysteries, which are important not just to improve the management of children with abnormal clotting, but because they are likely to contribute to improved care of thrombosis in adults as well. This talk will focus on novel methods being used to increase our understanding of developmental haemostasis and the interactions of the clotting system with clinically important anticoagulants. Initial studies of developmental haemostasis focussed on functional assays used in current clinical practice. However, more sophisticated quantitative and qualitative assessments of relevant proteins and specific measures of their binding activities with anticoagulant drugs, as well as other plasma proteins, (and diagnostic reagents) are now being used. Proteomics and metabolomics are increasingly relevant, including studies of the platelet binding and the platelet proteome, previously ignored aspects of the clotting pathway in children. In addition, new clinical studies are looking at the problems of thrombosis in children with a renewed focus. The traditional dogma of the necessity for RCTs may not be the most effective way to gather evidence at this point in time for many key questions that need to be answered. Large networks and alternative study designs are being implemented with good effect. Current ongoing studies in childhood stroke, post fontan surgery thrombosis prophylaxis, and central venous line associated thrombosis are excellent examples of differing study designs being utilised strategically with outstanding early results. Defining the natural history of the spectrum of thrombotic disease in children of different ages remains one of the most important pieces of the puzzle to be clarified. Only then, can true risk benefit analysis be performed. This presentation will describe a number of examples of the new approaches currently being used in pathophysiological research as well as clinical diagnostic and therapeutic research. For some, it is the case of the old becoming new again. The presentation will highlight the key areas that require further research and suggest approaches to tackling those questions.

FS 02.1

Significantly increased risk for venous thrombotic events in long-term pediatric, adolescent and young adult cancer survivors: a population-based study

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Background: With improving survival, longterm complications among pediatric and adolescent young adult (AYA) cancer survivors are being increasingly recognized. Venous thrombotic events (VTE) occurring during childhood and AYA cancers are well described and may place survivors at increased risk of recurrent VTE later in life. However, the incidence and risk factors for VTE in pediatric and AYA cancer survivors have not been studied.
Aim: To assess the incidence and risk factors for VTE among long-term childhood and AYA cancer survivors in comparison to control population.
Methods: A population-based cohort of long-term (≥ 5 years from diagnosis) cancer survivors identified through the provincial British Columbia (BC) Cancer Registry was used. All medically-necessary care is conducted within the public health system for BC residents and recorded in provincial registries and administrative databases. Hence,

the current study estimates are assumed to be an accurate approximation of the risk in an unselected total population of childhood and AYA survivors in post-treatment years.
For inclusion, a survivor had to be (i) diagnosed with cancer in childhood (age 0–14 years) or adolescence/young adulthood (age 15–24 years) between 1981 and 1999 (ii) alive on or after 1/1/1986 and (iii) linked to the BC health insurance plan registry in at least 1 year post-survivorship. Comparators consisted of 5-year birth cohort and gender-matched controls (10 per case) from the BC health insurance plan registry. Individuals were considered to have a VTE diagnosis if their physician-visit or hospitalization records indicated a VTE diagnosis (as coded by International Classification of Diseases Versions 9 or 10). Follow up (median 9 years, range 0–21 years) and outcome information was obtained from provincial health administrative files.
Results: 2857 survivors (1397 diagnosed in childhood) and 28,570 controls were assessed. The incidence of VTE was significantly increased in both childhood and AYA cancer survivors as compared to the controls: childhood 5.7% vs. 2.8% (Hazard Ratio (HR) 2.71, 95% CI 2.02–3.66) and AYA 7.0% vs. 4.9% (HR 1.92, 95% CI 1.46–2.5).
Several risk factors for VTE in survivors were identified. Survivors who experienced relapsed disease had a significantly increased risk (HR 3.26, 95% CI 1.79–5.92). Survivors who received chemotherapy, radiation, and surgery were nearly three times as likely to have a VTE than those with surgery alone (HR 2.91, 95% CI 1.16–7.30). Among childhood cancer survivors VTE risk was significantly increased in females (HR 1.61, 95% CI 1.02–2.52). Among AYA patients, carcinoma survivors ($n = 253$) had an increased risk of VTE as compared to lymphoma survivors (HR 2.26, 95% CI 1.06–4.83).
Summary: In this large study, we made a novel observation that VTE is an important complication in childhood and AYA long-term cancer survivors. The plausible hypothesis is that VTE (both symptomatic and asymptomatic) that develop during the treatment of cancer in these patients place them at an increased risk of recurrence. These observations underscore (i) the importance of developing strategies for early recognition, diagnosis and treatment of VTE in childhood cancer survivors and (ii) for primary prevention of VTE during cancer therapy.

FS 02.2
'Blood and bone thinning' in children on long-term oral anticoagulation: what a bone-scan can tell you

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Background and Aim: Chronic pediatric conditions may be complicated by low bone mass, measured by bone mineral density scans (BMD; i.e. *z*-score). Furthermore, children with chronic diseases are also at increased risk of developing thrombosis, requiring long-term anticoagulation (AC) with warfarin. Since warfarin may further jeopardize bone-health of a population already at risk for bone fragility, we wished to investigate bone-health outcomes in a cohort of such patients.
Methods: Our institutional protocol indicates children receiving warfarin for > 1 year undergo an initial BMD after the age of 5 years. Further management depends on the first results: group (G) 1 ($2.0 \leq z\text{-score} \leq -2.0$): bi-annual BMD; G2 ($-2.1 \leq z\text{-score} \leq -2.5$): annual BMD + biomarkers (Ca^{++}/Ph^{-}/PTH/1,25-vit.D); or G3 (z-scores < −2.5): annual BMD + biomarkers + spinal x-ray. For our analysis, patients on G2 and G3 were categorized as high-risk and G1, as low-risk. z-score s were deemed abnormal if < −2.0. Descriptive statistics and survival analysis were utilized. To minimize the influence of the per-protocol delay of younger patients to undergo a BMD scan, only patients commencing AC > 3 years (48/74) were included in survival analysis. The study was approved by the Institutional Ethics Review Board. Informed consent was waived.
Results: Seventy-four patients median age: 4.1 years (interquartile range [IQR] 2.0–10.1 at the time of starting long-term AC, M:F ratio 1.5:1), were followed-up at Sickkids, Toronto, between Jan/2003 and Dec/2011 and had at least one initial BMD scan, at a median of 3.6 years (IQR 1.5–7.5) from the time warfarin was started. Underlying conditions were: cardiac (85%), endocrine (8%), other reasons (7%), including rheumatic/renal diseases. The median AC duration was 7.8 years (IQR 5.2–12.4). 27/74 (36%) patients had additional LMWH therapy (median: 7.1 months [IQR 3.0–14.6]).
57/74 (77%) and 17/74 (23%) patients were categorized as low and high-risk groups, respectively. No statistical differences were found for age, sex, AC duration, dosing, previous LMWH, and time to 1st BMD. Only underlying condition was significant: whereas 80% of cardiac and 80% of other underlying conditions belonged to the low-risk group, 80% of endocrine patients were high-risk group ($P = 0.01$). Further investigation confirmed that the overall average z-score was −1.1, and −1.2 for cardiac and other conditions, and −3.9 for endocrine patients. Correlation between risk-group and condition was fair ($r = 0.4$).
Twenty-five patients (14 low vs. 11 high-risk) had an x-ray done. 7/25 (29%) suffered fractures; survival analysis revealed a significant difference in the time from AC-start to fracture between high and low-risk group (5.1 vs. > 15 years, log-rank $P = 0.032$).
13/48 (32%) patients reached an abnormal z-score at a median time from AC-start of 9.0 years (IQR: $3.5 \geq 15$ years). Log-rank test revealed no significant difference for sex ($P = 0.41$), preceding LMWH ($P = 0.27$), warfarin dose ($P = 0.94$), biomarkers abnormalities ($P = 0.41$), or underlying condition ($P = 0.06$). There was a statistically significant difference in the time from AC-start to abnormal z-score according to risk-group at presentation (high vs. low; 3.6 vs. > 15 years, $P < 0.0001$).
Conclusion: Children receiving long-term warfarin benefit from repeated bone health monitoring. Low-risk patients, defined as per their first BMD, appear to be stable over time. The first BMD also helps detecting children at higher risk of spontaneous bone fractures.

FS 02.3
Risk factors for the development of venous thromboembolism in childhood acute lymphoblastic leukaemia

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Background: Venous thromboembolism (VTE) frequently occurs during asparaginase (ASP) treatment in paediatric patients treated for acute lymphoblastic leukaemia (ALL). This complication causes significant morbidity. Identification of a susceptible population is crucial for effective thromboprophylaxis. However, risk factors for ALL-associated VTE have not been fully clarified.
Aims: The aim of this study was to investigate the incidence of VTE and to identify risk factors for the development of VTE in paediatric ALL patients treated according to the Dutch Childhood Oncology Group (DCOG) ALL IX or ALL X protocols.
Methods: A retrospective observational cohort study was performed for VTE among children (1–18 years), with newly diagnosed ALL receiving chemotherapy according to the ALL IX or ALL X protocol between February 1997 and January 2012. In the ALL IX protocol, patients were stratified into two risk groups: non-high risk (NHR) and high risk (HR). In the ALL X protocol, minimal residual disease was an important tool to stratify into three risk groups: standard risk (SR), medium risk (MR) and HR. All patients received central venous catheters: Broviac® or PAC®. Induction treatment included in the ALL IX protocol, day 0–49, dexamethasone and four doses of 6000 IU/m^2 ASP intravenously over a period of 2 weeks (day 29–40). The induc-

tion phase of the ALL X protocol, day 0–64, included prednisone and eight doses of 5000 IU/m^2 ASP intravenously over 3 weeks (day 12–33). Medical records were reviewed and all VTE were recorded. Furthermore, details of ALL diagnosis, treatment protocols, laboratory values of fibrinogen and antithrombin, and risk factors were recorded.
Results: Two hundred and five children (♂,n = 123; ♀, n = 82) treated according to the two different DCOG ALL protocols (ALL IX, n = 112; ALL X, n = 93) were included. The median age was 4.8 years (range 1–18 years). Totally, 19 children (9.3%; 95% CI: 5.3–13.3%) developed VTE; 9 (4.4%) during the induction phase and 10 (4.9%) during later treatment phases. In the induction phase, four VTE occurred in the central nervous system (45%) and five VTE were catheter-related (55%). During later treatment phases, two deep vein thromboses and eight catheter-related trombi occurred. During induction, univariate analysis showed that VTE was significantly more likely in children > 10 years of age (P = 0.031), in children treated according to the ALL X protocol (P = 0.012) and in children in MR or HR groups (P = 0.041). After induction, only MR and HR groups were associated with VTE (P = 0.005). Gender, body mass index, ALL-immunophenotype, type of catheter, antithrombin and fibrinogen levels, the presence of two or more of the following metabolic abnormalities: hyperuricemia, hyperkalemia and hyperphosphatemia, and infections appeared not to be associated with VTE. In the multiple regression analysis, after stepwise elimination, type of protocol (P < 0.0001) and risk group (P = 0.006) remained significantly associated with VTE during the induction phase.
Conclusion: VTE developed in about 9% of ALL paediatric patients. Protocol type including lower doses of ASP over a longer period and prednisone and the ALL protocol risk group were significant risk factors for the development of VTE.

FS 03 – Thrombosis and Haemostasis in the Asian-Pacific

FS 03.2

Registry of congenital atypical HUS in Japan

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Thrombotic microangiopathies (TMAs) are pathological conditions characterized by microangiopathic hemolytic anemia, destructive thrombocytopenia, and renal dysfunction (organ failure). Two typical phenotypes of TMAs are thrombotic thrombocytopenic purpura (TTP) and hemolytic anemia (HUS), both of which are either caused by congenital or acquired form. Now it is well established that most of TTP patients have deficient activity of ADAMTS13, a von Willebrand factor-cleaving protease, due to its gene mutations or acquired autoantibodies to this enzyme. On the other hand, a major population of HUS patients develops in association with hemorrhagic enterocolitis by Shigatoxin-producing *E. coli* infection. However, a minor population of HUS patients, who usually do not accompany with diarrhea and therefore referred to atypical (a) HUS, is caused by uncontrolled complement activation due to gene mutations involved in the alternative pathway, such as complement factor H (CFH), complement factor I (CFI), membrane cofactor protein (MCP), thrombomodulin (THBD), complement component 3 (C3), and complement factor B (CFB). In addition, CFH auto-antibodies with or without gene mutations of CFH related proteins (CFHRs 1~5) have also been reported to be the cause of aHUS. Diagnostic criteria for congenital aHUS used in Japan is almost comparable to that of US and Western countries, and consisted of the abovementioned clinical features of TMA and the exclusion criteria of more than 5% of normal for ADAMTS13 activity. Since 1998, Nara Medical University (NMU) has been functioning as a nation-wide referral center for TMA in Japan through the protein-based analyses including ADAMTS13 and hemolytic assays, in collaboration with National Cerebral & Cardiovascular Center (NCVC), which takes charge of the gene analyses responsible for TMAs. Through this cohort study, we made a large registry of 1149 TMA patients until the end of 2012, in which 49 were the patients with congenital TTP (Upshaw-Schulman syndrome) and 55 were congenital aHUS. Among the 55 aHUS patients, a set of complement regulatory gene analyses was performed on 29 patients (23 at NCVC and nine at other institutions including foreign countries). As consequence, the gene mutations responsible for aHUS were identified in 22 patients (22/29, 76%), but were not identified in the remaining seven patients (7/29, 24%). The number of gene analysis on aHUS patients is still small in our registry, but the presence of two rare and consistent mutations of C3 (I1157T) and CFH (R1215Q) have been presented.

FS 03.3

SMTP-7, a novel thrombolytic with an anti-inflammatory potential, improves primate thrombotic stroke with reduced hemorrhage risk: a role of soluble epoxide hydrolase inhibition

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Background: There is increasing evidence showing a close correlation between inflammation and neuronal damage/hemorrhagic transformation after acute ischemic stroke, thereby development of novel stroke therapy, hopefully with a drug having cerebroprotective/anti-inflammatory potential, is urgently needed. The thrombolytic SMTP-7, a small molecule that promotes plasminogen activation through relaxation of plasminogen conformation, has excellent therapeutic activities in cerebral infarction in several rodent models. Unexpectedly, unlike the standard thrombolytic stroke drug t-PA, SMTP-7 has much wider therapeutic time window and even suppresses cerebral hemorrhage, possibly by triggering anti-inflammatory responses. In the course of our study, soluble epoxide hydrolase (sEH), which plays a major role in the regulation of the inflammatory response, was identified as a target molecule of SMTP-7. We further demonstrated its unique mechanisms of action against intrinsic sEH, which can account for anti-inflammatory and cerebroprotective efficacy *in vivo*.
Aims: Our study aims at (i) evaluating SMTP-7 efficacy in several animal models, and (ii) investigating precise molecular mechanism of sEH inhibition by SMTP-7.
Methods: A monkey photochemical-induced thrombotic middle cerebral artery (MCA) occlusion model, as well as several other rodent models, was utilized to evaluate therapeutic potentials of SMTP-7. LC-MS analysis was performed to measure total dihydroxyeicosatrienoic acid (DHET) level; a fatty acid dioxide converted from the anti-inflammatory fatty acid epoxyeicosatrienoic acid by sEH in HepG2 cells in culture and mouse liver *in vivo*.
Results: In the monkey MCA occlusion model, SMTP-7 (10 mg/kg, i.v. infusion) increased post-infusion MCA recanalization rate (32.5-fold, P = 0.043) and ameliorated post 24 h neurological deficit (by 29%, P = 0.02), cerebral infarct (by 48%, P = 0.025), and even cerebral hemorrhage (by 51%, P = 0.013) as compared with saline-treated control. In normal monkeys, SMTP-7 did not affect general physiological and hemostatic variables including coagulation and platelet parameters. SMTP-7 inhibited sEH in a cell-free enzyme assay as well as in HepG2 cells. The IC_{50} value of 1.7 μM in HepG2 cells is well below the level of pharmacological plasma concentration of SMTP-7 (3–30 μM), suggesting that SMTP-7 inhibits sEH *in vivo*. Inhibitory effect on sEH by SMTP-7 (10 mg/kg, i.v.) was also observed in the liver (by 53% P = 0.033) derived from normal mice after 30â˜60 min post treatment. The findings observed in our rodent models suggested an importance of the drug's cerebroprotective/anti-inflammatory properties via the inhibition of intrinsic sEH function, as well as its

regulated profibrinolytic action, serving as a physiological on-demand system coping with thrombotic events.
Conclusions: SMTP-7 is effective in treating thrombotic MCA occlusion stroke in monkeys, which can be due, at least in part, to the thrombolytic activity as well as anti-inflammatory efficacy via the suppression of intrinsic sEH function, thereby resulting in amelioration of neurological deficits, infarct size, and even hemorrhagic transformation.

FS 03.4
ACTN1 mutations cause congenital macrothrombocytopenia

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Background: Congenital macrothrombocytopenia (CMTP) is a heterogeneous group of rare platelet disorders, characterized by a congenital reduction of platelet counts and abnormally large platelets, for which the causative genes are known only in approximately half the cases.
Aims: This study aims to identify novel causative genes for CMTP by next-generation sequencing.
Methods: We performed whole exome sequencing and targeted Sanger sequencing in CMTP cases in which a dominant mode of transmission had been suspected but no known responsible mutations had been documented.
Results: In 13 Japanese CMTP pedigrees, we identified six pedigrees (46%) with *ACTN1* variants, which co-segregated with CMTP. *ACTN1* encodes α-actinin-1, a member of the actin-crosslinking protein superfamily that participates in the organization of the actin-based cytoskeleton. Patients with variant *ACTN1* presented moderate macrothrombocytopenia with anisocytosis, but were either asymptomatic or associated with only a modest bleeding tendency. *In vitro* transfection experiments in Chinese hamster ovary cells demonstrated that mutant α-actinin-1 disrupted the normal actin-based cytoskeletal structure. Moreover, transduction of mouse fetal liver-derived megakaryocytes with patient-derived *ACTN1*-variants caused a disorganized actin-based cytoskeleton in megakaryocytes, resulting in the production of abnormally large proplatelet tips, which were reduced in number. In the entire cohort, *ACTN1* variants accounted for 5.5% of the dominant forms of CMTP cases and represent the fourth most common causative gene in Japanese individuals.
Summary/Conclusion: Our findings provide a novel insight of actin-based cytoskeleton in the pathogenesis of CMTP.

FS 03.5
Impaired haemostasis in Reelin-deficient mouse: a potential role of plasma Reelin in thrombin generation and fibrin clot formation

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Background: Haemostasis prevents excessive bleeding during vessel wall injury and is properly controlled by a balance between platelets, coagulation factors, and endothelial cells. Assembly of factor Xa/Va on activated platelet results in the burst of thrombin generation that plays an important role in converting fibrinogen to fibrin. Reelin is an extracellular glycoprotein that was first identified in mice with the characteristic of reeling gait. In addition to the central nervous system, Reelin is present in the plasma and interacts with platelets, facilitates platelet lamellipodia formation and F-actin bundling and enhances full spreading of platelets on fibrinogen. Nevertheless, whether plasma Reelin has any functional link with haemostasis *in vivo* has not yet been elucidated.
Aims: In the present study, the *reeler* ($Reln^{-/-}$) mouse was used as the model to explore the potential role of Reelin on haemostasis.
Methods: Heterozygous *reeler* ($Reln^{+/-}$) mice were interbred to generate wild type ($Reln^{+/+}$), and homozygous ($Reln^{-/-}$) *reeler* mice. Thrombelastography (TEG) and calibrated automatic thrombogram (CAT) assays were used to analyze the integrity of coagulation system. Prothrombin time (PT) and activated partial thromboplastin time (aPTT) were used to confirm the functional impact of Reelin deficiency on haemostasis.
Results: $Reln^{-/-}$ mice displayed a normal platelet number and size with a prolonged tail-bleeding time and an increase in rebleeding rate. TEG analysis revealed that $Reln^{-/-}$ mice was impaired in blood coagulation. Of the five TEG analytical parameters, R, K, and a-angle were similar for $Reln^{-/-}$ and control mice. In contrast, $Reln^{-/-}$ mice displayed a significant decrease in MA and CI which measures the strength of a clot and overall assessment of coagulability, respectively. In accordance with these findings, $Reln^{-/-}$ mice displayed a slight decrease in coagulation time when compared to the $Reln^{+/+}$ and $Reln^{+/-}$ mice in PT assays. The dH value that measured scattered light intensity change for $Reln^{-/-}$ mice was significantly decreased when compared to the $Reln^{+/+}$ and $Reln^{+/-}$ mice. For aPTT assays, $Reln^{-/-}$ mice displayed a five-fold increase in the coagulation time when compared to the $Reln^{+/+}$ and $Reln^{-/-}$ mice. The average dH for $Reln^{-/-}$ mice was significantly lower than the dH for $Reln^{+/+}$ and $Reln^{+/-}$ mice. A loosen fibrin clot structure was observed for the blood clot obtained from the $Reln^{-/-}$ mice as revealed by scanning electron microscopy. CAT analysis further indicated that Reelin deficiency resulted in a decrease in thrombin generation. Of the six CAT analytical parameters, the lag time and time to peak (ttpeak) were similar for $Reln^{+/+}$, $Reln^{+/-}$ and $Reln^{-/-}$ mice. Notably, $Reln^{-/-}$ mice displayed a significant decrease in the rate of thrombin generation, peak thrombin concentration, start tail (indicator of anticoagulant activity) and ETP (indicator for the total amount of thrombin generation). Impaired thrombin generation thereby contributes to the formation of fibrin clots with reduced strength for the Reelin-deficiency plasma.
Conclusions: This study represents the first report to define a novel function of plasma Reelin in thrombin generation and fibrin clot formation. Reelin is therefore suggested to be a key component in the regulation of coagulation cascade and haemostasis.

FS 04.4
Education in the developing world

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Thrombosis and haemostasis (T&H) is clearly emerging as a well-defined and growing subspeciality area. Although most of the professionals are specialists in Hematology, the field attracts a smaller number of professionals when compared to other subspecialties such as Oncohematology, Bone Marrow Transplant and Blood Transfusion. Furthermore, Thrombosis and Haemostasis is a complex field of Medicine, which requires education on clinical and laboratory training. This subspecialty is highly dependent on appropriate laboratory diagnosis, which can be expensive. This issue, in addition to few experts available and a lack of specialized centers makes the education on T&H in developing countries particularly challenging. An adequate

educational program in T&H should start at undergraduate levels of Medicine and related areas such as Pharmacy, Biochemistry, Biology and Biomedical Sciences. This strategy would attract young professionals to the field early on their carriers. Furthermore, training such as medical residency in Hematology, Internal Medicine and Clinical Pathology should address topics related to T&H with the aim of educating professionals with competence on diagnosis, treatment and research in the field. Indeed, in some countries (especially those located in Europe), a curriculum for T&H has been proposed and a role for a T&H specialist has been suggested. In the field of bleeding disorders, some countries have implemented training to educate medical professionals as hemophilia doctors. In the developing countries, these initiatives are still incipient and, therefore, a major effort is needed to implement education in T&H. In some countries, there is no Society of T&H, but of Hematology and its interest is, particularly, directed to the other fields of Hematology. In most of the cases, there is no T&H curriculum available during the medical residency in Hematology, Internal Medicine or Clinical Pathology. Lastly, the implementation of "state of art"diagnosis and treatment as well as research in T&H involves high costs which need public funding, not easily available. Some developing countries, however, have been capable of building good programs in T&H. In conclusion, education in T&H in developing countries is a major challenge. An appropriate approach needs training at early levels of education in the related fields with incentives, creation of a national curriculum for T&H, involvement of the related societies and close partnership with governmental bodies.

NS 01 – Nurses Symposium

NS 01.1

Adherence to prescribed treatment regimen is related to chronic pain among adolescent and young adults with moderate or severe hemophilia: Early results from the IMPACT QoL survey

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Background/Aim: Little data exist, especially for adolescents and young adults, about the relationship between adherence to prescribed hemophilia treatment regimens and chronic pain. We examined this relationship among moderate and severe hemophiliacs aged 13–25.

Methods: A convenience sample of hemophiliacs aged 13–25 completed an IRB-approved, cross-sectional, online survey that assessed, among other factors, regimen-specific adherence and chronic pain. Recruitment occurred at major hemophilia meetings, hemophilia treatment centers, and through social media from April through December of 2012. Adherence was assessed using the VERITAS-Pro and VERITAS-PRN for prophylactic and on-demand participants, respectively. VERITAS scores range from 24 (most adherent) to 120 (least adherent). Chronic pain was measured using the revised Faces Pain Scale (FPS-R). For purpose of analysis, chronic pain was dichotomized as *high* for those who reported their pain as 'moderate,' 'severe,' 'very severe,' or 'worst pain possible' (i.e. ≥ 4) and *low* for 'mild pain' or 'no pain' (i.e. < 4). Multivariable, parsimonious logistic regression models were constructed to assess factors associated with having high (vs. low) levels of chronic pain. Separate models were constructed to evaluate i) a combined VERITAS score among prophylactic and on-demand patients and ii) the VERITAS-Pro score among prophylactic patients only. Small sample size prevented analysis for on-demand (only) participants.

Results: Ninety-three adolescents and young adults with hemophilia completed the survey. Mild patients ($n = 13$) were excluded. Of the 80 participants (79 male) included in the final analysis, most had severe disease (91%), infused prophylactically (86%), and had Hemophilia A (91%). Fifty-one percent were aged 13–17 and most were white (76%), non-Hispanic (88%), and never married (93%). The majority (94%) had some type of health insurance, with the highest proportion having only commercial insurance (45%), only Medicaid (28%), or both (9%).

Mean VERITAS-Pro ($n = 69$) and PRN ($n = 11$) scores were 49.6 ± 12.9 (range 25–78) and 51.0 ± 11.6 (range 35–74), respectively. Chronic pain was reported as *high* for 35% of respondents (36% for prophylactic vs. 27% for on-demand, $P = 0.74$). Mean VERITAS-Pro scores for those with high and low chronic pain were 53.6 ± 12.3 vs. 47.4 ± 12.9, $P = 0.05$. VERITAS-PRN scores were similar across chronic pain status. Logistic regression analysis revealed that for each 10-point reduction (i.e. increase in adherence) in the combined VERITAS score (Pro and PRN) there was a 35% (OR = 0.65; 95% CI = 0.44, 0.96; $P = 0.03$) reduction in the odds of having high chronic pain. Among prophylactic respondents only, for each 10-point reduction in the VERITAS-Pro score there was a 39% (OR = 0.61; 95% CI = 0.39, 0.96; $P = 0.03$) reduction in the odds of having high chronic pain. Among prophylactic participants, the model also revealed that, compared to whites, non-whites were 4.42 (95% CI: 1.21, 16.1; $P = 0.02$) times as likely to report high chronic pain.

Summary/Conclusion: This study provides evidence that among adolescent and young adults with severe hemophilia, better adherence to prescribed treatment regimens (either prophylactic or on-demand) is associated with a significantly lower likelihood of having debilitating levels of chronic pain. Moreover, among prophylactic infusers, non-whites were > 4 times as likely as whites to report high levels of chronic pain.

NS 01.2

Effectiveness of patient education in VTE prevention: a nurse led collaborative local service improvement project

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Background: The National Institute for Health and Clinical Excellence (NICE) Quality Standard for venous thromboembolism (VTE) prevention (2010) states that all adult hospital in-patients and carers should receive both verbal and written information regarding VTE prevention on admission and discharge. Whilst much focus has been placed on VTE risk assessment and appropriate prophylaxis, it is widely acknowledged that nurses play a key role in patient education and this is central to delivering successful outcomes. The National Nursing and Midwifery Network (NNMN) for VTE prevention surveyed the current provision of educational information to evaluate its effectiveness.

Aims: To initiate a nurse-led collaborative local service improvement project across VTE Exemplar Centres to review and enhance the effectiveness of patient education for the prevention of VTE.

Methods: All VTE Exemplar Centres were invited to participate by contributing generic findings from a minimum of 10 patients interviewed at discharge from hospital. Patients were asked if they had received written and verbal information, along with 10 key questions relating to their level of understanding. Each participating hospital used its own standard literature. The Education Workstream Lead for the NNMN collated the findings.

Results: Thirteen centres collected data on 563 patients.

The median number of returns was 20 with a range of 10–226.

Content analysis identified 5 key themes relating to;

1) Provision of information:-patients who received leaflets didn't always read them, or found the leaflets too long or complex.

Patients that were given both written and verbal information had a better understanding of VTE prevention compared with either method alone.
2) Route of admission:-elective surgical patients were better informed than emergency surgical patients or medical patients.
3) Reducing risk of VTE:-patients had good understanding of how to reduce risks of VTE but had some significant misconceptions.
4) Appreciation of signs and symptoms of VTE:-patients more aware of those for DVT than PE.
5) Discharge information:-some patients were unsure of the duration of extended thromboprophylaxis and some were unsure who they should contact if they developed a problem after discharge.

Summary/Conclusions: The key themes identified from the collaborative work of 13 VTE Exemplar Centres across England highlight specific areas where efforts can be focused to improve the quality of patient education on VTE prevention. Whilst service improvement strategies will be implemented locally, the implications of these findings are useful for a wider audience and will be disseminated by the NNMN. This includes the promotion of;

combined provision of verbal and written information

reviewing the level and content of patient information for maximum effectiveness

strategies to empower nurses to lead service improvement through service evaluation, change management and re-evaluation

development of an aid memoire of key learning points to support the effective delivery of patient literature in alignment with the work of the National VTE Prevention Programme Board.

NS 01.3

An audit of the use of novel anticoagulants: Dabiagtran and Rivaroxaban for stroke prevention in AF

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In June 2012 our Acute Trust adopted the NICE TA for both Dabigatran and Rivaroxaban as an alternative for the treatment of non-valvular AF in stroke prevention. Funding was agreed with the local PCT and both drugs were added to the formulary. Prior to this all oral anticoagulation initiation was performed in the secondary care led anticoagulant service and had been for the past 15 years. We had an agreement with both the Trust and local PCT that the anticoagulant service would continue to provide counselling for patients who were commenced on either of the two novel anticoagulants and then further care would be overseen by the general practitioner. As part of our care we added these patients to our existing database and also drew up a proforma for monitoring these patients.

The decision which oral anticoagulant to prescribe is made by the referring clinician and anticoagulant staff use a decision aid to ensure the suitability of the proposed anticoagulant. This takes into account NICE criteria, patient age, baseline bloods including renal function, FBC and LFT's, concurrent medications, history of previous dyspepsia or gastric ulcer. To date we have 26 patients on Rivaroxaban (24/26 on 20 mg and 2/26 on 15 mg) and 17 on Dabigatran (11/17 on 150 mg BD and 6/17 on 110 mg BD).

After a 3 month period we contact all patients by telephone to establish whether the patient has had any problems taking the current medication. This includes: any problems with bleeding (if yes then date of episode, whether hospitalisation was required and any intervention needed), any renal problems (including checking that any relevant up-to date screening has been checked) and for patients taking Dabigatran if they have had any episodes of dyspepsia.

We are also recording if any of the patients have been cardioverted whilst on either of the novel anticoagulants.

23/43 patients have had 3 month follow up.

Of these, 19 (83%) reported no adverse event or side effects that warranted discontinuation of the drug.

4/21 (17%) of patients taking Dabigatran discontinued the medication.

2/4 discontinued due to SOB and chest pain (thought to be cardiac in origin), 1 switching to warfarin and the other Rivaroxaban (also discontinued – patient preference).

2/4 discontinued due to dyspepsia (one despite taking a prescribed PPI). One switched to Rivaroxaban and the other is currently not taking any anticoagulation.

Conclusion: It is important to keep a database of these patients so we can monitor for any side effects and support both the patient and primary care colleagues.

One of the issues seen in secondary care is a lack of awareness that these are anticoagulants which has resulted in poor prescribing, where Dalteparin has been prescribed as well as Dabigatran or Rivaroxaban. No harm has come to the patient as the error has been picked up by the pharmacist but there needs to be extensive continuing education relating all novel anticoagulants until their use is more familiar.

Effective communication across all teams is essential to minimise the risk of prescribing errors.

ORAL COMMUNICATIONS

OC 01.1

Prolylcarboxypeptidase promotes angiogenesis and vascular repair

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Background: Prolylcarboxypeptidase (PRCP) is a serine protease and endopeptidase on vasculature and kidney that degrades peptides with a Pro-X C-terminus (AngII, desArg9BK, $aMSH_{1-13}$). In kidney it is a major enzyme forming Ang-(1-7) (Am J Physiol Cell Physiol, In Press). PRCP hypomorphic mice (*PRCPgt/gt*) are lean, hypertensive, and prothrombotic (JCI 119:2130, 2009; Blood 117:3929, 2011). Although *PRCPgt/gt* are protected from metabolic insults (e.g. atherosclerosis), they have vascular inflammation. This injury is characterized by increased vessel ROS, reduced vasculo-protective transcription factors KLF2 and KLF4, reduced and uncoupled eNOS, reduced thrombomodulin with less protein C activation, and increased tissue factor and PAI-1.

Aims: Since PRCP levels influence vascular inflammation, we determined if PRCP also regulates vessel growth, migration, angiogenesis, and injury repair.

Methods: PRCP levels were manipulated in bovine aortic endothelial cells (BAEC) by siRNA and human PRCP cDNA (*hPRCP*) transfection. *hPRCP* was mutagenized at S179 and H455 (*hPRCPmut*). BAEC growth and proliferation were measured by counting and MTS color reaction. Cell ROS and apoptosis was assayed by dihydroethidium (DHE), HPLC, and annexin V. BAEC scratch assay assessed cell migration; aortic sprouts and matrigel plugs examined angiogenesis. Five mm punch biopsy wounds examined inflammation and repair angiogenesis. Femoral artery ligation examined ischemic injury repair angiogenesis. Femoral artery wire injury investigated vessel repair inflammation in $PRCP^{gt/gt}$ mice and $PRCP^{gt/gt}$ backcrossed in inflammation-protected MRP-$14^{-/-}$ mice.

Results: PRCP depletion by siRNA reduced BAEC growth, whereas transfection of *hPRCP* enhanced cellular proliferation. PRCP depletion is associated with increased ROS (by DHE and HPLC) and annexin V binding, and over-expression reduces ROS and apoptosis. Transfection of *hPRCP* or its active site mutant (*hPRCPmut*) rescued reduced cell growth after PRCP siRNA knockdown. These data indicate that PRCP levels directly affect cell growth, and this effect is not a function of its proteolytic activity. PRCP-depleted cells migrated less on scratch assay and murine $PRCP^{gt/gt}$ aortic segments had reduced sprout formation. Matrigel plugs in $PRCP^{gt/gt}$ mice had reduced hemoglobin content, less PECAM and NG2 staining, and a reduced NG2/PECAM ratio, indicating diminished angiogenesis. Skin wounds on $PRCP^{gt/gt}$ mice had delayed closure and reduced PECAM staining on day 7 but normal CD11b inflammatory response on day 2. $PRCP^{gt/gt}$ mice also had reduced reperfusion of the femoral artery after limb ischemia on days 7, 14 and 21 with decreased angiogenesis at day 28. After femoral artery wire injury, $PRCP^{gt/gt}$ mice had increased neointimal formation (neointima/media ratio) with increased CD45 leukocyte accumulation and Ki67 cellular proliferation. Mating $PRCP^{gt/gt}$ mice with MRP-$14^{-/-}$ mice reduced inflammation, cellular proliferation, and neointimal thickening after wire injury in the hypomorphic mice.

Summary: PRCP is known to regulate metabolism and vascular homeostasis. Deficiency of PRCP is associated with improved glucose tolerance and less insulin resistance but with sick blood vessels leading to hypertension and thrombosis. PRCP levels regulate endothelial cell growth, angiogenesis, and the response to vascular injury. This activity is not related to its protease function. This combined information suggests that PRCP is a unique regulator of vascular well-being and a target to promote vascular health.

OC 01.2

UPAR's domain 2 regulates single chain urokinase-mediated angiogenesis through beta-1-integrins and VEGFR2

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Background: The mechanism by which single chain urokinase (ScuPA) mediates angiogenesis is incompletely understood. Previous studies indicate that VEGF stimulation of VEGFR2 induces MMP-2 that activates ScuPA to tcuPA bound to uPAR to stimulate angiogenesis. Alternatively, soluble uPAR through its sequence $S^{88}RSRY^{92}$ stimulates angiogenesis through a protease-independent mechanism. CD146 stimulates angiogenesis through up-regulation of ScuPA, uPAR, VEGF and VEGFR2.

Aims: The purpose of this study is to map a ScuPA signaling pathway in endothelial cells that promotes angiogenesis and determine if it is similar to that described for factor XII (Blood 115:5111, 2010).

Methods: Cultured HUVEC (human umbilical vein EC), HMEC (human microvascular EC) and SIN$1^{-/-}$ MEFs treated with ScuPA, tcuPA, or APMSF-treated tcuPA were utilized for the signaling studies. Domain 2 (D2) of uPAR was modeled for the ScuPA and HK binding regions using Cn3D4.3 from NCBI. Peptides from uPAR's D2 and HK's domain 5 (D5) mapped ScuPA's binding region. MEK and PI3 kinase inhibitors characterized the mechanism for ScuPA-induced pERK1/2 and $pAktS^{473}$. Antibodies to beta-1-integrin, AG1478, and PP3 characterized the signaling pathways. siRNA to HER1-4 and VEGFR2 determined the communicating tyrosine kinase for ScuPA-induced signaling. ScuPA-induced cell proliferation and BrdU incorporation was determined. The pathway to ScuPA-induced *in vitro* sprouting and *in vivo* matrigel plug angiogenesis was determined.

Results: ScuPA (> 4 nM), APMSF-treated tcuPA or tcuPA induces pERK1/2 (MAPK44 and 42) and $pAktS^{473}$ in HUVEC or HMEC. ScuPA-induced activation of pERK1/2 is blocked by PD98059 or U0126 and $pAktS^{473}$ inhibited by Wortmannin or LY294002. ScuPA (32 nM) or protease-inhibited two-chain uPA stimulates pERK1/2 to the same extent, indicating that signaling is not dependent on enzymatic activity. ScuPA induces pERK1/2, but not $pAktS^{473}$, in SIN$1^{-/-}$ MEFs indicating that the two pathways are not identical and ScuPA interacts with uPAR independent of domain 1. Modeling of uPAR's crystal structure indicates that its domain 2 is on the surface and it does not interact with the ATF of ScuPA and uPAR's domain 1. Peptides from uPAR's D2 or HK's D5 or cleaved HK compete with ScuPA for induction of pERK1/2 and $pAktS^{473}$. A peptide to the integrin binding site on uPAR, a beta-1-integrin peptide that binds uPAR, antibody 6S6 to beta-1-integrin, the tyrosine kinase inhibitors AG1478 or PP3, and siRNA knockdown of VEGFR2, but not HER1-4, each block ScuPA-induced pERK1/2 and $pAktS^{473}$. ScuPA initiated cell proliferation is blocked by MEK and PI3 kinase inhibitors, antibody 6S6, and uPAR D2 and HK D5 peptides. ScuPA promotes aortic sprouts and matrigel plug angiogenesis in normal, but not uPAR deficient mouse aorta or mice, respectively, that is blocked by PD98059, LY294002, AG1478 or HKa.

Summary: These data suggest that ScuPA promotes endothelial cell proliferation and angiogenesis through a non-proteolytic signaling pathway, similar to factor XII, mediated by interactions involving uPAR's D2, beta-1-integrin, and VEGFR2. HKa's D5 peptides down regulate this pathway by, at least, blocking ScuPA binding to uPAR's D2. ScuPA, uPAR, VEGF, and VEGFR2 function in an autoregulatory and autocrine manner to promote angiogenesis.

OC 01.3

Junctional adhesion molecule-A modulates angiogenesis through transcriptional regulation of VEGF/VEGFR2 expression

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Background: The process of angiogenesis is associated with a number of human pathologies. Vascular endothelial growth factor (VEGF) is a potent angiogenic growth factor. VEGF expression is upregulated during ischemia and tumor growth. VEGF expression and thus VEGF-dependent angiogenesis are normally suppressed in healthy adults. The mechanism of this suppression is not known.
Aim: To show that junctional adhesion molecule-A (JAM-A), a tight junction protein expressed on endothelial cells, is an endogenous suppressor of VEGF-induced angiogenesis.
Methods: Using gene-targeted Jam-A null mice, we performed *in vivo* tumor growth, and a variety of angiogenesis assays such as MatrigelÒ plug and aortic ring sprouting. Wild-type (WT) mice were used as controls. Vascular permeability was assessed by Miles assay using Evans blue dye. Real-time PCR and Western blot analysis were used to assess gene expression.
Results: We found that murine melanoma tumor growth and associated angiogenesis were significantly augmented ($P < 0.001$) in Jam-A null mice compared to WT mice. Additionally, Jam-A null mice showed significantly enhanced ($P < 0.004$) vascular permeability. Furthermore, VEGF-, but not FGF-2-dependent angiogenesis is significantly augmented ($P < 0.001$) in the absence of Jam-A. Vascular endothelial cells isolated from Jam-A null mouse aorta showed significantly enhanced ($P < 0.05$) cell migration and tube-like structure formation ($P < 0.05$) in response to VEGF. Additionally, we found the plasma levels of VEGF in Jam-A null mice to be significantly ($P < 0.000001$) and age-dependently increased compared to WT mice. Furthermore, both mRNA and protein levels of VEGF and its receptor VEGFR2 were significantly increased ($P < 0.001$) in Jam-A null endothelial cells, suggesting that the VEGF/VEGFR2 signaling axis is augmented in the absence of Jam-A. To further confirm this finding, we injected anti-VEGFR2 (DC101) into the Jam-A null mice inoculated with murine melanoma (B16F0) cells. Inhibition of VEGF/VEGFR2 signaling by DC101 significantly reduced ($P < 0.00003$) tumor growth and associated angiogenesis in Jam-A null mice. Additionally, vascular permeability observed in Jam-A null mice was completely abrogated upon DC101 treatment. When tested if the expression of soluble Flt (sFlt), which is known to trap VEGF, is downregulated in the absence of Jam-A, we found no significant difference in sFlt mRNA expression in Jam-A null endothelial cells compared to WT. In order to determine the mechanism of this upregulation of the VEGF signaling axis, we tested the expression of hypoxia inducible factor-1a (HIF-1a) and inhibitor of DNA binding 1 (Id1), two transcription factors known to upregulate VEGF and VEGFR2 gene expression respectively. Interestingly, mRNA and protein levels of both HIF-1a and Id1 were significantly augmented ($P < 0.02$) in endothelial cells lacking Jam-A. Consistent with this finding, the overexpression of JAM-A in HUVECs attenuated the levels of Id1.
Summary: The results presented here suggest that JAM-A suppresses VEGF/VEGFR2 expression in endothelial cells by attenuating HIF-1a and Id1 expression, thus suppressing adult angiogenesis. During pathological conditions such as ischemia and tumor growth, it is possible that JAM-A levels are downregulated, thus supporting pathological angiogenesis.

OC 01.4

The intestinal microbiota triggers tissue factor-dependent vascular remodelling in the small intestine via the angiopoietin-1/Tie-2 pathway

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After birth the epithelial surfaces of the newborn are colonized by myriads of different bacteria that form one of the most densely colonized ecosystems on earth – the gut microbiota. We have used germ-free mouse technology to demonstrate that colonization with an intestinal microbiota promotes the formation of intricate capillary networks in the villi of the distal small intestine. The exact mechanisms that trigger mucosal vascular remodeling in the small intestine are largely unexplored.
We found that conventionally-raised (CONV-R) mice show wider villus structures and increased vascularization compared with germfree (GF) control mice. This is paralleled by increased angiopoietin-1 (Ang-1) mRNA levels and increased Tie-2 phosphorylation in the distal part of the small intestine. Furthermore, transcript levels of apelin (Apln), a regulator of vascular caliber size and cord hollowing that is regulated via the Ang-1/Tie-2 pathway are also increased in colonized mice. Isolated primary epithelial cells from CONV-R mice also showed increased Ang-1 mRNA levels. Since tissue factor (TF) has previously been implicated in angiogenesis and augments activation of protease-activated receptors (PARs) we blocked the intestinal TF function with an inhibitory antibody. Ex-germfree mice that were colonized with a gut microbiota (CONV-D) and treated with a functional inhibitory anti- TF antibody showed reduced vascularization and reduced Ang-1 mRNA levels compared to isotype-treated littermate controls. In line with this finding, decreased vascularization was observed in the small intestine of low TF mice that are expressing about 1% of human TF relative to mouse TF. The small intestinal epithelium is a major site of TF expression and both expression of fully N-glycosylated TF antigen and PAR1 expression was elevated in CONV-R mice compared with GF controls. TF expression could be induced by peptidoglycan treatment in the small intestinal epithelial cell line MODE-K. Increased TF procoagulant activity was also reflected by increased thrombin-antithrombin complex (TAT) levels and increased TF-dependent Factor Xa generation in CONV-R mice. Treatment of primary intestinal epithelial cells with thrombin resulted in increased phosphorylation of the TF cytoplasmic domain. In contrast, reduced TF cytoplasmic domain phosphorylation was noted upon inhibition of thrombin with hirudin in CONV-D mice and in CONV-D mice where TF was blocked with anti-TF suggesting that the TF cytoplasmatic domain could be involved in vascular remodeling in the small intestine. Indeed, we found small intestinal vascularization and expression of Ang-1 reduced in PAR1-deficient mice and in mice lacking the TF cytoplasmic domain. The functional importance of Ang-1 for vascular remodeling in the small intestine is further corroborated by inhibition of Ang-1 with the peptibody mL4-3, which resulted in inhibition of Tie-2 phosphorylation and in decreased villus vascularization.
Collectively, we revealed how the intestinal microbiota can activate vascular remodeling in the distal small intestine via the TF/PAR1/Ang-1 signaling axis. Apln could represent a novel factor involved in microbiota-induced intestinal vascular remodeling. Our findings exemplify how gut microbes can impact angiogenic signaling pathways to shape their habitat.

OC 01.5

Tissue factor rich endothelial-microparticles induce angiogenesis and post-ischemic revascularization

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Therapeutic angiogenesis may be a promising strategy for treating ischemia. However, the mechanisms of angiogenesis need to be further elucidated. Tissue Factor (TF) has shown to induce microvessel formation, but whether tissue factor-rich endothelial microparticles (TF+EMPs) released from activated endothelium induce angiogenesis has not been shown. Here we hypothesized that TF+EMPs trigger or facilitate angiogenesis by paracrine regulation. We used human dermal microvascular endothelial cells, human microvascular endothelial cells and human vascular smooth muscle cells. TF+EMPs shed from TF overexpressing migrating endothelial cells were used to perform *in vitro*, *ex vivo* and *in vivo* experiments. Endothelial cell activation and phenotype change, from quiescent to angiogenic, induces significant TF+EMPs release (0.8 ± 0.3 μg/μL vs 6.6 ± 0.5 μg/μL; $P < 0.001$). Released TF+EMPs showed autocrine and paracrine effects. TF+EMPs rapidly adhered to smooth muscle cells (SMC) in a β1-dependent fashion and increased SMC migration through β1-integrin, Rac1, and ERK1/2 activation independently of PAR2 signaling. Functionally, TF+EMPs up-regulated angiogenesis in aortic sprouting assays in a β1-integrin dependent pathway. Importantly, TF+EMPs induced angiogenesis in an *in vivo* model in which matrigel plugs containing TF+EMPs were implanted subcutaneously into nude mice. Finally, using a murine hindlimb ischemia, TF+EMPs enhanced collateral flow recovery and capillary formation in non-perfused adductor muscles ($N = 5$) in comparison to control buffer and EMPs without TF. The TF+EMPs -induced increase in *in vivo* angiogenesis operated through β1-integrin signaling via Rac1, and ERK1/2-dependent pathway. In conclusion, these experiments show that activated endothelial cells are able to further induce angiogenesis by releasing microparticles enriched in TF. These results suggest that TF-EMP could be a mechanism to overcome the consequences of arterial occlusion and tissue ischemia by promoting post-ischemic neo-vascularization and tissue reperfusion.

OC 01.6

Protease Nexin-1 regulates the retinal vascular development

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Angiogenesis is a process tightly controlled by a physiological balance between stimulatory and inhibitory signals for blood vessel growth, involved in reparative neovascularisation and in various ischemic and inflammatory diseases. Among the regulatory molecules, we recently identified serpinE2, or protease nexin-1 (PN-1), as an underestimated player of the angiogenic balance. PN-1 belongs to the serpin family of structurally related proteins that are present in the plasma or in tissues and play a central role in the regulation of protease activity. PN-1 is expressed by vascular cells, is secreted by platelets upon activation, and is often found overexpressed at sites of tissue injury. It is the most efficient tissue inhibitor of thrombin but also a powerful inhibitor of plasminogen activators, proteases largely involved in tissue remodelling.

We recently pointed out the anti-angiogenic activity of PN-1 *in vitro* on VEGF-induced HUVEC responses, *ex vivo* in the aortic ring assay and *in vivo* in the Matrigel plug assay, using PN-1 deficient mice. These effects are related to the interaction of PN-1 with endothelial cells glycosaminoglycans but do not require the anti-protease activity of the serpin. In this study, we used the well characterized postnatal vascular development of newborn mice retina, in which an organized bidimensional vascular architecture develops from the optic disk to the retina periphery, to further investigate the role of PN-1 in physiological angiogenesis and its mechanism of action. This vascular network can be observed by endothelial cell staining with markers such as isolectin B4. We observed that PN-1−/− retina display increased retinal vascularization in the postnatal period, with a rise in capillary thickness and density. Interestingly, the number of veins/arteries in the retina is increased in PN-1 deficient mice retina, from 4 to 5 in wild-type mice (WT), to 6–7 in PN-1−/− mice. Using transgenic (PN-1-lacZ) mice expressing X-Gal under the promoter of PN-1, we observed by X-Gal staining that PN-1 is expressed in the retinal vasculature. Retinal PN-1 expression, analyzed in WT whole retina lysates by immunoblotting, was elevated in the first postnatal days and progressively decreased to a very low level in adult retina. We did not observe any difference in the kinetics of retinal vasculature development, in VEGF expression, or in the overall retinal structure.

To go further in the mechanism of anti-angiogenic activity of PN-1, we performed antibody arrays on mice retina lysates, and PCR arrays on mice retina RNA. These studies identified angiogenesis-related factors that are differentially expressed in WT and PN-1-deficient newborn mice, in particular Midkine, Smad 5 and Sonic hedgehog, whose overexpression was confirmed by RT-PCR. We are now investigating how modification of expression of these factors could mediate the PN-1 anti-angiogenic effect.

Altogether, our results thus indicate that PN-1 limits physiological angiogenesis and has a real important anti-angiogenic potential. The characterization of its role in angiogenesis would not only increase our understanding in the functions of PN-1 in the development of diseases but may also provide important therapeutic strategies.

OC 02 – Anticoagulant Agents – Clinical Studies I

OC 02.1

Antithrombotic treatment of splanchnic vein thrombosis in the ISTH international registry: results of 6-month follow-up

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Background: Treatment of splanchnic vein thrombosis (SVT) is a clinical challenge due to heterogeneity of clinical presentations, increased bleeding risk and lack of evidences from clinical trials.

Aims: We carried out an international registry aimed to describe current treatment strategies and factors associated with therapeutic decisions in a large prospective cohort of SVT patients.

Methods: Between May 2008 and January 2012, consecutive SVT patients were enrolled in the registry and information on clinical presentation, risk factors, and therapeutic strategies was collected in an electronic database. Clinical outcomes during the first 6 months of treatment were documented. A two-year follow up is ongoing.

Results: Six hundred and thirteen patients from 12 countries were enrolled in the registry. Mean age was 53.1 (SD ± 14.8) years (range 16–85); 62.6% were males, 74.4% Caucasians. SVT occurred in the portal vein in 470 patients, in the mesenteric vein in266, in the splenic vein in 139, and in the supra-hepatic veins in 56; 38.8% of patients had multiple vein segments involved. In 29.8% of patients SVT diagnosis was incidental. Most common risk factors included cirrhosis (27.8%), solid cancer (22.3%), intra-abdominal inflammation/infection (11.5%), surgery (8.9%), and myeloproliferative neoplasm (MPN) (8.2%); in 27.6% of patients SVT was idiopathic.

During the acute phase, 471 (76.8%) patients were treated with anticoagulant drugs: unfractionated heparin (10.4%), low molecular weight heparin or fondaparinux (66.4%), vitamin K antagonists (VKA) (48.5%). Four patients received aspirin, 9 received thrombolysis. A total of 135 patients (22.0%) remained untreated. Of patients with incidentally diagnosed SVT, 61.1% received anticoagulant treatment.

Currently, information on treatment and clinical events occurred during the first 6 months of follow-up is available for 570 patients (96.1% of the population available for follow-up, since three centres participated in the baseline phase only). Baseline characteristics of patients with available follow-up were similar to those of patients with unavailable follow-up at the time of this analysis.

At 6 months, 79.2% of treated patients were still receiving anticoagulant treatment, while the remaining had stopped treatment earlier.

Venous thromboembolic events, including recurrent SVT and other site venous thromboembolism occurred in 26 patients (4.56%, 95% CI 3.06–6.70), 19 in treated patients (4.3%) and 7 in non-treated patients (5.5%). Major bleeding occurred in 18 patients (3.16%, 95% CI 1.94–5.04), 12 while on anticoagulant treatment (2.7%) and 6 in not-treated patients (4.7%). Death occurred in 55 patients (9.65%, 95% CI 7.41–12.45), 40 in treated patients (9.0%) and 15 in non-treated patients (11.8%).

Conclusions: The large majority of patients observed in our prospective cohort received anticoagulant treatment for their SVT, and 79.2% of them continued for at least 6 months. The incidence of both recurrent thrombosis and major bleeding events during this 6-month period is non-negligible, suggesting the need for a careful individual evaluation of the risks and benefits of anticoagulant treatment in SVT patients.

OC 02.2

What is the clinical impact of major bleedings with rivaroxaban? Results from the pooled EINSTEIN studies

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Background and aims: Rivaroxaban is one of the new oral anticoagulants, a member of the class of factor Xa inhibitors, that can be prescribed in a fixed dose, making regularly monitoring and dose adjustments unnecessary. In the EINSTEIN studies, rivaroxaban proved to be safe and effective in comparison to standard of care with enoxaparin/vitamin K antagonists (VKA; target INR 2.0–3.0) in treating acute deep vein thrombosis and pulmonary embolism and versus placebo for the prolonged treatment of both diseases. Nevertheless, as any anticoagulant, it carries a risk of bleeding. There is little knowledge on how bleedings during treatment with new oral anticoagulants develop. Therefore, all major bleedings during therapy with rivaroxaban and standard of care from the EINSTEIN studies were blindly assessed for their clinical impact and the measures that were required.

Methods: Two investigators (SM, HRB) performed a descriptive subgroup analysis on major bleedings in patients treated with either rivaroxaban (15 mg twice daily for the first 3 weeks, followed by 20 mg once daily) or standard of care. In the classification for *clinical presentation*, events were assigned to category one if the presentation was without any clinical emergency. The second category was reserved for all bleedings that could not be classified to any of the other three categories. Category three was used for bleedings presenting with great medical emergency; e.g. hemodynamic instability; or severe disability. The fourth category was applied to all bleedings with an (almost) immediate fatal outcome. The first category of the classification for *clinical course* was assigned to bleedings for which only standard measures were applied to treat discomfort. Bleedings requiring standard measures such as transfusions of erythrocytes, and straight forward interventions were placed in category two. Bleedings from the third category were described as life threatening requiring immediate and elaborate measures to avoid death. Category four comprised all bleedings for which death was unavoidable, so that no life saving attempts were made.

Results: Rivaroxaban-associated major bleeding events had a milder presentation (82% presented as categories one and two, versus 65% for standard of care). In 80% of all major bleeding events associated with rivaroxaban, the clinical course was also mild (category one and two, 68% of standard of care associated bleeds acted likewise).

Conclusions: Rivaroxaban associated major bleeding events appear to have a milder presentation, and to take a milder course in comparison to major bleeding events with standard of care, in patients who were treated for venous thromboembolism in the EINSTEIN studies.

OC 02.3

Subgroup analysis of the FONDACAST study comparing fondaparinux to low-molecular-weight heparin for the prevention of venous thromboembolism after an isolated, non-surgical below-knee injury

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Background: In a prospective, randomized, open-label study in 1349 patients requiring 21–45 days of immobilization (cast/brace), for below-knee, isolated, non-surgical injury, with at least one risk factor for venous thromboemboslism (VTE), once-daily s.c. fondaparinux 2.5 mg (1.5 mg if creatinine clearance 30–50 mL/min) was more effective than once-daily s.c. nadroparin 2850 aXa IU (0.3 mL) in preventing VTE, with no significant difference in terms of tolerability, both drugs being administered until mobilization (1).

Aims: To assess whether efficacy results were consistent across prespecified subgroups.

Methods: The incidence of the primary efficacy outcome up to complete mobilization plus 2 days, i.e. the composite of VTE [asymptomatic (ultrasonographically detected) or symptomatic deep-vein thrombosis (DVT) or symptomatic pulmonary embolism (PE)] or death was examined on the intent-to-treat population in pre-specified subgroups according to key demographic and below-knee injury characteristics, as well as potential risk factors for VTE. All events were blindly adjudicated by an independent committee. For each subgroup, treatments were compared using a Fisher's exact test. Odds ratio (OR) of fondaparinux/nadroparin and 95% confidence intervals (CI) were estimated using a univariate exact logistic regression model. The protocol was approved by independent Ethics Committees and written informed consent was obtained from all patients before inclusion.

Results: Overall, OR of fondaparinux/nadroparin in the subgroups examined were consistent with the overall study results on primary efficacy (OR: 0.30, 95% CI: 0.15–0.54, $n = 1170$). In each subgroup in which OR showed statistically significant difference between treatments in the risk of VTE or death, this risk was lower in fondaparinux-patients than in nadroparin-patients. OR were consistent according to age, gender, weight, body-mass index, or whether or not the patients have had previous VTE, a history of- or active cancer,

one, two or three risk factors for VTE, and whether or not they were receiving aspirin or antiplatelet agents at randomization. There were only 22 patients with a creatinine clearance < 50 mL/min, and no VTE events or death were reported in these patients. OR were also consistent irrespective of whether the patients had a bone fracture (n = 1041, OR: 0.31, 95% CI: 0.16–0.58) or not (n = 129, OR: 0.27, 95% CI: 0.00–2.51), the bone fracture was proximal (n = 60, OR: 0.60, 95% CI: 0.05–5.70) or distal (n = 981, OR: 0.29, 95% CI: 0.14–0.56), the injury was severe (n = 1066, OR: 0.31, 95% CI:0.16–0.57) or not (n = 104, OR: 0.42, 95% CI: 0.00–5.52), and the immobilization was rigid (n = 1050, OR=0.28, 95% CI: 0.14–0.52) or semi-rigid (n = 102, OR=1.04, 95% CI: 0.01–83.28).

Conclusions: In patients at risk of VTE immobilized following injury to the lower extremity, the statistically significant superior efficacy of fondaparinux 2.5 mg (1.5 mg if creatinine clearance 30–50 mL/min) *versus* nadroparin 2850 aXa IU (0.3 mL) in reducing the risk of VTE or death was consistent across all subgroups examined and with the overall study results.

1. Samama et al. Presented at the 58th Annual meeting of the SSC of the ISTH, Liverpool, 27–30 June 2012. Abstract HTC01.

OC 02.4

Effect of dabigatran etexilate on the risk of myocardial infarction and other cardiac events: a systematic review and updated dose-response meta-analysis of randomized controlled trials

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Background: Dabigatran etexilate (DE) is an oral direct thrombin inhibitor widely used for the prevention of venous thromboembolic events in major orthopedic surgery and in stroke prevention in patients with non-valvular atrial fibrillation. Early signals of an increased risk of myocardial infarction (MI) in clinical trials raised concerns about its use, particularly in patients with coronary disease.

Aims: We undertook an updated dose-response meta-analysis of randomized controlled trials (RCTs) to assess the possible association between the use of DE and the risk of MI or other cardiac events.

Methods: We conducted searches of the published literature, and a clinical-trials registry maintained by the drug manufacturer. Criteria for inclusion in our meta-analysis included all RCTs and the availability of outcome data for MI and cardiac events. Among the 318 unique references identified by all searches, there were 14 RCTs of which 13 fulfilled inclusion criteria. Three of them did not contribute to the analysis since there were no MI or other cardiac events reported. Stratification analyses by comparators (warfarin, placebo or enoxaparin) and doses of DE (150 mg *bid* and 110 mg *bid*) and sensitivity analyses were conducted. Statistical heterogeneity across trials was assessed with the Cochran Q-test and I^2 statistics.

Results: In the DE group, there were 292 MIs among the 23 839 exposed patients which represented an absolute risk of 1.23%. The overall odd ratio (OR) for MI was 1.32 (95% CI, 1.07–1.63; P = 0.010). When compared to warfarin, enoxaparin or placebo regimens, estimated OR for MI were 1.38 (95% CI: 1.08–1.77; P = 0.009), 0.96 (95% CI: 0.57–1.60; P = 0.869) and 1.70 (95% CI: 0.80–3.58; P = 0.165), respectively. In RCTs using the higher licensed DE dose (150 mg *bid*), the OR for MI was 1.45 (95% CI: 1.10–1.90; P = 0.008), and 1.41 (95% CI: 1.07–1.88; P = 0.016) when compared to any comparator or to warfarin, respectively. The sensitivity analyses preserved a significant increase of the MI associated with DE when compared with warfarin dropping one study at a time. The overall OR for MI was 1.33 (95% CI: 0.99–1.77; P = 0.057) for DE 110 mg *bid*. Additional analysis with a limited number of studies showed that there was no association between other cardiac events and DE based on 841 events. No publication bias was found and the observed heterogeneity was negligible.

Conclusions: This meta-analysis of RCTs provides robust evidence that DE is associated with a significantly increased risk of MI, especially at high dose (150 mg *bid*). No firm conclusion can be taken with the lower DE dose (110 mg *bid*) because of the limited number of studies included in this meta-analysis. Health care professionals and regulators should consider appropriate strategy to prevent such serious adverse drug reactions. The limitation of the use of DE in patients at high risk or suffering from coronary heart disease, the concomitant use of aspirin or switching to a FXa inhibitor should be carefully considered.

OC 02.5

Management of anticoagulation with vitamin k antagonists: can the time in therapeutic range (TTR) be used to optimize the interval between measurements?

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Background: The efficacy and safety of vitamin K antagonists (VKA) for the prevention of thromboembolism are dependent on the time for which the International Normalized Ratio (INR) is in the therapeutic range. This requires regular monitoring and appropriate dose adjustment. It has been reported that anticoagulation clinics should aim for a time in therapeutic range (TTR) between 70–80% to optimize benefit and minimize hemorrhagic and thrombotic complications.

Aims: The goal of our study was to evaluate quality of anticoagulation monitoring with the objective to adopt the new recommendations of interval between measurements of 12 weeks (according to 2012 guidelines of American College of Chest Physicians), instead of the current 8 weeks, using TTR determined by Rosendaal method.

Methods: We retrospectively analysed data of the last 6 years (September 2006 to June 2012) including patients that are regularly followed up at the outpatient Anticoagulation Clinic of a central hospital under anticoagulation for at least 8 weeks. 61 988 appointments corresponding to 2087 patients were analyzed. Patients were divided according to target INR in three groups: Group 1 with target INR 2-3, including 1927 patients corresponding to 54 325 appointments with mean age 65 ± 15 years, majority (44%) with atrial fibrilation; Group 2 with target INR 2.5–3.5, including 120 patients corresponding to 5754 appointments with mean age 54 ± 8 years, majority (88%) with mechanical heart valves; Group 3 with target INR 3–4, including 40 patients corresponding to 1909 appointments with mean age 41 ± 16 years, majority (65%) with antiphospholipid syndrome.

Results: In general, patients in Group 1 had TTR of 83%, patients in Group 2 had TTR of 74% and patients in Group 3 had TTR of 54%. Analyses of patients with longer mean interval between appointments (4–8 weeks interval) showed that there was no decrease of monitoring quality: patients in Group 1 had TTR of 86%, patients in Group 2 had TTR of 78% and patients in Group 3 had TTR of 64% (n.s.). Analysis of patients by age, using only patients from Group 1, showed that there were no differences in TTR in patients with less or equal to 80 years-old comparing to patients with more than 80 years-old (TTR 83% vs 82%, n.s.), showing that age *per se* is not a factor affecting monitoring quality.

Conclusions: In conclusion, patients with target INR of 2–3 and 2.5–3.5 had TTR that can be considered effective. Therefore this can be an indirect indicative that it would be safe to increase time between measurements to 12 weeks, as recommended by the new guidelines. Age should not be a limitation for this increase, at least in patients with target INR 2–3, which are the older ones in the present study. In patients

with target INR 3–4 we obtained the lowest value of TTR, probably due to proximity between therapeutic and toxic doses that can complicate VKA management. Therefore, for patients with target INR of 3–4 there is not enough evidence to support the increase of the interval between measurements.

OC 02.6

Cost-effectiveness of rivaroxaban for the treatment of pulmonary embolism and secondary prevention of venous thromboembolism – a UK perspective

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Background: The current standard of care for pulmonary embolism (PE) – initial treatment with low molecular weight heparin (LMWH) overlapping with, and followed by, a vitamin K antagonist (VKA) – suffers from limitations. Rivaroxaban, an oral, direct Factor Xa inhibitor, provides a single-drug approach that does not require LMWH bridging therapy, dose adjustment or routine laboratory monitoring. EINSTEIN PE compared rivaroxaban with LMWH/VKA for 3, 6 or 12 months of treatment post-PE. Rivaroxaban was shown to be non-inferior to LMWH/VKA therapy in the prevention of recurrent venous thromboembolism (VTE; hazard ratio 1.12, 95% confidence interval 0.75–1.68; $P = 0.003$) and resulted in a significant reduction in major bleeding (hazard ratio 0.49, 95% confidence interval 0.31–0.79; $P = 0.003$). These data indicate a potentially improved benefit-risk profile. On the basis of the EINSTEIN PE results, the European licence was extended to include treatment of haemodynamically stable patients with acute symptomatic PE.

Aim: To compare cost-effectiveness of oral rivaroxaban with standard of care for the treatment of PE and secondary prevention of VTE, from a UK payer perspective.

Methods: A Markov model was developed to explore cost-effectiveness over a patient's lifetime. Patients entering the model had experienced a PE and required 3, 6 or 12 months' treatment. A scenario evaluating high-risk patients requiring lifelong treatment was included.

Patients were exposed to treatment-specific risks of recurrent VTE and bleeding events. Associated mortality, post-thrombotic syndrome and chronic thromboembolic pulmonary hypertension were applied to both treatment arms. Inputs were derived from EINSTEIN PE and systematic literature reviews. To account for differences in patient risk profiles, rivaroxaban treatment effects were applied to the probability of events in patients treated with LMWH/VKA, according to intended treatment duration.

Economic inputs were based on costs from publicly available sources. An observational study provided resource use associated with routine laboratory monitoring. Consistent with EINSTEIN PE findings, a reduction in length of stay (LOS) was included for admitted patients treated with rivaroxaban. Quality-of-life (utility) inputs accounted for the impact of events and VKA treatment. Costs and benefits were discounted at 3.5% per annum. Extensive sensitivity analyses were undertaken.

Results: Rivaroxaban demonstrated per-patient cost savings of £396, £213 and £133 relative to LMWH/VKA therapy in patients treated with 3, 6 and 12 months' anticoagulation, respectively. Patients treated with rivaroxaban accrued more quality-adjusted life-years (QALYs; increases of 0.027, 0.013 and 0.019) compared with LMWH/VKA. Consequently, rivaroxaban dominated the comparator. Assuming a willingness-to-pay threshold of £20 000/QALY, rivaroxaban remained cost-effective in all sensitivity analyses. These included analyses in which no reduction in LOS or impairment to quality of life caused by warfarin administration was assumed. Probabilistic sensitivity analyses indicated that the likelihood of rivaroxaban being cost-effective was > 93% in all three groups. Rivaroxaban was cost-effective for patients requiring lifelong anticoagulation, with an incremental cost-effectiveness ratio of £7072/QALY.

Summary/Conclusions: The benefit-risk profile and single-drug approach of rivaroxaban improved QALY outcomes and reduced management costs. Rivaroxaban represents a cost-effective alternative to standard of care in the treatment of PE and secondary prevention of VTE from a UK payer perspective.

OC 03 – Clinical Issues in Haemophilia A

OC 03.1

Impact of sports on children with haemophilia in terms of their health status, health-related quality off life and physical performance

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Introduction: Sport is nowadays perceived as beneficial for children with haemophilia, as good muscle strength supports joints and may reduce bleeding frequency; by contrast psychological benefits are less known yet. On the other hand, there are still several obstacles towards sport activities such as worries of the patient/family and school teachers as well as limited knowledge of potential benefits related to different sport types. This study introduces the impact of sport on health status, health-related quality of life (HRQoL) and physical performance in children with haemophilia.

Methods: A cross sectional, multi-site study of boys aged 6–17 years with haemophilia A or B of any severity, current or past inhibitor was undertaken in children and their parents from HTCs in the UK. One hundred and twenty haemophilia families were invited to complete a questionnaire at routine review clinics including the following assessments using age appropriate questionnaires: socio-demographic data, questions about haemophilia, health-related quality of life (KINDL, Haemo-QoL), subjective physical performance (HEP-TEST-Q) and questions about sports activities. Additionally clinical data were collected from patient records; the orthopaedic status was assessed with the Petrini Haemophilia Joint Score.

Results: Eighty-four haemophilia boys (23 mild, 19 moderate, 42 severe) with a mean age of 11.52 years (SD = 3.4) were enrolled from 2 haemophilia centres in the UK (70% response rate). 92.3% had haemophilia A, 51.9% were severely affected; 66.3% received prophylactic treatment and had a mean Petrini Score of $M = 1.55 \pm 3.6$. 28.4% were classified overweight/obese according to their BMI in the respective age group. 90.5% participated in regular sporting activity, the majority 2–3 times/week at least twice a week (79.9%), mainly with friends (80%) and at school (80%). HRQoL in children was generally good, with highest impairments in boys aged 8–12 years. Children of younger age groups reported high impairments in the dimension 'sports & school', while adolescents showed highest impairments in social dimension such as 'perceived support' and 'friends'. Boys aged 8–17 years reported good physical performance ($M = 80.0$, SD=16.0) with highest impairments in the dimensions 'endurance' and 'mobility'.

Sedentary lifestyle and doing sport had no impact on children's health status. By contrast, children with a more sedentary lifestyle (watching > 1–2 h TV/playing computer games per day) had more haemophilia-related days lost ($M = 9.40$, SD = 7.1) than those with a less sedentary life style ($M = 3$, SD = 3.2) ($P < 0.032$). Sport had no impact on the HRQoL of younger boys; on the other hand older boys not doing sport showed more impairments in the HRQoL dimensions 'feeling' ($P < 0.014$) and 'family' ($P < 0.013$). Children doing sport reported better physical performance in all domains of the HEP-Test-Q ($P < 0.0001$).

Conclusion: Boys doing sport had a significantly better physical performance and HRQoL than boys not doing sport. Sedentary life style had a negative impact on the subjective physical performance and number of days lost of children. Participating in sport did not increase the risk of bleeding or developing target joints. Encouraging haemophilia boys to participate in sport will have a direct impact on their overall HRQoL. There is a further need of interventional studies confirming our findings.

OC 03.2

Intracranial haemorrhage in children with haemophilia A and B – interim analysis of the retrospective part of a multicentre study

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Background: In children with haemophilia A and B, the discussion on choice of therapy is focused on joint outcomes, even though intracranial haemorrhage (ICH) is a significant cause of death and morbidity in such patients. The risk of getting an ICH is 20–50 times higher in patients with haemophilia and affects 3–10% of patients with haemophilia treated on-demand. There are no recent studies on the incidence, morbidity and mortality of ICH on a well-defined prophylactic regimen.

Aims and objectives: Does prophylactic treatment reduce the frequency of intracranial haemorrhages? If ICH occurs, is the risk of sequelae lower?

Methods: Study group: Children (goal $n = 1000$), age < 18 years, with severe haemophilia A or B (FVIII/IX < 1%), without inhibitors who are on prophylactic treatment (defined as > 20 U/kg, minimum twice/week or prophylaxis three or more times a week), other prophylactic treatment (at least once a week) or on-demand.

Methodology: The study is a 3-year prospective survey of the cohorts with a 5-year retrospective part. Thirty-one international centres from PedNet (the European Paediatric Network for Haemophilia Management) and INPH (the International Network of Pediatric Hemophilia) are taking part in this multicentre study. The questionnaire is online (www.ich-hemophilia.se). The main questions are: mode of treatment, ICH or ICH-free period, technique for ICH imaging, preceding trauma and sequelae after ICH.

Results: At present, 796 patients have been recruited (median age 11.2 years, [range 1.9–20.0 year]) with a total of 3596 patient years: 2838 are on regular prophylaxis, 244 are on once/week prophylaxis and 514 are taking on-demand therapy. The present evaluation represents the 5-year retrospective part of the study. Nineteen events of ICH occurred in 17 patients with haemophilia A and two with haemophilia B. Three patients out of the 19 with ICH were on regular prophylaxis (two as a second bleeding after neonatal intracranial haemorrhage and one after trauma). Two patients with ICH were on prophylaxis once a week. The remaining 14/19 with ICH were treated on-demand, in three patients the ICH occurred in the neonatal period, and in six in the first year of life. The incidence of ICH in the whole cohort is 1.06/200 patient years versus 1 ICH/200 patient years in recent studies. The incidence for children taking on-demand therapy is 5.4/200 patient years, for children on full prophylaxis 0.21/200 patient years and for children on prophylaxis once a week 1.6/200 patient years.

Conclusion: The incidence of ICH in children on regular (at least twice/week) prophylaxis in this interim analysis of the retrospective cohort is 0.21/200 patient years compared to 5.4/200 patient years for the on-demand group. This strengthens the hypothesis that ICH occurs less frequently in patients on prophylaxis. Nine out of 19 ICH events occurred in the first year of life at an age when patients do not usually receive prophylaxis. If those patients are deducted from the analysis, the incidence is 1.9/200 patient years in the on-demand group, i.e. nine times higher compared to children on prophylaxis.

OC 03.3

Clinical risk factors in the development of inhibitors in non-severe hemophilia A patients: the first results of the INSIGHT case–control study

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Background: Inhibitor development is a major complication of treatment with factor VIII concentrates in non-severe hemophilia A and the etiology is only partly understood. It is important to identify clinical situations that elicit inhibitor development, as this enables the design of preventive strategies.

Aims: This case–control study investigates the association between clinical risk factors and inhibitor development in patients with non-severe hemophilia A.

Methods: From a source population of the INSIGHT study, including all 2711 non-severe hemophilia A patients (FVIII:C 2–40%) that were treated with FVIII concentrates in 33 European and 1 Australian center between January 1st 1980 and January 1st 2011, we selected 77 inhibitor patients (cases) and 230 control patients, matched for age and cumulative number of exposure days to FVIII concentrates. The reason for treatment (bleeding or surgery) and the intensity of treatment (1–2 exposure days (ED), 3–4 ED or ≥ 5 ED) were analyzed using a multivariate survival model with the exposure days to FVIII concentrates as time-variable.

Results: This first analysis of the INSIGHT case-control study includes 7793 exposure days in 307 patients, with a median age at first exposure of 22 years (inter quartile range (IQR) 6–44) and a median FVIII baseline level of 9 IU/dL (IQR 5–16). The 77 cases developed an inhibitor at a median age of 36 years (IQR 15–60) and after a median of 25 ED (IQR 12–40).

Neither intensity of treatment, nor surgery was independently associated with inhibitor occurrence. After adjustment for confounders (ethnicity, mild/moderate hemophilia A, family history and age) the adjusted odds ratios (aOR) for moderate bleed (3–4 ED) and major bleed (≥ 5 ED) compared to the reference of 1–2 ED, were 0.68 (95% confidence interval (CI) 0.08–5.85) and 0.66 (CI 0.24–1.79) and for moderate surgery (3–4 ED) and major surgery (≥ 5 ED) compared to ED in the absence of surgery were 4.25 (CI 0.74–24.47) and 1.04 (CI 0.38–2.83), respectively.

Factor VIII exposure at an older age showed a trend towards an increased risk of inhibitor development; aOR 1.82 (CI 0.79–4.17) in the age-group of > 60 years compared to patients who were exposed at younger ages (0–30 years).

Conclusions: In contrast to previous findings in patients with severe hemophilia, surgery was not independently associated with inhibitor development among patients with non-severe hemophilia A. This may reveal that exposure to therapeutic FVIII concentrates in patients with non-severe hemophilia always occurs in clinical situations with a certain threshold of tissue damage.

Factor VIII infusions at older ages seemed to convey a higher risk for inhibitors than infusion at younger ages. This may reflect that the immune system of older patients has been challenged relatively late in their life with therapeutic FVIII concentrates.

OC 03.4

Has the inhibitor incidence increased in severe hemophilia A from 1990 to 2009?

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Background: About 25% of patients with severe hemophilia A develop inhibitory allo-antibodies (inhibitors) towards infused factor VIII products. The risk of patients developing inhibitors depends on several genetic and non-genetic risk factors. Over the last decades, higher inhibitor incidences have been reported. It is unclear whether this is caused by a real increase in prevalence or by higher awareness and more frequent and sensitive testing.

Aim: To objectivate the perceived increased incidence for inhibitors.

Methods: The CANAL study collected data from patients born 1990–2000 with severe hemophilia until 50 exposure days. Similar data were collected in the RODIN study (2000–2010). Primary outcome for both studies was clinically significant inhibitor development, defined as ≥ 2 positive inhibitor titers combined with decreased *in vivo* factor VIII recovery. The secondary outcome was high-titer inhibitor development, defined as the occurrence of clinically relevant inhibitors with peak titers of ≥ 5 Bethesda Units (BU)/mL. A positive inhibitor titer was defined according to the cut-off level of the used inhibitor assay in each center's laboratory.

To make direct comparison on inhibitor incidence in these two patient cohorts, we selected only patients with severe hemophilia A (FVIII < 0.01 IU/mL) and a follow-up of at least 50 exposure days.

Results: In total 892 patients were included (319 selected in 1990–2000, 573 in 2000–2009). Excluding 51 patients born in 2009 (as 65% of them had not yet reached 50 exposure days), 245/863 patients (28.4%) had inhibitors, 174 of them (20.2% high-titer inhibitors. To compare different time periods, patients were divided into four 5-year periods; 1: patients born 1990–1994; 2: patients born 1995–1999; 3: patients born 2000–2004; 4: patients born 2005–2009. Total inhibitor development was as follows: period 1: 28/144 patients (19.4%); period 2: 47/175 patients (26.9%); period 3: 96/309 patients (32.1%); period 4: 74/264 patients (30.2%). High-titer inhibitor development did not increase significantly between 1990 and 2009: period 1: 24/144 patients (16.7%); period 2: 37/175 patients (21.1%); period 3: 66/309 patients (22.1%); period 4: 47/264 patients (19.2%). So, 85.7%, 78.7%, 68.8% and 63.5% of all patients with inhibitors had high-titer inhibitors.

Conclusions: The overall increase of inhibitor incidence in the period 1990–2009 is caused by the detection of more low-titer inhibitors, increasing to > 30% of all inhibitors in period 3 + 4. This could be explained by more frequent and sensitive testing. The high-titer inhibitor incidence, however, did not change significantly in the same time period, which seems to imply that high-titer inhibitors will be detected also with less frequent testing.

OC 03.5

Validation of the Extended Magnetic Resonance Imaging Scale for evaluation of joint status in adult patients with severe hemophilia A

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Background: Prophylactic replacement factor therapy reduces bleeding frequency and joint damage compared with on-demand treatment in patients with hemophilia. Because of varying treatment regimens used worldwide, adolescents with hemophilia enter adulthood with joint status ranging from little damage to severe deterioration. Most previous joint imaging scales for hemophilia have focused on earlier disease stages observed in younger patients. Adults with hemophilia, who may have more severe baseline joint damage, would benefit from an imaging scale with greater scale item detail that could evaluate a wide range of joint damage.

Aim: To demonstrate that the 45-point Extended Magnetic Resonance Imaging (eMRI) Scale, which scores soft-tissue changes, cartilage changes, and additive damage from all three bones in each joint (ie, ankles, elbows, knees), is a valid instrument for measuring joint status in adults with severe hemophilia A.

Methods: Six scale items (effusion hemarthrosis, synovial hypertrophy, hemosiderin, erosion, subchondral cysts, and cartilage loss) in two domains (soft tissue [range, 0–9 points] and osteochondral [range, 0–36 points]) were evaluated for each joint. eMRI scores were derived from baseline data from a randomized, controlled, parallel-group clinical trial (NCT00623480; SPINART). Scores were evaluated for linearity (through comparisons with the Colorado Adult Joint Assessment Scale [CAJAS] and the 17-point International Prophylaxis Study Group MRI scale) and reproducibility (through measurements of interreader variability and site variance). An item analysis assessed the internal reliability of items to consistently measure the health status of each joint.

Results: Patient eMRI scores correlated with age ($r = 0.58$), similar to the expected correlation of joint health with total lifetime bleeds ($r = 0.35$–0.68) for older scales and consistent with the expectation that all patients experience joint bleeds and older patients have more bleeds. eMRI scores demonstrated excellent between-reader agreement for the overall patient endpoint (intra-class correlation coefficient [ICC] = 0.88) and outstanding agreement for knee evaluations (ICC = 0.95). These agreement statistics are very close to those of older scales despite the increased complexity of the eMRI Scale and the increased burden to the reviewer. There was a strong linear relationship of the eMRI score with the CAJAS ($r = 0.7$). Internal reliability was good (overall Cronbach alpha, 0.75). There was minimal ceiling effect (5% maximum scores for all evaluated joints) compared with the 17-point MRI scale (20%). There was no apparent change in the ability of the eMRI Scale to evaluate joints regardless of the country where the image was acquired. Adding individual bone evaluations for each joint increased the sensitivity of the instrument to detect change at low scores.

Conclusions: Additive evaluation of bones articulating each joint and increased granularity of the extent of damage to joint cartilage increased sensitivity to change with the eMRI Scale compared with the 17-point MRI scale at low and high ends of the scale. Increased reviewer burden did not appear to add variability or affect the ability to discriminate between different levels of joint damage. Excellent to outstanding agreement between the two readers demonstrated reliability of the scale for use by different experienced radiologists.

OC 03.6

US retrospective database analysis on prevalence of cardiovascular comorbidities among hemophilia A patients

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Background: There is conflicting evidence in the published literature on whether patients with hemophilia in the United States have greater, reduced, or similar risks for cardiovascular disease compared with the general population.

Aims: To evaluate the prevalence of cardiovascular comorbidities among US male patients with hemophilia A relative to a general health-insured male population with similar patient characteristics.

Methods: Male patients with hemophilia A with continuous insurance coverage were identified by two International Classification of Diseases, Ninth Revision, Clinical Modification (ICD-9-CM) codes of

286.0 between January 1, 2007, and December 31, 2009 using the *MarketScan®* Commercial and Medicare Research Databases. Patients with hemophilia A were exact matched in a 1:3 ratio with males without a diagnosis of hemophilia A. Patients were matched by eligibility months in the study period, age, region, and plan type. The proportions of patients with evidence of cardiovascular comorbidities were determined for matched study cohorts using ICD-9-CM codes. Univariate descriptive statistics were used to describe demographics, clinical characteristics, and frequencies of comorbidities, with Student *t* tests and chi-square tests used to detect statistically significant differences in continuous and categorical variables, respectively.

Results: Of the study population, 2506 were grouped in the hemophilia A cohort and 7518 in the general cohort. The frequencies of hemorrhagic stroke (2.0% vs 0.5%; $P < 0.001$), ischemic stroke (1.9% vs 0.9%; $P < 0.001$), coronary artery disease (10.7% vs 5.8%; $P < 0.001$), myocardial infarction (0.8% vs 0.3%; $P = 0.001$), hypertension (22.6% vs 15.5%; $P < 0.001$), hyperlipidemia (16.2% vs 12.0%; $P < 0.001$), arterial thrombosis (12.2% vs 5.9%; $P < 0.001$), and venous thrombosis (4.3% vs 1.1%; $P < 0.001$) were significantly greater for the hemophilia A cohort compared with the general cohort. These results were consistent across most age groups.

Summary/Conclusions: This study is the first large, retrospective analysis to assess the prevalence of cardiovascular comorbidities among US patients with hemophilia A. These results, which contrast with other published data, indicate that among the US hemophilia population, cardiovascular comorbidities are more prevalent and appear earlier in life compared with the general male population, suggesting the need for enhanced screening for age-related comorbidities in the hemophilia community. Inherent limitations of claims database analyses include coding errors and inability to account for all disease- and treatment-related variables such as hemophilia severity, cardiovascular risk factors, and cardiovascular diagnostic and treatment variability. Despite the retrospective nature of these data, they may provide insight into specific risks associated with the frequent use of clotting factor replacement in otherwise hypocoagulable individuals; alternatively, the data may reflect the episodic hypercoagulability induced by infrequent replacement of large amounts of clotting factor protein, or they may indicate the necessity of implementing counterintuitive therapies to patients with hemophilia in certain scenarios (eg, antiplatelet therapy in those with high risk of atherosclerotic complications). Future studies are warranted to assess whether these databases and other registries reveal an accurate but worrisome trend toward increased cardiovascular and venous hypercoagulability in aging patients with hemophilia A.

OC 04 – Clinical Issues Related to Cancer and Haemostasis

OC 04.1

Type 1 plasminogen activator inhibitor (PAI-1) and risk of colorectal cancer in the european prospective investigation into cancer (EPIC)-Italy cohort

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Background: Experiments in KO and transgenic mice demonstrated that host-produced PAI is essential for cancer cell invasion and angiogenesis. High PAI-1 levels are predictive of poor survival for patients with different cancers. However, an involvement of circulating PAI-1 in the risk of cancer in human has never been demonstrated. We evaluated the association between PAI-1 and risk of colorectal cancer in 4 out of 5 of the (EPIC)-Italy cohort.

Materials and Method: We conducted a case-cohort study on 1028 (356 men; 672 women) participants in the EPIC-Italy cohort, by comparing subjects who developed colorectal cancer in a mean follow-up of 9.11 years.

Using a nested case-cohort design in the (EPIC)-Italy study ($n = 34\,148$), we identified a random subcohort ($n = 834$*) and incident colon cancer cases ($n = 194$ plus one case originated from the random subcohort*) occurring between baseline (1993–1997) and end of 2003 (Varese), of 2004 (Ragusa and Turin) or 2006 (Naples); PAI-1 levels were measured in citrated plasma collected at recruitment by ELISA (Hyphen Zymutest PAI-1, Intrumentation laboratories, Milan, Italy). The relative risk (RR) and 95% confidence interval (CI), adjusted by relevant confounders and stratified by center, were estimated by aCoxregression model using Prentice method.

Results: Individuals in the highest compared with the lowest quartile of PAI-1 had significantly increased risk of colorectal cancer (RR, 2.38; 95% CI, 1.45–3.90; *P* for trend < 0.001) after adjustment for sex, age and recruitment center. Additional adjustment for education, smoking status, BMI, physical activity did not modify the risk (RR, 2.45; 95% CI, 1.42–4.22; *P* for trend < 0.001). The risk of colorectal cancer increased by 33% for each increase in 1 standard deviation of PAI-1 levels.

Conclusions: Our data provide the first evidence for a link between high PAI-1 levels and an increased risk of colon cancer in the population.

OC 04.2

The risk of venous thrombosis in patients with malignancy is largely mediated through levels of factor VIII and von Willebrand factor

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Background: Venous thrombosis is a common complication in patients with cancer, but the pathophysiology underlying this association is unclear.

Aim: To assess the role of factor VIII and von Willebrand factor in the relation between cancer and venous thrombosis, overall and for different cancer types.

Methods: From a large case–control study (MEGA study), 4765 patients with a first venous thrombosis and 6149 partner controls or random digit dialing controls were included. At least 3 months after the thrombotic event, after discontinuation of the anticoagulant therapy, patients and controls were interviewed, and a blood sample was taken. With unconditional logistic regression, age and sex adjusted odds ratios (OR) with 95% confidence intervals for venous thrombosis were calculated for malignancy versus no malignancy. Mean levels of factor VIII (activity (IU/dL) as well as antigen (IU/dL)) and von Willebrand factor antigen (IU/dL) are given for participants with and without venous thrombosis and with or without an active malignancy. In mediation analyses we assessed the role of these haemostatic factors in the relation between cancer and venous thrombosis. Consent and ethical approval were obtained for this study.

Results: Among 4765 patients with venous thrombosis 453 individuals were known with active cancer (9.5%) as opposed to 106 in 6149 control participants (1.7%). The presence of a malignancy increased the risk of venous thrombosis about six-fold (OR 5.7 (95%CI 4.6–7.1), after adjustment for age and sex. Mean levels of factor VIII and von Willebrand factor were increased in venous thrombosis patients and in participants with a malignancy, with the highest mean levels in participants with both conditions present. For control participants without and with cancer and for venous thrombosis patients without and with

cancer mean factor VIII activity levels were respectively (111.9; 122.2; 139.6 and 157.2). Similarly, mean factor VIII antigen levels were respectively (116.1; 128.7; 155.1 and 178.1) and mean von Willebrand factor levels were respectively (111.4; 120.9; 147.3 and 177.2). In mediation analyses in which we analysed the effect on thrombotic risk of a malignancy conditional on the concentration of the haemostatic factors we found both factor VIII and von Willebrand factor to be mediators of the effect, with reduced odds ratios for the relation between cancer and venous thrombosis (adjustment for factor VIII activity: OR 2.8 (95%CI 2.0–4.0); factor VIII antigen: OR 2.8 (95%CI 1.9–3.9) and von Willebrand factor: OR 2.8 (95%CI 2.0–4.0)). Correction for the factors combined did not result in an additionally reduced risk [OR 2.8 (95%CI 1.9–3.9)]. Results were consistent for most of the common cancer types (haematological cancer, breast and prostate cancer), except for gastrointestinal cancer.

Conclusion: The increased risk of venous thrombosis in the presence of active cancer is largely explained through levels of factor VIII and von Willebrand factor. Further study is needed to determine the role of haemostatic factors in the relation between cancer and venous thrombosis for different cancer types and for further elucidation of the mechanism.

OC 04.3

Platelet count measured prior to cancer development is a risk factor for venous thromboembolism in cancer patients – the Tromsø Study

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Background: Platelets are essential in hemostasis, thrombosis and cancer progression. The platelet count is determined by both inherited and environmental factors, but is not associated with venous thromboembolism (VTE) in the general population. However, an elevated platelet count in cancer patients before start of chemotherapy has been associated with increased risk of venous thromboembolism (VTE). It is not known whether the risk of VTE by platelet count in cancer patients is causal or merely a consequence of the malignant disease.

Aims: To investigate whether platelet count measured prior to cancer development was associated with risk of VTE in subjects who developed cancer during follow-up and in subjects who remained cancer-free.

Methods: Platelet count and other baseline characteristics were measured in 25 175 initially cancer-free subjects who participated in the Tromsø Study, a prospective population-based study, in 1994–1995. The study was approved by the regional committee of research ethics, and participants gave their informed written consent. Incident cancer and VTE events during follow-up were registered up to September 1st, 2007. Subjects who developed cancer (cancer cohort) and subjects who remained cancer free (cancer-free cohort) where analyzed separately. In the cancer cohort, platelet count was measured on average 7.1 years before cancer development. The mean observational time was 9.8 years in the cancer cohort and 11.0 years in the cancer-free cohort. Cox-regression models were used to calculate hazard ratios (HRs) for VTE by platelet count with 95% confidence interval (CI). Platelet count was analyzed as a categorized (< 40th, 40–80th, and > 80th percentile) and as a continuous variable. The analyses were adjusted for age, sex, body mass index, smoking, mean platelet volume and white blood cell count.

Results: Overall, 1784 subjects developed cancer during follow up. There were 403 incident VTE events, of which 119 (30%) occurred in the cancer cohort (6.8 per 1000 person-years) and 284 (70%) in the cancer-free cohort (1.1 per 1000 person-years). In cancer patients, pre-cancer platelet count above the 80th percentile ($\geq 295 \times 10^9$/L) was associated with a 2.2-fold higher risk of VTE (HR: 2.16, 95% CI: 1.29–3.61) compared to platelet count below the 40th percentile ($< 235 \times 10^9$/L). When platelet count was treated as a continuous variable, the VTE risk increased by 32% (HR 1.32; 95% CI 1.12–1.48) per one standard deviation increase (1 SD = 56×10^9/L). In cancer-free subjects, no association was found between platelet count and VTE in either continuous (HR per 1 SD: 0.95, 95% CI 0.80–1.12) or categorized analyses (HR: 0.99, 95% CI: 0.681.43 in upper versus lower platelet category).

Conclusions: Platelet count measured several years prior to cancer development was associated with increased risk of VTE in cancer patients, whereas no association was found between platelet count and VTE in subjects who remained cancer free. Our findings suggest that platelets may play a role in the pathogenesis of cancer-related VTE.

OC 04.4

Chemotherapy-induced hypercoagulability and biomarkers for prediction of thromboembolic events in patients with metastatic testicular cancer

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The majority of patients with disseminated testicular cancer can be cured with combination chemotherapy consisting of bleomycin, etoposide and cisplatin (BEP). Because of this success, focus shifts to adverse effects of this treatment. The incidences of venous and arterial thromboembolism (VTE and ATE) during and after chemotherapy are up to 15%.

Low molecular weight heparin (LMWH) reduces VTE in cancer patients, but the incidence is too low to justify prophylaxis in most patients starting with chemotherapy.

To investigate the influence of BEP-chemotherapy administered to testicular cancer patients on coagulation and to estimate the predictive value of plasma levels of hemostatic proteins and activation markers in prediction of VTE and ATE within 2 years after treatment.

Patients with newly diagnosed metastatic testicular were included in this prospective cohort study, which was approved by the local ethical committee. Informed consent was obtained from all participants. Patients received 3 or 4 BEP-courses during 9–12 weeks. Serial measurements were performed at baseline, at the last day of the third BEP-course (day 56) and directly after chemotherapy. We assessed the Khorana-score and measured levels of the following hemostatic proteins: von Willebrand factor (VWF), fibrinogen, tissue-type plasminogen activator (tPA), plasminogen activator inhibitor 1 (PAI-1), soluble P-selectin (sP-selectin), coagulation factor VIII (FVIII) and D-dimer. Outcome parameters were symptomatic VTE or ATE or asymptomatic VTE or ATE discovered on staging CT-scans.

We included 74 patients with a median age of 31 years at start of chemotherapy. Eight patients (10.8%) developed thrombosis during or after chemotherapy, 4 had pulmonary embolisms (PE), of which 3 asymptomatic, and 4 had ATE (3 ischemic stroke, 1 asymptomatic splenal infarction), during a median follow-up of 3.2 years. Median duration from start of chemotherapy until development of VTE was 82 days (range: 32–278) and for ATE 85 days (range: 69–435).

Median baseline biomarker levels were not elevated. VWF, fibrinogen, FVIII, D-dimer and PAI-1 levels clearly increased during chemotherapy. Levels normalized to baseline 1 month after treatment, except for FVIII and VWF levels, which remained increased. High FVIII levels (> 150%) at baseline had a positive predictive value (PPV) of 50% (95%CI: 14–86) and a negative predictive value (NPV) of 92.3% (95% CI: 82–97), with a hazard ratio (HR) for TE of 4.7 (95%CI: 1–22) adjusted for age at start of chemotherapy. The Khorana-score had a low predictive value. Furthermore, a large increase between baseline and day 56 in both VWF ($\Delta > 86.5\%$) and D-dimer ($\Delta > 935$ ng/mL) had a PPV of 44.4% (95%CI: 15–77), a NPV of 95.2% (95%CI:

86–99) and a HR of 7.0 (95% CI: 2–33) for TE occurring after day 56. Hemostatic markers correlated with tumor markers.
BEP-chemotherapy in metastatic testicular cancer patients is associated with a hypercoagulable state. Elevated FVIII before start of chemotherapy and large increases in D-dimer and VWF levels during chemotherapy are associated with an increased risk of developing ATE or VTE. This study suggests that baseline FVIII is a predictor for future TE in testicular cancer patients.

OC 04.5

Prevalence of pulmonary embolism in patients with oncogene addicted advanced lung adenocarcinoma

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Background: Non-small cell adenocarcinoma (NSCLC) is associated with a higher risk of thromboembolic event in comparison with SCLC. Adenocarcinoma represent roughly 75% of NSCLC patients. Lung adenocarcinomas harboring EGFR and KRAS mutations as well as EML4/ALK rearrangements represent distinct subsets of this disease. EGFR or KRAS mutations and EML4/ALK rearrangements are predictors for response to therapy in patients with NSCLC, mainly adenocarcinoma. No data are available concerning the prevalence of pulmonary embolism in NSCLC patients with these mutations.
Aims: The aim of the study was to evaluate the prevalence of pulmonary embolism in patients with stage III B-IV NSCLC harboring EGFR and K RAS mutations as well as EML4/ALK rearrangements.
Method: Patients with stage IIIB or IV NSCLC referred to Division of Medical Oncology at the Perugia Hospital between 2008 and 2012 were included in the study. In these patients, contrast-enhanced CT scans of the chest were reviewed for the presence of pulmonary embolism by a panel composed by three radiologists. In the same patients, data on the oncogene mutations (EGFR, KRAS or EML4/ALK) were collected.
Results: A total of 209 patients with stage IIIB or IV NSCLC were included in the study. A histologic diagnosis of lung adenocarcinoma was done in 173 patients (82.7%). In 127 of these patients sequence analysis for know EGFR and K RAS mutation was performed. In this population 31/173 patients were EGFR mutated (17.9%), 27/173 were K RAS mutated (15.6%) and 17/173 were EML4/ALK rearrangements (9.8%). Forty-one patients with lung adenocarcinoma had a diagnosis of pulmonary embolism at CT scan (23.7%). Of these, 34.1% had no oncogene mutations in comparison with 28.8% of the patients without pulmonary embolism. Of the 41 patients with a diagnosis of pulmonary embolism 12.1% had an EGFR mutation and 12.1% a KRAS mutation, in comparison with 19.7% and 16.6% of patient without pulmonary embolism, respectively. In patients with lung adenocarcinoma, EML4/ALK rearrangements was observed in 19.5% among patients with pulmonary embolism and in 6.8% among patients without it. The risk of pulmonary embolism was 3.3-fold higher in presence of EML4/ALK rearrangements in comparison with no EML4/ALK rearrangements [OR: 3.3 (95%CI 1.2- 9.2)].
Conclusions: In patients with lung adenocarcinoma, the presence of EML4/ALK rearrangements seems to be associated with a high risk of pulmonary embolism and could help in identifying patients at particular high risk which might benefit from an antithrombotic prophylaxis. These preliminary date need to be confirmed by further studies.

OC 04.6

Using proteomics to identify new biomarkers for cancer-associated thrombosis: a pilot study

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Background: Thrombosis is a common and consequential problem in cancer patients but its mechanisms and predictors are incompletely understood. Proteomics is a powerful technique by which protein expression patterns can be measured, but there are limited data analyzing the plasma proteome of cancer patients for thrombotic outcomes.
Aims: To develop a reliable and reproducible method for studying differences in plasma protein expression amongst cancer patients receiving chemotherapy using proteomics.
Methods: We analyzed the plasma proteome of a cohort of cancer patients treated at the University of Rochester in this pilot study. Patients with lung or pancreatic cancer initiating chemotherapy were enrolled as part of an IRB approved protocol. Baseline and serial blood samples were collected and patients were followed prospectively for development of venous thromboembolism (VTE). Plasma samples collected in sodium citrate, adjusted to pH 7.0 and frozen at −80°C were thawed then depleted of albumin and immunoglobulin by sequentially applying them to a Cibacron blue resin and then protein G-Sepharose resin, in a batch mode. The partially depleted plasma was then size fractionated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The plasma was then processed in four mass ranges by gel isolation and in-gel trypsin digestion, followed by mass spectrometry analysis for each mass range separately. Each depleted plasma sample was subjected to an 80 min liquid chromatography-mass spectrometry (LC-MS/MS) run. Mascot searches were then conducted for protein matches with the NCBI human proteome. Search results for the study were uploaded into ProteoIQ and Scaffold software platforms and spectral counting was used for relative quantification of plasma proteins between study groups. Mean peptide expression level in patients with and without thrombosis was compared using two sample *t*-test.
Results: The cohort consisted of seven patients with primary lung cancer and eight patients with pancreatic adenocarcinoma. The average age of the population was 58 years of age and 53% (8/15) were female. The majority (80%, 12/15) had stage IV cancer. VTE developed in four patients during study follow-up consisting of two patients with deep vein thrombosis (DVT), one with pulmonary embolism (PE), and one with both DVT and PE. The average time from chemotherapy initiation to VTE was 37 days with range of 17–67 days. We were able to complete proteomic analysis as described above identifying 50 637 spectra representing 2145 unique peptide sequences from 149 proteins and 116 protein groups when applying thresholds requiring three peptide minimum and 95% probability of protein match. We identified nine proteins that were differentially expressed ($P < 0.05$) between patients with and without thrombosis including proteins known to be important for hemostasis and thrombosis like fibronectin and plasminogen.
Summary: A method for measuring plasma protein expression in cancer patients undergoing chemotherapy using proteomic techniques was successfully developed. This small pilot study was underpowered to accurately compare plasma protein expression profiles but we plan to apply these techniques in an ongoing study of a larger cohort of cancer patients with the goal of identifying predictive biomarkers and better elucidating mechanisms involved in this complex process.

OC 05 – Coagulation Factors VIII, IX and XI

OC 05.1

Residues of the 39-loop restrict the plasma inhibitor specificity of factor IXa

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Background: The two plasma inhibitors, protein Z-dependent protease inhibitor (ZPI) and tissue factor pathway inhibitor (TFPI), effectively inhibit the activity of FXa, however, neither inhibitor exhibits any reactivity with the homologous protease FIXa. We recently demonstrated that the residues of the 39-loop in FXa modulate the specificity of FXa interaction with plasma inhibitors. This loop in FXa is highly acidic and contains three Glu residues at positions 36, 37 and 39. On the other hand, this loop is shorter by one residue in FIXa (residue 36 is missing) and it contains a Lys and an Asp at positions 37 and 39, respectively.
Aim: The aim of this study was to investigate the molecular basis for the lack of the reactivity of FIXa with these plasma inhibitors and to determine whether the unique structural features of the 39-loop are responsible for the resistance of FIXa to inhibition by plasma inhibitors.
Methods: We used recombinant DNA methods to generate a FIX mutant in which residues of the 39-loop (residues 31–41) have been substituted with corresponding residues of FX. We expressed the FIX chimera in HEK-293 cells and following purification to homogeneity and activation to FIXa characterized its biochemical properties in established inhibition assays.
Results: We discovered that the FIXa chimera is highly susceptible to inactivation by both ZPI and TFPI. Thus, the inactivation rate of the chimeric FIXa by ZPI in the presence of protein Z, negatively charged membrane vesicles and calcium ions approached near the same diffusion-limited rate ($> 10^6$/M/s) that has been observed with the protein Z-dependent inhibition of FXa by ZPI. Interestingly, sequence alignments indicated that, similar to FXa, residue 37 is a Glu in mouse and that mouse FIXa is also susceptible to inhibition by the PZ-ZPI complex.
Conclusion: Our results suggest that structural features within residues of the 39-loop are primarily responsible for the resistance of FIXa to inhibition by plasma inhibitors ZPI and TFPI.

OC 05.2

Activated factor XI enhances procoagulant tissue factor activity on endothelial cells by cleaving tissue factor pathway inhibitor

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Background: Activated factor XI (FXIa) has been shown to be inhibited by aprotinin, a basic pancreatic trypsin inhibitor and analog of tissue factor pathway inhibitor (TFPI). TFPI is a kunitz-type protease inhibitor and the primary inhibitor of the tissue factor (TF)/activated factor VII (FVIIa)/activated factor X (FXa) complex on the surface of endothelial cells. Based on the fact that the catalytic domain of FXIa can bind to kunitz protease domain, we hypothesized that FXIa can inactivate TFPI to regulate the hemostatic function of endothelial cells, which may help explain why some congenital factor XI deficiencies can be associated with bleeding.
Aims: The aim of the present study was to determine whether FXIa regulates TF-dependent FX activation on endothelial cells.
Methods and Results: Conversion of FX to FXa in the presence of FVIIa was measured on the surface of activated endothelial cells using a chromogenic assay. Human umbilical vein endothelial cells (HUVEC) were stimulated for 5 h with tumor necrosis factor-α (TNFα) to induce TF expression and promote FX activation by the TF-FVIIa complex. The addition of a blocking anti-TF antibody inhibited the generation of FXa, indicating that the activation of FX is dependent of the TF-FVIIa complex. Our data show that treatment of TNFα-stimulated HUVECs with FXIa (0–100 nM for 2 h) led to increased TF-dependent activity in a FXIa concentration-dependent manner. We did not observe any hydrolysis of the FXa substrate in the absence of both FVIIa and FX, indicating that FXIa did not activate FX or cleave the chromogenic substrate. Time course studies (0–5 h) demonstrated that the increase in FXa activity in response to 50 nM FXIa was detectable after 1 h, with a maximum effect observed after 3 h. Induction of FXa generation required the presence of Zn^{++} and the proteolytic activity of FXIa. The serine protease inhibitor, aprotinin, blocked the effect of FXIa. We next determined whether FXIa cleaved TFPI from endothelial surface using a cell surface immunoassay. We observed that after FXIa treatment, the detection of the Kunitz 1 domain from TFPI was lost from endothelial cells surface in a FXIa concentration-dependent manner. The presence of aprotinin blocked the effect of FXIa. Using a cell surface immunoassay we observed that FXIa did not affect the total TF or TFPI antigen expression. The proteolytic cleavage of TFPI by FXIa on endothelial cells was characterized by western blot. Treatment of HUVECs with FXIa resulted in a reduction in the 50-kDa form of TFPI and an increase in the 38-kDa species of TFPI as a function of FXIa concentration, suggesting that FXIa cleaves TFPI in the endothelial cell microenvironment.
Summary/Conclusions: We propose that in addition to FXI's well established role in the stabilization of hemostatic plugs via increased thrombin generation by activation of FIX, FXIa could support hemostasis by limiting the TFPI-mediated inactivation of the TF/FVIIa-dependent extrinsic pathway on endothelial cells. The identification of TFPI as a substrate for FXIa may have pathophysiological implication for the regulation of TF/FVIIa activity during hemostasis and thrombosis.

OC 05.3

Role for coagulation factor XI during pneumococcal pneumonia independent of factor XII activation

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Background: Severe bacterial infections such as pneumococcal pneumonia are associated with activation of coagulation. Coagulation can play an active role in the containment and elimination of bacteria by the fibrin network. Fibrin can be formed via two distinct coagulation pathways, the tissue factor (extrinsic) pathway or the intrinsic pathway both converging in the formation of thrombin. Factor XI (FXI) is the key component of the intrinsic pathway and can be activated either via factor XII (FXII), part of the contact system, or via thrombin that is generated via both coagulation pathways.
Aim: To determine whether the intrinsic coagulation pathway is involved in pneumococcal pneumonia and whether this is dependent on FXII activation.
Methods: Pneumonia was induced by intranasal inoculation with 5×10^5 CFU of *S. pneumoniae* serotype 2 (D39) in male wildtype (WT), FXI-KO and FXII-KO mice. Mice were sacrificed 12 or 48 h following infection, blood, organs and bronchoalveolar lavage fluid (BALF) were collected to determine bacterial loads, inflammatory markers and pathology.
Results: Bacterial loads in lung, blood and distant organs were significantly higher at 48 h in FXI-KO mice. In line, 48 h following infection

increased lung cytokine and chemokine levels were detected in FXI-KO mice. Several parameters of lung damage were assessed revealing significantly increased pathology in FXI-KO mice mainly caused by presence of endothelialitis. Endothelial damage was confirmed by enhanced plasma and lung E-selectin levels. No differences in any of the examined parameters were observed between WT and FXII-KO mice. In a separate experiment BALF was collected following induction of pneumonia. Although FXI-KO mice again showed higher bacterial counts in blood and distant organs, BALF bacterial loads and leukocyte influx were comparable between WT and FXI-KO or FXII-KO mice.

Conclusion: FXI deficiency increased bacterial dissemination during *S. pneumoniae* induced pulmonary infection, while FXII deficiency did not influence the host response. These results indicate that FXI protects against pneumococcal pneumonia via a mechanism independent of FXII activation. The critical role for intrinsic coagulation during pneumococcal pneumonia most likely involves thrombin-mediated activation of FXI.

OC 05.4

Regulation of human factor XI by a hepatic microRNA

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Background: Plasma levels of factor XI (FXI) are variable in healthy population. Indeed, high levels of FXI increase the risk of thromboembolic disease or may suppose a septic survival advantage. Despite intensive research in recent years, the genetic and environmental factors that regulate FXI expression are still largely unknown. Recently, microRNAs (miRNAs) have appeared as novel regulators of protein expression in physiological and pathological processes.

Aim: To evaluate the regulation of FXI by miRNAs in human liver.

Methods: A miRNA array was performed using total RNA extracted from two healthy human liver samples. Mature hepatic miRNAs potentially regulating human *F11* 3′UTR mRNA were selected using four algorithms. Human liver carcinoma cells (HepG2) were transfected with miR-181a-5p mimic or non-specific scrambled control (Scr). The effect of miRNA overexpression was assessed by analyzing *F11* mRNA and intra- and extracellular FXI by qRT-PCR and western blot, respectively. Human colon cancer cells deficient for Dicer (HCT116-DK) were used for luciferase assays. One hundred fourteen healthy livers were analyzed to quantify mRNA levels as well as miR-181-5p by qRT-PCR.

Results: *In silico* prediction yielded six miRNA candidates that might regulate FXI expression. We focused on miR-181a-5p because it has the highest probability to bind to *F11* mRNA. Transfection of HepG2 cells with miR-181a-5p mimic compared with Scr provoked a significant decrease of FXI levels both, intracellularly (100% *vs.* 53 ± 16%; $P = 0.02$); and extracellularly (100% *vs.* 71 ± 7%; $P = 0.04$). This miRNA also caused a statistically significant decrease of *F11* mRNA (100 ± 17% *vs.* 71 ± 9%, $P = 0.03$). Luciferase assays in HCT116-DK cells demonstrated a direct interaction between miR-181a-5p and 3′UTR of *F11* mRNA since miR-181a-5p provoked a decrease of the luciferase activity in comparison with Scr (100 ± 17% *vs.* 71 ± 27%, $P = 0.04$). To validate these results *ex vivo*, we evaluated the potential correlation between the expression of miR-181a-5p and *F11* mRNA in 114 liver samples. Our results showed an inverse and significant correlation between *F11* mRNA levels with miR-181a-5p levels (Pearson's = −0.23; $P = 0.017$). In contrast, the lack of correlation between miR-181a-5p and *F9* mRNA levels, which may be expected according to *in silico* predictions, is important to strengthen the specificity of miR-181a-5p on *F11*mRNA levels. Actually, both FXI and FIX plasma levels have been previously shown to parallel, and our data also demonstrated that mRNA levels of these two factors strongly correlated in healthy livers (Pearson's = 0.477; $P < 0.001$).

Conclusions: The present study identifies miR-181a-5p as the first endogenous modulating agent of FXI. Our results strongly suggest that miR-181a-5p takes part in the complex regulation of FXI and might contribute to the inter-individual plasma variability of this key hemostatic factor. Our results demonstrate that the effect of miR-181a-5p on *F11* mRNA levels is highly specific. Further studies are necessary to identify potential associations of miR-181a-5p levels with disorders involving FXI, mainly thrombosis.

OC 05.5

Abnormal plasma clot structure and stability distinguish bleeding risk in patients with severe factor XI deficiency

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Background: Factor XI (FXI) deficiency is a rare, autosomal, recessive disorder. Many patients with low plasma FXI levels are asymptomatic or exhibit only mild bleeding; however, others experience severe bleeding usually associated with trauma. Interestingly, neither FXI antigen nor activity levels, nor routine activated partial thromboplastin time (APTT) assays, are able to predict bleeding risk in FXI-deficient patients.

Aims: (i) Characterize the formation, structure and stability of plasma clots from patients with severe FXI deficiency, and (ii) Determine whether these measurements distinguish asymptomatic patients ('non-bleeders') from those with a defined clinical history of bleeding ('bleeders').

Methods: Sixteen Israeli severe FXI-deficient patients were divided into 'bleeders' (patients who bled following one or two tooth extractions) or 'non-bleeders' (patients who underwent two or more tooth extractions that were not associated with bleeding) ($n = 8$ patients per group). Groups were similar with respect to gender and age. Informed consent was obtained in compliance with the medical ethics committee. Whole blood was collected via venipuncture into sodium citrate and corn trypsin inhibitor. The first 5 mL of blood were discarded, and platelet-poor plasma was prepared by sequential centrifugation of whole blood. Clot formation was triggered by mixing recalcified plasma with dilute tissue factor and phospholipids in the absence or presence of tissue plasminogen activator and/or thrombomodulin. Clot formation, fibrinolysis, and fibrin network structure were measured by turbidity and laser scanning confocal microscopy, respectively.

Results: 'Non-bleeders'and 'bleeders' had similar low levels of FXI (3.0 ± 1.4 *versus* 3.3 ± 3.0%, respectively), and normal levels of platelets, factor VIII, factor XIII, von Willebrand factor, fibrinogen, and thrombin activatable fibrinolysis inhibitor. 'Non-bleeders' and 'bleeders' had similar prothrombin times (105 ± 13 and 104 ± 16 s, respectively) and prolonged APTT (56 ± 6 and 68 ± 24 s, respectively). Compared to 'non-bleeders,' 'bleeders' exhibited lower clot stability in the presence of tissue plasminogen activator, with significantly longer lag times (1020 ± 399 *versus* 693 ± 245 s, respectively, $P < 0.04$), smaller turbidity change (0.40 ± 0.12 *versus* 0.58 ± 0.14 O.D., respectively, $P < 0.008$), and reduced area under the turbidity curve (674 ± 210 *versus* 1176 ± 386 arbitrary units, respectively, $P < 0.004$). Compared to 'non-bleeders,' 'bleeders' also exhibited significantly lower fibrin network density than 'non-bleeders' (177387 ± 22610 *versus* 234574 ± 42621 arbitrary units, respectively, $P < 0.005$). In the presence of thrombomodulin, differences were even more profound; 7 of 8 'bleeder' patients failed to form a clot, whereas only 3 of 8 'non-bleeder' patients did not clot in the same time.

Summary/Conclusions: Plasma clot structure and stability assays distinguished 'non-bleeder' and 'bleeder' patients: compared to

'non-bleeders,' FXI-deficient patients with a history of bleeding exhibited reduced fibrin network density and lower plasma clot stability. Plasma clot formation assays may provide information on hemostatic mechanisms in FXI-deficient patients and have clinical utility in predicting bleeding risk in these patients.

OC 05.6
The endothelial lectin CLEC4M is a novel clearance receptor for factor VIII

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Introduction: Plasma levels of the glycoprotein coagulation factor VIII (FVIII) represent a balance between synthesis and clearance, where elevated FVIII represents a risk factor for thrombosis, and decreased FVIII is associated with the bleeding disorder hemophilia A. However, the mechanisms which regulate FVIII clearance remain incompletely characterized. Previous studies have demonstrated that FVIII interacts with the lectin receptor DC-SIGN. Additionally, we have previously identified the DC-SIGN homolog CLEC4M as a clearance receptor for the FVIII binding partner von Willebrand Factor (VWF).
Aims: We hypothesize that CLEC4M is a clearance receptor for Factor VIII, which can bind and internalize FVIII alone or as a complex with VWF.
Methods: There is no murine ortholog for CLEC4M and thus hepatic CLEC4M expression was induced in normal C57/BL6 mice through hydrodynamic injection of CLEC4M cDNA in the pLIVE vector. Four days post-injection, VWF:Ag was quantified through ELISA and FVIII:C was measured with a chromogenic assay. Binding and internalization of FVIII by HEK293 cells stably expressing CLEC4M was characterized with an enhanced-sensitivity ELISA and immunofluorescence. Binding of FVIII to recombinant CLEC4M-Fc fusion protein was assessed with a modified ELISA.
Results: Plasma levels of VWF:Ag and FVIII:C were decreased by 46 and 44% respectively in mice expressing CLEC4M as compared with controls ($N = 17$, $P = 0.0094$, $P = 0.0110$). Expression of CLEC4M by hepatocytes (10–30% positive) from hydrodynamic injections was confirmed through immunohistochemistry.
Using a modified ELISA, CLEC4M-Fc bound to murine rVWF and rFVIII. CLEC4M-Fc also bound to human plasma-derived FVIII-VWF complex, human full-length rFVIII, and B-domain deleted rFVIII; binding of CLEC4M-Fc to B-domain deleted FVIII was decreased by 50% relative to full-length on a per unit basis ($P = 0.0061$). Binding was dose-dependent, saturable, calcium-dependent, and reversible. Binding was inhibited by the mannose polysaccharide mannan ($P < 0.0001$), and competed by 50% with soluble FVIII ($P = 0.0086$) or FVIII-VWF complex ($P = 0.01$). Binding was blocked with a polyclonal anti-FVIII antibody (90%, $P < 0.0001$), and with monoclonal antibodies to A2 (30%, $P = 0.0316$), C1 (50%, $P = 0.0004$), and C2 (45%, $P = 0.0029$) domains. Removal of FVIII N-linked, and total glycan content attenuated binding to CLEC4M-Fc by 75% and 62% respectively ($P < 0.0001$).
Immunofluorescence demonstrated that binding of recombinant FVIII was increased on 293 cells that stably expressed CLEC4M relative to cells that did not express CLEC4M. Internalization of FVIII by CLEC4M-expressing cells was confirmed with Z-stack analysis and 3D reconstruction. CLEC4M-expressing cells exposed to physiological levels of FVIII and the VWF-FVIII complex had a two-fold increase in levels of FVIII in cell lysates in a dose and time-dependent manner relative to controls as measured by ELISA. The CLEC4M-mediated increase in FVIII levels in cell lysate could be attenuated by 50% with mannan ($P = 0.0013$).
Conclusion: CLEC4M binds FVIII alone or as part of the VWF complex, and facilitates receptor-mediated endocytosis of FVIII and/or VWF-bound FVIII. Characterization of the interaction between CLEC4M and FVIII may lead to better understanding as to how plasma levels of FVIII are regulated.

OC 06 – Fibrinogen

OC 06.1
A new mechanism for modulation of fibrin formation by shear: the knob–hole interactions display 'catch-slip' kinetics

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Background: Fibrin polymerization, which underlies the formation of clots and thrombi, is driven by intermolecular interactions between knobs 'A' and complementary holes 'a'. These interactions dictate the rate of fibrin formation and provide the fibrin network with mechanical stability, which determines how clots and thrombi respond to mechanical force, such as blood shear. Many aspects of fibrin formation and properties remain unknown, including those associated with impaired blood flow. We hypothesized that the A:a knob-hole bonds display a complex dynamic behavior when subjected to mechanical stress, perhaps due to existence of multiple bound states and/or a broad interface extending beyond the N-terminal α chain motif (GPR) and the complementary pocket in the γ-nodule.
Aims: To resolve the mechanism of A:a knob-hole interactions in fibrin by precise nano-mechanical measurements of formation and dissociation of A:a knob-hole bonds at the single-molecule level.
Methods: We used an optical trap-based system to measure bond lifetimes corresponding to the forced dissociation of single receptor-ligand pairs under a constant tensile force. Briefly, a microscopic ligand-coated latex bead is trapped by a focused laser beam and repeatedly brought into contact with a receptor-coated pedestal. When the ligand (fibrinogen or fragment D, bearing holes 'a') on the bead binds to the receptor (monomeric fibrin or fibrin fragment E, bearing knobs 'A') on the pedestal, the bead is displaced from the trap center to generate a controlled pulling force. The time needed to separate the surfaces is then measured to characterize the interaction properties.
Results: We investigated the dependence of the average bond lifetime of the A:a knob-hole complex on tensile force and found it to follow an unusual non-monotonic behavior. The strength of knob-hole interactions first increased with forces up to 40 pN and then decreased with larger forces. Normally, bond lifetimes diminish with force ('slip' bonds). An increase of bond lifetime with force ('catch' bonds) is a rare and counterintuitive phenomenon, demonstrated for few receptor-ligand pairs. The A:a knob-hole 'catch-slip' behavior was revealed by interacting monomeric fibrin with fibrinogen as well as fragments desA-E/desAB-E with fragments D. The control for specificity was fibrinogen-fibrinogen binding/unbinding without knobs 'A' and the use of knob 'A'-mimicking inhibitory GPRPam peptide. The negative control for 'catch' bonds was a streptavidin-biotin complex, which displayed a pure 'slip' bond behavior with a monotonic decrease of the bond lifetimes with pulling forces from 10 to 50 pN.
Summary/Conclusion: The finding that single A:a knob-hole bonds show dual 'catch-slip' response to mechanical tension is a highly significant result, suggesting a complex energy landscape underlying these bimolecular interactions. This, in turn, implies that the A:a knob-hole bonds are heterogeneous and are characterized by multiple bound states, which dissociate following different unbinding pathways. The 'catch-slip' behavior of the A:a interactions may be a mechanism for shear stress-related modulation of fibrin formation, since the bond lifetime increases with force over a certain range. This behavior may underlie the predisposition to venous or arterial thrombosis and other complications associated with alterations in normal blood flow.

OC 06.2

DNA methylation profiling of the fibrinogen gene landscape in human cells and during mouse and zebrafish development

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Background: Fibrinogen is the soluble precursor of fibrin, the central blood clotting agent in wound healing. Two sets of the three polypeptide chains Bß, Aα and γ form hexameric fibrinogen. The chains are encoded by the genes *FGB, FGA* and *FGG*, which are clustered on a 50 kb region in humans with cluster conservation seen across terrestrial vertebrates. Fibrinogen gene expression is limited to hepatocytes and several studies indicate a coordinated regulatory mechanism. Transcription factor binding sites have been analyzed in all three promoter regions with hepatocyte nuclear factor-1 (HNF-1) and CCAAT-box/enhancer-binding protein (C/EBP) being recognized as key contributors to basal fibrinogen expression. Identifying additional mechanisms contributing to the coordinated liver-specific expression of the fibrinogen genes may provide explanations for the variability of fibrinogen blood levels.

Aims: The role of DNA-methylation in gene regulation has focused on CpG-rich 'islands' that are present in approximately 70% of protein-coding gene promoters. The three fibrinogen gene promoters are CpG-poor and do not contain CpG islands. CpG-methylation in CpG-poor gene promoters is thought to be dynamic with low methylation correlating with tissue-specific gene expression, but its direct effect on gene regulation as well as the role of non-promoter CpG methylation are not clear. To investigate the role of CpG-methylation in the regulation of fibrinogen gene expression this study aims to characterize the methylation status of CpGs across the fibrinogen locus in human cells as well as in developing mouse and zebrafish tissues.

Methods: For a representative overview of CpG-methylation we used DNA-methylation sequencing to analyze 33% (144 CpGs) of CpGs in the human fibrinogen locus with a focus on promoter regions, gene bodies and the liver enhancers CNC12 and PFE2 recently identified in our laboratory. We analyzed the methylation status of the fibrinogen locus in mouse and zebrafish tissues in relation to changing fibrinogen expression levels during development. Finally, we determined whether trimethylation of H3K4, a mark of active promoters which strongly correlates with hypomethylated CpGs, changes in the fibrinogen promoters during development.

Results: We observed low CpG-methylation in the three fibrinogen promoters, CNC12 and PFE2 in human fibrinogen-expressing samples (HuH7, HepG2, primary hepatocytes). In a gene reporter assay, CpG-methylation in the *FGA* promoter reduced promoter activity, suggesting a direct repressive function for CpG-methylation in the fibrinogen locus. In mouse and zebrafish livers CpG-methylation around fibrinogen genes reduced during development. This lowering appears to be preceded by increased fibrinogen expression and tri-methylation of H3K4 (H3K4me3) in fibrinogen promoters.

Summary/Conclusion: Our data support a model where changes in hepatic transcription factor expression and histone modification provide the switch for increased fibrinogen gene expression in the developing liver which is followed by reduction of CpG methylation.

OC 06.3

The B subunit for coagulation factor XIII accelerates the crosslinking of fibrin in human plasma

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Background: Coagulation factor XIII (FXIII) is a proenzyme of plasma transglutaminase consisting of two catalytic A subunits (FXIII-A) and two non-enzymatic B subunits (FXIII-B). FXIII-B used to be thought to have an inhibitory effect on the activation of FXIII, primary in purified system *in vitro*. We previously found that administration of FXIII-B into FXIII-B-knockout mice increased plasma level of FXIII-A *in vivo*. In addition, an addition of FXIII-B into plasma of FXIII-B-knockout mice enhanced crosslinking of fibrin *in vitro*.

Aim: The role(s) of FXIII-B in crosslinking of fibrin was explored in human.

Methods: Recombinant human FXIII-B (rFXIII-B) was prepared by a baculovirus expression system. Fibrin-crosslinking reaction was examined in citrated plasma by the addition of thrombin and calcium chloride. Generated fibrin clot was collected by centrifugation and subjected to SDS-PAGE followed by protein staining or Western blotting using antibodies for target proteins. FXIII remaining in the supernatant was quantified by ELISA.

Results: The addition of rFXIII-B into FXIII-B-deficient plasma *in vitro* accelerated crosslinking between fibrin-gamma chains. FXIII-B was co-immunoprecipitated with fibrinogen from FXIII-A-deficient plasma, although FXIII-A did not precipitate with fibrinogen from FXIII-B-deficient plasma. In normal plasma, FXIII-A was incorporated into fibrin clot, and FXIII-B was released from the clot dependent on the crosslinking of fibrin gamma-chains. In contrast, FXIII-B-deficient plasma showed reduced incorporation of FXIII-A into fibrin clot, and decreased cleavage of the activation peptide (AP) of FXIII-A by thrombin. Both the reduced FXIII-A-incorporation into fibrin clot and the decreased AP-cleavage by thrombin were restored by the addition of rFXIII-B.

Conclusion: These results strongly suggested that FXIII-B led FXIII-A to an adapted site of fibrinogen to the effective cleavage of the activation peptide by thrombin and to immediate crosslinking of gamma-chains. The binding sites of both fibrinogen and FXIII-B should be determined for further understanding the mechanism of fibrin-crosslinking by the possible cooperative action(s) of FXIII-A and FXIII-B.

OC 06.4

Fibrin formation under flow on biomimetic tissue factor microparticles

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Background: The interplay between thrombin generation, fibrin polymerization and flow determines the structure and stability of a thrombus. In purified systems containing only thrombin and fibrinogen, it has been shown that fibrin polymerization can only occur under a narrow set of conditions that are defined by the rate of thrombin generation and the shear rate [Neeves, *Biophys J* 98, 2010]. What is unknown is how the surface concentration of TF affects fibrin formation over a wide range of flow conditions and how coagulation factor deficiencies influence this phenomenon.

Aims: The objective of this work was to quantify fibrin formation and thrombin generation under flow as a function of TF surface concentration and shear rate in a plasma-based assay.

Methods: Tissue factor bearing particles were synthesized by coating 1 μm silica beads with 0.5, 5, or 50 molecules TF/μm^2 in a lipid bilayer (phosphatidylserine:phosphatidylcholine 30:70). The particles were patterned as 100 μm spots on a glass substrate using a microblotting technique to define a focal 'injury.' Normal pooled plasma, prothrombin, FVIII, FX and FXI deficient plasmas were perfused over the TF spots at wall shear rates of 100, 250, 500 and 1000/s for 10 min. Fibrin formation and thrombin generation were measured in real-time by epifluorescence using labeled fibrinogen and the thrombin substrate boc-VPR-AMC, respectively. Following the assay, fibrin gels were either (i) fixed and further imaged by confocal or scanning electron microscopy or (ii) digested by plasmin to quantify the amount of fibrin deposited using a D-dimer ELISA.

Results: As the shear rate was increased (100, 250, 500 and 1000/s) the initiation times to visible fibrin formation was prolonged (38 ± 8, 72 ± 10, 150 ± 13 and 216 ± 35 s). The rate of fibrin formation (1719, 1367, 351 and 97 RFU/s) and the final fibrin deposition (20, 18, 1.6, and 1.4 ng) decreased with increasing shear rate. Similarly, the diameters of the fibrin fibers decreased (162 ± 49, 99 ± 20, and 58 ± 15 nm). Thrombin generation was similar for all shear rates, except 50/s, were it was five-fold higher. This suggests above 50/s coagulation is kinetically limited, and below 50/s it is transport limited. Similar trends were observed at 50 molecules of TF/μm^2 except fibrin deposition was significantly higher. Fibrin fibers orient in the direction of flow. At shear rates of 50, 100, 250, 500 and 1000/s the median fiber angles were 46.3°, 34.8°, 17.8°, 13.5°, and 5.3° with respect to the direction of flow (0°). Preliminary studies using FVIII and FXI deficient plasmas resulted in approximately 50% and 40% less fibrin deposition compared to NPP, respectively, at 100/s. As expected, there was no observable fibrin formation in prothrombin and FX deficient plasmas.
Conclusions: A plasma-based flow assay was developed to measure transient fibrin deposition and thrombin generation. We found that for a given TF concentration, flow profoundly influenced fibrin deposition, fiber diameter, fiber orientation and local thrombin concentration.

OC 06.5

Evidence that fibrinogen gamma′ regulates plasma clot structure and lysis, and relationship to cardiovascular risk factors in black Africans

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Background: Fibrinogen γ' is known to influence fibrin clot structure in purified experimental models but little is known regarding its influence on clot structure in plasma. Furthermore, the environmental and biological factors that affect its concentration are poorly described.
Aims: Our aim was to analyse whether γ' fibrinogen, total fibrinogen, and γ'/total fibrinogen ratio relate to plasma clot structure and lysis in a large population-based study of apparently healthy black South Africans. We also aimed to analyse the relationship between γ' fibrinogen and γ'/total fibrinogen ratio and traditional cardiovascular risk factors.
Method: We analysed fibrinogen γ', total fibrinogen concentration, γ'/total fibrinogen ratio and fibrin clot structure and lysis, determined through the use of turbidity analysis, in 2010 apparently healthy black South African men and women, aged 35–65, who formed part of the South African arm of the international PURE study and related them to traditional CVD risk factors.
Results: Fibrinogen γ' generally increased with increasing fibrinogen concentration, however a decreased γ'/total fibrinogen ratio was found at the highest total fibrinogen concentrations. Clot maximum absorbance increased with total fibrinogen and fibrinogen γ', in agreement with the formation of clots composed of increased fibrin material. However, the γ'/total fibrinogen ratio was associated with decreased maximum absorbance, in agreement with clots made of thinner fibrin fibres. Clot lysis time showed a stronger relationship with fibrinogen γ' than with total fibrinogen, whereby increased fibrinogen γ' delayed clot lysis times. Traditional CVD risk factors (excluding fibrinogen) explained 20% and 3% respectively of the variance in fibrinogen γ' and the γ'/total fibrinogen ratio, with CRP making the biggest contribution. More than 50% of the variance in fibrinogen γ' and γ'/total fibrinogen ratio is explained by factors other than total fibrinogen concentration or other traditional CVD risk factors.
Conclusions: These data show that physiological levels of γ' fibrinogen influence fibrin clot structure and lysis in plasma and that factors other than fibrinogen, likely involved in the inflammatory response, regulate plasma γ' fibrinogen concentration. Our findings support future studies of the role of γ' fibrinogen in thrombosis.

OC 06.6

Transcriptome analysis of the miR-29-mediated control of fibrinogen gene expression

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Background: Fibrinogen is a critical component of hemostasis. In humans, circulating fibrinogen levels are highly variable, ranging from 1.5 to 3.5 g/L. Increased plasma fibrinogen is a risk factor for cardiovascular disease, including stroke and deep vein thrombosis. Basal fibrinogen expression is regulated HNF-1 and C/EBP transcription factors and, in the acute phase response, in response to inflammatory signals such as interleukin-6 (IL-6) and glucocorticoids. However, the molecular mechanisms underlying the coordinated transcription of the five major fibrinogen mRNAs remain unclear. Previously, we have demonstrated the potential role of microRNAs (miRNAs) in the fine-tuning of fibrinogen expression and shown that the overexpression of a single miRNA family (hsa-miR-29abc) member can inhibit the expression of the five major fibrinogen transcripts and fibrinogen protein production by 40–60%. The miR-29 family does not directly target the fibrinogen genes to control their expression, therefore the mechanism is more likely to be the result of an indirect interaction via regulatory transcripts or transcripts for regulatory proteins. Identifying a master regulator of fibrinogen gene expression is vital to understanding plasma fibrinogen variation in normal populations and its contribution to hemostasis in humans.
Aims: The aim of this study is to uncover novel pathways that regulate the levels of circulating fibrinogen. We aim to identify and characterize key members of a fibrinogen regulatory network and to understand the extent to which microRNAs actively regulate liver-expressed or hemostasis-related genes within this network.
Methods: HepG2 cells were transfected in biological replicates with 30 nM of miR-29c precursor miRNA or a scrambled negative control miRNA, and untransfected cells were used as an additional control. At 48 h post-transfection, total RNA was extracted and RNA-seq libraries were prepared using the Illumina TruSeq RNA v2 and Small RNA kits. mRNA and small RNA transcriptomes were sequenced to an average read depth of 70 M combined reads per sample on an Illumina Hi-Seq 2000. Bioinformatic analysis was performed using the 'Tuxedo' pipeline comprising the tophat, cufflinks and cuffdiff software packages.
Results: Our analysis revealed more than 2900 transcripts that are differentially expressed following miR-29c overexpression in HepG2 cells compared to cells containing the negative control miRNA precursor or untransfected HepG2 cells. Of this subset of transcripts, 1212 were significantly up-regulated (log2 fold-change 0.5–5.6) and 1746 were down-regulated (log2 fold-change 0.5–4.4).
Summary/Conclusions: miR-29c-mediated repression of gene expression in HepG2 cells significantly altered the mRNA abundance of several transcription factors that regulate fibrinogen gene and protein expression. In addition, we observed significant changes in key members of the coagulation cascade and the fibrinolysis pathway, as well as a number of related miRNAs. Our analysis reveals several strong candidates that are likely to act as upstream regulators of fibrinogen, and provides insight into mechanisms that may be exploited to finely control fibrinogen biosynthesis.

OC 07 – Haemostatic Factors and Arterial Vascular Disorders

OC 07.1

Impact of incident venous thromboembolism on future risk of arterial thrombotic disease

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Background: Growing evidence support a possible association between venous thromboembolism (VTE) and subsequent arterial thrombotic events (myocardial infarction (MI) and ischemic stroke). However, the evidence is established either by findings in selective patient cohorts (e.g. patients who survived pulmonary embolism) or from a large population-based prospective registry study unable to adjust for obvious confounders such as body mass index (BMI).
Aims: To study the association between incident VTE and future risk of arterial thrombotic events adjusted for obvious confounders, and to determine the population attributable risk (PAR) of arterial thrombotic disease by VTE in a large prospective cohort of subjects recruited from the general population.
Methods: Individual participant data from the Tromsø Study (Tromsø) (screening visits in 1994–1995) and the Diet, Cancer and Health Study (DCH) (screening visits 1993–1997) were merged. Thus, our prospective cohort comprised information of 81 687 subjects, aged 24–97 years without a previous history of VTE or arterial thrombotic diseases at inclusion. Information on age, BMI, diabetes, smoking, hypertension, hypercholesterolemia, physical activity and education was obtained by physical examination, blood samples and questionnaires. All first-time events of VTE, MI and ischemic stroke during follow-up were identified and validated. Subjects were followed from baseline to the date of an incident arterial event, death or migration or to the end of the study period [April 30, 2008 (DCH) and December 31, 2010 (Tromsø)]. Cox regression models with age as time scale and VTE as a time-dependent variable were used to determine risk estimates. The analyses were adjusted for age, sex, BMI, diabetes, smoking, hypertension, hypercholesterolemia, physical activity and education. The study was approved by the regional committees for research ethics in Tromsø and Aarhus and all subjects gave their informed written consent.
Results: There were 6344 incident arterial thrombotic events (3666 MIs and 2678 ischemic strokes) and 1208 incident VTE events during a median of 12.2 years of follow-up. Subjects suffering from a VTE event had a 35% higher risk of a future arterial event (adjusted HR 1.35; 95% CI 1.09–1.66). Stratified analyses revealed that the association between VTE and subsequent arterial thrombosis applied particularly to women and those below 65 years of age. Women below 65 years with VTE had 3.6-fold higher risk of MI (adjusted HR 3.62; 95% CI 1.49–8.80) and 3.2-fold higher risk of ischemic stroke (adjusted HR 3.16; 95% CI 1.18–8.47). No association between VTE and subsequent arterial thrombosis was found in men above 65 years of age. The population attributable risk (PAR%) of arterial thrombosis by incident VTE was only 0.9%, and the VTE event explained 64% of the risk (attributable risk) for arterial thrombosis in VTE patients.
Conclusions: Our prospective cohort study implies that women and subjects below 65 years of age with incident VTE have increased risk of future arterial thrombotic disease, including myocardial infarction and ischemic stroke. However, only 1% of the arterial thrombotic events in the population can be attributed to VTE.

OC 07.2

Procoagulant tumor-derived microparticles: phenotypic and functional approaches for their detection in an *in vitro* model of tumor cells-containing blood

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Background: Tumor-derived microparticles (tumor-MP) can be expected in the peripheral blood of cancer patients and these may often be highly pro-coagulant due to expression of phosphatidylserine (PS) and tissue factor (TF). Their measurement in plasma may thus help evaluating thrombotic risk and even serve for the early diagnostics of hidden tumors. However, these rather elusive tumor-MP are not yet easily detected and efforts are still needed to define optimal techniques.
Aims: (i) To use an *in vitro* model of cancer cells admixed in whole blood to generate large volume plasma samples containing highly procoagulant TF+ tumor-MP. (ii) To compare phenotypic and functional approaches in tumor-MP detection and evaluate possible interactions between normal blood cells and admixed tumor cells.
Methods: Cultured cells from a human colon cell line (CY1) expressing high amounts of TF (> 200 000 molecules/cell, as measured by Quantitative Flow CytoMetry (FCM)) were spiked at different levels (0, 10, 100 and 1000 cells/μL) into ACD-anticoagulated blood issued from transfusion bags (100 mL per level), or the corresponding platelet poor plasma (PPP) or platelet rich plasma (PRP) as controls. After a 3 h incubation period at 37 °C with gentle agitation, the platelet-rich buffy-coat and PPP were obtained (2500 g 15 min) and the platelet free plasma (PFP, again 2500 g 15 min) recovered and frozen as aliquots. Buffy-coat cells (WBC+Plt) with/without tumor cells (CY1) were analyzed in 4-color FCM using anti-EPCAM-FITC, CD14-APC and CD45-PC5 to discriminate WBC and tumor cell subsets, plus a series of PE-labeled MAbs to study various markers including TF (CD142), platelet- (Plt), monocyte-, granulocyte- and lymphocyte-related markers plus tumor-associated markers (EGFr, CEA, E-cadherin) and shared antigens (e.g. CD9, 24, 61, HLA). TF-linked procoagulant activity of PFP was tested using a FXa generation assay on their washed MP (2 × 24 000 ***g*** 1 h). Distribution of MP subsets in each PFP was studied by 5-color IF in CMF using a 3 laser instrument (Gallios, Beckman-Coulter) set-up for standardized size-related cut-off at 0.3 μm-eq. using Megamix-Plus FSC beads (BioCytex). The three major subsets were delineated with AnnV-FITC (PS+ MP), CD41-PC7 (PMP), CD235a-APC-A750 (Ery-MP) and CD15-Pac.Blue (Leu-MP) plus a large series of PE-MAbs as described above.
Results: In spiked blood samples, unusual cell-surface markers highly expressed on CY1 cells (e.g. Epcam, TF, CD9, E-cadherin…) also appeared on all WBC subsets and platelets. This suggested an active generation of tumor-MP, some of which adhering to blood cells. This was confirmed by a high, spiking level-dependent, TF-dependent, procoagulant activity. No major difference of activity was noted between PFP derived from PPP, PRP or whole blood samples spiked with tumor cells at the same level, suggesting that in all three cases the procoagulant activity came from the generated tumor-MP without major influence of WBC, red cells or platelets.
Conclusions: When incubating whole blood (or plasma) with TF+ tumor cells, a high amount of TF+ tumor-MP is released which harbour antigens absent from blod-borne MP. Although some adhere to WBC and Plt, most of them remain in the plasma, thus generating high levels of TF-dependent procoagulant activity.

OC 07.3

High on-treatment platelet reactivity in patients with unprotected left main disease treated by percutaneous coronary intervention: the ALMA (angioplasty of left main – lariboisiere) registry

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Background: the clinical benefit of platelet antiaggregation monitoring to tailor antiplatelet therapy is highly debated. The apparent clinical benefit is influenced by the patient population: higher is the thrombotic risk, more sensitive is the outcome on the exact degree of platelet inhibition.
Aims: In patients with unprotected left main disease (ULMD), angioplasty is emerging as an alternative technique. High on-treatment platelet reactivity (HOPR) despite aspirin and/or thienopyridines is associated with acute events after angioplasty. We aimed to determine the rate and potential clinical impact of HOPR in patients treated by angioplasty for ULMD.
Methods: One hundred and twenty-five consecutive patients referred for angioplasty of ULMD prospectively were included in a monocentric registry (ALMA). For the first 64 patients (ALMA-1), angioplasty was performed under aspirin and clopidogrel treatment without platelet reactivity assessment. For the last 61 patients (ALMA-2), platelet reactivity assessment (light transmission aggregometry triggered by arachidonic acid and ADP and measurement of VASP index by platelet flow cytometry) were systematically performed before angioplasty: in case of HOPR for aspirin, aspirin was given twice daily and in case of HOPR for clopidogrel, daily clopidogrel dose was doubled or replaced by prasugrel.
Results: Overall, patients mean age was 69 ± 13 y.o., 37% were diabetic, 37% had NSTEMI and 62% had multivessel disease. Mean SYNTAX score was 22.8 ± 9.2. In ALMA-2, 28% patients had HOPR for aspirin and 29% patients had HOPR for clopidogrel. Aspirin twice daily was given in 28% of patients, clopidogrel double dose in 6% and prasugrel in 23%. Compared to ALMA-1, the rate of 1-year MACCE was decreased in ALMA-2 (8.2 vs. 20.8%; $P = 0.04$) as a composite endpoint of cardiovascular death and stent thrombosis (0.0 vs. 8.3%; $P = 0.02$).
Conclusion: HOPR for aspirin and clopidogrel is frequent in patients undergoing angioplasty for ULMD and appears to be strongly associated with subsequent major events, adaptatation of the dual antiplatelet therapy to platelet inhibition monitoring is correlated with a more favourable clinical outcome.

OC 07.4

FXIII levels and different major adverse cardiac events (MACEs) in acute myocardial infarction: a potential prognostic biomarker

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Background: FXIII fall in the early phases of myocardial infarction (MI) could impair heart healing, cause cardiac rupture and anomalous left ventricular remodelling. Monitoring of FXIII levels in the acute phases of MI could be considered a novel prognostic biomarker together with the classical ischemic routine biomarkers. Thus, FXIII might predict healing suggesting implications in prognosis and therapy.
Aims: We recently recognized acute FXIII drops in virtually all patients during the first post-MI days. This behaviour had a wide range of distribution (Δ-drop ranged from 10% to > 50%). Our objective is to investigate if FXIII drop, and/or its late recovery, could be considered a novel MI marker, and if a FXIII prognostic threshold could be recognized.
Methods: Informed consent and approval medical ethics committee were obtained. We recruited 334 MI patients (72% male) from the Coronary Care Unit of Hospital-University of Ferrara. We performed 1-year follow-up and the combined end-point was re-MI, death, heart failure, u-angina, and stroke/ictus (MACEs). FXIII level was assessed by a commercial kit [Instrumentation Laboratory, Italy] at the recruitment (t_0) and every 24-h for five additional days (t_{1-5}) post-MI. Control samples were drawn at 30-day (t_{30}) to have steady state FXIII levels.
Results: Globally, 25.5% of patients experienced MACE (re-MI, 3.4%; death, 10.5%; heart failure, 8.0%; u-angina, 2.7%; stroke/ictus, 1%). In the group of MACE(+) FXIII was: t_0 90.43 ± 31.1; t_{4-5} 77.2 ± 28.7; t_{30} 97.84 ± 25.9. In the group of MACE(−) FXIII was: t_0 100.7 ± 30.8; t_{4-5} 85.6 ± 25.23; t_{30} 106.9 ± 25.7. All the comparisons were statistically significant and the output was that MACE(+) group had the highest FXIII falls: (MACE+ vs MACE-: $P_{t0} = 0.0086$; $P_{t4-5} = 0.03$; $P_{t30} = 0.06$). Within group comparisons showed similar Results (MACE+: $_{t0}$ vs $_{t4-5}$, $P = 0.01$; MACE−: $_{t0}$ vs $_{t4-5}$, $P = 0.0001$). Interestingly, the FXIII drop behaviour was very different according to the different end points considered. In particular, re-MI cases strongly diverged from the other MACEs, being apparently not affected by the fall of FXIII levels [FXIII% lowest mean value (t_{4-5}): 92.5 ± 31.5]. On the contrary, cases who died within the first weeks after MI, presented with significant lower FXIII levels since the recruitment (t0), and the lowest FXIII% mean value (t_{4-5}) was 69.5 ± 28.5.
Conclusions: Post-MI MACEs still remain critical influencing prognosis and survival. FXIII could be considered a candidate molecule in cardiovascular diseases, and its employment is strongly promising. Understanding whether FXIII level monitoring may be useful to select cases with poor clinical outcome could pave the way to consider FXIII as a tailored treatment to improve myocardial healing, recovery of functions and survival.

OC 07.5

Effect of factor XIII polymophisms on the risk of myocardial infarction

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Background: Coronary artery disease (CAD) is still a major health issue in the Western world in both women and men. In our previous study elevated factor XIII (FXIII) levels were demonstrated to be associated with increased risk of myocardial infarction (MI) in females, but not in males. Among FXIII polymorphisms Val34Leu polymorphism of FXIII A subunit (FXIII-A) has been intensively investigated and meta-analysis of the reported data demonstrated a protective effect against myocardial infarction. Moreover, in the Hungarian population FXIII-A Val34Leu polymorphism was protective against MI in patients with elevated fibrinogen levels. FXIII-A Tyr204Phe polymorphism was reported to be associated with decreased FXIII levels and Phe204 was associated with a mild increased risk of ischemic stroke in young women. The His95Arg polymorphism in FXIII B subunit (FXIII-B) gene was found to be a risk factor of venous thrombosis. FXIII-B Intron K nt29756 C>G polymorphism leads to a novel splice acceptor site and a protein 15 amino acid longer at the C-terminus. It may have a profound effect on disease susceptibility, however no study on cardiovascular patients has been reported, as yet.
Aims: Investigation of the role of FXIII-A Tyr204Phe, FXIII-B His95Arg and IntronK (nt29756C>G) polymorphisms in cardiovascular diseases.

Methods: Consecutive patients (n = 900) with suspected CAD were subjected for coronary angiography and recruited for the study. Among them, patients without significant coronary stenosis and the history of myocardial infarction were considered as clinical controls (n = 276). Age and sex matched individuals (n = 1000) representing the Hungarian general population were also enrolled. FXIII-A Tyr204Phe, FXIII-B His95Arg and intron K nt29576C>G polymorphisms were detected by real time PCR using melting point analysis and FRET detection on a LightCycler 480 instrument (Roche). Clinical and laboratory data were analyzed by SPSS 19.0 statistical package.
Results: The rare allele frequencies of the FXIII-A Tyr204Phe, FXIII-B His95Arg and IntronK (nt29756C>G) polymorphisms in the general Hungarian population were 0.02, 0.08 and 0.15, respectively, which are similar to the results for Caucasians available in the HapMap database. The allele frequencies in the clinical controls did not differ significantly from the respective values in the general population. Individuals carrying the rare allele of FXIII-B IntronK polymorphism had significantly ($P < 0.0001$) lower FXIII activity (94 ± 21% vs. 103 ± 21%) and $FXIIIA_2B_2$ antigen (20.7 ± 4.7 mg/L vs. 23.1 ± 5.0 mg/L) levels compared to individuals homozygous for the frequent allele. FXIII-B IntronK polymorphism decreased the risk of MI by 60% in patients with fibrinogen level in the upper tertile (OR, 0.37; 95% CI, 0.17;0.84, P = 0.017). Moreover, an interactive effect was demonstrated between FXIII-B IntronK and FXIII-A Val34Leu polymorphisms; OR for MI in the case of double carriers with elevated fibrinogen was 0.031 (95% CI, 0.01;0.21, $P < 0.0001$).
Conclusions: The effect of certain FXIII subunit polymorphism on the rik of MI prevails only at elevated fibrinogen level. FXIII-B Intron K polymorphism decreases FXIII level and is protective against MI in patients with fibrinogen level in the upper tertile.

OC 07.6

D-dimer levels are differently associated with the risk of acute coronary syndrome in men and women of the european prospective investigation in the cancer (EPIC)-Italy cohort

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Background: Fibrin D-dimers can be considered as a measure of fibrin turnover, being the balanced products of formation (via thrombin) and lysis (via plasmin) of crosslinked fibrin. We evaluated the association between D-Dimers and risk of acute coronary syndrome (ACS) in four out of five centers of the EPIC-Italy study.
Materials and Method: We conducted a case-cohort study on 1467 (712 men; 755 women) volunteers of the EPIC-Italy cohort, by comparing subjects who developed ACS (MI fatal or nonfatal, coronary revascularization, sudden death) in a mean follow-up of 11.93 years.
Using a nested case-cohort design in the EPIC-Italy study (n = 34 148), we identified a random subcohort (n = 830) and incident acute coronary syndrome cases (n = 634 plus 13 cases originated from the random subcohort) occurring between baseline (1993–1997) and end of 2006 (Varese and Naples), of 2007 (Ragusa) or 2008 (Turin); D-Dimer levels were measured in citrate plasma, utilizing HemosIL, an automated latex immunoassay on IL coagulation System ACL9000. Inter and intra-day CV were 7.6% and 5.4% respectively. The relative risk (RR) and 95% confidence intervals (CI), adjusted by relevant confounders and stratified by center, were estimated by aCoxregression model using Prentice method.
Results: Individuals in the highest compared with the lowest quartile of PAI-1 had significantly increased risks of ACS (RR: 2.50; 95%CI: 1.54–4.06; P for trend < 0.001) in univariate analysis; adjustment for sex, age, education, smoking habit, BMI, total physical activity, hypertension, diabetes and hyperlipidemia treatment, did not modify the association (RR: 2.62; 95%CI:1.50–4.55) (P for trend = 0.005). The risk of ACS increased by 44% for each increase in 1 standard deviation of D-Dimers. No association was found in women.
Conclusions: Our data support the evidence for a link between D-dimers and ACS in men but not in women.

OC 08 – Inflammation – Clinical Studies

OC 08.1

Prognostic role of MIR146A polymorphisms in atrial fibrillation

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Background: Atrial fibrillation (AF) is a prevalent disease in which the underlying atherosclerosis, a chronic inflammation of the vessel wall with monocytes driving the initiation and progression of atherosclerotic lesions, increases the incidence of arterial thrombosis. At this time, there are limited biomarkers able to forecast new thrombotic events in anticoagulated patients with AF. In this framework, microRNAs (miRNAs) have emerged as critical players in cardiovascular biology. In particular, miR-146a-3p is recognized as an important negative regulator of inflammation. To date, neither the role of miR-146a-3p nor the role of functional polymorphisms (miRSNPs) rs2431697 (~1 kb upstream of *MIR146A*) and rs2910164 (within *MIR146A*), both affecting miR-146a-3p levels in monocytes, have been related with cardiovascular disease.
Aim: To evaluate the prognostic role of functional *MIR146A* miRSNPs in a large cohort of anticoagulated permanent AF patients.
Methods: We studied 904 patients (50% male; median age 76) with permanent AF who were stabilized for at least 6 months (INR = 2–3) CHADS2DS2-VASc score was calculated. Patients were followed-up for 2 years [median = 957 days (range 789–1078)] and adverse cardiovascular events (stroke, acute coronary syndrome, and acute heart failure) were recorded. MiRSNPs were genotyped by Taqman® analysis. *In vitro* studies were performed in immunoselected monocytes from healthy controls homozygous for the two genotypes of rs2431697 (n = 9 of each genotype). Monocytes were activated with10 ng/mL LPS, previously shown to increase miR-146a-3p levels. MiR-146a-3p and *IL6* mRNA levels were measured by qRT-PCR.
Results: The frequencies of these two miRSNPs in AF patients were similar to those reported in Caucasians (ancestral alleles: rs2431697, C = 0.48; rs2910164, G = 0.74). MiRSNP rs2910164 had no association with adverse cardiovascular events. However, on multivariate analysis (adjusted by CHADS2DS2-VASc score) we found that TT genotype of rs2431697 was associated with adverse cardiovascular events (HR, 95%CI: 1.56, 1.04–2.33; P = 0.030). In order to better define the functional effect of miR-146a, we studied the effect of rs2431697 in miR-146a-3p levels in monocytes from healthy subjects. After 2 h activation, LPS induced an increase of miR-146a-3p levels, which was slightly higher in CC carriers (increase *vs.* no LPS; TT = 15%, CC = 27%). Interestingly, after 24 h incubation, monocytes from CC individuals had a 65% increase of miR-146a-3p levels while TT individuals only increased their levels by 28%. These data suggest that TT individuals when submitted to an inflammatory stress may be prone to a highest pro-inflammatory state due in part to lower levels of miR-146a-3p. Indeed, *IL6* mRNA levels, previously shown to

be influenced by miR-146a-3p, were inversely correlated with those of miR-146a-3p. Thus, after 24 h of LPS activation, TT monocytes showed a higher increase of *IL6* expression than CC (52% *vs*. 26%).
Conclusions: Our study established *MIR146A* rs2431697 as a prognostic biomarker for adverse cardiovascular events in anticoagulated AF patients. According to our data, patients with AF who carry the TT genotype of rs2431697 might have a minor response to pro-inflammatory signals than CC, resulting in reduced levels of miR-146a-3p and a final increased production of pro-inflammatory targets such as IL-6, which might contribute to the higher risk of adverse cardiovascular events of these patients.

OC 08.2

Inflammatory markers and metalloproteinases profiles predict death in in the acute phase of ischemic stroke treated with tissue plasminogen activator thrombolysis

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Background: Inflammatory mediators and metalloproteinases (MMPs) are altered in the acute phase of ischemic stroke and play a detrimental role on severity and hemorrhagic transformation of ischemic brain lesions after thrombolysis.
Aim: This study aimed to evaluate the effect of inflammatory and MMPs profiles on mortality in stroke patients submitted to thrombolysis.
Methods: Blood was taken at baseline and 24 h after thrombolysis from 327 patients (mean age 68, mean NIHSS 11.9) with acute ischemic stroke. Circulating molecules were measured using Bio-plex suspension array system [MMP-1, MMP-8, MMP-9, tissue inhibitor of metalloproteinase -1 (TIMP-1), C-reactive protein (CRP), haptoglobin, alpha2-macroglobulin (A2M), interleukin-1 receptor antagonist (IL-1RA), interleukin-6 (IL-6), interleukin-10 (IL-10), interleukin-12 (IL-12), tumor necrosis factor-alpha (TNF-alpha), vascular endothelial growth factor (VEGF)]. Baseline values and delta median values [(post tPA-baseline)/baseline] of each parameters were analyzed in 3 month-survivors and non-survivors.
Results: Baseline levels of CRP, haptoglobin, A2M, IL-10, IL-12 and IL-6 and delta values of MMPs 1, 8, 9 and TIMP-1 were significantly different in patients who died with respect to survivors [CRP: 8.25 (2.17–15.49) mg/L vs 2.96 (1.44–8.35) mg/L; haptoglobin: 3.12 (1.24–10.70) mg/mL; A2M: 2.26 (1.83–4.23) mg/mL vs 1.78 (1.24–2.60) mg/mL; IL-6: 6.25 (4.16–11.53) pg/mL vs 4.01 (2.16–7.90) pg/mL; $P < 0.01$ respectively, IL-10: 3.58 (1.16–16.70) pg/mL vs 9.80 (2.99–23.10) pg/mL; IL-12: 18.20 (9.23–42.10) pg/mL vs 24.20 (11.70–52.90) pg/mL, $P < 0.05$ respectively], whereas IL-1RA, TNF-alpha and VEGF levels [IL-1RA: 16.0 (4.98–65.40) pg/mL vs 17.44 (10.3–29.80) pg/mL; TNF-alpha: 1.61 (0.17–3.00) pg/mL vs 2.40 (0.58–5.67) pg/mL; VEGF: 82.2 (43.70–130.5) pg/mL vs 105.8 (56.40–203.7) pg/mL] did not significantly differ between non-survivors and survivors. Adjusting for age, sex, glycemia, baseline NIHSS, history of atrial fibrillation, or congestive heart failure, history of inflammatory diseases or infections occurred within the last 7 days before stroke onset, only deltaMMP-9 and baseline A2M remained significantly and independently associated with 3 month-death [OR (95% CI): baseline A2M: 1.49 (1.12–2.00); deltaMMP9:1.58 (1.11–2.26), $P < 0.01$]. ROC analysis demonstrated that the addition of baseline A2M and deltaMMP-9 (model 2) to a model that included factors known to affect the outcome (model 1) significantly improved the area under the curve for the detection of mortality in ischemic stroke patients [model 1: AUC = 0.82 (95% CI 0.75–0.90); model 2: AUC = 0.88 (95% CI 0.83–0.93), $P < 0.05$].
Conclusion: our findings suggest that A2M and deltaMMP-9 are significant and independent markers of mortality and that may be used to improve prediction of unfavourable outcome in the clinical setting of ischaemic stroke patients treated with thrombolytic therapy.

OC 08.3

Circulating nucleosomes and neutrophil activation as a measure of the formation of Neutrophil Extracellular Traps (NETs) during sickle cell painful crisis

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Background: Sickle cell disease (SCD) is characterized by recurrent acute vaso-occlusive painful crisis frequently leading to SCD related complications, like acute chest syndrome. The complex pathophysiology of the vaso-occlusive painful crisis is mediated by activation of endothelial cells, adhesion of sickled erythrocytes and polymorphonuclear neutrophils (PMN), oxidative stress, coagulation activation and an increased release of inflammatory mediators, resulting in ischemic organ damage. Recently, PMN have been demonstrated to form Neutrophil Extracellular Traps (NETs) upon activation. During this process, DNA and DNA-binding proteins are extruded from neutrophils exposing a mesh consisting of nucleosomes, histones and neutrophil proteases. NET formation has been shown to propagate coagulation in sepsis and deep venous thrombosis. Moreover, nucleosomes and histones exposed on NETs have been shown to be strongly cytotoxic to endothelial cells. Beside the exposure on NETs, nucleosomes can be actively released into the circulation from dead cells. Circulating nucleosomes detected in sepsis have been reported to correlate with markers of coagulation and inflammation as well as with organ dysfunction and mortality. *The aim* of this case–control study was to assess plasma levels of circulating nucleosomes and neutrophil activation as evidenced by human neutrophil elastase-α_1-antitrypsin (EA) complexes, as indirect measure of NET formation in plasma.
Methods: After obtaining informed consent, nucleosomes and EA complexes were measured using ELISA in blood samples of race matched controls (24), sickle cell patients during steady state (74) and sickle cell patients with painful crisis (70). Markers of inflammation and endothelial activation were also measured. For statistical analysis, patients were divided in two groups: patients with the relatively severe genotypes HbSS and HbSβ^0-thalassemia (HbSS/HbSβ^0-thal) and patients with the relatively milder HbSC and HbSβ^+-thalassemia genotypes (HbSC/HbSβ^+-thal). The study protocol was approved by the local medical ethical committee.
Results: Plasma levels of nucleosomes in both patients with HbSS/HbSβ^0-thal and HbSC/HbSβ^+-thal were significantly higher during painful crisis (median; IQR, 20.2 U/mL: 8.9–129.0, $P < 0.001$; 11.7 U/mL: 5.1–67.7, $P = 0.045$) as compared to those in steady state (6.0 U/mL: 3.0–9.8; 7.1 U/mL: 4.6–9.6). EA levels were higher in HbSS/HbSβ^0-thal and HbSC/HbSβ^+-thal patients during painful crisis (75.1 ng/mL: 56.5–102.4, $P < 0.001$; 62.0 ng/mL: 48.0–96.7; $P = 0.051$) as compared to levels during steady state (45.7 ng/mL: 34.7–59.7; 50.2 ng/mL: 33.3–67.7), but just failed to reach statistical significance in HbSC/HbSβ^+-thal patients. During painful crisis, EA levels correlated with levels of nucleosomes in both HbSS/HbSβ^0-thal (Sr = 0.55, $P < 0.001$) and HbSC/HbSβ^+-thal patients (Sr = 0.90, $P \leq 0.001$). In steady state HbSS/HbSβ^0-thal patients, levels of nucleosomes correlated with endothelial markers sVCAM-1 and vWF:Ag (Sr = 0.421, $P = 0.003$; Sr = 0.452, $P = 0.001$). Six patients who developed an acute chest syndrome during painful crisis were among those with the highest nucleosome and EA levels. A significant (Sr = 0.441, $P < 0.001$) correlation was found between levels of nucleosomes and duration of hospitalization.
Conclusion: We demonstrate increased levels of circulating nucleosomes in sickle cell patients with painful crisis reflecting NET formation which strongly correlates with PMN activation and disease severity.

OC 08.4

Activated protein C inhibits lung inflammation in asthma patients after intrabronchial allergen challenge

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Background: Allergic lung inflammation in asthma is associated with a pulmonary prothrombotic and antifibrinolytic state. Activated Protein C (APC) exerts anticoagulant, pro-fibrinolytic and anti-inflammatory effects that could be beneficial in allergic lung inflammation. House dust mites (HDM) are common allergens and natural carriers of lipopolysaccharide (LPS). LPS exposure is strongly associated with asthma severity.
Aim: To investigate the effect of recombinant human (rh)APC on allergic lung inflammation provoked by intrabronchial HDM and LPS administration in asthma patients.
Methods: A double-blind, randomized, placebo-controlled trial was conducted in 24 HDM allergic asthma patients. Male and female, mild to intermittent asthma patients of 18–45 years of age were eligible for screening. Asthma medications were discontinued 2 weeks before the study. Patients received intravenous rhAPC (24 μg/kg/h, $n = 12$) or placebo ($n = 12$) for 11 h. Four hours after the start of the infusion a first bronchoscopy was performed to challenge one lung segment with saline (serving as control) and a contralateral segment with a combination of HDM (50 biological units) and LPS (75 ng). Eight hours after provocation (1 h after stopping rhAPC or placebo) a second bronchoscopy was conducted to obtain bronchoalveolar lavage fluid (BALF) from challenged segments. The protocol was approved by the institutional Medical Ethics Review Committee and all patients gave written informed consent. Comparisons were done by Wilcoxon or t-test where appropriate.
Results: Patient characteristics were similar at baseline, including lung function tests and biochemical screening. RhAPC treatment resulted in increased plasma APTT values compared to placebo ($P < 0.05$). Cell counts in BALF were increased after HDM+LPS challenge compared to saline ($P = 0.001$) primarily as a consequence of eosinophil and neutrophil influx. RhAPC significantly reduced total cell counts in BALF by 43% ($P < 0.05$ versus placebo), which was due to a decrease in neutrophil influx ($P < 0.05$) and accompanied by an attenuated rise in BALF levels of elastase-1-antitrypsin complexes (indicative of neutrophil degranulation, $P < 0.01$). HDM+LPS challenge caused a procoagulant response in the bronchoalveolar space of both treatment groups, as reflected by increased BALF levels of D-dimer, thrombin-antithrombin complexes (TATc) and plasminogen-activator inhibitor type I (PAI-1). In addition, HDM+LPS exposure resulted in increased protein leakage in BALF, local activation of the complement system (increased BALF C3a, C3bc and C4bc levels) and local release of nucleosomes (all $P < 0.05$ versus saline). Relative to placebo, rhAPC decreased PAI-1 ($P < 0.05$), C3b ($P < 0.05$) and nucleosome ($P < 0.05$) levels in BALF. Remarkably, intravenous rhAPC did not significantly impact on BALF D-dimer or TATc levels.
Conclusions: Bronchial allergen provocation in asthma patients results in local activation of coagulation and multiple inflammatory pathways. Intravenous rhAPC inhibited distinct inflammatory responses in this human model of acute allergic inflammation without influencing coagulation. These data indicate that rhAPC exerts anti-inflammatory effects in the lungs of asthma patients by mechanisms that do not rely on its anticoagulant properties.

OC 08.5

Increased adhesive properties of neutrophils and inflammatory markers in VTE patients with residual vein occlusion and high D-dimer levels

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Background: Venous Thromboembolism (VTE) is a multifactorial disease that affects 1–3:1000 individuals worldwide. The venous thrombus develops via a multicellular process on the surface of the endothelium and presents a laminar structure comprised of layers of platelets, leukocytes, erythrocytes and fibrin. The relationship between inflammation and coagulation is bidirectional, and has been mainly evaluated through protein interactions between pro-inflammatory cytokines and elements of the coagulation cascade. Inflammatory cells such as neutrophils, have not been previously correlated with thrombotic or procoagulant processes.
Aims: To evaluate the adhesive properties of neutrophils, erythrocytes and platelets, as well as the expression of neutrophil adhesion molecules in patients with VTE, correlating them with markers of the systemic inflammatory response, and with the presence of residual vein obstruction (RVO) and higher D-dimer.
Methods: Study group consisted of thirty chronic VTE patients (1–6 years after the acute episode) followed in our outpatient clinic, as well as age, gender and ethnic background-matched healthy individuals. Adhesive properties of neutrophils, erythrocytes and platelets were determined by a static adhesion assay using ligands such as fibrinogen (FB) and fibronectin (FN). The expression of neutrophils adhesion molecules (CD11a, CD11b, CD18) was evaluated by flow cytometry. Levels of inflammatory markers (IL-6, IL-8, TNF-α, PCR) were evaluated by ELISA and nephelometry. RVO was evaluated by Doppler ultrasound.
Results: No significant difference could be observed in the platelets (basal: 16.37% vs. 14.59%, $P = 0.309$; and stimulated with thrombin: 33.45% vs. 26.62%, $P = 0.200$) and erythrocytes adhesion (7.28% vs 7.49%, $P = 0.859$) between chronic VTE patients and healthy individuals. Interestingly, in patients with a higher risk of recurrent VTE (defined by the presence of high levels of D-dimer) and RVO, a significant increase in neutrophils adhesion compared to healthy individuals was observed (24.68% vs. 19.07%, $P < 0.05$). Inflammatory markers (IL-6, IL-8, TNF-α and CRP) were also significantly elevated (2.08 pg/mL vs 0.90 pg/mL, $P = 0.01$; 28.72 pg/mL vs 16.46 pg/mL, $P = 0.02$; 4.50 pg/mL vs 2.11 pg/mL, $P = 0.04$; 0.35 pg/mL vs 0.14 pg/mL, $P = 0.09$, respectively) in this subgroup compared to patients with a standard risk of recurrent VTE. Adhesive properties of neutrophils was correlated with IL-6 ($r = 0.3815$ and $P = 0.0375$) and D-dimer levels ($r = 0.3831$ and $P = 0.0367$). Neutrophils adhesion molecules (CD11a, CD11b and CD18) were not altered in any of the groups.
Conclusions: Our results suggest that, after an acute episode of VTE, patients do not exhibit increased adhesive properties of platelets and erythrocytes. However, neutrophils adhesive properties were increased in patients with higher D-dimer levels and RVO, independently of the expression of neutrophil adhesion molecules. A hypothesis for this increase could be due to alterations in affinity of surface adhesion molecules to their ligands, as a consequence inflammatory processes associated with the hypercoagulability that is characteristic of this disease.

OC 08.6

Does platelet activation mediate pathogenesis of malaria infection?

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Background: Platelet activation by malaria parasites is thought to cause pathology in malaria infection by acting as a bridge for infected red blood cells to adhere to the endothelium leading to blockage of the blood vessels in the brain, and in turn cerebral malaria. This event has most commonly been investigated using the *Plasmodium berghei* ANKA mouse model that causes experimental cerebral malaria (ECM) in C57BL/6 mice. Platelet depletion in mice using an anti-platelet monoclonal antibody can protect against death from ECM, but only when performed within the first few days of infection. This early effect of protection suggests that platelets mediate lethal inflammation in acute malaria infection, rather than act to enhance blood vessel blockage in chronic infection.

Aims: Investigate the role of platelets in mediating inflammation and pathology in experimental cerebral malaria.

Methods and Results: Platelet depletion using monoclonal antibodies involves clearance of antibody-opsonized platelets. Fc triggering by the opsonized platelets and subsequent IL-10 production by macrophages may offer an alternative explanation for the protection offered by platelet depletion in these experiments. IL-10 can act to down-regulate the anti-malarial inflammatory response. Consistent with this hypothesis, following antibody removal of platelets the number of splenic T cells secreting protective IL-10 significantly was increased. Indeed, unlike antibody-mediated platelet removal, inactivation of platelets using aspirin or Plavix© neither protected against the manifestations of ECM nor increased the numbers of IL-10-producing T cells in the spleen.

To ascertain whether platelet presence truly alters the inflammatory landscape of mouse malaria infection we analyzed the effects of platelet depletion on malaria pathogenesis and immune response using a novel non-antibody-mediated method of platelet depletion. In this model, megakaryocyte and platelet-limited ectopic expression of the simian diphtheria toxin (DT) receptor allows for selective and nearly complete platelet depletion following DT administration. Upon DT administration platelets were ablated. However, unlike in antibody-mediated platelet depletion, no protective effect against the development of ECM was observed upon DT-mediated platelet ablation. Similarly, IL-10 production was unaffected.

Conclusions: Our results suggest that studies showing a protective effect in ECM of platelet removal by mAb depletion may in fact reflect an artifact of the method of platelet depletion, and indicate that platelets do not play a critical role in the pathogenesis of ECM.

OC 09 – Novel platelet Inhibitors

OC 09.1

Discovery of novel GPVI receptor antagonists by structure-based repurposing

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Background: Current strategies for managing cardiovascular risk caused by platelet aggregation rely largely on the use of thienopyridines and aspirin. Although these drugs are effective in a large number of people, they have limitations. Increased bleeding risk and drug 'resistance' underpins a drive for new antiplatelet therapeutics. Under pathological conditions, collagen exposure leads to the recruitment and activation of platelets leading to the formation of an occlusive thrombus. Platelet activation by the primary platelet collagen receptor, GPVI, is central to cell activation. Perturbation of collagen-GPVI interactions using GPVI-Fc fusion proteins or single chain antibody fragments (scFvs) can reduce thrombus formation *in vivo* with minimal effects on bleeding, highlighting GPVI as a viable target for antiplatelet therapy.

Aims: We hypothesised that the GPVI-collagen interaction is amenable to perturbation by small molecules and that disruption of this receptor-ligand interaction would be sufficient to reduce inhibit platelet activation and aggregation. Drug repurposing is being fiercely promoted and supported by academic, government and industrial partners to reduce the bottle neck associated with traditional drug discovery approaches and accelerate the bench-to-bedside transition. We therefore used to a structure-based repurposing approach by docking a database of FDA approved drugs into the GPVI receptor ligand binding site.

Methods: For *in silico* docking, the crystal structure of human platelet glycoprotein VI (PDB ID 2gi7) was processed using our own set of parameters and used to dock the NIH Clinical Collection into the GPVI ligand binding site, with an emphasis on the electrostatic environment around Lys 41. The docking was performed using Fred 2.2.5 and a database of ~900 compounds with conformers pre-generated using Omega 2.2.3; molecules were docked and scored with FRED's default consensus scoring. We evaluated biologically the top 20 compounds from the *in silico* screen using both Ca^{2+} release and platelet aggregation assays in washed human platelets.

Results: Fourteen out of the top 20 compounds inhibited CRP-induced Ca^{2+} release by 50% or more. The ability of these compounds to elicit dose-dependent effects on GPVI-mediated Ca^{2+} release and platelet aggregation was further assessed and three compounds were found to inhibit the GPVI receptor in a selective and dose-dependant manner, with IC_{50} values of between 40 mM and 400 nM. Global tyrosine phosphorylation following GPVI activation was reduced by all three compounds as assessed by western blotting; no effect was observed on thrombin- or ADP-induced platelet activation. Our most potent and selective inhibitor, torasemide, inhibited thrombus formation in whole human blood under physiological rates of shear.

Summary/Conclusions: We have identified the first selective small molecule inhibitors of GPVI. By coupling *in silico* screening with medium-throughput approaches to assess platelet function, we were able to quickly and effectively identify novel small molecule inhibitors of the GPVI-collagen interaction. Most importantly, our lead compound, torasemide, can inhibit thrombus formation on collagen under physiological conditions *in vitro*, validating our approach and supporting our hypothesis. These compounds will serve as starting points for the development of new, better antiplatelet agents.

OC 09.2

Parmodulins target the intracellular side of PAR1 to selectively interfere with Gaq but not Ga12/13 signaling and block thrombus formation, but not hemostasis

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Background: Protease-activated receptor-1 (PAR1) serves a critical role in coupling the coagulation cascade to platelet-mediated thrombosis. Small molecules that inhibit at the binding site of the tethered ligand of PAR1 have been developed and recently tested in clinical trials. However, bleeding complications including hemorrhagic stroke have been observed.

Aim: Our goal is to develop allosteric PAR1 modulators that bind outside of the PAR1 ligand binding pocket, stabilizing alternative confor-

mations of the receptor and interfering with procoagulant PAR1 signaling without having significant effects on hemostasis.

Results: We have performed two independent high throughput screens of 16 000 and 300 000 compounds, respectively, to identify molecules that inhibit PAR1-mediated platelet granule release. We now demonstrate that these molecules act by a common mechanism, defining a new class of PAR1 inhibitors termed parmodulins. The first screen identified an alkylated quinolone that inhibits PAR1-mediated platelet activation with an IC50 of 4 μM (parmodulin-1; aka JF5, PNAS, 108:2951). The second screen identified a 1,3-diaminobenzene that inhibits PAR1 with an IC50 of 0.3 μM (parmodulin-2; aka ML161, ACS Med Chem Lett, 3:232). Both compounds failed to inhibit the binding of a radiolabeled PAR1 peptide agonist, [3H]TRAP, to the ligand binding pocket of PAR1 on platelet membranes. In contrast, an orthosteric PAR1 inhibitor, SCH79797, blocked binding of [3H]TRAP to PAR1. To determine whether these allosteric PAR1 modulators act on the extracellular or intracellular face of PAR1, we tested the ability of the antagonists to inhibit signaling in COS7 cells overexpressing different PARs. Both parmodulins and SCH79797 inhibited $[Ca^{2+}]i$ flux induced through PAR1, but not PAR4. We next constructed a PAR1 (amino acids 1–365),4(amino acids 334–385) chimera consisting of the extracellular and transmembrane loops of PAR1 and the C-terminus of PAR4 distal to the DPXXY motif. Parmodulins failed to inhibit activation of the PAR1,4 chimera. In contrast, the orthosteric PAR1 inhibitor SCH79797 inhibited the chimeric receptor. Together with the [3H]TRAP binding studies, these results indicate that parmodulins act at the cytosolic face of PAR1. Parmodulins inhibited PAR1-mediated $[Ca^{2+}]i$ flux, aggregation, and secretion. However, they failed to block PAR1-mediated shape change, signaling through Rho and src kinase in platelets, or inhibit $G_{\alpha 12}$-mediated loss of barrier function elicited by a PAR1 agonist in MDCK cells overexpressing $G_{\alpha 12}$. Thus, parmodulins inhibit signaling downstream of $G_{\alpha q}$, but not that downstream of $G_{\alpha 12/13}$. Although parmodulin-2 failed to inhibit human PAR4, it did inhibit platelet aggregation stimulated through mouse PAR4, which differs from human PAR4 at the cytosolic face. When tested in a murine model of thrombus formation following laser-induced injury of cremaster arterioles, parmodulin-2 (5 mg/kg) reduced platelet accumulation during thrombus formation by 72%. However, parmodulin-2 (5 mg/kg) failed to affect bleeding times in a tail clip assay of hemostasis.

Conclusions: These studies define a class of small molecules that target the intracellular face of PAR1 to selectively modulate downstream signaling and inhibit thrombosis, but not hemostasis. The approach of targeting its intracellular face represents a novel approach in the development of PAR1 antagonists and has broader implications for modulating GPCR function in disease states.

OC 09.3

Antiplatelet and antithrombotic activity of 2NTX-99, a novel molecular entity combining dual thromboxane inhibition with NO-donor properties

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Background: Dual thromboxane (Tx) antagonists (Tx-synthase inhibitors/Tx receptor antagonists) have superior antiplatelet effects and a stronger antithrombotic activity in animal models than aspirin. This is in part due to the increase of intraplatelet cAMP deriving from the enhanced formation of prostaglandin (PG)I2 and PGD2. Nitric oxide (NO) is a naturally occurring vasodilator and platelet inhibitor provided also with antiatherosclerotic properties. NO exerts most of its biological effects by raising intracellular cGMP levels though the stimulation of soluble guanylyl cyclase (sGC). Intraplatelet cGMP and cAMP synergize in inhibiting platelet function. It seems thus logical to combine the pharmacologic effects of dual Tx-antagonists with those of a NO-donor to get a stronger antiplatelet and antithrombotic effect. A potential benefit of NO supply has previously been shown with molecules in which a NO-donor was added to aspirin or to statins. Picotamide, a dual Tx antagonist, has been structurally modified giving a new orally active molecule, 2NTX-99.

Aims: Aim of the present studies was to test whether the combination of a dual Tx inhibitor with a NO-donor provides additive effects on platelet activation *in vitro* and on thrombosis *in vivo*.

Methods: Platelet function was evaluated by platelet adhesion to collagen under flow (3000/s); arachidonic acid (AA)-, U46619- and collagen-induced platelet aggregation; AA-induced TxA2 production; serum TxB2 generation; platelet release of b-TG and intraplatelet cGMP. The antithrombotic effect of 2NTX-99 was evaluated in a model of pulmonary tromboembolism in mice induced by the injection of collagen+ epinephrine, U46619 or hardened rat red blood cells (HRBC).

Reference compounds were picotamide, isosorbide mononitrate (ISMN) or sodium nitroprusside (SNP), pure NO donors, 2NTX-101 (the denitrated metabolite of 2NTX-99).

Results: 2NTX-99 reduces dose-dependently platelet adhesion, similar to ISMN, an effect reversed by ODQ, a sGC-inhibitor while picotamide only marginally reduces platelet adhesion, an effect not modified by ODQ.

2NTX-99 inhibits U46619-induced platelet aggregation (IC50 = 9.8 ± 1.5 μM) significantly more than 2NTX-101 and picotamide (IC50 = 182.4 ± 1.5 μM and 452.8 ± 1.6 μM, respectively); similar results were observed with AA and collagen.

2NTX-99 significantly inhibited the release of b-TG from AA-stimulated platelets with an IC50 of 14.7 ± 0.3 μM; picotamide was also effective (IC50 = 29 ± 0.5 μM) while 2NTX-101 was not. 2NTX-99 inhibited the formation of TxB_2 by AA-stimulated platelets (IC50 = 3.77 ± 0.16 μM) significantly lower than picotamide (IC50 = 21 ± 0.2 μM) and 2NTX-101 (IC50 = 32 ± 0.3 μm). Serum TxB2 was inhibited by 2NTX-99 more efficiently than by picotamide or 2NTX-101 (IC_{50} = 5.42 ± 0.23 μM; 20.23 ± 0.26 μM and 40.7 ± 2.2 ± 2.2 μm, respectively). Finally, intraplatelet cGMP was enhanced dose-dependently by 2NTX-99 (500 μM: +330%) and by SNP (100 μM: +370%), an effect completely abolished by ODQ, while it was not changed by picotamide or 2NTX-101.

Serum TxB2 was dose- and time-dependently inhibited in mice treated with 2NTX-99. 2NTX-99 administration (300 mg/kg) significantly enhanced plasmatic levels of NO2/NO3, the final metabolites of NO (2NTX-99 = 127 ± 5.3 μM vs control 34 ± 3 μM, $P < 0.01$), while picotamide and 2NTX-101 were ineffective. Finally, 2NTX-99 reduced dose-dependently platelet pulmonary thromboembolism induced by all the thrombogenic stimuli used while picotamide was less effective in these models.

Summary/Conclusion: In conclusion, the combination of a dual Tx antagonist with a NO-donor provided enhanced antiplatelet and antithrombotic effects.

OC 09.4

Delayed targeting of CD39 to thrombus with an activated-platelet antibody allows for effective antithrombotic treatment without prolonging bleeding times

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CD39, a NTPDase with strong antithrombotic properties, has previously been shown to be protective in models of stroke, transplantations, pulmonary embolism and myocardial infarction by hydrolyzing/removing the platelet agonist ADP and thus inhibit ADP receptor-mediated platelet activation. However, CD39's high potency comes with an increased bleeding risk, as noted with ADP receptor inhibitors in clinical use. We hypothesized that a delayed targeting CD39 to

thrombi will allow localized enrichment to effective concentrations at the growing thrombus despite a low systemic and thus safe concentration with a low risk of bleeding side effects.
CD39 was recombinantly fused to a single-chain antibody (scFv) specific to the active conformation of GPIIb/IIIa on activated platelets. This targeting was shown by binding to microthrombi on flow adhesion assays. Targeted CD39 (Targ-CD39) was significantly more effective at preventing ADP-induced platelet activation (flow cytometry) and platelet aggregation assays using light transmission aggregometry, than its non-targeted control (NT-CD39, CD39 fused to a non-functional scFv). In a mouse model of ferric chloride-induced carotid artery thrombosis NT-CD39, while being protective against vessel occlusion, showed a significant bleeding risk as demonstrated by mouse tail transection. Targ-CD39 concentrates at the thrombus site, allowing a lower dose to be administrated. A dose ~10× lower is able to prevent vessel occlusion to a similar extend as the high dose NT-CD39. An equimolar dose of NT-CD39 at this low concentration is ineffective at preventing vessel occlusion. The improved efficiency in preventing thrombosis as a result of the targeting also means that the systemic dose of CD39 administered as part of the Targ-CD39 construct is below that causing a bleeding time prolongation in mice.
Interestingly, intravital microscopy reveals that the scFv binding to platelets comes with a delay of up to 40 s, representing the time it takes for GPIIb/IIIa to become activated on adhering platelets. This allows for the build up of a sealing platelet layer before CD39 becomes enriched and thereby blocks platelet aggregation and thrombus formation.
Delayed targeting CD39 to its desired site of action enables administration of such a low concentration as to avoid the previously observed bleeding tendencies while still being a highly effective antithrombotic drug. Thus, enriching CD39 to activated platelets at thrombi prevents the previously limiting bleeding side effects and advances CD39 towards potential clinical use.

OC 09.5

Endocannabinoids limit collagen-induced platelet activation and restrict aggregate formation under flow

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Background: With a worldwide growing number of aging persons, the incidence of cardiovascular disease is rising. As a result, there is an increasing need for alternative antithrombotic strategies. Neurons and hematopoietic cells in the bone marrow express endocannabinoid receptors, through which endogenous endocannabinoid agonists adjust their cellular functions. Intriguingly, circulating platelets also present endocannabinoid receptors on their surface.
Aims: To investigate the effects of endocannabinoids on platelet function.
Methods: We studied the influence of the endogenous endocannabinoid anandamide on platelet aggregation, α-granule release, and activation of glycoprotein IIb/IIIa by light transmission aggregometry and flow cytometry. Platelet spreading and aggregate build up were analyzed by real-time video microscopy under flow. Moreover, we studied the effects of anandamide on calcium mobilization and Syk phosphorylation during platelet activation. Finally, we investigated the influence of *Cannabis sativa* consumption by human volunteers on *ex vivo* platelet activation and thrombus formation under flow.
Results: We here report that anandamide inhibits platelet aggregation and α-granule release in response to both collagen and arachidonic acid, while it does not affect platelet activation through thrombin receptor PAR-1. Anandamide reduces IIb/IIIa activation and inhibits platelet spreading on fibrinogen under flow. Moreover, anandamide disturbs aggregate formation under flow over immobilized collagen at arterial shear rates. While anandamide reduces thrombin-induced calcium mobilization by approximately 50%, it fully prevents calcium mobilization in response to collagen. We investigated this observed additional influence of anandamide on the collagen pathway in further detail. We subsequently found that collagen-induced Syk phosphorylation is perturbed by anandamide, which can be attributed to the inability of Syk to associate to the Fc-gamma receptor. In comparable fashion to these *in vitro* studies, collagen-induced platelet aggregation and aggregate formation under flow *ex vivo* was impaired in whole blood of human donors that had consumed *Cannabis sativa* for 10 consecutive days. During this period, no symptoms of a bleeding tendency were observed in these donors, nor were they previously reported for the use of *Cannabis sativa*. This defective platelet responsiveness was fully restored after a wash-out period of 10 days.
Conclusions: Endocannabinoid receptor agonists have the potential to negatively regulate platelet activation and aggregate formation both *in vitro* and *in vivo*. Understanding the mechanisms involved may lead to the development of new strategies for the treatment of thrombotic disease.

OC 09.6

Extracellular fibrinogen-binding protein (Efb) from *Staphylococcus aureus* inhibits fibrinogen binding, platelet aggregation and whole blood thrombus formation

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Background: *Staphylococcus aureus* releases a variety of extracellular factors that participate in the infection and pathogenesis processes induced by this bacterium. Amongst these factors, Efb has been shown to directly bind to both platelets and fibrinogen, leading to the inhibition of platelet aggregation *in vitro* and the reduction of haemostasis *in vivo*. Although the molecular mechanism underlying the inhibition of platelet aggregation by Efb remains to be elucidated, this bacterial protein represents a promising antiplatelet agent for the development of a novel antithrombotic treatment.
Aims: In this study we aimed to assess the efficacy of Efb as an antiplatelet agent *in vitro* and investigate its mechanism of action.
Method: In order to locate the protein domain responsible for the antiplatelet properties of Efb, we generated different constructs representing the N-terminal and C-terminal regions of the protein. N-terminal and C-terminal constructs and full length Efb were tested for their ability to impair the aggregation of platelets *in vitro* in response to different physiological stimuli, including thrombin, collagen, ADP, and the thromboxane A2 analogue U46619. The aggregation response of platelets resuspended in either plasma or isotonic physiological buffer (washed platelets) was tested by turbidimetry. The same constructs were also analysed for their ability to interfere with washed platelet static adhesion and whole blood thrombus formation under physiological flow conditions. Fibrinogen binding and the activation of the main platelet receptor for fibrinogen integrin αIIbβ3 (or inside-out signalling) were assessed by flow cytometry in the presence of the Efb constructs using a fluorochrome-labelled fibrinogen and an activation-dependent antibody, respectively. Finally, the effect of Efb constructs on integrin αIIbβ3 downstream signalling (also known as outside-in signalling) was investigated by phospho-specific immunoblotting.
Results: Two independent antiplatelet domains were identified corresponding to two previously reported fibrinogen binding domains, one located in the N-terminus and the other in the C-terminus of Efb. Both truncation constructs and full length Efb require the presence of fibrinogen in the platelet suspension to exert their antiaggregatory effect in response to thrombin, ADP and U46619, whilst the responses to different collagen concentrations were not affected. Surprisingly, whole blood thrombus formation onto fibrinogen- or collagen-coated surfaces were equally inhibited by Efb and the truncation mutants, possi-

bly suggesting that fibrinogen-mediated platelet-platelet binding is critical for thrombus formation but not for aggregation in response to collagen. Flow cytometry experiments confirmed that fibrinogen binding induced by physiological stimulation of platelets was inhibited by N-terminal and C-terminal constructs and by full length Efb. Experiments on inside-out and outside-in signalling of integrin αIIbβ3 suggested that the Efb constructs act by interrupting the positive feedback initiated by integrin αIIbβ3 binding of fibrinogen and Src family kinase activation.

Summary/Conclusions: In summary, this project confirmed the antiplatelet properties of Efb, localised the antiplatelet activity in two distinct fibrinogen-binding domains of Efb, and characterised the effect of these domains on platelet functional responses and signalling. Overall, due to its strong antiplatelet activity, Efb is a promising candidate for the development of novel antithrombotic drugs.

OC 10 – Platelet Granule Secretion

OC 10.1

Role of novel SNARE proteins syntaxin 8, VTI1A and VTI1B in regulating platelet secretion and function

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Background: A number of novel roles for platelets beyond traditional haemostasis and thrombosis have emerged in recent years, including inflammation, cancer metastasis and lymphatic and arterial development. Since many cytokines and growth factors are stored in platelets, granule secretion may play a role in these processes. Despite its importance, the exact mechanisms of platelet secretion are unclear, although the ubiquitous SNARE machinery and the v-SNARE VAMP8 in particular are thought to be essential. We sought to identify potential VAMP8 interacting proteins and elucidate their role in platelet secretion. Syntaxin-8 (STX8), VTI1A and VTI1B have all been reported to interact with VAMP8 in other cells. In addition, recent transcript evidence confirmed the presence of all three mRNAs in both human and mouse platelets.

Aims: We aimed to characterize the novel SNAREs STX8, VTI1A and VTI1B in platelets and identify their roles in secretion and, in turn, platelet function.

Methods: STX8, VTI1A and VTI1B global mouse knockouts were used for analysis of platelet function and granule secretion in particular. Parameters measured included ATP secretion, aggregation, P-selectin expression, β-hexosaminidase secretion and *in vitro* thrombus formation.

Results: STX8, VTI1A and VTI1B were all expressed in human and mouse platelets. Several interactions between VAMP8, STX8, VTI1A and VTI1B were also confirmed by co-immunoprecipitation.

The roles of STX8, VTI1A and VTI1B were investigated using platelets from mice deficient in these proteins. Platelet number or mean platelet volume, as well as dense and alpha granule numbers and glycoprotein surface expression levels were unchanged between *Vti1a*−/−, *Vti1b*−/− or *Stx8*−/− and their wild type counterparts.

Dense granule secretion was significantly impaired in response to low (0.05–0.075 U/mL) thrombin concentration in *Stx8*−/− platelets, suggesting an important role for STX8 in platelet secretion. In contrast, no difference in α-granule or lysosome secretion was observed, supporting the proposed SNARE selectivity for different granules. Interestingly, despite VTI1B interaction with VAMP8 in resting and activated platelets, there was no difference in any of the granule secretion in *Vti1b*−/− or *Vti1a*−/− platelets. This may reflect existence of different SNARE complexes in platelets or functional redundancy between SNAREs.

Stx8−/− platelets also showed a defect in aggregation in response to thrombin. This defect was rescued by exogenous ADP in a $P2Y_{12}$-dependent manner, suggesting that the reduced aggregation was due to the reduced dense granule secretion observed.

Vti1a−/− and *Vti1b*−/− platelets aggregated to thrombin and formed thrombi *in vitro* normally, further suggesting that different SNAREs play different roles in platelet activation.

Finally, the reduction in dense granule secretion in *Stx8*−/− platelets was also reversed by co-stimulation with exogenous ADP. This was also dependent on $P2Y_{12}$ signalling, suggesting complex interactions between platelet signalling and SNARE-mediated secretion.

Conclusions: Importantly, STX8 regulates platelet dense granule (but not α-granule or lysosome) secretion and in turn aggregation in response to low concentrations of thrombin. This defect can be overcome by exogenous ADP indicating the synergistic relationship between a $P2Y_{12}$ signalling pathway and SNARE machinery in platelets, and reveals further the molecular mechanism by which granule secretion in platelets is tightly and specifically controlled.

OC 10.2

Alpha-granule proteins are localized in a 'cap' on the surface of procoagulant platelets to promote their incorporation into aggregates

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Background: Strong platelet activation leads to the formation of a procoagulant subpopulation that is often called 'coated platelets'. It is characterized by increased phosphatidylserine (PS) expression, high α-granule protein retention on their membranes, and inactivated integrin $\alpha_{IIb}\beta_3$. Because of this lack of active integrins, it is unclear how these platelets can get incorporated into thrombi and fulfil their procogulant function.

Objective: We investigated the structure, function and formation mechanisms of the α-granule protein 'coat' of the PS-expressing platelets.

Methods: Platelets were isolated from blood of healthy donors or patients with bleeding disorders (Glanzmann's thrombasthenia, Bernard-Soulier syndrome, dysfibrinogenemia, factor XIII deficiency, kindlin 3 deficiency) using centrifugation and gel filtration. They were activated with different agonists (in the presence of various inhibitors where indicated), labeled with fluorescently labeled antibodies or annexin V, and analyzed with flow cytometry and Nipkow disc confocal microscopy. In addition to isolated platelets, whole blood thrombi were formed on collagen in parallel-platelet flow chambers at venous and arterial shear rates, and different platelet subpopulations in thrombi were characterized with confocal microscopy.

Results: Confocal microscopy revealed that fibrin(ogen) and thrombospondin colocalized as a 'cap', a single patch on the PS-positive platelet surface, with thrombospondin. The surface fibrin(ogen) was strongly decreased in the presence of fibrin polymerization inhibitor GPRP, and also in platelets from a patient with dysfibrinogenemia involving a polymerization defect. In contrast, a fibrinogen-cleaving protease ancistron increased the amount of fibrin(ogen) and thrombospondin retention on the surface of the PS-positive platelets stimulated with collagen-related peptide. Transglutaminases also induced the cap formation, but platelets from patients with factor XIII deficiency had normal fibrin(ogen) retention, and a pan-transglutaminase inhibitor T101 only mildly decreased it. In aggregates, the fibrin(ogen) cap was located at the point of attachment of the procoagulant platelets. Without this 'cap', their ability to aggregate was drastically decreased. Because of this one-sidedness of their aggregation, procoagulant platelets were predominantly distributed in a necklace-like fashion along the outer face of both stirring-induced aggregates and whole blood thrombi formed under venous and arterial shear conditions.

Conclusions: These data show that the fibrin(ogen)-covered 'cap', predominantly formed as a result of fibrin polymerization, is a critical mechanism that allows 'coated' (or rather 'capped') platelets to become incorporated into thrombi despite their lack of active integrins.

OC 10.3
Functional regulation of platelet membrane systems by the F-BAR protein PACSIN2

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Proteins of the Bin-Amphiphysin-Rvs (BAR) and Fes-CIP4 homology BAR (F-BAR) families bind and deform membranes, promoting tubular invaginations that are reminiscent of the platelet open canalicular system (OCS) and megakaryocyte (MK) demarcation membrane system. Here we investigated the role of the F-BAR protein PACSIN2 in platelets and MKs, as PACSIN2 is the only F-BAR protein known to bind to the cytoskeletal and scaffold protein filamin A (FlnA), deficiency of which results in macrothrombocytopenia (Falet et al. J Exp Med 2010; Jurak Begonja et al. Blood 2011). Human and mouse platelets contained 1 μM PACSIN2, but not its paralogs PACSIN1 or PACSIN3. Spinning disk laser fluorescence confocal microscopy revealed a specific association between PACSIN2 and tubular membrane structures in human and mouse platelets. However, PACSIN2 did not co-localize with secretion organelles or dense tubular system markers, i.e. CD62P, CD63, LAMP1, and SERCA3. Endogenous PACSIN2 and over-expressed EGFP-PACSIN2 associated with tubular membrane structures in mouse bone marrow-derived MKs, reminiscent of the DMS. Immunoprecipitation of PACSIN2 from human platelet lysates pulled down FlnA. The interaction required FlnA immunoglobulin-like repeat 20 and an unstructured stretch at the tip of PACSIN2F-BAR domain, and was functionally relevant, as FlnA potentiated the membrane tubulation activity of PACSIN2F-BAR domain *in vitro*. Further, PACSIN2 localization was disrupted in FlnA-null platelets and MKs. PACSIN2-null mice had a mild thrombocytopenia, enlarged spleens, and increased spleen and bone marrow megakaryopoiesis. PACSIN2-null mice had a bleeding disorder, as evidenced by tail bleeding time. PACSIN2-null platelets had an abnormal morphology, with more and narrower OCS channels, compared to WT platelets. Further, PACSIN2-null platelets had increased activation of their collagen receptor, the integrin β1, in response to thrombin. Together, the data indicate that PACSIN2 orchestrates the intracellular membrane architecture of platelets and MKs in cooperation with FlnA, and regulate the function the integrin β1 in platelets.

OC 10.4
Identification of secretion-related PKC substrates in platelets by a proteomic and pharmacological approach

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Background: Platelet cell surface receptors induce intracellular signalling events leading to the activation of protein kinase C (PKC), the major regulator of platelet function. Upon stimulation, platelets release a wide variety of mediators, thereby promoting thrombus formation. Of the PKC family members expressed in platelets, the conventional isoforms PKCα and PKCβ have a positive regulatory role in the secretion of granules, and genetic deletion of these isoforms in mice has been shown to result in impaired secretion and thrombosis. The underlying molecular pathway from PKC to secretion, however, is poorly understood.
Aims: We aimed to identify substrates of conventional PKC isoforms in human platelets which are potentially involved in platelet secretion and thrombus formation.
Methods: The kinetics of dense and α-granule secretion were assessed by luminometry and flow cytometry, respectively. We performed immunoprecipitations with a phosphoserine PKC substrate antibody followed by mass spectrometry to identify proteins phosphorylated by PKC. Candidate proteins were validated as PKC substrates by western blotting. Protein function was investigated using a pharmacological approach. Platelet aggregation, granule secretion, and integrin αIIbβ3 activation were assessed.
Results: There was a clear difference between the kinetics of dense and α-granule release, peaking at 1 and 10 min after platelet activation, respectively. Phosphoserine PKC substrate phosphorylation was maximal after 2 min and was markedly impaired by the broad-spectrum PKC inhibitor BIM-IX. One of the candidate proteins identified by mass spectrometry as a potential PKC substrate was cytohesin-2. We showed that cytohesin-2 phosphorylation was induced by platelet stimulation and blocked by BIM-IX. The cytohesin inhibitor SecinH3 significantly enhanced platelet dense granule secretion and aggregation, but did not affect α-granule secretion or integrin αIIbβ3 activation.
Summary/Conclusion: The proteomics-based approach employed here is an effective strategy for identifying PKC substrates in platelets. It will serve as the basis for further molecular studies to investigate the functional significance of the phosphorylation events downstream of PKC. We identified cytohesin-2 as a PKC substrate in platelets and our results suggest it to be a negative regulator of dense granule secretion.

OC 10.5
Platelet granule release patterns under flow

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Background: Recent studies have suggested that alpha granules are heterogeneous both in morphology and protein content and this heterogeneity may give rise to differential release kinetics. A strictly timed release of granule contents is important for the platelet contribution in modulating haemostasis and inflammatory responses. The exact mechanism by which platelets regulate the fine-tuning of alpha granule release is not known.
Method: To better understand the mechanism of potential differential secretory patterns, particularly under flow, we have used live cell imaging and electron microscopy tomography to visualize granule release profiles.
Results: We present data on alpha granule dynamics in flow in relation to their secretory behaviour. Platelets were allowed to adhere to fibrinogen and von Willebrand Factor (VWF) under low shear conditions (300/s), and granule dynamics were monitored using time laps video microscopy. While a subpopulation of the alpha granules are mobile and move continuously along microtubules in the cell's periphery, others remain stationary located in the central region. A subset of VWF-containing granules was selectively targeted towards downstream exit sites where they fuse with the platelet membrane and release VWF as strings in the direction of the flow. Furthermore immunoelectron microscopy cross-sections of release profiles show that CD63 positive exosomes are secreted both at basolateral and luminal sites. These observations indicate that cargo release occurs at multiple sites and is probably under control of shear forces. Shear forces also induce the formation of membrane tethers. Surprisingly, platelet granules are able to move into extending membrane tethers thereby contributing to a spatial segregation of alpha granule secretory events. Electron tomography analysis of platelet release profiles revealed fusion of tubular alpha granules with the plasma membrane, but no fusion of dense tubular system membranes.
Conclusion: Together, our observations suggest that shear-driven granule motion and differential release may be tightly connected. We are currently developing ways to capture short-lived fusion events and cargo release patterns under physiological flow conditions at high spatial resolution.

OC 10.6

Platelet dense granule secretion is required for infarct progression, but not for intracranial haemostasis in the ischaemic brain

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Background: Ischaemic stroke is a frequent and serious disease with limited treatment options. Previous studies indicated that inhibition of early steps of platelet adhesion may offer a novel and safe treatment strategy in acute stroke. In contrast, inhibition of platelet aggregation by GPIIb/IIIa blockade did not reduce infarct size but induced severe intracerebral haemorrhage. The pathophysiological role of amplification mechanisms during platelet activation in the setting of acute ischaemic stroke has not been determined.

Aims: The aim of this study was to determine the functional significance of platelet dense granule secretion for infarct progression and intracerebral haemostasis in ischaemic stroke.

Methods: We generated mice deficient in Munc13-4 (*Unc13d−/−* mice) and assessed the function of their platelets by flow cytometry, aggregometry, flow adhesion assays and biochemical methods. Further, these mice were subjected to models of arterial thrombosis and the transient middle cerebral artery occlusion (tMCAO) of ischaemic stroke.

Results: In line with reports on *Unc13dJinx* mice, *Unc13d−/−* mice were unable to secrete dense granule content and displayed infinite tail bleeding times. Further, ablated dense granule secretion resulted in severely defective thrombus occlusion in two *in vivo* models of arterial thrombosis. Remarkably, *Unc13d−/−* mice displayed reduced infarct sizes and significantly better neurological outcome at 24 h after induction of tMCAO and this protection was not associated with increased intracranial bleeding risk.

Summary/Conclusion: Collectively, our data argues for a critical role of dense granule secretion in infarct progression, but not for safeguarding the brain vasculature following focal cerebral ischaemia. This indicates that interference with amplification processes of platelet activation might be an effective, yet safe therapeutic strategy in ischaemic stroke.

OC 11 – Platelet Ion Channels and Protein Kinases

OC 11.1

Chloride channels regulate platelet calcium signalling and procoagulant activity

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Background and Aims: Thrombin generation is central to arterial thrombosis. Activated platelets expose a phosphatidylserine (PS)-containing procoagulant surface that accelerates thrombin generation at sites of vascular damage. Plasma membrane phosphatidylserine is normally restricted to the inner leaflet of resting platelets. However, a sustained increase in intracellular calcium concentration during platelet activation triggers phospholipid scrambling and PS exposure in the outer leaflet.

The molecular identity of the calcium-dependent scramblase is not known. Mutations in TMEM16F have been found in patients with Scott syndrome, whose platelets do not expose PS and are poorly procoagulant. However, exactly what role TMEM16F has in phospholipid scrambling is unclear. Since related TMEM16 family members form chloride channels, we tested whether it was possible for a chloride channel to regulate platelet PS exposure.

Methods: Platelet phosphatidylserine exposure was determined by annexin V binding and flow cytometry. Intracellular calcium signalling was monitored in fura-2-loaded platelets. Changes in membrane potential were detected by $DiBAC_3(4)$ fluorescence.

Results: Platelet phosphatidylserine exposure, measured by annexin V binding in response to thrombin plus collagen-related peptide (C+T), was inhibited by about 50% by a range of chloride channel blockers, suggesting a role for chloride channels. C+T-induced phosphatidylserine exposure was similarly reduced in chloride-free saline. Moreover, chloride channel blockers reduced platelet-dependent thrombin generation. However, phosphatidylserine exposure induced by ionomycin, a calcium ionophore, was unaffected by chloride channel block, suggesting that chloride channels are not involved in responding to increased intracellular calcium, but may perhaps instead regulate agonist-induced calcium signalling. Consistent with this idea, chloride channel blockers substantially reduced C+T-induced calcium signalling. This was due to inhibition of calcium entry but not intracellular calcium release.

To understand how chloride channels regulate platelet calcium signalling, we investigated their role in platelet membrane potential. Using a membrane potential-sensitive fluorescent dye, we found that C+T induced hyperpolarization within 1 min. This was completely blocked by chloride channel block or by absence of extracellular chloride, and instead the platelets depolarized. We propose that chloride entry through chloride channels induces platelet hyperpolarization. This acts to maintain the driving force for calcium entry through plasma membrane calcium channels, leading to a sustained increase in intracellular calcium concentration. This is necessary to trigger full PS exposure and promote thrombin generation. In the absence of chloride-mediated hyperpolarization, agonist stimulation leads to platelet membrane depolarization. This reduces further calcium entry and limits platelet calcium signalling and phosphatidylserine exposure.

Summary/Conclusion: We have demonstrated a novel role for chloride channels in regulating platelet phosphatidylserine exposure through control of membrane potential and calcium entry. Importantly, our data suggest that a chloride channel is unlikely to be the scramblase itself, but instead a major regulator of platelet PS exposure through its control of platelet calcium signalling.

OC 11.2

Transient receptor potential channels (TRPCs) contribute to platelet phosphatidylserine exposure

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Background and Aims: Phosphatidylserine (PS) exposure on platelet surfaces is essential for generation of thrombin in thrombosis. The mechanisms underlying this process remain elusive, although sustained cytosolic Ca^{2+} signalling forms one of the major regulatory pathways. Platelets express at least two major Ca^{2+} entry pathways: store-operated Ca^{2+} entry (SOCE) that is activated by intracellular Ca^{2+} store depletion, and a less well-defined 'store-independent' Ca^{2+} entry pathway, which can be activated by DAG analogues. We aimed to define the nature of the store-independent pathway and to understand how these different Ca^{2+} entry pathways contribute to PS exposure.

Results: Using gene knockout mouse and pharmacological approaches we show the store-independent pathway involves Na^+ entry through TRPC6 and TRPC3 that is coupled to Ca^{2+} influx via reverse-mode Na^+/Ca^{2+} exchange (NCX). This Ca^{2+} entry pathway is stimulated in an agonist-specific manner by thrombin but not by collagen-related peptide (CRP), although it appears to make little contribution to Ca^{2+} signalling or PS exposure unless SOCE is blocked. However,

when thrombin and CRP are combined, the TRPC6/3-NCX pathway synergises with SOCE to promote sustained Ca^{2+} signalling and PS exposure.

Conclusion: We propose that SOCE and TRPC6/3-NCX-dependent store-independent calcium entry provide distinct pathways that may be critical to allow appropriate PS exposure in a stimulus-specific, context-dependent manner. The requirement for stimulation of thrombin and collagen pathways suggests that the TRPC6/3-NCX pathway may be part of a coincidence detection system.

OC 11.3

The Scott syndrome protein anoctamin 6 (TMEM16F) regulates multiple cell death responses including membrane phospholipid scrambling in platelets

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Background: Anoctamins (Ano) are a family of predicted transmembrane proteins that mediate ion conductances. Ano1 (TMEM16A) has been proposed to act as Ca^{2+}-dependent Cl^- channel. Recently, Ano6 (TMEM16F) was identified as an essential component for Ca^{2+}-dependent phospholipid scrambling and hence, of phosphatidylserine exposure in non-vital platelets. Dysfunctional mutations in TMEM16F have been linked to the Scott syndrome, a rare bleeding disorder characterized by impaired Ca^{2+}-dependent phospholipid scrambling of platelets and other blood cells. Murine Ano6 has been proposed to act as a Ca^{2+} channel, and was found to generate a Ca^{2+}-dependent Cl^- channel in murine macrophages.

Aim: Establish the phenotype of blood cells from anoctamin-deficient mice and of platelets from a Scott patient, compound heterozygous for two TMEM16F mutations.

Methods: Ano6-deficient mice were generated by homologous recombination using the AW-0382 stem cell clone or the PAC-379 clone (PAC library RPCL21). Ano1-deficient mice were described before. Platelets and erythrocytes were studied from wildtype and heterozygously or homozygously deficient mice. Immortalized B-cell lines were used from two Scott syndrome patients ($Scott^{UK}$ and $Scott^{USA}$). Platelets and red cells were examined from $Scott^{UK}$.

Results: Ano1-deficient mice were vital, and their platelets were without phenotype. In two independent laboratories, breeding of heterozygous Ano6 animals, generated from AW-0382 stem cells, failed to produce homozygous mutant offspring, due to early embryonic lethality. Some of the unborn embryos showed major bleeding or exencephaly. Heterozygous platelets were without phenotype, in particular showed unchanged Ca^{2+}-dependent (ionomycin or convulxin/thrombin) phospholipid scrambling. Homozygous Ano6-deficient mice, generated from the PAC-379 clone, were born at 30% of the expected ratio. Their platelets were greatly reduced in Ca^{2+}-dependent phospholipid scrambling, bleb formation and integrin closure, but were moderately reduced in apoptosis-dependent (ABT-737) phospholipid scrambling. Their red cells were also impaired in Ca^{2+}-dependent phospholipid scrambling. While heterozygous platelets were normal, heterozygous red cells demonstrated reduced scrambling activity.

Platelets from $Scott^{UK}$ were impaired in Ca^{2+}-dependent phospholipid scrambling, bleb formation and integrin closure. Apoptosis-dependent scrambling was far less affected. Similarly, Ca^{2+}-dependent phospholipid scrambling was absent in $Scott^{UK}$ red cells, and in $Scott^{UK}$ and $Scott^{USA}$ B-cells, while apoptosis-dependent scrambling was normal.

At various voltage protocols, Ca^{2+}-dependent chloride and cation conductances were abolished in $Scott^{UK}$ and $Scott^{USA}$ B-cells, suggesting an important role for Ano6 in Ca^{2+}-dependent chloride and cation channel activities. Apoptosis (ABT-737) did not provoke such conductances. In control B-cells, chloride currents were abrogated by a range of specific chloride channel inhibitors. However, neither these inhibitors nor depletion of extracellular chloride affected Ca^{2+}-dependent phospholipid scrambling in B-cells or platelets.

Summary/Conclusion: In mouse, homozygous Ano6 deficiency is partly lethal, but platelets from the surviving mice phenocopy the platelets from Scott syndrome patients. In blood cells from mice and patients, Ano6 appears to be involved in a range of Ca^{2+}-dependent cell death responses, namely phospholipid scrambling, membrane bleb formation, integrin closure and chloride/cation channel currents. We conclude that Ano6 is an essential regulatory protein controlling these but not in apoptotic cell responses.

OC 11.4

Ste20 kinase MINK is involved in platelet function and thrombus formation

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Background: Ste20 kinase MINK is involved in many important cellular processes such as cell growth, rearrangement of cytoskeleton, and cell motility. However, the role of MINK in platelets is still unclear.

Aims: To determine the role of MINK in platelet activation and thrombus formation.

Methods: *In vivo*, the tail-bleeding assay and ferric chloride-induced mesenteric arteriole thrombosis model were used to exam the role of MINK in hemostasis and thrombosis. *In vitro*, the ability of platelet adhesion and spreading were tested by a microfluidic perfusion assay and spreading assay, respectively. Platelet aggregation and ATP secretion were monitored by aggregometry. We also employed immunoblotting to detect signaling pathway.

Results: In tail-bleeding assay, MINK(−/−) mice exhibited longer bleeding time than wild-type mice (589.0 ± 68.1 s vs 406.6 ± 70.5 s). In a ferric chloride-induced mesenteric arteriole thrombosis model, vessel occlusion time were two times longer in MINK(−/−) mice than in wild-type mice. In an *in vitro* microfluidic whole-blood perfusion assay, thrombus formation on a collagen matrix under arterial shear conditions was significantly reduced for MINK(−/−) platelets. Moreover, MINK(−/−) platelets demonstrated an impaired aggregation and secretion in response to low doses of thrombin *in vitro*. Furthermore, platelet spreading on fibrinogen was largely hampered in MINK (−/−) platelets, the difference could be attributed to impaired ADP secretion upon MINK deficiency. Signaling events associated with MINK appeared to be involving protein kinase C, p38, and ERK2. Hence, MINK may be an important signaling molecule that mediates MAPK signaling and participates in platelet biology and thrombus formation.

Conclusions: MINK is involved in platelet activation an thrombus formation *in vivo* and *in vitro*.

OC 11.5

Characterisation of platelets lacking the p110a PI3K isoform and its role in primer mediated enhancement of platelet activation

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Platelets are found to be hyperactive in a range of diseases such as obesity and type 2 diabetes mellitus. It is thought that one contributing factor to this is the priming of platelets by increased levels of circulating growth factors. These growth factors can't induce platelet activation alone but are able to enhance the actions of physiological platelet

stimuli in a PI3K-dependent manner. We have previously demonstrated that IGF-1-mediated enhancement of platelet activation is blocked in the presence of the p110α PI3K isoform inhibitor PIK-75. Therefore targeting p110α could be a potential strategy for reducing platelet hyperactivity.

To address the role of p110α in platelet function and in primer-mediated enhancement of platelet function, we used the *Pf4-Cre* system to generate megakaryocytic lineage specific p110α knock-out mice. We confirmed that p110α was absent in platelets from p110α$^{-/-}$ mice, whereas expression levels of p110β, p110δ and p110γ were normal. In addition, we found no changes in association of p110β and p110δ with p85 or in expression of membrane receptors. The loss of p110α had no significant effect on PAR-4 and collagen-related peptide-induced aggregation, integrin activation, a-granule secretion and Akt phosphorylation. This suggests that p110α plays no or a redundant role in platelet activation downstream of PAR-4 and GPVI.

Similar to findings in human platelets, pre-treatment of mouse platelets with the primers TPO, IGF-1 or IGF-2 resulted in enhanced PAR-4-mediated integrin activation and platelet aggregation. Surprisingly, the potentiatory effect of these primers on platelets lacking p110α was comparable to that of wild-type platelets. Furthermore TPO and IGF-2-induced Akt phosphorylation was still present in p110α$^{-/-}$ platelets, whereas IGF-1-mediated Akt phosphorylation was slightly reduced. As there may be redundancy between the p110α and p110β isoforms, we also treated platelets with the p110β inhibitor TGX-221. However, TGX-221 only partially inhibited TPO enhanced integrin activation in p110α$^{+/+}$ and p110α$^{-/-}$ platelets. In contrast, the pan-PI3k inhibitor wortmannin blocked the effect of primers in wild-type and p110α$^{-/-}$ platelets.

In summary, we demonstrate that p110α plays a minor or redundant role in platelet activation by PAR-4 and GPVI. In addition, we rule out that p110α is the sole PI3K isoform involved in primer-mediated enhancement of platelet activation, suggesting that redundancy between PI3K isoforms is an important mechanism by which platelet function is controlled.

OC 11.6

The Class II PI3K, PI3K-C2a, regulates internal membrane reserves and biomechanical integrin aIIbβ3 adhesive function in platelets

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Background: Phosphoinositide 3-kinases (PI3Ks) are a family of eight broadly-expressed enzymes important for signalling in a range of cells, including platelets. Much is known regarding the function of Class I PI3Ks, and p110β inhibitors are in clinical development as anti-platelet agents. In contrast, little is known about the function of Class II PI3Ks.

Aims: To examine the role of Class II PI3Ks in platelet function.

Methods and Results: We detected expression of two of the three Class II PI3Ks – PI3K-C2α and PI3K-C2β, but not PI3K-C2γ – in human and mouse platelets *via* Western blot. Platelets from PI3K-C2β$^{-/-}$ mice functioned normally in all assays examined. We generated PI3K-C2α$^{-/-}$ mice, which died *in utero* prior to haematopoiesis, preventing analysis of platelet function. To overcome this, we generated a novel RNAi-based *in vivo* mouse model in which inducible expression of a shRNA against PI3K-C2α in adult mice reduced protein expression in platelets to < 5% of normal levels. These PI3K-C2α-deficient mice exhibited significantly impaired haemostasis (increased tail bleeding time) and thrombosis (unstable occlusion of electrically injured carotid artery) in *in vivo* models. These phenotypes were reproduced in wild-type mice reconstituted with PI3K-C2α-deficient haematopoietic cells, strongly suggesting they resulted from defective platelet function. Despite this, agonist-induced platelet activation and accumulation of the PI3K lipid products (PtdIns(3)P and PtdIns(3,4)P_2) were normal in PI3K-C2α and combined PI3K-C2α/β deficient platelets, suggesting PI3K-C2α does not play an acute signalling role in platelets. Strikingly however, transmission electron microscopy revealed that platelets from PI3K-C2α-deficient mice had a 37% increase in size and altered distribution of the open canalicular membrane system. Detailed analysis of the adhesion behaviour of PI3K-C2α-deficient platelets revealed that haemodynamic shear stress induced significant changes in the structure of the open canalicular system that was associated with enhanced biomechanical adhesive function of the major platelet integrin $\alpha_{IIb}\beta_3$, and led to increased platelet-fibrinogen interactions and accelerated thrombus growth. These dysregulated thrombi were highly unstable, leading to thromboembolism from an immobilized collagen substrate.

Conclusions: Our studies define a novel role for the Class II PI3K, PI3K-C2α, in the haemostatic and thrombotic function of mouse platelets and suggest that PI3K-C2α regulates the function of cell surface platelet adhesion receptors by maintaining plasma membrane structure and/or function. Further, these studies provide the first example of a signalling enzyme regulating plasma membrane reserve and linked to biomechanical cell adhesion.

OC 12 – Recurrent Venous Thrombosis – I

OC 12.1

Secondary prevention of recurrent venous thromboembolism: systematic review and meta-analysis of bleeding complications among patients receiving anticoagulation

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Background: The risk of recurrent venous thromboembolism (VTE) in patients with unprovoked VTE following the completion of an initial 3–6 month course of anticoagulation is approximately 10% in the subsequent year. In non-high bleeding risk patients, the American College of Chest Physicians recommends long-term anticoagulation in unprovoked VTE patients. As a result, clinicians are faced with the challenge of balancing risks and benefits of prolonged anticoagulation. Warfarin, aspirin and the novel oral anticoagulants (rivaroxaban, apixaban, dabigatran, and ximelagatran) have all been evaluated for the secondary prevention of recurrent VTE. Herein, we meta-analysed the safety profiles of anti-thrombotic therapy options in VTE patients.

Aim: To report the major bleeding and fatal bleeding rates in patients receiving oral anticoagulants (warfarin, direct Xa inhibitors and direct thrombin inhibitors) or ASA, and placebo/observation for the secondary prevention of recurrent VTE.

Methods: A systematic literature search was conducted to identify potential studies on MEDLINE, Embase, and Cochrane Central Register of Controlled Trials. Pooled proportions and their associated 95% confidence intervals (CI) for major bleeding and fatal bleeding were calculated.

Results: Fourteen randomized controlled trials (warfarin = 7; dabigatran = 2; rivaroxaban = 1; apixaban = 1; ximelagatran = 1; ASA = 2) met all the inclusion criteria and 11, 922 patients were included in our analysis. The rates of major and fatal bleeding among patients randomized to observation or placebo was 0.39%/ 100 patient-years (95% CI, 0.24–0.58; I^2 = 0%) and 0.12%/ 100 patient-years (95% CI, 0.04–0.23; I^2 = 0%), respectively. The rates of major bleeding and fatal bleeding for warfarin (target INR 2-3) were higher than placebo at 1.65%/ 100 patient-years (95% CI, 1.07–2.36; I^2 = 50.7%) and 0.087%/ 100 patient-years (95% CI, 0.024–0.19; I^2 = 0%) respectively. The rates of major and fatal bleeding for ASA were comparable to

placebo with a rate of 0.58%/100 patient-years (95% CI, 0.28–1.0) and 0% (95% CI, 0–0.29) respectively. Similarly, patients receiving a novel oral anticoagulant (oral direct Xa or thrombin inhibitor) had rates of major and fatal bleeding comparable to placebo. Patients receiving direct Xa inhibitors (apixaban or rivaroxaban) had major and fatal bleeding rates of 0.39%/ 100 patient-years (95% CI, 0.097–0.88; I^2 = 51.5%) and 0% (95% CI, 0–0.16; I^2 = 0%) respectively, whereas patients receiving a direct thrombin inhibitor (dabigatran or ximelagatran) had rates of 0.67%/ 100 patient-years (95% CI, 0.42–0.98; I^2 = 0%) and 0% (95% CI, 0–0.096; I^2 = 0%) for major and fatal bleeding episodes, respectively.

Summary/Conclusions: Rates of major and fatal bleeding among VTE patients receiving ASA or novel anticoagulant therapy for secondary prevention of VTE are low. Warfarin is associated with a higher risk of major bleeding than placebo. However, it appears that bleeding rates for novel anticoagulants and ASA are no different than placebo.

OC 12.2

Risk profile and clinical outcome of symptomatic isolated subsegmental pulmonary embolism

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Background: Improved imaging techniques have led to an increased detection of subsegmental pulmonary embolism (SSPE). The clinical significance of SSPE is often doubted by clinicians and remains to be determined.

Aims: To investigate whether SSPE forms a distinct subset of thromboembolic disease compared to more proximally located pulmonary embolism (PE).

Methods: In this, *post-hoc* analysis of two prospective outcome studies, 3703 consecutive patients with clinically suspected PE were included. Patients with SSPE were contrasted to patients with more proximal PE, and to those in whom PE was ruled out, as regards the prevalence of thromboembolic risk factors and the 3-month risk of recurrent venous thrombembolism (VTE), bleeding complications, and mortality.

Results: PE was confirmed in 748 patients, of whom 116 (16%) had SSPE; in 2995 patients PE was ruled out. No differences were seen in the prevalence of VTE risk factors, the 3-month cumulative risk of recurrent VTE (3.6% vs 2.5%; P = 0.42) and mortality (10.7% vs 6.5%; P = 0.17) between patients with SSPE and those with segmental or more proximal PE. Multivariate analyses demonstrated age > 60 years (OR 1.6; 95%CI: 1.07–2.42), recent surgery (OR 2.3; 1.23–4.20), estrogen use (OR 2.5; 1.34–4.81) and male gender (OR 2.1; 1.38–3.32) to be independent predictors for SSPE, when compared to patients without PE.

Conclusions: In contrast to common belief that SSPE represents a clinically less severe subset of PE, this study shows that patients with SSPE mimic those with more proximally located PE as regards their risk profile and clinical outcome.

OC 12.3

The REVERSE I and II studies: impact of using. Men continue and HERDOO2 clinical decision rule to guide anticoagulant therapy in patients with first unprovoked venous thromboembolism

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Background: Whether or not to continue oral anticoagulants (OAC) after short-term therapy for unprovoked VTE is one of the most important unanswered questions in the clinical care of patients with venous thromboembolism (VTE). In REVERSE I (RI), a multinational prospective cohort study, we developed a clinical decision rule (CDR) to identify low risk patients with unprovoked VTE who could safely discontinue OAC after completing 5–7 months of OAC: 'MEN continue and HERDOO2'. This CDR labels all men as high risk of recurrent VTE as well as women with 2 of the following: (i) Hyperpigmentation, Edema or Redness (HER) on exam in either leg, (ii) VIDAS D-Dimer>250 ug/L, (iii) Obesity-BMI > 30 or (iv) Older age > 65. We are validating the rule by applying the CDR in a second ongoing multi-national cohort study (REVERSE II (RII)) where low risk patients discontinue anticoagulants and high risk patients are recommended to continue anticoagulants (but may discontinue).

Aims: We sought to compare the overall 1-yr risk of recurrent VTE in the REVERSE I patients (all discontinued OAC at 5–7 months) to patients enrolled in REVERSE II who completed 1-yr follow-up prior to August 2012 (continuation or discontinuation of OAC informed by 'MEN continue and HERDOO2').

Methods: REVERSE I and II are multi-national prospective cohort studies of first unprovoked VTE patients. Symptomatic suspected major recurrent VTE (proximal DVT and/or PE) during 1-yr follow-up off of OAC was investigated with reference to baseline imaging and then independently adjudicated and compared between studies.

Results: Baseline characteristics of RI and RII patients were comparable: RI: 664 enrolled participants, mean age of 53 (range 17–95) and 49% were female; RII: 673 included in this analysis, mean age of 55 (range 18–93) and 49% female. In RI, 183 (28%) participants were classified as low risk (all discontinued anticoagulants) and 481 (72%) were classified as high risk (all discontinued anticoagulants). In RII, 177 (26%) participants were classified as low risk (156/177, (88.1%) discontinued anticoagulants) and 496 (74%) were classified as high risk (436/496, 87.9% continued anticoagulants). In RI, out of 239 suspected VTE, 64 were adjudicated as recurrent VTE during 1 year follow-up, resulting in an annual risk of recurrent VTE of 10.7% (95% CI: 8.2–13.6%). In RII, out of 81 suspected VTE, 17 were adjudicated as recurrent VTE during 1 year follow-up; an annual risk of recurrent VTE of 2.6% (95% CI:1.5–4.1%). Hence, using the CDR in RII resulted in an 8.1% absolute reduction in annual risk of recurrent VTE compared to RI. This 8.1% absolute recurrent VTE reduction was at a cost of five major bleeds in RII patients during 1 year follow-up (i.e. 0.8% (95% CI: 0.2–1.8%). It is also notable that use of the CDR resulted in a 66% reduction in suspected recurrent VTE over 1 year follow-up (RII: 81 vs RI: 239).

Summary/Conclusions: Use of the 'Men continue and HERDOO2' clinical decision rule to guide anticoagulant therapy results in significantly fewer suspected recurrent VTE and fewer adverse events of confirmed recurrent VTE or bleeding.

OC 12.4

D-dimer and ultrasound in combination italian study (DULCIS) to establish the optimal duration of anticoagulation for venous thromboembolism on behalf of the DULCIS investigators

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Background/Aims: to evaluate the efficacy and safety of a procedure employing the evaluation of residual vein obstruction (RVO) and D-dimer to establish the individual risk of recurrence and thus the necessity to prolong or stop anticoagulation after idiopathic deep vein thrombosis (DVT) and/or pulmonary embolism (PE). The specific aims of the study were: (i) to obtain a recurrence rate < 5% per year in the first and second year after anticoagulation is suspended according to the procedure (ii) to allow anticoagulation suspension in at least 40% of all subjects.

Materials and Methods: Multi-centre management study in which D-dimer was measured after at least 3 months of anticoagulation in patients with RVO < 4 mmin case of a previous DVT and/or normal pulmonary arterial pressure with echocardiography in case of previous PE and those who had undergone at least 12 months of therapy for previously altered RVO. If D-dimer was below age and gender specific cut-offs, anticoagulation was stopped and D-dimer was re-assessed after 15, 30, 60 and 90 days. If all the D-dimer measurements were below the cut-offs, anticoagulation was definitely stopped and patients were followed-up for two years and recurrent proximal DVT and/or PE were recorded. If one of these D-dimer measurement was above the cut-offs, anticoagulation was resumed for at least 6 months and patients were then re-evaluated.

Results: Thousand and two out of 2501 screened patients (40%) were enrolled in 17 centres. Of these, 516 (47%) stopped anticoagulation for normal D-dimer with 26 recurrences (3.7% patient-years; 95% CI: 2–5%). In 371 subjects in whom anticoagulation was resumed, 18 major or clinically relevant bleeds were observed (4.8%; 95% CI:3.8%). In 115 patients in whom D-d was altered but anticoagulation was not resumed, 12 recurrent events were observed (7.7% patient-years; 95% CI:11–12%).

Conclusions: A strategy based on RVO and D-dimer allows stopping anticoagulation in 47% of patients with a low risk of recurrence after a first episode of idiopathic VTE.

OC 12.5

D-Dimer levels over time and the risk of recurrent venous thromboembolism: an update of the Vienna Prediction Model

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Background: Patients with unprovoked venous thromboembolism (VTE) have a high recurrence risk and are candidates for extended anticoagulation. However, many patients stay recurrence free and are unnecessarily exposed to anticoagulants. The Vienna Prediction Model has been developed to discriminate patients with unprovoked VTE with a low recurrence risk from those with a high risk based on the patient's sex, the location of VTE, and D-Dimer, but allows risk assessment only at one single time point (3 weeks after anticoagulation).

Aim: to update and expand the model based on a larger number of events and a longer observation time, in order to assess the recurrence risk also from time points later than 3 weeks after anticoagulation on.

Methods: We analysed the data set of the Austrian Study on Recurrent Venous Thromboembolism, a prospective cohort study in patients of legal age with a first VTE who had received anticoagulants for 3–18 months. Patients with VTE provoked by surgery, trauma, pregnancy, or female hormone intake; with a natural inhibitor deficiency, the lupus anticoagulant, or cancer were excluded. The study end point was recurrent symptomatic deep vein thrombosis (DVT) and/or pulmonary embolism (PE). We integrated D-Dimer levels measured at several time points after anticoagulation with the patient's sex and location of VTE. We generated nomograms to calculate individual risk scores and cumulative recurrence rates from 3 weeks, 3, 9, 15 and 24 months on after discontinuation of anticoagulation using a dynamic landmark competing risks regression approach. The ethics committee approved the study and all patients gave written informed consent.

Results: One hundred and fifty-nine of 738 patients had recurrence during a mean follow-up of 6 years. The cumulative probability of recurrence was 5.5% (95%CI 3.9–7.2%) after 1 year and 18.4% (95% CI 15.4–21.4%) after 5 years. D-Dimer levels varied between patients, but did not substantially – albeit statistical significantly ($P < 0.001$) – increase over time. The updated model has two improvements: we accounted for the competing risk of death or informative drop out by competing risks regression, and we considered various time points of prediction rather than predicting just once after anticoagulation. Subdistribution hazard ratios (95% CI) dynamically changed from 3 weeks to 3, 9, 15 and 24 months from 0.29 (0.19–0.43), 0.31 (0.21–0.46), 0.37 (0.24–0.55), 0.43 (0.27–0.68) to 0.55 (0.32–0.94) in women vs. men, from 1.60 (0.84- 3.05), 1.58 (0.83–3.00), 1.54 (0.80–2.96), 1.49 (0.74–3.00) to 1.43 (0.64–3.20) in patients with proximal DVT or PE compared to distal DVT, and from 1.37 (1.23–1.66), 1.36 (1.14–1.62), 1.34 (1.14–1.58), 1.32 (1.11–1.58) to 1.29 (1.02–1.63) per doubling D-Dimer levels. We created nomograms based on subdistribution hazard ratios from the multivariable dynamic model to predict the recurrence risk from 3 weeks, 3, 9, 15 or 24 months on after anticoagulation. A web-based calculator allows risk assessment from random time points on between 3 weeks and 24 months.

Conclusions: The updated Vienna Prediction Model integrates patient's sex, location of first VTE and serial D-Dimer measurements and allows prediction of recurrent VTE at a random time point after discontinuation of oral anticoagulation.

OC 12.6

Recurrent venous thrombosis in premenopausal women: effect of hormonal contraceptive use

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Background: There is a large body of literature available on hormonal contraceptive use and the risk of a first venous thrombotic event. Despite guideline recommendations to discontinue hormonal contraceptive use after a thrombotic event, still a sizeable proportion of women continue or start using hormonal contraceptives after a venous thrombosis. The risk of recurrent venous thrombosis in women using hormonal contraceptives has not been studied extensively. The aim of this study was to evaluate the effect of hormonal contraceptive use on the recurrence risk in premenopausal women.

Methods: Patients with a first venous thrombosis included in the MEGA case-control study between 1999 and 2004, were followed for a recurrent venous thrombotic event up to 2009 ($N = 4731$). Included in the current analyses were premenopausal patients with a first venous thrombosis and for whom detailed information was available on hormonal contraceptive use during follow-up ($N = 702$). Time-dependent Cox-proportional hazards models were used to estimate hazard ratios

(HR) with 95% confidence intervals (CI), adjusted for age and BMI at baseline. Both the risk of recurrent venous thrombosis with hormonal contraceptive use at time of the first event and with hormonal contraceptive use during follow-up were calculated, as well as risks of recurrence for different types of hormonal contraceptives. Consent and ethical approval were obtained for this study.

Results: Seven hundred and two premenopausal women with a first venous thrombosis were followed for a total of 4673 woman-years (median 7.0 years; range, 12 days to 9.9 years) during which 74 recurrent events occurred resulting in a recurrence rate of 15.8 (95%CI: 12.6–19.8) per 1000 woman-years. Hormonal contraceptive use at the first thrombotic event was not associated with the risk of recurrent venous thrombosis ((HR 0.8, 95%CI: 0.5–1.5) for users versus non-users at the first event). Two hundred and ten women used hormonal contraceptives, mainly orally administered, during follow-up with a total follow-up of 545 woman-years. They experienced 21 recurrent thrombotic events resulting in a rate of 38.5 per 1000 woman-years, which was triple the rate of non-use during follow-up (HR 2.8, 95% CI: 1.7–4.7). Recurrence rates for women using combined oral contraceptives during follow-up ($N = 180$) were influenced both by the dose of ethinylestradiol as well as the type of progestagens. None of the women ($N = 20$, 70 woman-years of follow-up) using a levonorgestrel-releasing intra-uterine device during follow-up developed a recurrent venous thrombosis.

Conclusion: Hormonal contraceptive use after a first venous thrombosis increases the risk of a recurrent venous thrombotic event. When a non-hormonal contraceptive is excluded after careful consideration a levonorgestrel-releasing intra-uterine device may be a safe contraceptive to use after a venous thrombosis.

OC 13 – Von Willebrand Disease

OC 13.1

Genetic variations determine von Willebrand factor levels in patients with von Willebrand disease from the WiN study

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Background: Von Willebrand disease (VWD), the most common inherited bleeding disorder, is caused by reduced concentration or activity of von Willebrand factor (VWF) and is characterized by recurrent mucocutaneous bleeding. There is a large variation in VWF levels in VWD patients, even in individuals with identical mutations. Recently, a meta-analysis of genome-wide association studies identified genes other than the VWF gene that contribute to the variation in VWF levels in healthy individuals. We hypothesize that genetic variations in these other genes may also influence VWF levels in individuals with VWD and thereby affect the bleeding phenotype.

Aims: To investigate the effect of genetic variations in STXBP5, SCARA5, ABO, STAB2, STX2, TC2N and CLEC4M genes on VWF levels in a large cohort of patients with moderate and severe type 1 and type 2 VWD and to determine the influence on bleeding phenotype.

Methods: We genotyped 709 patients with moderate and severe type 1 and type 2 VWD, defined as VWF levels ≤ 30 U/dL, from a nationwide cross-sectional study (Willebrand in the Netherlands – WiN study). Seven SNPs in STXBP5 (6q24), SCARA5 (8p21), ABO (9q34), STAB2 (12q23), STX2 (12q24), TC2N (14q32) and CLEC4M (19p13) were analyzed. We studied the relationship of variation in these SNPs with VWF:Ag, VWF:CB, VWF:Act and FVIII:C levels in 604 patients with type 1 ($n = 364$) and type 2 ($n = 240$) VWD, in whom centrally measured VWF levels were available. Exclusion criteria were pregnancy and the recent use of desmopressin or replacement therapy. VWF levels were adjusted for age, sex and blood group. We also studied the influence of variation in these SNPs on bleeding phenotype, which was determined using the validated Tosetto Bleeding Score. Consent and ethical approval is obtained.

Results: In type 1 VWD patients rs9390459 in STXBP5 was significantly associated with VWF:Ag levels: ($\beta = -0.03$ IU/mL per allele [95%CI −0.06 to −0.00], $P = 0.049$). Rs868875 in CLEC4M was associated with both VWF:Ag ($\beta = -0.04$ IU/mL [95%CI −0.08 to −0.01], $P = 0.023$) and VWF:Act levels ($\beta = -0.06$ IU/mL [95%CI −0.11 to −0.00], $P = 0.034$). In type 2 VWD patients rs9390459 in STXBP5 was significantly associated with VWF:CB ($\beta = -0.04$ IU/mL [95%CI −0.07 to −0.01], $P = 0.015$). Interestingly, in the type 1 index patients ($n = 195$) an association between FVIII:C and STAB2 ($\beta = -0.06$ IU/mL [95%CI −0.12 to −0.00], $P = 0.048$) and in type 2 index ($n = 72$) patients an association between SNP rs2402074 in TC2N and VWF:Ag levels ($\beta = -0.09$ IU/mL [95%CI −0.17; −0.02], $P = 0.014$) was found. Previously, we observed an association between VWF levels and bleeding score (*de Wee et al, Thromb Haemost 2012*). Although there is a modest effect of genetic variants on VWF levels, we did not observe a clear association between bleeding score and VWF level decreasing alleles.

Summary/Conclusion: In type 1 and type 2 VWD patients, genetic variants in STXBP5, CLEC4M and TC2N are associated with VWF:Ag, VWF:CB and VWF:Act levels. These genetic determinants seem not to be associated with the bleeding phenotype.

OC 13.2

Incidence of large VWF gene deletions and duplications in the French cohort of 1182 patients with von Willebrand disease (VWD)

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Background: The French reference center for VWD (CRMW) organizes a national biologic platform for the phenotype/genotype characterization of VWD. The inclusion criteria are: type 3 phenotype (VWF levels < 5 IU/dL), type 2 phenotype (discrepancy between VWF antigen and VWF functional activities), and type 1 phenotype limited to VWF levels < 30 IU/dL (as VWF levels above this threshold are usually not in linkage to*VWF* gene). Patients with a clear diagnosis of acquired von Willebrand syndrome were excluded. After an exhaustive sequencing of the 52 exons (including intron-exon boundaries), the 5' and 3′UTR and the promoter of *VWF* gene, at least one deleterious sequence variation was identified in 1153 patients (635 families) (69% with type 2, 23% with type 1, 5% with type 3 and 3% with an undetermined phenotype). However, the direct sequencing failed to detect any causative mutation in 29 patients (17 index cases (IC)), and revealed only one heterozygous genetic defect discrepant with the phenotypic severity in six patients (5 IC).

Aims and Methods: To search in these 35 patients (22 IC) a large *VWF* gene deletion/duplication using multiple ligation-dependent probe amplification (MLPA) (kits MRC Holland).

Results: Nine distinct large gene alterations (6 novel*) were identified at a heterozygous state in 22 patients (10 IC): deletion spanning exons 1–3, exons 6–18*, exons 19–20*, exons 32–34*, exons 33–34, whole

gene deletion; duplication exon 6*, exons 35–37*, exons 38–42*. These defects were detected: in three patients (2 IC) with type 3 VWD (associated to a nonsense mutation on the other allele); in one patient with type 2A(IIC) VWD (associated to a splice mutation on the other allele); in eight carriers of type three VWD with type 1 phenotype (one IC); in 10 patients (6 IC) with either type 1 or undetermined phenotype showing VWF levels between 7 and 25 IU/dL. No *VWF* gene defect was detected in seven unrelated patients with a type 1 phenotype (all with blood group O and VWF levels around 30 IU/dL); also, the abnormality of the second allele was not detected in two unrelated patients with type 3.

Conclusions: Large *VWF* gene alterations occur in about 2% of our large cohort of patients, and around 5% of patients with type three VWD. Hypothesis concerning the deleterious process of these defects are suggested. Some of these deletions/duplications may create a frame shift and then abolish *VWF* gene expression (feature of type 3 mutations). Some others may conserve the frame but the truncated or the longest translated VWF affects the structure of the multimeric VWF and provides a dominant negative effect interacting with the normal VWF allele. These deletions/duplications can hamper the correct dimerization or multimerization process and subsequent extracellular secretion of VWF inducing a type 1 VWD. Finally, only seven patients out of our 1182 patients (~0.5%) remain without identified *VWF* gene abnormality. Our results demonstrate that the screening of large deletions and duplications is an essential way to explain some VWD phenotype.

OC 13.3

Severity of bleeding tendency in von Willebrand disease is associated with von Willebrand factor string formation

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Background: von Willebrand disease (VWD) type Vicenza is caused by a p.Arg1205His von Willebrand factor (VWF) mutation. The characteristics of the phenotype are very low levels of plasma VWF, often in the presence of ultra-large VWF multimers. Rapid clearance has been implicated in the pathogenesis, however, other still unresolved mechanisms may add to the bleeding tendency. Two VWD type Vicenza patients from the same family, one heterozygous for VWF mutation p.Arg1205His and the other compound heterozygous for VWF mutations p.Arg1205His and p.Arg924Gln, showed remarkable differences in their bleeding tendency with Tosetto bleeding scores of respectively 3 and 18.

Aim: To understand the molecular mechanism underlying the disparate bleeding severity in the two VWD type Vicenza patients.

Methods: Blood outgrowth endothelial cells (BOECs) were isolated from peripheral blood. Storage and secretion of VWF was analyzed in patient-derived BOECs and in transfected HEK293 cells. VWF strings were characterized under both static and flow conditions. This study complies with the Declaration of Helsinki, and informed consent and ethics approval were obtained.

Results: VWF mutants p.Arg1205His and p.Arg924Gln were stored normally in (pseudo-)Weibel-Palade bodies (WPB) both in transfected HEK293 cells and in BOECs. Compared to normal BOECs, histamine-induced release of VWF propeptide (VWFpp) from BOECs of the two VWD patients was reduced (70% of total VWFpp from the normal BOECs versus 40% from the two patients' BOECs). Upon stimulation with histamine, approximately 30 VWF strings per 10 imaging fields were released from BOECs derived from a healthy donor or from the patient heterozygous for p.Arg1205His, whereas the quantity was less than on average 5 strings per 10 imaging fields for BOECs derived from the patient compound heterozygous for p.Arg1205His and p.Arg924Gln ($P < 0.01$). Instead, many extracellular VWF 'patches' appeared on the cell surface of BOECs derived from the compound heterozygous patient, indicating that VWF did not unfold when WPB fused with the cell-membrane to form secretory pods. Furthermore, the platelet binding ability of VWF strings released from the two patients' BOECs appeared somewhat reduced under flow conditions (0.12 ± 0.06 platelets per μm string for patients' BOECs versus 0.15 ± 0.07 platelets per μm string for normal BOECs; $P < 0.05$). The pathogenic nature of p.Arg1205His and p.Arg924Gln was also evaluated in transfected cells. In HEK293 cells, p.Arg1205His led to a significant reduction in total production of VWF, basal and regulated secretion of VWF, and to formation of shorter VWF strings. Similarly, p.Arg924Gln reduced regulated secretion of VWF and resulted in shorter VWF strings. The impairment in VWF production and secretion caused by p.Arg1205His was only partially corrected upon co-transfection of wild type VWF or p.Arg924Gln.

Conclusions: Synergistic effects of p.Arg1205His and p.Arg924Gln on VWF string formation may contribute to the more severe phenotype in the compound heterozygous patient. Failure of unfolding VWF into strings is a new pathogenic mechanism for VWD that remains undetected by the common diagnostic VWF assays.

OC 13.4

Characterisation of large in-frame deletions contributing to type 1 VWD pathogenesis in the MCMDM-1VWD study

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Introduction: Type 1 von Willebrand disease (VWD1) results from a partial quantitative deficiency of von Willebrand factor. VWD1 mutations are largely missense, but also include small nucleotide insertions/deletions, splice and nonsense mutations. Investigation into the contribution of heterozygous exon deletions to VWD1 pathogenesis within the MCMDM-1VWD cohort, using multiplex ligation-dependent probe amplification (MLPA) analysis led to identification of six heterozygous deletions. Three novel deletions, involving exons 3 (p.G19_G74del), 32–34 (p.V1820_C1948delinsS) and 33–34 (p.G1874_C1948del) were identified in addition to three cases with a previously reported exon 4–5 deletion among 150 index cases (4%). The index cases with exon 3, 32–34 and 33–34 deletions had bleeding scores of 10, 6 and 4, VWF:Ag levels of 31 12 and 32 IU/dL, VWF:RCo of 20, 11 and 23 IU/dL and VWF:CB of 31, 12 and 15 IU/dL, respectively. Phenotype was more severe for these deletions than previously reported for the exon 4–5 deletion. The exon 32–34 deletion was associated with a slight multimer abnormality.

Aim: To characterise and determine deletion breakpoints and the mechanism by which the deletions exert an effect on VWF protein synthesis and secretion.

Methodology: Heterozygous deletion breakpoints were characterised through haplotyping of intronic SNPs followed by long-range PCR. *In silico* analysis of breakpoint regions was used to investigate repetitive elements and DNA sequence motifs that might explain mechanisms of deletion formation. VWF deletion mutants were created using site-directed mutagenesis. Recombinant vectors were transiently-transfected into HEK293T cells and Renilla was used as a transfection control. Conditioned media and cellular lysates were collected 48 h post-transfection and analysed by VWF:Ag ELISA, comparing to wild-type expression.

Results: Heterozygous deletions resulting in in-frame loss of exons 3 (c.56-335_220+1585), 32–34 (c.5455+277_5842+978delinsTGGACACA) and 33–34 (c.5620+872_5842+2440delinsGCAGCATAAGCATAAAGC) ranged in size from 1.5 to 5.2 kb. Breakpoints were mapped to intronic regions of *VWF* and deletion-specific PCR demonstrated fully penetrant dominant inheritance in each family. Breakpoint junctions for the exon 3 deletion lay within *Alu*-repeat elements; motif analysis revealed a topoisomerase I cleavage site adjacent to the 5′ junction, inferring single stranded annealing (SSA) as the likely deletion mechanism. Microhomology mediated end-joining (MMEJ) was hypothesised as the mechanism for deletion of exons 32–34 and 33–34. Expression of recombinant mutant VWF rVWFdel3 and rVWFdel32-34 demonstrated secretion reduced by 81% and 87% ($P < 0.0001$) respectively in the homozygous state in comparison with rVWFwt, with no significant increase in intracellular retention. Both deletions also resulted in significantly ($P < 0.0001$) reduced secretion (53% and 61%, respectively) in the heterozygous state. Additionally, there was reduction in intracellular VWF for rVWFdel3 (21%, $P < 0.05$) but a 41% ($P < 0.05$) increase in intracellular VWF for rVWFdel32-34 in the heterozygous state.

Conclusion: Molecular characterisation of VWD1 deletions indicates that SSA between *Alu*-repeat elements, as well as MMEJ are causal. *In vitro* expression data from this study suggests that deletions of exons 3 and 32–34 do not affect VWF secretion as significantly as reported for the exon 4–5 deletion (98% homozygous and 86% heterozygous). However, these in-frame VWF deletions do result in significantly decreased secretion of heterozygous mutant VWF.

OC 13.5

Distribution of the von Willebrand disease Types In 337 patients followed by a single hemophilia center since 2002: a comparison of von Willebrand disease patients classification after 10 years

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Background: von Willebrand Disease (VWD) is the most frequent bleeding disorder and it is caused by quantitative (type 1 and 3) or qualitative (type 2) defects of von Willebrand factor (VWF). Type 2 VWD is further divided into four categories: 2A, 2B, 2M and 2N. The accurate classification of VWD patients requires the evaluation of several laboratory tests.

Aims: To compare the current distribution of VWD types diagnosed at Angelo Bianchi Bonomi Hemophilia and Thrombosis Center (Milan, Italy) with the distribution obtained 10 years ago.

Methods: A total of 337 VWD patients referred to our center from 2002 through 2012 have been fully characterized. Laboratory diagnosis of VWD was made by VWF antigen (VWF:Ag), VWF ristocetin cofactor, factor VIII coagulant activity, VWF collagen binding, multimeric analysis in low and medium resolution gels, VWF propeptide/VWF:Ag ratio, VWF binding to FVIII, ristocetin induced platelet agglutination, intra-platelet VWF evaluation and in type 2 and 3 molecular characterization. We considered 0.6 the cut off of VWF:RCo/Ag ratio to differentiate type 1 from type 2 VWD. Patients with 'supranormal' multimers in plasma and R1205H mutation were classified as type 1 'Vicenza'.

Results: VWD type diagnosis was made on the basis of the revised classification of the ISTH Subcommittee on VWF (J Thomb Haemost 2006;4:2103). The distribution of the VWD population in the years 2002 and 2012 are reported as number of cases (%, percentage). 2002: type 1: 108 (34.2%); type 2A: 45 (14.3%); type 2B: 35 (11%); type 2M: 105 (33.2%); type 2N: 1 (0.3%); type 3: 22 (7%), total 316 (100%). 2012: type 1: 113 (33.5%); type 2A: 105 (31.2%); type 2B: 58 (17.2%); type 2M: 28 (8.3%); type 2N: 7 (2.1%); type 3: 26 (7.7%), total 337 (100%).

Discussion: The main difference between the two populations is the inversion in the percentages of type 2A and 2M patients. This finding appears to be related to the more precise approach to laboratory diagnosis due to the introduction of additional and more sensitive tests and to the improvement of multimeric analysis performed at low and medium resolution gels. Indeed, the identification of more VWD type 2A cases in 2012 was also due to the better characterization of a subgroup of type 2A IIE patients. These subjects, previously misclassified as types 1 or 2M, were revaluated thanks to their abnormal triplet structure and the presence of the mutations in the D3 domain. The reduced number of type 1 VWD patients was compensated by the fact that patients previously classified as type 2M Vicenza have been included now as type 1 Vicenza, following the most recent classification published in 2006. In conclusion, a detailed characterization of VWF variants have tendency to highlight dysfunctional defects reducing the number of patients with normal VWF multimeric pattern, like type 1 or 2M. The clinical significance of this VWD heterogeneity is still under investigation and may support or not the use of such comprehensive characterization.

OC 13.6

Type 2N von Willebrand disease (VWD): one variant, two diseases? Analysis of the French cohort

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Aims: Type 2N VWD is defined by a markedly decreased binding of VWF to FVIII, recessively inherited. Since its first description in France in 1989 this VWD variant has been reported in many countries. In order to better redefine the clinical and biological characteristics of these patients (pts) compared to original publications we reviewed the records of pts identified as type 2N VWD by the French Reference Center of VWD since May 2007. Only pts with a ratio FVIII/VWF:Ag < 0.6 were enrolled. Further phenotypic and genotypic analyses were centralized. The clinical data were collected by questionnaire or site monitoring.

Results: Seventy nine pts (70 families) were recruited (F/M ratio: 1.6). The median (range) age was 39 (7–85) y.o. All the pts have a low FVIII:C level and FVIII/VWF:Ag ratio:15 (2–43) IU/dL and 0.29 (0.01–0.55) respectively. Genetic analysis showed that the R854Q mutation is involved in 70 pts (89%) (Group A) who are either homozygous (32) or compound heterozygous for another type 2N mutation (7) or a null allele (31). The 9 other pts (Group B) are homozygous for another type 2N mutation (4) or a null allele (5). The FVIII:C level and FVIII/VWF:Ag ratio are much more decreased in these last patients: 4 (2–15) IU/dL and 0.07 (0.01–0.41) compared to Group A pts: 20 (5–45) IU/dL and 0.31 (0.12–0.56) respectively (median and range). By contrast there is no significant difference between pts who are homozygous for R854Q or compound heterozygous for another type 2N mutation or null allele (not shown). The bleeding score (BS) was calculated (Tosetto) in 40 pts, but the results are currently available in 27. The median (range) BS is much less lower in 23 Group A pts: 5 (−1 to 14) compared to four Group B pts: 15 (6–24). The age in both groups is comparable. Clinical history showed that Group B pts were mostly diagnosed during childhood and experienced much more haemarthrosis and life threatening hemorrhages than Group A pts. Those pts had an adulthood-onset disease and exhibited mainly post surgery bleedings. The response to desmopressin was tested in 52 pts. Good response was defined as FVIII:C > 50 IU/dL 2 h after desmopressin. All the 48 Group A pts but one (R854Q/T791M) were found

to be good responders. None of the four tested pts in Group B responded to desmopressin.

Conclusions: The presence or absence of the mutation R854Q in pts with type 2N VWD allows a clear distinction between two groups. In the 1st group (R854Q present) pts have only a moderate FVIII:C deficiency and lower FVIII:C/VWF:Ag ratio. They have little spontaneous bleeding events and good response to desmopressin. The 2[d] group (R854Q absent) is much rarer (11% in our series) but much more severe: pts have a very low FVIII:C defect (< 5 IU/dL) and FVIII:C/VWF:Ag ratio with early and severe bleeding manifestations and no response to desmopressin. Type 2 N VWD encompasses two strongly different phenotypic and clinical patterns closely related to the genotype.

OC 14 – Acquired Bleeding Disorders

OC 14.1

Treatment of acute bleeding episodes in acquired haemophilia with recombinant activated factor VII (rFVIIa): analysis from 10-year Japanese post-marketing surveillance

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Background: Acquired haemophilia (AH) is a rare, spontaneous bleeding disorder caused by autoantibodies, usually against factor VIII. Control of acute bleeding is the first priority, and patients are treated with bypassing agents such as recombinant activated factor VII (rFVIIa, NovoSeven®). However, the data for the use of rFVIIa for AH is limited. To investigate the safety and efficacy of rFVIIa for the treatment of AH, data from Japanese post-marketing surveillance was collected.

Methods: A multi-centre, non-interventional, observational study was conducted from May 2000 to March 2010. Under routine clinical practice, the dosing regimen of rFVIIa was recorded, and clinical efficacy was evaluated as 'markedly effective' (clinical improvement within 8 h), 'effective' (clinical improvement within 8–12 h), 'moderate' (clinical improvement in > 12 h), or 'ineffective'. Adverse events were also recorded.

Results: Data from 132 patients were collected, including 57 (43%) females and 75 (57%) males. The mean values for patient characteristics were as follows: age, 67.9 years; FVIII activity, 4.1 IU/dL; and FVIII inhibitor titre, 101.1 Bethesda units/mL. Among 371 bleeding episodes, muscle bleeds were most common (40%), followed by subcutaneous haemorrhages (14%), joint bleeds (9%), haematuria (5%), and gastrointestinal bleeds (4%). Overall, 92% of bleeding episodes improved following treatment with rFVIIa (41% 'markedly effective', 10% 'effective', and 41% 'moderate'). Median (mean) treatment was of 2.0 (2.9) days' duration, with 3.0 (11.6) injections of 93.2 (99.5) μg/kg rFVIIa. Disseminated intravascular coagulation was reported in two patients; an 87-year-old female with rheumatoid arthritis and an 81-year-old male with pneumonia. Bowel necrosis was reported in a 70-year-old male with intracerebral haemorrhage, complicated with pneumonia. These three cases were determined by the reporters to be related to rFVIIa administration.

Conclusions: These data represent the largest report of rFVIIa use in AH from a single country. rFVIIa provided adequate haemostasis for the treatment of AH. Thromboembolic events were observed in elderly patients with severe complications.

OC 14.2

Treatment of bleeding episodes in acquired haemophilia with activated prothrombin complex concentrate: what is the optimal therapy?

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Background: Acquired Haemophilia A (AHA) is a rare condition associated with severe bleeding and high rate of mortality. Activated Prothrombin Complex Concentrate (aPCC) was described as being useful to treat AHA patients. Doses and duration of treatment are still a matter for debate.

Aims: To retrospectively compare, in a small Italian cohort of bleeding AHA patients, the efficacy of two different strategies of treatment with aPCC in the prevention of haemorrhagic relapses: acute phase treatment only vs acute phase plus low-dose prophylaxis.

Methods: All patients with AHA consecutively admitted to PaduaUniversityHospitaland PaviaUniversityHospitalbetween April 2008 and August 2012 were considered for enrolment. Information on the characteristics and treatment of bleeding episodes was collected according to the use of aPCC in the acute phase and subsequently during 4 weeks after the initial treatment. For all patients written informed consent was obtained.

Results: Sixteen bleeding episodes in fourteen AHA patients treated with aPCC for bleeding recurrence were considered. Eight were major haemorrhagic episodes (according to Schulman S et al. JTH 2005;3:692–4) and eight were minor bleeding. Ten bleeding episodes were treated with the use of aPCC limited to the acute phase. Meanwhile, in the other six episodes the acute phase treatment was followed by a low-dose prophylaxis with aPCC. Comparing the two strategies, no difference in the initial dose of drug used was found. In particular, for major bleeding, patients were treated with aPCC 205 ± 7.1 Ukg/day (mean M ± standard deviation SD) in the former regimen vs 162.2 ± 33.3 Ukg/day in the low-dose prophylaxis regimen (P = 0.137); and, for minor bleeding, with an initial mean dose of 64 ± 40.4 Ukg/day in the acute-phase treatment versus 85 ± 10 Ukg/day in the latter (P = 0.38). Low-dose prophylaxis was maintained with a mean dose of 82.5 ± 46.0 Ukg/day and for a mean period of 9.5 ± 2.1 days in case of major haemorrhage and with a mean dose of 28.5 ± 9.3 Ukg/day and for a mean period of 12.25 ± 10.7 days in minor episodes. Five cases of bleeding relapse were observed in patients treated with aPCC only during the acute phase and no bleeding events in AHA patients treated with low-dose prophylaxis strategy occurred. A two-fold increase in the risk of haemorrhagic recurrence in patients who did not receive the prophylactic treatment was found (OR 2.18, 95% confidence interval [CI]: 1.11–4.28, P = 0.02). This risk was not affected by adjustment for possible confounders (age, gender, type of bleeding, levels of inhibitor titre and factor VIII activity). No thromboembolic complication or other adverse events were reported in both treatments. In all patients the acute phase treatment with aPCC was started in association with immunosuppressive treatment (i.e. steroids ± cyclofosfamide) to obtain inhibitor eradication.

Conclusions: A low-dose prophylaxis regimen with aPCC in bleeding AHA patients seems to prevent the risk of haemorrhagic relapses. This approach appeared to be safe and well tolerated. Larger and prospective studies are needed to better clarify the role of this strategy.

OC 14.3

In vivo hemostatic potency of a factor VIII function-mimetic, bispecific antibody to factors IXa and X (ACE910) against on-going bleeds in an acquired hemophilia A model

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Background: We previously reported that a bispecific antibody to factors IXa and X, hBS23, mimicked the function of factor VIII (FVIII) even in the presence of FVIII inhibitors, and exerted preventive hemostatic activity *in vivo* (Nature Medicine 2012). By the further molecular engineering of hBS23, we identified an improved bispecific antibody, ACE910, which presented higher *in vitro* cofactor activity than hBS23 and *in vivo* hemostatic activity even against on-going bleeds (Early results were presented at the ASH meeting 2012).
Aims: To elucidate the *in vivo* hemostatic potency of ACE910, by examining its dose-dependent hemostatic effect against on-going bleeds.
Methods: A non-human primate model of acquired hemophilia A was established by injecting anti-primate FVIII neutralizing antibody which was not cross-reactive to porcine FVIII. When bleeds emerged following an artificial bleed-inducing procedure, either ACE910 (0.3, 1 or 3 mg/kg, $n = 4$ for each dose), recombinant porcine FVIII (rpoFVIII; 3.4 or 10 U/kg, $n = 4$ for each dose) or no test item ($n = 6$) was intravenously administered. rpoFVIII was additionally administered twice daily on the following 2 days. Bleeding symptoms, progressive anemia and expansion of bruised area, were monitored for 3 days.
Results: In this *in vivo* model, rpoFVIII 10 U/kg (twice daily) showed significant hemostatic effect against on-going bleeds, whereas the hemostatic effect of rpoFVIII 3.4 U/kg (twice daily) was not detected clearly. Since the minimal target plasma level against on-going bleeds, 10 to 20 U/dL of FVIII:C, would be achieved with rpoFVIII 10 U/kg but not with 3.4 U/kg, this reestablished model was found validated well in terms of the reactivity to FVIII. In this model, a single bolus of ACE910 1 or 3 mg/kg presented a hemostatic activity comparable to rpoFVIII 10 U/kg (twice daily) against on-going bleeds. The hemostatic effect of a single bolus of ACE910 0.3 mg/kg was not clearly detected. The mean plasma concentrations of ACE910 just after the intravenous administration were 6.6, 26 and 61 μg/mL in the ACE910 0.3, 1 and 3 mg/kg groups, respectively.
Conclusion: ACE910 demonstrated hemostatic effect against on-going bleeds in a non-human primate model of acquired hemophilia A. The hemostatic potency of a single bolus of ACE910 1 or 3 mg/kg was comparable to that of rpoFVIII 10 U/kg (twice daily).

OC 14.4

The prevalence and severity of shear stress-associated acquired von Willebrand factor abnormality in patients with mitral regurgitation

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Background: Loss of the highest molecular weight multimers (HMWM) of von Willebrand factor (VWF) due to high intravascular shear stress has been well recognized in patients with aortic stenosis. However, the prevalence and severity of such a HMWM loss has not been thoroughly studied in patients with mitral regurgitation (MR).
Aims: In this study, we examined the prevalence and severity of VWF HMWM loss in patients with various degrees of MR.
Methods: A total of 47 patients (47% male and median age, 69.5 years) with MR were studied and their clinical and laboratory data were collected. Nineteen patients had severe organic MR, and 28 had mild or moderate MR. Separate seventeen patients were status post mitral valve replacement (MVR). Blood samples were tested for VWF antigen (VWF:Ag), VWF activity by enhanced immunoturbidity method (VWF:Lx), VWF multimer analysis and HMWM densitometry quantification (VWF:M) (Am J Cardiol. 2013;111:374–81), and ADP cartridge closure times of platelet function analysis (PFA-CADP). These laboratory results were compared with the severity of MR measured by regurgitatant volume (RV).
Results: The prevalence of loss of HMWM in patients with various degrees of MR was as follows: severe MR, 79%; moderate MR, 57%; mild MR, 21%. Two patients with severe MR developed Heyde syndrome. Conversely, only 6% of patients post MVR had loss of HMWM. RV significantly correlated with PFA-CADP ($r = 0.66$, $P < 0.001$), VWF:M ($r = -0.60$, $P < 0.001$) and VWF:Lx/Ag ratio ($r = -0.39$, $P = 0.007$). Over a median of 11.3 months, VWF multimer ratio ($P = 0.001$) and PFA-CADP ($P = 0.007$) were predictors of freedom from death ($n = 1$) or mitral valve surgery (MVS, $n = 15$). In the 15 patients who had MVS, post-operative samples revealed significant resolution of VWF abnormality indicated by marked improvement of VWF:M ($P < 0.001$), PFA-CADP ($P < 0.001$) and VWF:Lx/Ag ratios ($P < 0.01$).
Conclusions: Surprisingly the loss of VWF HMWM is quite common in patients with moderate -severe MR, and resolves after MVS. Heyde syndrome can occur in patients with severe MR. Since the degree of HMWM loss significantly correlates with MR severity, PFA-CADP, VWF:Lx/Ag ratio and VWF:M are potentially useful diagnostic and prognostic biomarkers in patients with MR.

OC 15 – ADAMTS-13

OC 15.1

ADAMTS13 meets von Willebrand factor strings: a single molecule approach

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Background: Single molecule fluorescence microscopy (SMFM) is a powerful technique to explore the individual nano-scale behavior of molecules. SMFM allows direct visualization of molecular motions and observation of diversity in a population of molecules, normally hidden in classical bulk experiments. We previously demonstrated that platelet-decorated VWF strings attached to the endothelium are cleaved multiple times by ADAMTS13 preferentially at sites of local elongations. However, it is currently unknown how ADAMTS13 locates a binding site on these long VWF strings. New insights in the working mechanism of ADAMTS13 on these strings could be revealed by visualizing the molecular motion of single ADAMTS13 enzymes.
Aim: To set-up a SMFM experiment to track the movement of individual ADAMTS13 enzymes on platelet-decorated VWF strings, hence increasing our understanding of individual ADAMTS13 enzymes at work.
Methods: Fluorescently labeled catalytically inactive ADAMTS13^{E225Q}-Atto647N (5.7 nM) was perfused over endothelial cell-anchored VWF strings in a parallel-plate flow chamber at a shear stress of 2.5 dyne/cm^2. VWF strings were indirectly visualized by green fluorescent labeling of platelets (100 nM Dioc). A customized SMFM microscopy set-up was developed to visualize individual ADAMTS13 enzymes.
Results: Single ADAMTS13 enzymes were detected by exciting ADAMTS13^{E225Q}-Atto647N with a powerful laser, using high magnifications (~2000×) and a sensitive EM-CCD camera. Due to nm scale visualization necessary for ADAMTS13 detection but not warranted for the observation of long VWF strings a two camera set-up was developed allowing the use of different magnifications to selectively detect platelet-decorated VWF strings and single ADAMTS13^{E225Q}

enzymes. Autofluorescence background signals from endothelial cells were reduced by performing excitation in total internal reflection-like mode. This set-up allowed us to visualize single ADAMTS13 binding to the strings in real-time. To now analyze enzyme trajectories and ADAMTS13 binding sites on these VWF strings customized single particle tracking software was developed. Initial analysis of ADAMTS13^{E225Q} binding to platelet-decorated VWF strings (n = 39) in real time under flow revealed three different kinds of motion. Enzymes could slow down without arrest on the VWF string (slow down), slow down and stop for only two to five frames (60 ms exposure time) (stop & go) or bind to the VWF string for a longer time (stop).

Conclusions: We were able to visualize the binding of single ADAMTS13 enzymes to platelet-decorated VWF strings. Further detailed analysis of all ADAMTS13 trajectories and binding sites will allow us to elucidate how ADAMTS13 preferentially localizes its binding site on these strings and how this correlates with ADAMTS13 binding at sites of local elongations.

OC 15.2

Spatially distinct regulation of von Willebrand factor by ADAMTS13 at the sites of platelet accumulation

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Background: von Willebrand Factor (VWF) is a large multimeric glycoprotein that mediates platelet adhesion and subsequent platelet aggregation to the damaged blood vessel wall in both physiological hemostasis and pathological thrombosis. The metalloprotease ADAMTS13 regulates the functional activity of VWF appropriate for normal hemostasis by reducing VWF multimer size. The cleavage site for ADAMTS13 in the VWF A2 domain is inaccessible in the globular configuration of the protein and requires the application of tensile force mediated by platelets and hydrodynamic shear to facilitate cleavage.

Aims: Newly released ULVWF is anchored on endothelial cells and cleaved by ADAMTS13. Other potential sites where ADAMTS13 might regulate VWF size include in the circulation and at the sites of vessel injury. At this latter location, ADAMTS13 is hypothesized to regulate thrombus growth and prevent vessel occlusion. However, direct evidence to support this role is limited. To address this issue, we developed a mouse model system to directly visualize the involvement of ADAMTS13 in the thrombogenic process under flow conditions.

Methods: Full-length mouse ADAMTS13 cDNA was cloned into pcDNA3.1 and mCherry was cloned at the C terminus of ADAMTS13 with a 12AA linker. Recombinant mADAMTS13-mCherry protein was produced via HEK293T cells by transient transfection. mADAMTS13-mCherry was pre-incubated with mCherry antibody and goat anti-rabbit IgG-Alexa568 30 min before the following flow chamber experiment. Whole blood obtained from ADAMTS13 knockout mice with added mADAMTS13-mCherry was perfused into a collagen coated flow chamber at various shear rates (500/s, 2500/s, 7500/s). Platelets and ADAMTS13 were visualized and quantified with a Quorum WaveFX- X1 spinning disk confocal system. After the perfusion, thrombi were fixed and immunostaining was performed to further analyze the distribution of platelets, VWF and ADAMTS13.

Results: ADAMTS13-mCherry was visualized only under the highly elevated shear condition (7500/s) in the real-time flow system where the signal was mainly on the formed thrombi. It was also occasionally detected on the collagen surface, where it was associated with nearby platelets and resembled VWF strings in appearance. Image analysis of the thrombus demonstrated 42.1, 36.1, and 23.3% reductions in size with the addition of ADAMTS13 at 7500, 2500, and 500/s, respectively. Further analysis by immunostaining of the thrombus demonstrated a 2.3-fold-increase of ADAMTS13 total area at 7500/s compared to that at 2500/s. Moreover, 45.4% and 37.7% of this increase was associated with the top and middle layers of the thrombus. In contrast, at the low shear condition (500/s), ADAMTS13 signal was very limited to only 6% compared to 7500/s.

Summary/Conclusion: Direct visualization of ADAMTS13 was achieved in our flow chamber system using fluorescence-tagged ADAMTS13. Our multi-colour immunostaining revealed enhanced ADAMTS13 activity in the top and middle layers of thrombus at a highly elevated shear rate and was associated with reduction of thrombus size. It is speculated that the platelet surface contributes, along with platelet tethering and increasing hydrodynamic shear force, to accelerate VWF cleavage and limit thrombus size. These results have characterized a shear-dependent, spatially distinct regulatory mechanism for ADAMTS13 that limits thrombus growth directly at the sites of platelet accumulation.

OC 15.3

ADAMTS13 is autoinhibited by distal thrombospondin-1 (T) or CUB domains and is activated allosterically by VWF or antibodies against ADAMTS13 domain T8

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Background: ADAMTS13 is a metalloprotease that cleaves von Willebrand factor multimers. Genetic or autoimmune deficiency of ADAMTS13 causes thrombotic thrombocytopenic purpura (TTP), which is characterized by microangiopathic hemolytic anemia, thrombocytopenia, and tissue damage caused by microvascular thrombosis. ADAMTS13 is a multidomain protein with metalloprotease (M), disintegrin-like (D), thrombospondin-1 (T), Cys-rich (C) and spacer (S) domains, followed by seven T domains and two CUB domains. ADAMTS13 binds native VWF (Kd ≈70 nM), which requires ADAMTS13 domains T8-CUB1 and VWF domain D4. ADAMTS13 truncated after the spacer domain (MDTCS) does not bind native VWF but binds to unfolded VWF domain A2 (Kd ≈30 nM).

Aim: The regulation of ADAMTS13 is poorly understood, and ADAMTS13 has no known inhibitors *in vivo*. The multidomain structure of ADAMTS13 suggests that distal T or CUB domains might regulate its activity. We sought to characterize ADAMTS13 auto-regulatory mechanisms.

Methods: ADAMTS13 activity assays were performed with FRETS-rVWF71, a fluorogenic ADAMST13 substrate with increased sensitivity. Product generation was monitored as an increase in fluorescence emission at 660 nm upon excitation at 635 nm. Assays included 50 mM Bis-Tris or HEPES at the indicated pH, 150 mM NaCl, 10 mM $CaCl_2$, and varying concentrations of VWF. Recombinant ADAMTS13 and MDTCS were expressed in HEK293 cells and chromatographically purified. Plasma ADAMTS13 was from pooled normal plasma. Monoclonal antibody 14D2 recognizes ADAMTS13 domain T8. Plasma samples from 65 patients with TTP and ADAMTS13 activity < 20% were obtained through approved human studies protocols.

Results: At pH 6, ADAMTS13 and MDTCS had equal and maximal specific activity. As pH was increased toward pH 7.4, MDTCS activity remained roughly constant but full-length plasma ADAMTS13 activity decreased progressively to one-fourth of the activity observed at pH 6, suggesting that distal ADAMTS13 T-CUB domains inhibit proteolytic activity under physiological conditions. Recombinant ADAMTS13 behaved similarly. When plasma samples from patients with TTP were assayed for inhibitors, one contained an antibody that increased ADAMTS13 activity three-fold to almost equal the activity of MDTCS, with an activation titer of 9 U at pH 7.4. This activating antibody had no effect on ADAMTS13 activity at pH 6, or on

MDTCS activity at either pH 6 or pH 7.4. We screened 18 anti-ADAMTS13 monoclonal antibodies and found that 0.8 μg/mL MAb 14D2, with an epitope in T8, activated ADAMTS13 2.2-fold at pH 7.4 but did not further increase the activity of ADAMTS13 at pH 6. Increasing concentrations of VWF (up to 125 μg/mL, or 500 nM) progressively activated ADAMTS13 1.8-fold at pH 7.4 but not at pH 6, and had no significant effect on MDTCS activity.

Summary/Conclusion: ADAMTS13 is regulated by substrate-induced conformational changes. Full-length ADAMTS13 is autoinhibited by distal T-CUB domains and is activated allosterically by VWF, probably by binding to VWF domain D4. Activation by VWF is mimicked by low pH or by antibodies that bind ADAMTS13 domain T8. ADAMTS13 bound to native VWF has increased proteolytic activity and may contribute disproportionately to the cleavage of VWF multimers under shear stress *in vivo*.

OC 15.4

A comprehensive mutagenesis screen defines the substrate recognition landscape within VWF for ADAMTS13

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Background: The metalloprotease ADAMTS13 regulates the multimeric structure and adhesiveness of von Willebrand Factor (VWF). Deficiency in ADAMTS13 activity can lead to thrombotic thrombocytopenic purpura (TTP), a devastating microvascular thrombopathy.

Aims: The aim of this study was to comprehensively investigate the interaction of ADAMTS13 with its substrate VWF73, a fragment of the VWF A2 domain, to gain insights into ADAMTS13 specificity.

Methods: A mutant VWF73 substrate phage display library was developed consisting of 30 million independent clones with an average mutation frequency of 3% at each position. This library should contain VWF73 variants corresponding to every possible single amino acid substitution at every position as well as most combinations of double amino acid substitutions. This library exhibited proper substrate consumption kinetics when reacted with ADAMTS13, although accumulation of uncleaveable phage at later time points caused variation to predicted pseudo first order kinetics.

High throughput deep sequencing using the Illumina platform was used to provide unprecedented information of how each mutation influenced VWF73 proteolysis by ADAMTS13. Maximal enrichment of uncleavable phage was achieved by reacting 25 nM ADAMTS13 with the mutant VWF73 phage library for 10 hr and isolating uncleaved phage by immunoprecipitation. Thirty-five million sequences were obtained for both the selected uncleaved phage and the starting phage population and enrichment was analyzed using the ENRICH software package.

Results: Overall, the uncleaved phage exhibited enrichment for mutations compared to the unselected library at the majority of VWF73 positions. However, mutation frequencies remained unchanged at R1597, A1600, A1612, Q1667, and R1668, indicating that mutations at these positions did not inhibit proteolysis by ADAMTS13. Also, the interval between T1608-S1613 exhibited only modest increases in mutation frequency, indicating that these positions were also tolerant to mutations.

Mutations surrounding the Y1605-M6066 scissile bond tended to inhibit proteolysis compared to mutations at other positions on the substrate. Positions such as L1603, V1604, Y1605 exhibited an increase mutation frequency, indicating mutations at these positions inhibited proteolysis by ADAMTS13. Position V1607 exhibited the greatest increase in mutation frequency following selection, suggesting that this is an important amino acid in VWF substrate recognition. However, the P1' residue, M1606, was comparably permissive to mutations, indicating a less-critical role in substrate recognition. Positions I1616 and P1620 also exhibited increased mutation frequency after selection, indicating potential exosite interactions.

Focusing on single amino acid substitutions, L1619G exhibited a log2 enrichment ratio (L2ER) of 3.9, among the most deleterious mutations we discovered, indicating a potentially important role for this amino acid in regulating VWF73 recognition by ADAMTS13. However, L1619A had a L2ER of 1.8, suggesting that ala-scanning mutagenesis does not serve as a proxy for other substitutions.

Summary and Conclusions: Coupling comprehensive mutagenesis with high throughput deep sequencing revealed a detailed substrate recognition landscape for VWF73 that could not previously be assessed with standard technology. These data will be useful in defining important substrate recognition domains on VWF that may lead to the development of more sensitive ADAMTS13 activity assays and improved understanding of ADAMTS13 protease function and TTP pathogenesis.

OC 16 – Anticoagulants – Basic

OC 16.1

Effect of rivaroxaban with or without acetylsalicylic acid on thrombus formation in an *ex vivo* perfusion chamber – an open-label, randomized study in healthy subjects

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Background: Antiplatelet agents are effective for the secondary prevention of cardiovascular events in patients with acute coronary syndrome, but there is still a substantial residual risk of recurrence. Previous evidence has shown that co-administration of antiplatelet and anticoagulant therapies can further reduce the risk of cardiovascular events, although the risk of bleeding was increased with these combinations. Rivaroxaban, an oral, direct Factor Xa inhibitor, has been shown to be effective in the prevention and treatment of several thromboembolic disorders.

Aims: To investigate the effect of single doses of rivaroxaban (5–20 mg), with or without acetylsalicylic acid (ASA), on thrombus formation in an *ex vivo* perfusion chamber at low and high shear rates, representing conditions in the venous system and stenosed arteries, respectively.

Methods: Fifty-one healthy subjects were enrolled in this randomized, two-way crossover (for treatment with rivaroxaban with and without ASA) and parallel-group study for comparison between the different dosing regimens of rivaroxaban and ASA plus clopidogrel (EudraCT number: 2007-002345-21). The treatment groups were: (A) rivaroxaban plus ASA: rivaroxaban 5, 10 or 20 mg on day 0 and ASA once daily (od) on 4 consecutive days (day −3 to day 0; 300 mg loading dose followed by 100 mg); (B) rivaroxaban alone at 5, 10 or 20 mg on day 0; and (C) clopidogrel plus ASA: clopidogrel od on 4 consecutive days (day −3 to day 0; clopidogrel 300 mg loading dose followed by 75 mg) and ASA od on 4 consecutive days (as in group A). Thrombus formed in the perfusion chamber was measured as D-dimer levels (for fibrin deposition) and P-selectin content (for platelet deposition), performed at the time of maximum plasma concentration of rivaroxaban and at the maximum effect of ASA or clopidogrel. Pharmacodynamic parameters measured from plasma included inhibition of Factor Xa activity and measurement of prothrombin time, activated partial thromboplastin time and endogenous thrombin potential.

Results: Rivaroxaban reduced fibrin deposition in the perfusion chamber thrombus as measured by D-dimer levels, which were decreased by 9%, 84% and 65% at low shear rate and 37%, 73% and 74% at high

shear rate after rivaroxaban doses of 5, 10 and 20 mg, respectively. Steady-state ASA with rivaroxaban 5 mg caused a greater reduction in D-dimer levels (63%) at low shear rate. Co-administration of ASA and clopidogrel was associated with a 30% decrease in D-dimer levels at low shear rate and a 14% decrease at high shear rate. No conclusive effect was observed for thrombus P-selectin content across the treatment groups owing to large variations in the results. The other pharmacodynamic parameters measured in plasma were similar to previous studies, and co-administration of ASA and rivaroxaban had no additional influence.

Summary/Conclusions: Rivaroxaban dose dependently inhibited *ex vivo* thrombus formation under low and high shear rates. Co-administration of ASA had a significantly additive effect on the antithrombotic action of low-dose rivaroxaban, which further supports clinical findings in patients with acute coronary syndrome.

OC 16.2

Analysis of the interaction of anticoagulants on points of care (POC) tests for urine from patients on therapy with dabigatran and rivaroxaban

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Backgroud: The amount of new oral anticoagulants (NOACs) such as dabigatran and rivaroxaban may be necessary to determine in specific patient populations. Point of care (POC) methods were currently developed to analyse direct thrombin (international patent application No. PCT/EP2012/002540) and direct factor Xa inhibitors (international patent PCT/WO2012/069139A1) in urine. NOACs as well as conventional anticoagulants (except vitamin K antagonists) are excreted into the urine. Interactions of the anticoagulants may occur on the POC tests for dabigatran and rivaroxaban. POC test have the advantage over tests from plasma to be non-invasive, repetitive and rapid. They show when the NOAC is not any more on board and help to decide when to start or when to increase the dose the conventional anticoagulant.

Aim: We aimed to quantify the interaction of heparin, low-molecular-weight heparin (nadroparin), hirudin, and argatroban of the thrombin and factor Xa specific POC tests using urine samples on treatment with dabigatran or rivaroxaban.

Background: Urine samples were obtained from patients on treatment with 2 × 110 mg or 2 × 150 mg dabigatran (all $n = 15$) daily, 10 mg od rivaroxaban ($n = 15$), from 30 patients on treatment with low-molecular-weight heparin nadroparin (LMWH), and from five controls. All studies were accepted by the local university ethical board and patients gave written informed consent prior to participation. Urine samples of patients with dabigatran and controls were spiked with 0.0, 0.1–1.0 units of unfractionated heparin (UFH), 0.0 0.1–1 mg/mL hirudin and up to 6 mg/mL argatroban. From these samples the POC test for dabigatran was performed. Urine samples of patients with rivaroxaban or controls were spiked with similar concentrations of nadroparin and fondaparinux. Samples with UFH, nadroparin and fondaparinux were analysed without and with addition of 1 IU antithrombin/mL (AT) per mL urine. The POC test for rivaroxaban was performed from all these samples and from urine samples of patients under therapy with LMWH. The urine samples were incubated for 15 min in the POC device and the colour of the samples were judged as positive or negative.

Results: The POC tests for dabigatran and rivaroxaban were positive in all patients on therapy and negative in controls. UFH, LMWH, and fondaparinux did not indicate any interaction in all experimental settings. The POC test for rivaroxaban was negative in all patients on treatment with LMWH without addition of AT and was positive in some patients after addition of AT. High concentrations of hirudin and argatroban showed some interaction with the POC test for dabigatran.

Conclusion: The specificity of the POC assays for dabigatran and rivaroxaban in urine is high and no interactions occur in the absence or presence of AT for all heparins and heparin derived anticoagulants in patients on treatment with LMWH. Only for patients receiving hirudins and argatroban the POC test for dabigatran should not be used.

OC 16.3

Human clinical trials evaluating protein disulfide isomerase as an antithrombotic arget: pharmacodynamic and pharmacokinetic studies of oral quercetin and isoquercetin

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Background: Protein disulfide isomerase (PDI) regulates both platelet accumulation and fibrin generation during thrombus formation following vascular injury in animal models. A high-throughput drug screen previously identified the flavonoid quercetin-3-rutinoside (and related analogs) as potent inhibitors of PDI activity *in vitro* and *in vivo*.

Aims: In anticipation of clinical trials to evaluate PDI as a novel antithrombotic target, we performed a pharmacokinetic study to assess whether oral quercetin or isoquercetin achieves therapeutic concentrations and pharmacodynamic studies to measure PDI inhibitory activity.

Methods: A total of 10 healthy volunteers were enrolled and received either quercetin 500 mg or isoquercetin 500 mg as a single dose. Serial phlebotomies were performed at 0 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h, and 24 h. Total quercetin (aglycone) in plasma was measured by HPLC following enzymatic hydrolysis. In order to assess the pharmacodynamic inhibition of PDI following oral administration of the flavonoids, we developed a plasma-based assay utilizing a fluorescence-based eosin probe coupled to glutathione, with relative fluorescence measured in the presence of PDI.

Results: Isoquercetin demonstrated improved bioavailability (maximal concentration was 3.45 mM 95% CI 0.52–6.39 mM, Tmax 2.6 h, half-life 9.14 h, AUC 17.54) compared with quercetin aglycone (Cmax 0.77 mM 95% CI 0.4–1.12 mM, Tmax 3.8 h, half-life 10.79 h, AUC 5.14). The formulation of isoquercetin or quercetin with ascorbic acid (500 mg) to prevent oxidation did not alter pharmacokinetic profiles. Significant PDI inhibitory activity in plasma was detected following the ingestion of isoquercetin but not quercetin. The PDI inhibitory activity in plasma increased significantly by 4 h following the oral administration of isoquercetin ($P = 0.001$) with a mean difference of 59% relative to baseline. By 6 h, the mean PDI inhibitory activity neared baseline values (mean difference of 16% compared to baseline).

Conclusions: These studies provide proof-of-principle for the use of quercetins to inhibit extracellular PDI in humans and represent an important first step in the development of a novel class of antithrombotic agents. We conclude that oral isoquercetin has improved bioavailability compared with quercetin in healthy adults and results in detectable PDI inhibition as measured by a novel plasma based PDI assay. The antithrombotic efficacy of isoquercetin will be evaluated in cancer-associated thrombosis for high risk cancer populations and in individuals with antiphospholipid syndrome.

OC 16.4

Aptamer inhibition of an exosite of Factor (F)Xa or thrombin synergizes with non-aptamer inhibition of the catalytic site of FXa or thrombin, respectively

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Background: Aptamers that inhibit factor (F)Xa and thrombin function as reversible anticoagulants but achieve less intense anticoagula-

tion than recombinant (r)-tick anticoagulant peptide (TAP), r-hirudin, or heparin. 11F7t, an RNA FXa aptamer, binds a FXa exosite in a manner that inhibits FXa-FVa binding. ARC 183, a DNA thrombin aptamer, and R9D-14T, an RNA thrombin aptamer, each bind thrombin's substrate binding exosite, thereby inhibiting thrombin-substrate binding. In contrast, r-TAP and r-hirudin inhibit FXa and thrombin, respectively, by binding both an exosite and the catalytic site of FXa or thrombin, respectively. Those findings suggest that a univalent catalytic site inhibitor of FXa or thrombin might augment the anticoagulant intensity of an exosite-binding aptamer.

Aims: To test two hypotheses: (1) 11F7t plus a FXa catalytic site inhibitor will synergize; and (2) ARC183 or R9D-14T plus a thrombin catalytic site inhibitor will synergize.

Methods: One hundred and eighty-minute (min) whole blood thromboelastography (TEG) assays.

Results: r-TAP (2.0 μM), r-hirudin (3.67 μM), or heparin (5 U/mL) prevented clot initiation for > 180 min in whole blood TEG assays. The aptamers 11F7t (4.0 μM), ARC 183 (7.5 μM), and R9D-14T (10.0 μM), the small molecule FXa direct catalytic site inhibitors PRT54004 (10.0 μM), rivaroxaban (7.5 μM), and edoxaban (7.5 μM), the FXa indirect catalytic site inhibitor fondaparinux (4.63 μM), or the small molecule thrombin direct catalytic site inhibitor argatroban (98.3 μM) only prolonged clot initiation time to between 14 and 147 min (~2 to ~20 times control). In contrast, the combinations 11F7t (0.5 μM) plus PRT54004 (2.0 μM), 11F7t (2.0 μM) plus rivaroxaban (5.0 μM), 11F7t (2.0 μM) plus edoxaban (2.0 μM), 11F7t (0.5 μM) plus fondaparinux (1.16 μM), ARC 183 (5.0 μM) plus argatroban (5.0 μM), and R9D-14T (10.0 μM) plus argatroban (10.0 μM) each prevented clot initiation for > 180 min.

Summary/Conclusion: A FXa or thrombin aptamer plus a FXa or thrombin catalytic site inhibitor, respectively, synergize to anticoagulate whole blood. A FXa or thrombin catalytic site inhibitor may intensify the reversible anticoagulant effect of a FXa or thrombin aptamer in a clinical setting, such as cardiopulmonary bypass, that requires a high level of anticoagulation.

OC 17 – Clinical Relevance of Microparticles

OC 17.1

Circulating microparticles and thrombin generation phenotypes in the Prospective Observational Multicenter Major Trauma Transfusion (PROMMTT) study: correlation with coagulopathy and survival

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Background: Trauma-induced coagulopathy (TIC) following severe injury and shock is associated with increased bleeding, morbidity, and mortality. Severe injury may result in alteration of cellular phenotypes and release of cell-derived, procoagulant microparticles (MP). Circulating MPs are predominantly of platelet origin and support thrombin generation (TG) and clotting.

Aims: We hypothesized that trauma patients requiring transfusion would have different MP and TG phenotypes than controls, and these differences would be attenuated in TIC and non-survivors.

Methods: The PROMMTT study enrolled 1245 acute trauma patients at ten major level 1 USA trauma centers, of which 196 had blood samples collected for cellular and TG studies. The study was approved by the Institutional Review Boards and by the United States Army Human Research Protection Office. Blood samples were collected at hospital admission. Twenty normal subjects were analyzed for comparisons. Samples were analyzed by the Calibrated Automated Thrombogram to assess TG, and by flow cytometry for MP counts and cellular origin using marker-specific antibodies for platelets (PMP, CD41, CD62P), leukocytes (LMP, CD45), erythrocytes (RMP, CD235a), endothelial cells (EMP, CD51, CD146), tissue factor (TFMP, CD142), and Annexin V (AVMP). These data were analyzed with comprehensive demographic, injury, outcome, and other PROMMTT clinical data. Coagulopathy was defined as either INR ≥ 1.3 or APTT ≥ 35 s.

Results: The median cohort age was 41 (±18.7), 74% were male, Injury Severity Score was 26 (IQR 17, 34). Coagulopathy was noted in 41.6% by INR and 20.5% by APTT criteria. Admission endogenous thrombin potential (nM thrombin*min), thrombin peak (nM) and TG rate (nM/min) were significantly higher in study patients compared to controls (1539, 358, 247 vs. 1184, 208, 61, respectively; all $P < 0.001$). Flow cytometry revealed significant differences in both the MP counts/μL and their relative phenotypic distributions between patients and controls. Study patient EMP levels were 20-fold higher compared to controls (473 vs. 23, $P < 0.001$). RMP, LMP, and TFMP were also higher (407, 362, 181 vs. 197, 185, 67, respectively; $P < 0.001$).

INR-defined coagulopathic patients had significantly lower thrombin peak, TG rate, AVMP and PMP levels (314, 201, 1900, 1762 vs. 384, 272, 2616, 2326, respectively; all $P < 0.01$) compared to non-coagulopathic patients. APTT-defined coagulopathic patients additionally had significantly longer TG lag time, and a lower TFMP (57 vs. 206, $P < 0.001$).

When analyzed by the outcome, patients who died within 24 h of admission had significantly lower thrombin peak (321 vs. 364, $P < 0.01$), TG rate (199 vs. 256; $P < 0.02$), and AVMP, PMP, RMP, and TFMP compared to survivors (1962, 1732, 234, 112 vs. 2432, 2196, 434, 197, all $P < 0.01$). Patients who died within 12 h had the lowest thrombin peak (304) and PMP (1613).

Conclusion: Severe injury results in a significant endothelial activation and increased circulating cell-derived microparticles and thrombin generation. However, a combination of elevated EMP, lower PMP, RMP and TFMP, and lower TG correlates with coagulopathy and poor outcome at 24 h, offering the possibility of indices for interventions.

OC 17.2

Microparticle-dependent plasmin generation predicts the outcome of septic shock patients

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Background: Septic shock (SS) occurs in 10 to 20% of patients in intensive care unit with a high mortality rate (30–50%). It is a systemic response to an infection associated with an hypotension resistant to vasoactive molecules and organ failures. The mechanisms imply a leucocytes and vascular inflammatory response associated with activation of the coagulation and abnormal fibrinolysis. Cellular activation is closely related to the vesiculation process which results in the release of microparticles (MP) from membranes. Over the past decades the role of these small vesicles in activation of the clotting cascade has been well recognized. High level of circulating leukocyte-derived MP (LeuMP) have been reported in SS. Moreover, we recently demonstrated that LeuMP support a plasmin generation capacity (MP-PGC) sustaining a fibrinolytic activity in the circulation.

Aims: Therefore, we hypothesized that MP-PGC is vectorized by MP in SS patients and counterbalances the risk of micro-thrombosis in those with a favorable outcome.

Methods: Thirty-two patients with proved SS were included in the study. All informed consents were obtained and the study was

approved by a local medical ethics committee. Patients outcome was recorded at 30 days. Consistent with the literature the mortality was 37%. MP were purified from SS plasma samples and characterized for plasminogen activators content, activity and cellular origin using chromogenic test, zymography, ELISA, flow cytometry and selective depletions. A new immuno-magnetic separation (IMS)-based bioassay was developed and validated to reproducibly measure the MP-PGC in plasma samples. MP-PGC was measured within the first 24 h after diagnosis on platelet free plasma of SS patients.

Results: Plasmin generation was found on MP purified from SS plasma. This MP-PGC was dependent on a C-terminal lysine binding of plasminogen and the expression of the uPA-uPAR system on the MP surface. LeuMP from granulocyte origin (CD15+) were the major MP subpopulation supporting this activity. Using a new and sensitive IMS-based method, we found that MP-PGC of healthy donors was 4.5 ± 2.8 mDO/min, $n = 20$. In contrast, SS patients showed heterogeneous profile of MP-PGC varying from 0.3 to 600 mDO/min. Interestingly, we observed significant lower MP-PGC values in the patients who died compared to those who survived (median [25–75% interquartile range] = 1.41 [0.89–2.33] mDO/min vs 3.82 [1.84–8.53] mDO/min, respectively, $P = 0.0017$). Thus, among patients with MP-PGC values above 1.65 mDO/min, 85% survived while 75% of patients with MP-PGC below this cut-off value died.

Summary/Conclusion: We demonstrated that MP-PGC is supported by circulating LeuMP in SS patients and predicts the outcome of SS patients. High MP-PGC was associated with survival suggesting that MP dependent fibrinolytic activity may counterbalances the systemic pro-coagulant state found in SS. Thus, MP-PGC not only provides clues for a more comprehensive view on the role of MP in the haemostatic equilibrium but also puts forward the basis for a potential new biomarker that needs to be evaluated on a larger scale.

OC 17.3

Characterization of microparticle numbers and cellular origin in human endotoxemia using high-sensitivity flow cytometry

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The processes of inflammation and coagulation are intimately linked. The interplay of these two systems may be pertinent to a variety of disease states, such as sepsis and sickle cell disease. Endotoxemia causes an acute systemic inflammatory response with concurrent activation of coagulation. Circulating microparticles (MPs), particularly those bearing tissue factor (TF^+-MPs), have been studied using this model, where it has been shown that MP-TF activity increases acutely after intravenous endotoxin administration. To date, however, there are no comprehensive data documenting changes in total MP numbers and sub-sets according to cellular origin. We hypothesized that total MP numbers detected by flow cytometry would increase in parallel with MP-TF activity in the endotoxemia model, and that the magnitude of this increase would vary by cellular origin. To test this hypothesis, 16 healthy individuals were infused with LPS (4 ng/kg body weight) and blood samples were collected in 3.2% sodium citrate at four time points (pre-, 3-, 6- and 24 h post- infusion). Platelet free plasma (PFP) was obtained by two sequential centrifugations at 2500 $\boldsymbol{g} \times 15$ min, and aliquots were frozen at −80°C until analysis. MP-TF activity was determined using an in-house chromogenic assay detecting TF-dependent Xa generation on the MP fraction of plasma as previously described. MP enumeration and characterization was performed on a high-sensitivity flow cytometer (Stratedigm S1000Ex) using an MP size gate of 0.2–0.9 μm by polystyrene bead equivalents. Within the MP gate, total MPs were defined as Annexin+, platelet MPs (PMPs) as CD41+/Annexin+, monocyte MPs (MMPs) as CD14+/Annexin+, RBC MPs (RMPs) as CD235+/Annexin+, and endothelial MPs (EMPs) as CD31+/CD41- events. Circulating nucleosomes (DNA-histone complexes) were also measured using a commercial ELISA kit (Roche). As previously reported, MP-TF activity increased from baseline with a peak at 3 h (baseline: 0.06 ± 0.01 pg/mL; 3 h: 0.51 ± 0.05, $P < 0.001$; 6 h: 0.47 ± 0.06, $P < 0.001$; mean ± SEM). Increases in circulating nucleosomes paralleled that observed with MP-TF activity, peaking at 3 h and returning to baseline by 24 h (3 h: 1.4 ± 0.12-fold increase, $P = 0.001$; 6 h: 1.4 ± 0.12-fold increase, $P = 0.004$; mean ± SEM). The number of MPs generally peaked later at 6 h and was significant for total MPs (baseline: 677 [317, 1432]; 6 h: 1287 [694, 2952], $P = 0.046$; median [IQR]), RMPs (3 h: 155 [85, 377]; 6 h: 275 [157, 611], $P = 0.046$) and PMPs (baseline: 94 [77, 176]; 6 h: 232 [137, 966], $P = 0.046$). Similar increases were noted for MMPs (baseline: 78 [44, 245]; 6 h: 121 [72, 243], $P = 0.317$) but did not reach significance. No significant changes were noted for EMP numbers. Together these data further characterize the pathophysiologic processes observed in the human endotoxemia model and are the first comprehensive analysis of MPs utilizing high-sensitivity flow cytometry. This is also the first description of an acute increase in nucleosomes following endotoxin exposure to our knowledge, which may be clinically relevant in inflammatory disorders with thrombotic risk given their recently discovered association with activation of coagulation and thrombus formation.

OC 17.4

Microparticle-associated tissue factor activity is associated with disease severity in patients with E. coli urosepsis

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Background: Blood microparticles may serve as highly mobile carriers of pro-inflammatory mediators and procoagulant proteins. Microparticles expressing tissue factor (MP-TF) are of special interest. Tissue factor (TF) is thought to play an important role in sepsis and inhibition of TF in primates attenuates disease morbidity and mortality. In a kinetic study of healthy volunteers challenged with purified *E. coli* lipopolysaccharide, we demonstrated that microparticles bearing active TF are concurrently released with markers of inflammation. Interestingly, the subject with the most prominent clinical response to endotoxin also had the highest MP-TF activity. We hypothesized that inflammatory conditions caused by an intact pathogen similarly induces shedding of MP-TF.

Aim: We investigated whether MP-TF activity correlated with disease severity and bacteraemia in a large patient cohort with *E. coli* urosepsis. In addition, we examined whether MP-TF activity decreased upon improvement of the infection and explored the relation of MP-TF activity with blood monocyte count, procalcitonin (a marker for bacterial infection) and markers of endothelial activation.

Methods: During a five-year period, we enrolled 215 consecutive patients without relevant comorbidity presenting with culture proven community-acquired *E. coli* urosepsis at the emergency departments of seven hospitals and affiliated primary healthcare centers. Patients provided informed consent and the study was approved by the medical ethics committees of all participating centers. Cultures of blood and urine were available for all patients. We measured MP-TF activity, sE-selectin, sVCAM-1, monocyte count and procalcitonin in blood collected immediately upon admission, and determined APACHE II disease severity scores. All patients received antibiotic treatment and MP-TF activity was again measured on day 3.

Results: Median MP-TF activity in the 215 septic patients (mean age 51 years, 73% female) was higher than in healthy controls (125 vs. 50 fM Xa/min; $P < 0.0001$), and was the highest in the 48 bacteraemic patients (206 fM Xa/min). Median MP-TF activity was significantly higher in all APACHE II disease severity score categories > 4 compared to the lowest category (299 fM Xa/min for 15–17, 164 fM Xa/min for 10–14, 144 fM Xa/min for 5–9, and 97 fM Xa/min for 0–4 scores; all P-values < 0.01). Furthermore, MP-TF activity decreased during antibiotic treatment from a median value of 116 at admission to 82 fM Xa/min on day 3. MP-TF activity upon inclusion correlated significantly, but weakly with markers of endothelial activation ($r = 0.28$ and 0.30 for sE-selectin and sVCAM-1, respectively; $P < 0.0001$) and showed an inverse correlation with monocyte count ($r = 0.23$; $P = 0.0142$). Moreover, MP-TF activity correlated with procalcitonin levels ($r = 0.35$; $P < 0.0001$).
Conclusion: In this large cohort of urosepsis patients reflecting daily clinical practice, we showed that MP-TF activity is related to disease severity and bacteraemia in patients with *E. coli* urosepsis. MP-TF activity correlated to procalcitonin, and decreased upon resolution of the infection (day 3). We speculate that monocytes are the source of MP-TF and that sE-selectin and sVCAM-1 reflect disease severity rather than an endothelial origin of MP-TF.

OC 18 – Coagulation Factors XI and XII

OC 18.1

APC-resistant factor V restores impaired coagulation resulting from deficient factor XI-mediated feedback activation

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Background: The positive factor Va/VIIIa/IXa feedback reactions by which thrombin propagates its own production are essential to robust clot formation, which is underscored by the fact that defects or deficiencies therein lead to hemophilia. We have previously demonstrated that these feedback loops are regulated in a highly interdependent manner, as absence of the factor VIII/IX-dependent feedback results in premature inactivation of factor V (FV) by activated protein C (APC). Interestingly, APC-resistant FV was shown to have the potential of counterbalancing this secondary defect by impairing the APC system and correcting thrombin generation.
Aim: Here we examined whether the factor XI (FXI)-mediated feedback loop is also tightly linked to FV regulation by assessing if APC-resistant FV can overcome the attenuated coagulation in FXI deficiency.
Methods: Constitutively active FV-810 (Pro811-Gy1491 deleted) and FV-810-QQ, in which the main APC cleavage sites Arg306 and Arg506 are substituted by Gln, were expressed and purified. Coagulation was initiated by low tissue factor, and thrombin activity and tPA-induced fibrinolysis were assessed.
Results: Consistent with its bleeding phenotype, thrombin generation was reduced in FXI deficiency. Surprisingly, whereas triggering the protein C pathway by adding thrombomodulin (TM, 10 nM) or APC (2 nM) partially reduced thrombin formation in normal plasma, thrombin production became fully abolished in FXI-deficient plasma. This suggests that, similar to deficiencies in the FVIII/FIX-dependent feedback loops, a lack of FXI activity renders the plasma more sensitive to the APC system. APC-resistant FV could counterbalance this defect, as addition of FV-810-QQ (1 U/mL) increased thrombin formation substantially in FXI-deficient plasma, whereas FV-810 had no effect. In a clot lysis assay, addition of increasing TM concentrations (0–10 nM) confirmed enhanced sensitivity to the APC system as clot formation was severely prolonged in FXI-deficient plasma (40 min. vs. 5–6 min. in normal plasma). Even though fibrinolysis was inhibited by TM-mediated thrombin-activatable fibrinolysis inhibitor (TAFI) activation in both plasmas, the maximum inhibition attained in FXI deficiency was much lower as compared to normal, concurring with previous observations. Subsequent supplementation with FV showed that, being constitutively active, both variants normalized the clot time in FXI-deficient plasma. Clot lysis times, on the other hand, were fully restored upon adding FV-810-QQ to plasma lacking FXI activity; addition of FV-810 showed no effect in both plasmas. This implies that APC-resistant FV may contribute to enhanced TAFI activation, thereby bypassing the need for upstream feedback activation reactions. This was supported by assessing the clot lysis time using varying concentrations of a TAFI inhibitor (carboxypeptidase inhibitor), which demonstrated that supplementation of FXI-deficient plasma with FV-810-QQ increased activated TAFI to the level of normal plasma.
Conclusions: These data show that similar to deficiencies in the FVIII/FIX-dependent feedback loop, a defect in the thrombin-mediated FXI activation pathway leads to a dramatic reduction in thrombin formation due to action of the protein C pathway. Whereas constitutively active normal FV can partially restore impaired coagulation, its APC-resistant counterpart has the potential of rescuing both clot formation and stability, thereby providing a potential alternative for hemophilia treatment.

OC 18.2

Two novel inhibiting factor XI antibodies prevent cessation of blood flow in a murine venous thrombosis model

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Background: Coagulation factor XI is a potential target for anticoagulation. Factor XI seems promising because of its major role in thrombosis and relatively minor role in hemostasis. This might imply that inhibition of factor XI can prevent thrombosis without causing bleeding.
Aim: To investigate the antithrombotic properties of two novel inhibiting factor XI antibodies (αFXI-175 and αFXI-203).
Methods: The *in vitro* properties of both antibodies were analyzed using standard clotting assays (PT/aPTT) and calibrated automated thrombography. For the *in vivo* model we used factor XI knock out mice on a C57BL/6 background, in which factor XI plasma levels were restored with purified human factor XI. Thrombosis was induced by applying a 10% ferric chloride solution to the vena cava inferior for 3 min. Time to occlusion was analyzed with a tissue perfusion monitor. A tail bleeding assay was used to investigate the safety of both antibodies.
Results: Both antibodies dose-dependently prolonged coagulation in normal plasma in the aPTT. Using calibrated automated thrombography, both antibodies inhibited thrombin generation initiated via the intrinsic pathway. In contrast, upon tissue factor (TF)-initiated thrombin generation, αFXI-203 did not inhibit thrombin generation, while αFXI-175 was only able to inhibit thrombin generation at low concentrations of TF (up to 1 pM). The vena cava inferior remained patent for 25 min in mice treated with αFXI-175 and for 12.5 min in αFXI-203 treated animals, which was significantly longer than in placebo treated animals (5 min, $P < 0.05$). Both antibodies did not prolong the bleeding time or caused severe blood loss in a tail bleeding assay.
Conclusions: Two inhibitory antibodies against factor XI were generated with different specificities. Both antibodies prevented cessation of blood flow in a murine thrombosis model without inducing a bleeding tendency.

OC 18.3

Factor XI acts by mechanisms at least partially independent of thrombin-induced platelet activation in a mouse model of arterial thrombosis

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Background: Factor XI (FXI) is a coagulation factor that can be activated by Factor XIIa (contact pathway) to trigger coagulation and thrombin generation. FXI can also be activated by thrombin as part of a positive feedback loop. Once generated, thrombin cleaves fibrinogen and protease-activated receptors (PARs) to form fibrin and activate platelets, respectively. Fibrin and activated platelets together form a thrombus. FXI-deficient patients and mice exhibit little or no spontaneous bleeding, but appear to be protected against some forms of thrombosis.

Aims/Methods: The primary objective of these studies was to understand the mechanism(s) by which Factor XI deficiency is protective against thrombosis without significantly impairing hemostasis. More specifically, we employed mouse genetics to explore whether there are differential actions of FXI on platelet activation and fibrin formation in the settings of hemostasis and thrombosis.

Results: FXI knockout mice showed no spontaneously bleeding or increased blood loss in a tail bleeding assay, but exhibited remarkable protection in an arterial thrombosis model (2.8 M $FeCl_3$-induced carotid injury). Par4 knockout mice, which have platelets that show no responses to thrombin, showed increased tail blood loss but only modest protection against thrombosis under the same conditions. Mice lacking both Par4 and Factor XI were indistinguishable from mice lacking only Factor XI in our arterial thrombosis model and indistinguishable from Par4 knockouts in the tail blood loss assay. Thus, Factor XI contributes to arterial thrombosis in a manner that is at least partially independent of Par4 and platelet activation by thrombin. Furthermore, Factor XI function appears to be unnecessary in a mouse model of hemostasis even in a sensitized setting in which thrombin-mediated platelet activation has been eliminated.

Thrombin-dependent fibrin formation is an obvious candidate for the platelet-independent role of Factors XI in the thrombosis model. FibAαEK mice carry a mutant form of fibrinogen that cannot be converted to fibrin but fully support platelet aggregation. Preliminary data suggest that the fibrinogen-AαEK variant can support hemostasis and that the FibAαEK mice are comparable to control littermates in the carotid thrombosis under the conditions employed above. While fibrin formation may not be strictly required for hemostasis or arterial thrombosis, it is possible that fibrin formation will be required in the setting of Par4 deficiency and defective platelet activation, and that loss of both fibrin formation and platelet activation due to decreased thrombin generation will account for the dramatic protection seen in FXI-deficient mice. Alternatively, FXI may contribute to hemostasis and thrombosis by mechanisms independent of thrombin generation and thrombin-dependent fibrin formation and platelet activation.

Conclusion: Protection of Factor XI-deficient mice in a model of arterial thrombosis is at least partially independent of thrombin-induced platelet activation. To assess the possibility that fibrin formation accounts for this activity, a comparison of FXI deficient mice to *Par4*:*FibAαEK* and *FXI:FibAαEK* double mutant mice and to mice treated with inhibitors of thrombin generation is in progress.

OC 18.4

A model for binding of factor IX to the factor XIa apple 3 domain

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Background: Factor XIa (fXIa) cleaves factor IX (fIX) at the Arg^{145}-Ala^{146} bond to form the intermediate factor IXα (fIXα), and then at the Arg^{180}-Val^{181} bond, to form the protease factor IXaβ (fIXaβ). As catalytic efficiency for Arg^{180}-Val^{181} cleavage is seven-fold greater than Arg^{145}-Ala^{146} cleavage, fIXα does not accumulate. A fIX binding exosite on the fXIa A3 domain is central to this mechanism. The fIX Gla-domain is also required for fIX activation by fXIa, and may be the structural element that binds to fXIa.

Aims: To identify structures on the fXIa A3 domain and fIX Gla-domain involved in fIX activation by fXIa.

Methods: Wild type fXIa ($fXIa^{WT}$), fXIa with the A3 domain (amino acids 182–265) replaced with the prekallikrein A3 domain (fXIa-PKA3), and fXIa with alanine substitutions for amino acids 183–185 ($fXIa\text{-}Ala^{183\text{-}185}$) were expressed in HEK293 fibroblasts. FXIa-PKA3 was used as a scaffold to reintroduce fXI sequence into PKA3 to determine the minimal number of fXIa residues needed for restoration of fIX activation. FIX molecules with segments of the Gla-domain replaced with factor VII sequence were also expressed. The fXIa proteases were tested for their ability to activate recombinant and plasma fIX.

Results: FXIa-PKA3 cleaves the fIX Arg^{145}-Ala^{146} and Arg^{180}-Val^{181} bonds with 60- and 4500-fold lower catalytic efficiency, respectively, than $fXIa^{WT}$, due to loss of the fIX/fIXα binding site on the A3 domain. $FXIa\text{-}Ala^{183\text{-}185}$ displayed a comparable defect, indicating residues 183–185 are required for exosite function. Residues 183–185 are at the N-terminus of the A3 domain, and contribute to formation of a charged patch and hydrophobic pocket on the A3 surface. Replacing PK residues at positions 183–185 in fXIa-PKA3 with the corresponding fXIa residues partially restored fIX activation (~5% activity of fXIaWT). The N- and C-termini of A3 are in proximity due to the Cys^{182}-Cys^{265} bond at the ends of the domain. Introducing fXI residues at the C-terminus of fXIa-PKA3 (residues 260–264), in addition to fXI sequence at 183–185, completely restored fIX activation. Replacement of fIX Gla-domain residues 4–11 (the Ω-loop) with the corresponding sequence from factor VII resulted in a defect in activation by $fXIa^{WT}$ similar to the one observed when fXIa-PKA3 activates fIX.

Summary/Conclusion: Amino acids 183–185, and 260–264 are closely associated in fXIa, and contribute to formation of charged and hydrophobic regions on the A3 domain surface that are not predicted to be present in the prekallikrein A3 domain. These structures likely form a fIX binding site on fXIa. The protease domain in zymogen fXI covers this region of A3, consistent with the observation that fIX does not bind to the zymogen. Conformational changes that occur during fXI conversion to fXIa must expose this area to permit fIX binding. Binding of the fIX Gla-domain through its Ω-loop to phosphatidylserine-rich surfaces contributes to K_m for fIX activation by factor VIIa-tissue factor. The fIX Ω-loop may mediate a similar interaction with the fXIa A3 domain that is required for initial recognition of fIX by fXIa.

OC 19 – Coagulation – I

OC 19.1

Circulating histone-induced thrombosis leads to circulatory and respiratory failure

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Background: Extensive cell damage associated with coagulation activation and the development of circulatory and respiratory failure are common features in critically illness but the underlying pathophysiological mechanisms remain unclear. During cell death, nuclear proteins and histones in particular, are released into the circulation and cause toxicity through thrombin generation, platelet aggregation, endothelial damage and cytokine release. This recent discovery may increase the understanding of the fundamental pathologies of critical illness and deliver improved patient management approaches for these unmet health challenges.
Aim: Investigate the hypothesis that extracellular histones serve as mediators of distant organ damage after extensive cell death.
Methods: To investigate this further, we used *in vivo* experimental models and clinical samples from patients with severe non-thoracic trauma.
Results: Cytokines and markers for endothelial damage (sTM) and coagulation activation (TAT) significantly increased immediately after trauma or histone-infusion in mice. Cardiac function was impaired in histone-infused mice with increased pulmonary pressure and right ventricular size. Pathological examination showed that lungs were the predominantly affected organ with severe edema, multicocal alveolar hemorrhage, microvascular thrombosis and neutrophil congestion. Histone treated mice developed dyspnea and cyanosis as a manifestation of pulmonary and cardiac failure and died within 2 h. Pathological changes and mortality could be prevented by anti-histone antibodies. Clinically, circulating histone levels surged significantly immediately after injury to levels that were toxic to cultured endothelial cells ($\geq$ 50 μg/mL). The high levels were significantly associated with the incidence of acute respiratory failure and SOFA scores, as well as sTM (median: 4.6, quartile: 3.6, 5.5 ng/mL) and TAT levels (median: 81.2, quartile: 33.7, 110.3 ng/mL).
Conclusions: This work has elucidated a new mechanism for multiple organ failure in critically ill patients and proposes future translational intervention with rapid assays to monitor circulating histone levels and anti-histone therapies to reduce thrombosis and improve the pulmonary microcirculation. Overall, anti-histone therapy could potentially protect against the development of multi-organ failure and improve overall outcome in many critical illnesses.

OC 19.2

An essential role of factor XI-feedback activation for hemostasis in embryonic development

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Background: The classical cascade model of plasma coagulation proposes that activated factor XII initiates thrombin formation via activation of its substrate factor XI (FXI). Recent studies have shown that factor XII-mediated FXI activation has a crucial role for thrombosis in mouse models. In contrast, the revised model of coagulation proposes that FXI is predominantly activated by thrombin, which is generated upon activation of the tissue factor pathway (feedback activation). This concept, however, has poorly been tested *in vivo*.
Aims: Our studies analyze the concept of FXI-feedback activation in plasma and in genetically altered mice.
Methods and Results: Minute amounts of particle-bound thrombin triggered thrombin production in plasma both in the absence of tissue factor and in the presence of active site inhibited factor VIIa. Thrombin did not activate factor XII and thrombin generation was nearly abolished by an anti-FXIa antibody and in FXI-deficient plasma. Surface bound thrombin induced complex formation of FXI, with its major inhibitor C1 inhibitor, even in factor XII-deficient plasma. To analyze functions of FXI-feedback activation *in vivo* we crossed tissue factor-deficient mice (low tissue factor) with mice deficient in the intrinsic pathway proteases factors IX, XI and XII, respectively. Factor IX null mice have a bleeding phenotype (hemophilia B), whereas FXI and factor XII deficient mice have a normal hemostatic capacity. Combined deficiency in tissue factor and factor IX resulted in the intrauterine death of embryos due to hemorrhage. In contrast, factor XII/tissue factor-deficient mice were viable and their bleeding phenotype was unchanged as compared to deficiency in tissue factor alone. Unexpectedly and in contrast to factor XII/tissue factor-deficiency phenotype, combined deficiency in FXI/tissue factor was lethal. Less than 5% of expected offspring with deficiency in both factors were born alive. At late developmental stages FXI/tissue factor-deficient embryos were found in the Mendelian distribution range, indicating that most embryos died during delivery. Morphological and histological analysis of these embryos revealed normal growth but hyperemia in livers, edema and fibrin deposition in hearts and frequent hemorrhages in abdominal or thoracic cavities and in placentas.
Conclusions: Our studies show that FXI-feedback activation exists *in vivo* and support a crucial role of this pathway for hemostasis in neonatal development.
Disclosure of interest: None declared

OC 19.3

Whole blood minimal tissue factor triggered thrombelastometry and calibrated automated thrombogramme are useful tools for the evaluation of the global effect of antithrombotic treatment

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Background: Routine blood coagulation tests (PT and aPTT) and the anti-Xa activity evaluate a partial aspect of the global antithrombotic activity of the anticoagulant drugs and are used for the monitoring of treatment with VKA, UFH and LMWHs. Thromboelastometry and thrombogramme are performed in more physiologically relevant conditions in the presence of low TF concentration and might offer additional information for the global anticoagulant effect of the anticoagulant agents.
Aim of the Study: We investigated the sensitivity of whole blood thromboelastometry and thrombin generation (TG) assay to detect the effect of LMWH or VKA treatment.
Materials and Methods: Patients receiving prophylaxis (n = 100) or treatment (n = 50) with enoxaparin or VKA (n = 100) were studied. Minimal TF-TEM in whole blood (WBminTF-TEM) was performed according to previously published method. TG was done in PPP prepared from the same WB samples and assessed with Thrombinoscope assay. For patients receiving enoxaparin the dose, time of injection and blood collection were recorded. The anti-Xa activity in PPP and the INR were also measured. The control group (n = 40) consisted of healthy individuals with normal PT and aPTT. The upper and lower normal limit (in the control group – UNL/LNL) and upper and lower

therapeutic limit for each group of treated patients and the inter-individual variability of the response (iCV) was calculated for each assay.
Results: Table 1 shows the values of WBminTF-TEM and TG parameters in the control group and in the patients' groups. Both tests were sensitive to the anticoagulant effect of the studied drugs. Enoxaparin induced dose-dependent alterations on both assays. Treatment with LMWH or VKA induced similar modifications on both assays. For each assay the iCV range was 6–30% in the control group, 11–45% in LMWHprophylaxis group, 25–80% in LMWHtreatment group and 9–79% in VKA group. In 10% of patients no modification of thromboelastogram or thrombogram was observed although the anti-Xa activity or INR were within the expected range.
Conclusion: WBminTG-TEM and TG are global coagulation assay sensitive to detect the anticoagulant effect of prophylactic and therapeutic doses of LMWH and that of treatment with VKA. Both assays demonstrate the presence of a variable biological response to the treatment which is not predicted by the levels of the anti-Xa activity and the INR.

OC 19.4

Human VKORC1 mutations (His28Gln, Trp59Leu, Val66Met) investigated by the new cell-based assay exhibit warfarin resistance phenotypes not detected by the 'classical' DTT-driven VKOR assay

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Background: Coumarins are globally used as oral anticoagulants for prevention and therapy of thrombosis and embolism since the early 1950s. However, clinical use of coumarins is difficult due to risk of bleeding and high interindividual dose variation including coumarin resistance. In most cases, oral anticoagulant resistance (OACR) originates from mutations in the enzyme vitamin K 2,3-epoxide reductase complex subunit 1 (VKORC1), that is the molecular target of coumarins.
Aim: Because investigation of VKORC1 variants by the 'classical' dithiothreitol (DTT)-driven VKOR assay does not reflect patient phenotype well, we used the cell-based assay to study human VKORC1 (hVKORC1) mutations (His28Gln, Trp59Leu, Val66Met). This assay implements coexpression of human coagulation factor IX (hFIX) together with hVKORC1 in HEK293T cells and uses secreted hFIX activity as surrogate marker to report wild-type and variant hVKORC1 inhibition by warfarin.
Methods: hVKORC1 and hFIX cDNA were cloned into a bicistronic vector. Afterwards, OACR-associated hVKORC1 mutations (His28Gln, Trp59Leu, Val66Met) were introduced by site-directed mutagenesis and the respective constructs were transfected into HEK293T cells. The expression medium was supplemented with vitamin K (5 ng/μL) and different concentrations of warfarin (0.01–1 μM). Seventy-two hour after transfection FIX activity was measured.
Results: In the absence of warfarin, basal FIX activities for the coexpressed hVKORC1 variants were similar to that of wild-type hVKORC1 and decreased according to the warfarin concentration. Dose response curves were fitted and half maximal inhibitory concentrations (IC_{50}) for warfarin were calculated for wild-type hVKORC1 as well as for His28Gln, Trp59Leu, and Val66Met hVKORC1 variant (24.7 nM, 71.6 nM, 1857.9 nM, 133.6 nM, respectively).
Conclusion: In contrast to the 'classical' DTT-driven VKOR assay, mutation His28Gln, Trp59Leu, and Val66Met exhibit a warfarin resistance in our cell-based system. Furthermore, calculated IC_{50} values for hVKORC1 variants allow us to rank the severity of the respective mutation. Thus, His28Gln exhibit a mild resistance, Val66Met a moderate resistance and Trp59Leu a total resistance phenotype. Additionally, IC_{50} values for the OACR-associated variants divided by that for wild-type VKORC1 might be a first approximation for increased warfarin dose requirements for patients carrying the mutation *in vivo*. Based on our *in vitro* results, patients bearing His28Gln, Trp59Leu, and Val66Met are expected to require 2.9-, 5.4-, and 75-fold greater mean warfarin dosage in order to achieve the same VKORC1 inhibition levels as patients with wild-type VKORC1. These data correspond well to the clinical phenotype reported for the respective patients.
In conclusion, our results from the cell-based assay accurately reflect *in vivo* OACR patient phenotype. In addition, this assay is a good tool for further studies on VKORC1 function.

OC 20 – Diagnosis of Primary Venous Thrombosis

OC 20.1

Is it useful to image both legs in patients with suspected deep vein thrombosis? A retrospective chart review

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Background: Compression ultrasonography (CUS) is the cornerstone of the diagnostic workup in patients with suspected deep vein thrombosis (DVT). However, significant variations exist in clinical practice between centers and/or countries, as highlighted by recent studies comparing single whole-leg to serial proximal ultrasonography. Much less data exist on the need for bilateral leg imaging. Many centers systematically perform a bilateral leg ultrasonography, while imaging is restricted to the symptomatic leg(s) in other centers.
Aims: To assess the yield of bilateral leg ultrasonography in patients with suspected DVT.
Methods: We retrieved the charts of all patients who underwent venous ultrasonography inGeneva University Hospital, Switzerland, between Jan 1, 2009 and Dec 31, 2012. Patients undergoing CUS for another reason than a clinical suspicion of DVT were excluded. For each patient, we extracted information on general demographic information, main risk factors for DVT, clinical probability as assessed by the Wells' score for DVT, side of clinical suspicion, and CUS results. All patients were investigated using bilateral whole-leg CUS.
Results: Out of > 3000 patients referred with suspected DVT, 467 patients (mean age 64 (SD 20) years, female 52%) had proximal or distal DVT confirmed by CUS. Main risk factors were immobilization (32%), cancer (23%), personal history of venous thromboembolism (22%), estrogen use (8%) and family history of venous thromboembolism (8%). DVT was suspected on the left side in 255 (55%), on the right side in 187 (40%), and on both sides in 23 (5%) patients; this information was missing in two patients. A DVT was found only in the symptomatic leg(s) or in one of the two symptomatic legs in 442 patients (95%). In 19 patients (4%), a DVT was found in the asymptomatic leg as well as in the symptomatic leg. Of note, 15 of these 19 patients had cancer, and they represented 14% of cancer patients with DVT. Finally, in four patients (0.9%), CUS was negative on the side of the suspected DVT but positive on the opposite side. All four were below-knee muscular thromboses (three soleal and one gastrocnemial).
Summary/Conclusions: Systematic imaging of both legs in patients with suspected DVT is unnecessary. An examination of the contralateral leg may be worthwhile in cancer patients found to have DVT as this test could be used as a baseline imaging test in case of future suspected DVT in this population with a high risk of recurrent thrombosis.

OC 20.2

Safety of ruling out pulmonary embolism (PE) in pregnancy by computed tomography pulmonary angiography (CTPA)

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Background: Pulmonary embolism (PE) is one of the leading causes of maternal mortality. An accurate diagnosis of PE is of critical importance in order to adequately treat diagnosed PE on one hand, and to prevent morbidity and mortality by starting anticoagulant treatment without indication on the other hand. Numerous studies have investigated clinical decision rules, D-dimer and imaging tests in order to establish the most efficient diagnostic algorithm. However, pregnant patients have been systematically excluded from these studies. Clinical decision rules established in non-pregnant patients cannot be applied to pregnant patients since items in these scores are either physiologically more common, e.g. tachycardia, or seldomly present, e.g. malignancy. D-dimer tests as a single-test is advised against in non-pregnant patients and furthermore D-dimer levels rise physiologically during pregnancy accounting for a lower specificity. Objective imaging tests are therefore the cornerstone of diagnosing PE in pregnancy. Prospective studies on the performance of these tests to exclude the diagnosis in pregnancy are lacking.

Aim: To investigate the performance of ruling out PE by CTPA in pregnant patients with a clinical suspicion of PE.

Methods: We performed a prospective study of consecutive pregnant patients with a clinical suspicion of PE, conducted in three centers in the Netherlands from February 2004 through December 2012. We excluded women who used anticoagulant treatment. A CTPA was performed and in case of an intravascular filling defect, the diagnosis of pulmonary embolism was established and anticoagulant treatment was started. In case of a normal result or an alternative diagnosis, pulmonary embolism was ruled out and anticoagulant treatment was withheld. In case of an inconclusive result, management was left to the discretion of the treating physician. All patients were followed for 3 months to assess the occurrence of symptomatic or fatal venous thromboembolism (VTE). Consent and ethical approval was obtained.

Results: A total of 149 pregnant patients were enrolled and six patients were excluded because of use of therapeutic Low-Molecular-Weight-Heparin (LMWH) for more than 24 h. The median age of the 143 included patients was 30.7 years (IQR 27.3–35.2) and 10 patients (7.0%) had a history of previous VTE. PE was diagnosed in six patients (4.2%). CTPA excluded PE in 129 patients (90.2%) and was inconclusive in eight patients (5.6%), of whom one was treated with anticoagulation therapy. None of the 136 patients in whom PE had been ruled out and who were not treated with anticoagulants experienced VTE in the 3-month follow-up (incidence 0.0%, 95% upper confidence interval 2.7%).

At time of submitting this abstract, a few patients had not completed the 3-month follow-up because this date is behind the abstract submission date.

Summary/Conclusions: A negative CTPA safely rules out pulmonary embolism in pregnant women with a clinical suspicion and can be used as a first-line test.

OC 20.3

Cost-effectiveness of ruling out pulmonary embolism in primary care using the Wells rule and D-dimer testing

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Background: Referral of all patients suspected with PE presenting in primary care for diagnosis is inefficient since the prevalence of the disease is only 10–15%. The Amsterdam Maastricht Utrecht Study on ThromboEmbolism (AMUSE-2) recently showed that a diagnostic strategy that combines the Wells rule for pulmonary embolism (PE) with a negative D-dimer test result can safely and efficiently exclude PE in primary care. This abstract presents the results of a cost-effectiveness study that evaluates whether the AMUSE-2 strategy will reduce health care costs without considerable loss of quality adjusted life years (QALY).

Aims: To assess the cost-effectiveness of a diagnostic strategy that combines the use of the Wells rule with qualitative point-of-care D-dimer testing to select primary care patients with suspected PE for referral to secondary care, compared to referral of all patients with suspected PE.

Methods: A Markov-type cost-effectiveness model with a 5-year time horizon and a 6-month cycle time was used to compare the AMUSE-2 strategy to referral of all patients with suspected PE to secondary care. Transition probabilities were derived from the AMUSE-2 study (2007–2010) and the literature. Societal costs and health state utilities were obtained from literature. The Markov model consisted of six mutually exclusive health states: no PE, post 1st PE, post recurrent PE, chronic thromboembolic pulmonary hypertension, post central nervous system bleed, and death. The AMUSE-2 strategy consisted of two sub-strategies: the Wells rule with a cut-off point of < 2 combined with qualitative point-of-care D-dimer testing and the Wells rule with a cut-off point of ≤ 4 combined with qualitative point-of-care D-dimer testing. The model was evaluated using cohort simulation. Uncertainty was assessed in a probabilistic sensitivity analysis. Cost effectiveness acceptability curves (CEAC) were constructed.

Results: Compared to referral of all patients both AMUSE-2 strategies resulted in lower costs and less QALYs. The AMUSE-2 strategy with a cut-off point of < 2 resulted on average in a saving of €903 82 and a QALY loss of 0.03 as compared to referral of all. The strategy with a cut-off point of ≤ 4 showed on average a saving of €1198 76 with a QALY loss of 0.10 compared to referral of all. The ICER is €32747 for cut-off point < 2 compared to referral of all patients. For cut-off point ≤ 4 compared to cut-off point < 2 the ICER is €4318. The CEAC curves show that the AMUSE-2 strategy with Wells-score cut-off point < 2 has the highest probability of being cost-effective even for ceiling ratios over €100.000

Conclusions: The Amuse-2 strategy based on the Wells rule for PE and a qualitative point-of-care D-dimer test to exclude PE in primary care is cost-effective compared to referral of all patients suspected of PE in primary care when using a cut-off point of < 2 for the Wells rule.

OC 20.4

D-dimer relevance score

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Background: Some clinical conditions (age, cancer…) increase the risk of false positive D-dimer results, decreasing test utility. In these conditions some clinicians elect not to measure D-dimer and proceed directly to computed tomography pulmonary angiography (CTPA).

Aims: To help physicians to determine the utility of D-dimer measurement, we derived and internally validated a score for risk of false positive D-dimer. We analyzed an arbitrary D-dimer relevance threshold of at least 10% probability of negative D-dimer.

Methods: Secondary analysis of three prospectively collected databases of PE suspected patients (2 European, 1 American). Only patients with highly sensitive quantitative D-dimer measurement were included. The study population (n = 4537) was randomly divided into two groups. Patients with PE were excluded to derive a score of risk of false positive D-dimer. In the derivation group, variables statistically significantly associated with PE in univariate analyses were included in a multivariable logistic model. Points were assigned according to regression coefficients. The score was applied to the validation group. Its clinical applicability was evaluated in patients with low or moderate clinical probability (i) assessed by three methods (Wells score, revised Geneva score and gestalt) (ii) in our European and American populations. Receiver operating characteristic curve analysis was performed and the area under the curve (AUC) assessed.

Results and Discussion: The final score contained ten variables: Female sex (1 point), Age 65–84 years (4 points), Age ≥ 85 years (8 points), Personal history of venous thromboembolism (1 point), Surgery within 4 weeks (2 points), Active malignancy (3 points), Pregnancy or Postpartum within 4 weeks (4 points), Heart rate ≥ 95/' (1 point), O2 saturation < 95% (2 points), Temperature ≥ 38.5 °C (3 points). AUC was 0.76 (CI: 0.73–0.78). There was a regular decrease in the proportion of negative D-dimers with increasing score values. Scores ≤ 8 were associated with at least 10% probability of a negative D-dimer. In clinical application (patients with low or moderate clinical probability), results were similar regardless of how clinical probability was assessed, and in both continents. The proportion of patients with a score above 8 was 5% in the overall population and 10.2% vs. 3.1% respectively in the European and American populations ($P < 0.001$). This difference appears to be mainly explained by the difference in PE prevalence (13.3% vs. 3.2%) and by differences in patient characteristics between these two populations.

The score might lead physicians who always measure D-dimer to think about alternative strategies when the probability of a negative D-dimer result is low (score > 8). On the other hand, this was the case in only 5% of patients; thus the score should also and mainly lead sceptical physicians to measure D-dimer more frequently, instead of directly performing CTPA in patients with one criterion thought to be associated with a high risk of false positive test.

Conclusion: The score gives information about the expected proportion of negative D-dimer and enables clinicians to make better informed decisions about whether to measure D-dimer or not in PE suspected patients.

OC 21.1

Bimolecular interactions of platelet factor 4 (PF4) with HIT-pathogenic versus non-pathogenic anti-PF4/heparin antibodies

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Background: Heparin induced thrombocytopenia (HIT) is a thrombotic complication of heparin therapy mediated by antibodies to complexes between platelet factor 4 (PF4) and heparin or cellular glycosaminoglycans. However, only a fraction of patients with these antibodies develop HIT, implying that only a subset of anti-PF4/heparin antibodies is pathogenic. The basis for the difference in the capacity of anti-PF4/heparin antibodies to activate platelets/monocytes and induce thrombosis *in vivo* are not revealed by conventional bulk techniques, such as ELISA, and remains unclear.

Aims: To elucidate the intrinsic PF4-binding properties of HIT-like monoclonal antibodies (KKO) vs. non-pathogenic antibodies (RTO) at the single-molecule level to avoid effects of avidity and other auxiliary intermolecular interactions.

Methods: We measured the strength of binding of surface-attached bivalent intact KKO and RTO with PF4 utilizing optical trap-based force spectroscopy. Briefly, a KKO- or RTO-coated latex bead was trapped by the laser beam and brought into intermittent contact with the PF4-coated pedestal. Rupture force signals following repeated contacts between the protein-coated surfaces were collected and displayed histograms. The binding probability reflected the association rate, while the peak of forces (binding strength) reflected the forced dissociation rate.

Results: In control experiments with tetrameric WT PF4 and either KKO or RTO, we identified weak interactions arising from PF4-PF4 bonds, which were difficult to separate from antibody-PF4 interactions. To study antibody-PF4 binding specifically, we prevented rupture of PF4-PF4 bonds by chemical cross-linking of the PF4 tetramers. The specificity of rupture forces generated by the surface-bound cross-linked PF4 and KKO or RTO was confirmed by competitive inhibition experiments in the presence of the free Fab fragments or PF4. Single-molecule binding was confirmed by the dependence of binding probability on surface density. Modeling of the force histogram revealed that KKO-PF4 interactions were about 10-fold faster than RTO-PF4 (kon' = 4.36 vs. kon' = 0.45/s, respectively) and the apparent equilibrium dissociation constants differed ~10-fold (Kd' = 7.1 × 10^{-4} and Kd' = 6.4 × 10^{-3}, respectively) with similar force-free off-rates ($koff$ = 0.0031 and $koff$ = 0.0029/s). The KKO-PF4 interactions were somewhat stronger, as reflected by the position of the force peak (84 ± 5 vs. 78 ± 4 pN, $P < 0.05$). In contrast to the WT PF4, KKO and RTO showed lower and similar binding probabilities to cross-linked $PF4^{K50E}$, which forms few oligomers, although the binding strength was again slightly higher for KKO vs. RTO.

Summary/Conclusions: The data indicate that KKO has significantly higher affinity for PF4 oligomers than RTO. In combination with the ability of KKO to promote super-oligomerization of the PF4 tetramers, these data suggest an amplification reaction, in which KKO, unlike RTO, binds preferentially to PF4 tetramers (with or without heparin), enhances their further polymerization and becomes more and more avid as the antigenic complexes grow in size. The results of the single-molecule rupture force measurements indicate that there is a fundamental difference in the antigen binding mechanisms between pathogenic and non-pathogenic anti-PF4 antibodies that underlie their distinct pathophysiological behaviors/profiles. These studies suggest that epitope specificity may contribute to the pathogenic potential of some anti-PF4 antibodies.

OC 21.2

Serological investigation of 20 patients re-exposed to heparin with a previous history of heparin-induced thrombocytopenia (HIT)

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Background: Sparse data exist regarding the frequency, timing and magnitude of the anti-platelet factor 4/heparin (PF4/H) immune response 'and associated risk of recurrent heparin-induced thrombocytopenia (HIT)' 'among patients who have recovered from HIT but who are subsequently re-exposed to heparin. One report (Poetzsch et al. N Engl J Med 2000;343:509:515) found that among 10 patients with previous HIT who underwent cardiac surgery with unfractionated heparin (UFH), *none* developed antibody recurrence by anti-PF4/H enzyme-immunoassay (EIA), even though ~50% of post-cardiac surgery patients would be expected to form anti-PF4/H antibodies (and ~10% expected to form platelet-activating antibodies by serotonin-release assay [SRA]). The true risk of HIT recurrence' 'whether higher, about the same, or potentially even lower than in patients without previous HIT' 'remains uncertain.
Aims: To determine the frequency, timing, and magnitude of the anti-PF4/H immune response, and the frequency of recurrent HIT, among patients with well-documented previous HIT who were re-exposed to heparin.
Methods: We studied 20 patients with previous HIT (definite [4Ts $\geq$ 4, SRA+], n = 16; probable [4Ts $\geq$ 4, EIA+; SRA not done], n = 4) who underwent repeat heparin re-exposure (UFH, n = 19; LMWH, n = 1), occurring 4.4 year (mean [range, 8 week to 13.5 year]) post-HIT diagnosis. We examined serial platelet counts for HIT recurrence, and the SRA and isotype-specific anti-PF4/H EIAs for antibody recurrence, testing serial post-exposure blood samples (median, nine samples/patient to day 30 post-rechallenge [last sample, median 11 days after re-exposure]).
Results: One patient (5%) developed recurrent HIT, confirmed by seroconversion from negative baseline to strong-positive SRA (%serotonin-release = 95%/100%/100%/5% at 0/0.1/0.3/100 U/mL UFH) and strong-positive EIA-IgG (2.74 OD units; normal < 0.45) following cardiac surgery and while receiving postoperative thromboprophylaxis with fondaparinux (2.5 mg/day); this patient's clinical course was unusual (2009 HIT: onset day 7, platelet nadir = 20, platelet recovery by ~70 days, deep-vein thrombosis (DVT), generalized maculopapular rash, full recovery with therapeutic-dose fondaparinux 7.5 mg/day), virtually recapitulating his prior HIT episode 11 year earlier (1998 HIT: also post-cardiac surgery, onset day 7, platelet nadir = 26, recovery by ~50 days, DVT/pulmonary embolism, generalized maculopapular rash, full recovery with therapeutic-dose danaparoid). Despite no other patient developing recurrent HIT, 8/17 (47%) post-cardiac/vascular surgery re-exposed patients developed seroconversion to a positive SRA, and 10/17 (59%) demonstrated an anti-PF4/H immune response (IgG>IgA>IgM), even though none of these 17 patients continued to receive UFH or LMWH postoperatively. None of three patients who received a repeat course of UFH or LMWH for a medical indication developed recurrent antibodies. Interestingly, there was no evidence for speedier antibody formation among eight patients in whom timing of antibody detectability after re-exposure could be compared with the timing of onset of their prior HIT episode. Summary/Conclusions
Patients with a previous history of HIT who undergo repeat heparin exposure are at considerable risk of repeat formation of platelet-activating anti-PF4/H antibodies (positive SRA), although in the absence of continuing heparin administration the risk of HIT appears to be low. We also report the first case of recurrent HIT despite the strategy of limiting repeat UFH to a brief intraoperative re-exposure (this patient recovered fully with therapeutic-dose fondaparinux).

OC 21.3

Impact of polymorphisms affecting ACP1, which codes for a protein tyrosine phosphatase, on levels of antibodies to platelet factor 4/heparin complexes

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Background: Heparin-induced thrombocytopenia (HIT) results from an atypical immune response to platelet factor 4/heparin complexes (PF4/H), with rapid synthesis of platelet-activating IgG antibodies that activate platelets *via* FcgRIIa receptors. The reasons explaining why only a subset of patients treated with heparin develop IgG to PF4/H complexes, and why most patients with these antibodies do not develop HIT, have not been fully defined. The immune response in HIT involves both B and T cells, and protein tyrosine phosphatases (PTPs) are crucial for regulating antigen receptor-induced lymphocyte activation and FcgR-dependant pathways. Therefore, several studies have showed that functional polymorphisms affecting PTPs such as *PTPN22* and *ACP1* coding LYP and LMPTP respectively were associated with autoimmune diseases.
Aim: To investigate whether an association between polymorphisms in *PTPN22* (1858C/T) or *ACP1* (A, B, C alleles) and the development antibodies to PF4/H and HIT may exist.
Patients and Methods: Eighty nine patients with definite HIT (positive PF4-specific ELISA (HAT® GTI) and serotonin release assay) and two control groups were studied. The first control group (Ab^{neg}) consisted of 179 patients who had undergone cardiopulmonary bypass (CPB) with high doses of heparin, without Abs to PF4/H post-operatively. The second control group (Ab^{pos}) consisted of 160 patients who had also undergone CPB and developed significant levels of PF4-specific antibodies, but without HIT. Genotypes of *PTPN22* 1858C/T (rs2476601), *ACP1* 228C/T (rs11553742) and 254C/T (rs11553746) were defined by the PCR-HRM method. The *ACP1* A, B, C alleles were defined by combining the analysis of both SNP 228C/T and 254C/T.
Results: The frequency of *PTPN22* 1858T alleles was similar in HIT patients (11%) and controls (12% and 11%), whether they had developed antibodies to PF4 or not. In contrast, the *ACP1* A allele was less frequent in patients with antibodies to PF4, whether they had developed HIT (25%) or not (27.5% in Ab^{pos} controls), than in Ab^{neg} subjects (37%, P = 0.001). The A, B and C alleles *of ACP1* had previously been associated with the synthesis of distinct active LMPTP isoforms exhibiting different catalytic properties. In this regard, the genotypes known to exhibit the highest LMPTP enzyme activity i.e. AC, BB and BC, were more frequent (66.5%) in patients with high levels of antibodies to PF4/H complexes (A_{405} > 2) than in the others (52%, P = 0.03). Therefore, these genotypes appeared increase the risk of anti-PF4/H antibody formation in heparin-treated patients (OR 1.8; 95% CI 1.2–2.6, P = 0.005 after comparing Ab^{pos} + HIT vs. Ab^{neg}).
Conclusion: LMPTP regulates ZAP70 and lymphocyte activation by enhancing T-cell antigen receptor-dependant signaling and our study supports that *ACP1* genotypes associated with strong enzymatic activity of this PTP may increase the intensity of immune response to heparin-modified PF4.

OC 21.4

Endocytotic mechanisms contributing to the internalization of ADAMTS13 by macrophages

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Background: Acquired thrombotic thrombocytopenic purpura (TTP) is a severe disorder characterized by the production of autoantibodies directed against ADAMTS13, a metalloproteinase that regulates plate-

let adhesion and aggregation through cleavage of ultra-large von Willebrand factor (UL-VWF) multimers. At present the cause of antibody formation is unknown. We have previously shown that ADAMTS13 is efficiently internalized and presented on MHC class II by dendritic cells (Sorvillo et al, Blood 2012, 2013), suggesting a possible role of CD4+ T cells in the initiation of the autoimmune reactivity towards ADAMTS13. Activation of T cells leads to production of autoantibodies directed against ADAMTS13. Macrophages may participate in the immune response to ADAMTS13 and may also accelerate the clearance of ADAMTS13.

Aim: Here we investigated endocytotic mechanisms contributing to the uptake of ADAMTS13 by macrophages.

Methods: Human monocyte-derived macrophages (MDMs) were used to monitor the uptake of fluorescently labelled recombinant ADAMTS13 by flow cytometry. Macrophages were treated with different pharmacological inhibitors such as mannan, EDTA, heparin and dextran sulphate. In addition, an established cell-permeable inhibitor of clathrin-mediated endocytosis was used for these studies. The involvement of cellular receptors was addressed using blocking antibodies or siRNA mediated gene silencing. Uptake of ADAMTS13 by macrophages was also monitored by confocal microscopy.

Results: A time- and concentration dependent endocytosis of ADAMTS13 was observed when MDMs were incubated with fluorescently labeled ADAMTS13. Confocal microscopy studies revealed partial colocalization of ADAMTS13 with early endosomes. Recognition and/or uptake of antigens by MDMs occurs through two main pathways: receptor-mediated endocytosis and macropinocytosis. In order to identify the mechamism that mediates ADAMTS13 endocytosis we evaluated uptake of fluorescently labeled ADAMTS13 after pre-incubation of the cells with dynasore. Significant reduction of endocytosis was observed suggesting that ADAMTS13 uptake proceeds via clathrin mediated endocytosis. Internalization of ADAMTS13 was blocked upon addition of mannan and EDTA suggesting a possible role of C-type lectins (CLRs). We have recently demonstrated that the macrophage mannose receptor (MR) is involved in endocytosis of ADAMTS13 by human monocyte derived dendritic cells (Sorvillo et al, Blood 2012). However, uptake of ADAMTS13 by MDMs was not affected by the addition of different sugars or by a monoclonal antibody directed towards the MR. Furthermore siRNA silencing of MR did not significantly reduce uptake suggesting the involvement of other type of receptors. Interestingly, we observed a robust inhibition of ADAMTS13 endocytosis upon incubation with heparin and dextran sulphate.

Summary/Conclusion: Taken together our data suggest that internalization of ADAMTS13 by macrophages proceeds via a mechanism that is dissimilar from that previously defined in dendritic cells. We anticipate that the endocytic uptake by macrophages promotes the clearance of ADAMTS13 from the circulation.

OC 22 – Inhibitors in Haemophilia A – I

OC 22.1

Exploration of biomarkers for early recognition of FVIII inhibitor development in previously untreated severe hemophilia A patients: Hemophilia Inhibitor PUP Study and beyond

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Background: The development of neutralizing antibodies (FVIII inhibitors) is the major treatment complication of current care for patients with hemophilia A. Despite progress in understanding the regulation of immune responses against FVIII in preclinical animal models, no substantial improvement in the treatment of patients has resulted from animal studies.

There is a demand for biomarkers that allow early recognition of FVIII inhibitor development in previously untreated patients (PUPs) during first exposure days to FVIII. Likewise, suitable biomarkers are missing that facilitate early recognition of FVIII inhibitor eradication during Immune Tolerance Induction therapy (ITI).

Aim: Establish suitable technologies that facilitate the search for early biomarkers of inhibitor development and inhibitor eradication in patients. These technologies are to be optimized for small blood volumes and should be applicable to a multi-center setting of clinical studies.

Methods: A set of assays was established to extensively characterize antibodies against FVIII and to identify FVIII-specific CD4+ T cell signatures. Antibody assays not only include the differentiation of Ig isotypes and IgG subclasses but also allow for the assessment of apparent affinities of FVIII-specific antibodies to facilitate monitoring of affinity maturation of antibodies during the evolvement of FVIII inhibitors. Furthermore, DNA-based epigenetic marker analysis for different lymphocyte populations is included that should be suitable for monitoring the immune status of patients.

Results: We will present a set of proof of principle data that include the prevalence and in-depth characterization of FVIII-specific antibodies (Ig isotypes, IgG subclasses and apparent affinity) found in healthy individuals and in different cohorts of severe hemophilia A patients (with and without FVIII inhibitors as well as after successful ITI). High affinity FVIII-specific IgG1 and IgG4 occur to be the predominant antibodies in inhibitor patients, whereas low affinity FVIII-specific IgG1 and IgG3 can be found in selected healthy donors and in non-inhibitor patients. Interestingly enough, no FVIII-specific IgG4 was detected in healthy individuals (n = 600) from different geographies or in patients without FVIII inhibitors. Furthermore, CD4+ T-cell signatures indicate a FVIII-specific modulation of the expression of inflammatory genes such as *MYD88* in FVIII inhibitor patients in the course of successful ITI.

Conclusions: We developed and validated a set of technologies that will allow the identification of early biomarkers associated with FVIII inhibitor development and inhibitor eradication in patients with hemophilia A. Their application in the ongoing Hemophilia Inhibitor PUP Study (HIPS) has the potential to uncover new mechanisms of FVIII inhibitor development during early exposure days to FVIII.

OC 22.2

European monitoring of inhibitor development in haemophilia A and B

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Introduction: Inherited bleeding disorders are rare and monitoring side effects of treatment with FVIII or IX concentrates in haemophilia A and B requires multicentre, preferably international collaboration.

Aim: The European Haemophilia Safety Surveillance (EUHASS) was set up as a prospective registry monitoring adverse events, including inhibitor formation.

Methods: The EUHASS project started on 1-10-2008, patient numbers according to diagnosis and treatment status were reported annually, specifying patients who completed 50 exposure days. Adverse events were reported every quarter using a web-based form. Inhibitors were defined as two positive inhibitor tests according to the local laboratory.

Results: During the first three years, the number of reporting centres increased from 45 to 69. 250 Previously Untreated Patients (PUPs) with haemophilia A, and 40 with haemophilia B reached 50 exposure days. In total, 12470 (haemophilia A) and 2206 (haemophilia B) patient years were monitored in Previously Treated Patients (PTPs). Inhibitor development in PUPs with severe haemophilia A occurred in 71/250 (26%; 95% confidence interval: 21–32%) patients and in 3/39 [8%; 95% CI (2–21)] patients with haemophilia B. Inhibitor development for FVIII concentrates was similar for recombinant (65/250 = 26%, 95% CI 21–32) and plasma derived (6/40 = 15%, 95% CI 6–30) FVIII products. For PTPs, 26 new inhibitors were recorded in the registry, the rate of inhibitor development was 0.2/100 patient years (95% CI 0.1–0.3) for severe haemophilia A, 0.05/100 patient years (95% CI 0–0.3) for severe haemophilia B. Statistically significant differences between concentrates were not observed during the first 3 years of EUHASS. Results from all first 4 years of data will be presented during the ISTH congress.
Conclusion: Results from the first 3 years of the EUHASS registry showed that inhibitor formation is the main side effect of haemophilia treatment, occurring in 26% of PUPs with severe haemophilia A and 8% with severe haemophilia B. The inhibitor rates for PTPs with severe haemophilia A and B were 0.2 and 0.05 per 100 patient years, respectively.

OC 22.3

The change of Treg cells and serum BAFF level in the development of anti-factor VIII antibiotics in hemophilia A mice model

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Objectives: Hemophilia A is an X-linked bleeding disorder. The most serious complication of replacement therapy was production of antibodies. Regulatory T cells (Tregs) play a critical role in maintenance immune homeostasis and inhibitor formation. B cell-activating factor (BAFF) is involved in the survival and maturation of B cell and plays a critical role in most of immune responses. The purpose of this study was to investigate the relationship between the percentage of Treg and BAFF level in the emergence of anti-FVIII antibodies in animal model.
Methods: Hemophilia mice (C57BL/6 and 129 mix background, Exon 16 knockout), were intraperitoneally injected with 2 IU (~80 IU/Kg) of human recombinant FVIII (rFVIII) (Baxter) at 4 consecutive weeks. The mice serum and plasma were sampled before injection and after 4 consecutive weekly injections. Total anti-FVIII antibody titres and serum BAFF concentration were determined by ELISA. The functional activity of FVIII antibodies was measured using modified-Bethesda assay. The percentage of Treg cells were stained with specific antibodies conjugated with fluorochromes (CD4-FITC, CD25-APC, FoxP3-PE), and analysed with a FACSCalibur fluorescence activated cell sorter (FACS) using CELL QUEST software (BD Biosciences Pharmingen). The difference between those experimental groups was evaluated by one-way ANOVA using PRISM.
Results: Four subsequent weekly intraperitoneal injection of 2 IU rFVIII successfully induced high titer of anti-FVIII antibodies (216–309 μg/mL; 38–152 BU).We found in the rFVIII treated group, BAFF level and Treg cells increased before high-titer anti-FVIII antibodies formation, then the BAFF level decreased while the antibodies significant increased. High-titer anti-FVIII antibodies developed after Treg cells dropped.
Conclusions: In rFVIII treated group, high-titer anti-FVIII antibodies developed after brief BAFF and Treg cells surged. Our investigated results indicated that BAFF and Treg cells may play a role in early anti-FVIII inhibitor formation. Design of Treg positive cell-based therapy or anti-BAFF treated experiment, may characterize the mechanism between the Treg cells and BAFF in the inhibitor immune response. BAFF and Treg targeting strategy might prevent or reduce its occurrence.

OC 22.4

Restricted specificity of a recombinant anti-idiotypic antibody in protecting human factor VIII against anti-C2 inhibitory antibodies

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Background: Anti-idiotypic antibodies (anti-id's) are generated against naturally circulating antibodies, mimicking somehow the structure of the protein initially targeted by the circulating antibodies. Natural anti-id's directed against anti-factor VIII (FVIII) antibodies can be retrieved in normal population but also in hemophilia A patients, in particular in those that generated inhibitors. Anti-id's were shown to participate in the success of immunotolerance protocols in hemophilia A patients with inhibitors.
Aim: In order to gain further insight in the mechanism governing this effect, we decided to assess the ability of recombinant anti-id's in protecting FVIII to inhibitors.
Methods: Four couples of FVIII inhibitors/anti-idiotypic antibodies were generated. Two inhibitors directed against the C1 domain (Le2E9 and RHD5) or the C2 domain (B02C11 and VDR2C5) were generated by immortalizing B-cells from hemophilia A patients using Epstein-Barr Virus. Antibodies from these cell lines were isolated and characterized. These antibodies were used to generate anti-id's in mice. Anti-id's against Le2E9, RHD5 and B02C11 were characterized and prepared as chimeric by fusing the variable regions with human IgG1/k constant regions. The recombinant chimeric anti-id antibody 14C12, raised against the anti-C2 inhibitor BO2C11, closely mimics the structure of the entire C2 domain. It was thus used as a model to evaluate an anti-id protective action with regard to the C2 domain.
Results: As evidenced by flow-cytometry, the anti-id antibodies specifically recognized the B-cell line expressing the inhibitors against which they were raised. In contrast, they were unable to cross-react with the B-cell line which expressed a non-related inhibitor directed against a different or the same FVIII domain. The potential of 14C12 in alleviating inhibitor actions was then assessed in a thrombin generation assay. The reaction was induced by tissue-factor (0.5 nM) in FVIII-deficient plasma reconstituted by 1 U/mL FVIII. Anti-id 14C12 (ratio varying up to 250:1) was able to significantly restore the amount of thrombin generated in the presence of BO2C11 inhibitor. An endogenous thrombin potential higher than 50% of a control without inhibitor was obtained. In contrast, 14C12 restored no more than 12%, 17% and 3% respectively of the thrombin generated in the presence of VDR2C5, or in the presence of the commercial ESH8 or ESH4 monoclonal anti-C2 antibodies. In the presence of a sheep polyclonal anti-FVIII antibody (adjusted at 20 BU/mL), or in two plasmas from hemophilia A patients with inhibitors (7 BU/mL and 20 BU/mL), 14C12 anti-id restored only 3%, 10% and 0% respectively of the endogenous thrombin potential.
Summary/Conclusions: This data showed that anti-id's seem to specifically and efficiently target the inhibitory antibody against which they were established. Despite mimicking a complete protein domain, such as 14C12, anti-id cannot efficiently inhibit other inhibitors directed against the same protein domain. These results therefore suggest the need for an extended anti-idiotypic network in down-modulating *in vivo* a pre-established complex inhibitory response against factor VIII.
Technical help: This data was obtained with the technical help of Carole Pirckher, Linda Baptista and Dominique Grenier

OC 23 – Intrinsic Pathway of Coagulation

OC 23.1

Two cleavage sites in factor XII direct its diverging activities

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Introduction: When factor XII (FXII) binds to activating surfaces, it becomes catalytically active in an elusive event. Intriguingly, some surfaces trigger both coagulation and inflammation, whereas others only trigger inflammatory responses. FXII contains three closely positioned kallikrein cleavage sites; two of which reside within the catalytic domain and one within the heavy chain. During digestion of FXII by plasma kallikrein, the 80 kDa zymogen is first activated into α-FXIIa (80 kDa), where after its catalytic domain (β-FXIIa; 28 kDa) is released from its heavy chain. β-FXIIa is not procoagulant, but it does retain its inflammatory capacity. This suggests that a cleavage intermediate of FXIIa drives coagulation.

Aims: To investigate whether separate isoforms of FXIIa affect coagulation and inflammation with varying capacities.

Methods: The essential arginines within the three kallikrein cleavage sites (R334, R343, R353) and combinations thereof, were replaced with alanines (A) by site-directed mutagenesis. Subsequently, we studied the capacity of these mutants to directly cleave chromogenic substrates for kallikrein and thrombin. In plasma, we studied their capacity to trigger kaolin-induced prekallikrein activation, thrombin generation and coagulation.

Results: In line with earlier work, we found that cleavage at R353 is essential for induction of FXII activity, as none of the mutants containing R353A displayed any activity. Next, we identified that further proteolytic steps modulate the molecular functions of FXIIa. Upon activation, purified FXII mutant R343A is less capable of directly cleaving chromogenic kallikrein substrate and also has a reduced capacity to activate prekallikrein in plasma compared to WT FXII. This R343A mutant also converts less thrombin substrate and triggers a prolonged time to peak in a kaolin-driven thrombin generation assay in plasma (38 ± 2 min,: WT 26 ± 3 min). These findings suggest that a reduced thrombin-like activity of FXIIa may underlie a reduced capacity to activate factor XI and support thrombin generation.

In contrast, prevention of heavy-chain dissociation in FXII mutant R334A accelerates surface-induced coagulation and thrombin generation (21 ± 2 min; WT 26 ± 3 min), as well as prekallikrein activation and conversion of kallikrein substrate.

Finally, FXII R334A/R343A double mutant has a normal capacity (comparable to WT) to convert chromogenic kallikrein substrate and activate prekallikrein in plasma. Interestingly, this mutant shortens coagulation times in clotting assays (compared to WT), directly converts more thrombin substrate, and further accelerates thrombin generation (18 ± 1 min).

Conclusions: Together, our data indicate that cleavage intermediates of FXIIa have different enzymatic capacities. Where a primary cleavage at R353 yields a thrombin-like FXIIa, further cleavage at R343 modulates its function from a thrombin-like into a kallikrein-like enzyme. Subsequently, these activities can be amplified via the intrinsic pathway or the kallikrein-kinin system, respectively. A final cleavage at R334 dissociates the catalytic domain to restrict autoactivation.

OC 23.2

FXIIa enhances fibrinolysis in addition to plasminogen activators

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Background: Previously we have shown that activated coagulation factor XII (α-FXIIa) is able to bind to both fibrinogen and fibrin, and alter the structure and properties of a fibrin clot. In the presence of α-FXIIa, a denser clot is formed with thinner fibers, lower maximal turbidity and increased clot stiffness (Blood 2011, PMID = 21828145), typical for clots which are difficult to lyse. Conversely, the proteins of the contact system and the fibrinolytic system show a high degree of homology and FXIIa can directly convert plasminogen into plasmin, contributing to increased fibrinolysis.

Aims: The aim of this study was to investigate the contribution of α-FXIIa to clot stability and fibrinolysis.

Methods: Under static conditions, fibrin formation and fibrinolysis were determined simultaneously by turbidity and plasmin generation tested with S-2251. Fibrin formation and fibrinolysis of clots formed under flow conditions in a Chandler loop were determined through plasmin generation and fluorescent measurements of incorporation and release of fibrinogen Alexa Fluor® 488. The influence of α-FXIIa on clot structure and on fibrinolysis was visualized via scanning electron microscopy (SEM) and the fiber thickness was analyzed using Fibermetrics.

Results: Fibrin clots formed in the presence of α-FXIIa had a lower maximal turbidity than clots formed in the absence of α-FXIIa independent of tPA concentration, indicating a denser fibrin structure with thinner fibers. Furthermore, we observed that α-FXIIa was able to directly convert plasminogen into plasmin and to reduce the clot lysis time at all tPA concentrations tested (0–1500 pM). Simultaneous assessment of plasmin generation (with the chromogenic substrate S-2251) alongside turbidity measurements, showed earlier onset of plasmin generation in the presence of α-FXIIa. Fibrinolysis of clots formed under flow conditions, revealed that incorporation of α-FXIIa, together with plasminogen, into the clot accelerated clot break-down and increased plasmin generation by tPA, compared to clots prepared in the absence of α-FXIIa. Electron microscopy revealed that fibrin fibers were thinner if α-FXIIa was present during clot formation: in the presence of α-FXIIa on average the fibers were between 120 and 140 nm, in the absence between 140 and 160 nm. When we initiated fibrinolysis by adding tPA (150 pM and 300 pM) during clot formation, the fiber thickness did not change in the absence of α-FXIIa. However, in the presence of α-FXIIa the average fiber thickness increased at 300 pM tPA.

Summary/Conclusion: We observed that α-FXIIa is able to convert plasminogen into plasmin and that the additional plasmin generated enhances fibrinolysis. This was the case irrespective of whether tPA was present during clot formation or added after to initiate fibrinolysis. Visualisation by SEM showed that in the presence of α-FXIIa, the fibrin clot consisted of thinner fibers, however, during fibrinolysis the fiber thickness of the remaining fibers increased. The fiber thickness did not change during fibrinolysis in the absence of α-FXIIa. We postulate that FXIIa first enhances the clot structure during clot formation and thereafter helps initiate fibrinolysis.

OC 23.3

Direct inhibition of FXa by TFPI independent of TF-FVIIa contributes to the down-regulation of coagulation

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Background: Tissue factor pathway inhibitor (TFPI) is a Kunitz-type serine protease inhibitor that down-regulates the extrinsic coagulation pathway by inhibiting the tissue factor-factor VIIa complex (TF-FVIIa). In the process of TF-FVIIa inhibition TFPI first binds to and inhibits Factor Xa (FXa) forming a binary TFPI-FXa complex in a reaction that is stimulated by protein S. The TFPI-FXa complex subsequently forms a quaternary complex with TF-FVIIa and blocks the initiation of coagulation. Although TFPI can directly inhibit FXa, this inhibition is generally considered not to be physiologically relevant because the plasma concentrations of other FXa inhibitors e.g. antithrombin (AT = 2.5–5 μM) are several orders of magnitude higher than the TFPI concentration (0.25–0.5 nM).
Aims: To investigate whether direct inhibition of FXa by TFPI, independent of TF-FVIIa, contributes to the down-regulation of coagulation.
Methods: Inhibition of FXa by TFPI and AT in model systems was determined in the presence of calciumchloride, phospholipids and protein S, using a chromogenic substrate to monitor FXa. Inhibition of FXa by TFPI in plasma was determined by measuring thrombin generation curves triggered with FXa, RVV-X, FXIa or FIXa in plasma that contained a cocktail of anti TF/anti FVIIa antibodies to inhibit any contribution of traces of TF that might be present in plasma to the initiation of coagulation. The TF-independent contributions of TFPI and/or protein S to the down-regulation of thrombin generation were quantified after neutralisation of TFPI and protein S with an anti TFPI cocktail or anti protein S antibodies.
Results: Comparison of the inhibition of FXa by physiological concentrations of AT (2.5 μM) and TFPI (0.25 nM) shows that TFPI is a better FXa inhibitor ($t_{1/2}$ = 2.5 min) than AT ($t_{1/2}$ = 3.2 min). Both anti-TFPI and anti-protein S antibodies enhanced thrombin generation in plasma triggered with RVV-X, FXa, FIXa or FXIa. Depending on the trigger and trigger concentration used anti-TFPI and anti-protein S antibodies decreased the lag time and/or increased the peak height of thrombin generation. At high trigger concentrations neither anti-TFPI nor anti-protein S antibodies enhanced thrombin generation. TFPI and protein S titrations in TFPI- and protein S-depleted plasma in which thrombin formation was initiated with triggers other than TF confirmed that both TFPI and protein S express TF-independent anticoagulant activity.
Conclusions: Our observations indicate that TFPI, despite its low plasma concentration, expresses anticoagulant activity in the absence of TF. Kinetic experiments in model systems at plasma TFPI concentrations show that direct inhibition of FXa by TFPI can account for the TF-independent anticoagulant effect of TFPI on thrombin generation. Neutralisation of protein S with anti-protein S antibodies also enhances thrombin generation with triggers other than TF. This suggests that TFPI requires protein S for expression of TF-independent anticoagulant activity in plasma. Inhibition of thrombin generation by TFPI independent of the TF pathway is an important observation since coagulation on endothelial cells, which mainly proceeds via the intrinsic pathway, becomes susceptible to down-regulation by TFPI through direct inhibition of FXa.

OC 23.4

A microfluidic model of *in vitro* hemostasis that potentiates thrombin generation via the intrinsic pathway of coagulation

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Background: Microfluidic models of hemostasis rarely incorporate both platelet adhesion and coagulation, despite their strong connections. Traditionally, PPACK, a small molecule inhibitor of thrombin, is used to study platelet deposition to adhesive proteins under flow. Recently, we have demonstrated a model of platelet adhesion and coagulation functioning together under flow using a procoagulant surface of collagen type I with immobilized tissue factor-bearing vesicles (TF). Here we describe a model of hemostasis that does not rely on extrinsic coagulation to generate thrombin.
Aim: We sought to develop a microfluidic model of platelet adhesion and coagulation under flow utilizing the intrinsic pathway to trigger thrombin generation.
Methods: Whole blood (WB) was drawn from healthy or hemophiliac donors into 4 μg/mL Corn Trypsin Inhibitor (CTI), an inhibitor of activated factor XII (FXIIa). This quantity of CTI was chosen as it was below the saturating levels described by our lab earlier, but provided 30–45 min before clotting was observed in a test tube. Platelet counts and Factor VIII or IX levels were collected for hemophiliac donors. Samples were treated with fluorescently conjugated monoclonal antibodies against platelet CD41a and fibrin. Within 5 min of the blood draw, labeled samples were perfused over a collagen type I surface in a microfluidic device consisting of eight individual channels at 100/s. Platelet adhesion and fibrin accumulation were measured in 30 s intervals over a 20 min time period.
Results: Platelets deposited onto the collagen surface from healthy WB samples within 30 s. Onset of fibrin generation required a minimum of 6 min for all healthy donors and was donor dependent, with a maximum time of 9 min. Maximum platelet and fibrin generation was typically observed between 10–15 min, resulting in full channel occlusions. Addition of Factor VIII (FVIII) inhibitory antibodies abolished fibrin generation in healthy donors, confirming that intrinsic coagulation was the trigger for thrombin generation. In patients with hemophilia A and B who ranged from severe to mild disease, platelet deposition measured after 10 min of perfusion correlated strongly with FVIII levels but not with platelet count. Fibrin generation required a minimum of 5% of normal FVIII levels, but was highly variable above this threshold.
Conclusions: We have developed a model of hemostasis under flow that combines platelet adhesion to a collagen surface and intrinsically activated coagulation. We demonstrated robust generation of fibrin on platelet deposits in healthy donors, which was absent when antibodies against FVIII were used or samples from hemophiliacs with low (< 5%) FVIII/IX levels were considered. We have also demonstrated that platelet deposition is a strong function of FVIII levels, likely due to the potent activating potential of thrombin.

OC 24 – Platelet and Coagulation Interaction

OC 24.1

A new role for integrin outside-in signaling in the regulation of platelet packing density, solute transport and fibrin deposition following vascular injury *in vivo*

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Background: Prior studies have demonstrated that platelet activation within a hemostatic plug is heterogeneous. We recently demonstrated

that the platelet mass which forms after penetrating injuries is composed of distinct regions defined by the extent of platelet activation and other properties (Stalker, et al. Blood 2013). At least two regions are apparent: a core of fully activated and degranulated platelets immediately adjacent to the site of injury, which is overlaid by a shell of less activated platelets that have not undergone a-granule secretion. Platelets in the core are packed more closely together, facilitating contact-dependent signaling and restricting the entry of plasma-borne molecules. Aims and Methods

Here we used high-resolution confocal intravital imaging to test two hypotheses. The first hypothesis is that platelet accumulation and activation create distinct microenvironments within the different regions of a hemostatic plug. The second hypothesis is that integrin outside-in signaling plays a role in shaping the structure and transport properties of the core region. Hemostatic thrombus formation was observed in mouse cremaster muscle arterioles in response to a penetrating injury. Fluorescently-labeled albumin and dextran were used to measure the spaces between platelets (a measure of porosity) and the velocity of solute transport within the thrombus.

Results: When measured over the full thrombus, porosity and solute transport velocity were reduced in the spaces between platelets compared to the blood flow outside of the thrombus. An analysis by regions showed that this reduction was greater in the core than in the outer shell of loosely adherent platelets. To examine the impact of integrin outside-in signaling, we utilized mice in which two Tyr residues in the cytoplasmic domain of the β chain of $\alpha_{IIb}\beta_3$ integrin were replaced with Phe (diYF). Prior studies have shown that this double substitution impairs outside-in signaling and causes unstable platelet aggregation. It also results in defective fibrin clot retraction despite normal fibrinogen binding. We found diYF mice formed smaller hemostatic plugs following vascular injury. The decrease in total platelet accumulation was specifically due to reduced formation of the core region, while platelet recruitment and retention in the shell region were unaffected. We also found a decrease in fibrin generation, indicating a reduction in thrombin activity within the hemostatic plug.

Conclusions: Taken together, these results support the hypothesis that tight platelet packing provides a protected microenvironment in which bioactive molecules such as thrombin may accumulate to support platelet activation. They also identify a novel role for $\alpha_{IIb}\beta_3$ outside-in signaling in the structure of the hemostatic response and the regulation of hemostatic plug formation. The authors acknowledge support from the NHLBI and the American Heart Association.

OC 24.2

Rap signaling is central to the pro-adhesive and pro-coagulant platelet response

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Background: The small GTPase Rap1B, which accounts for ~90% of Rap GTPases in platelets, plays a critical role for platelet activation at sites of vascular injury. In our previous work, we have identified signaling by the guanine nucleotide exchange factor CalDAG-GEFI (CDGI) and the ADP receptor P2Y12 as the main pathways leading to the activation of all Rap GTPases in platelets. Platelets lacking functional CDGI and P2Y12 were strongly defective in all major platelet responses, including phosphatidylserine exposure and microvesiculation.

Aim: In this study, we aimed to better understand how Rap1B and other Rap GTPases contribute to platelet activation. We therefore evaluated the pro-adhesive and pro-coagulant response of platelets from *Rap1b−/−* and clopidogrel-treated *CalDAG-GEFI−/−* (CDGI/clopidogrel) mice.

Methods: Integrin activation (JON/A-PE), granule secretion (anti-P-selectin), and PS exposure (annexinV) were assayed by flow cytometry. Platelet-dependent thrombin generation was measured by calibrated automated thrombography (CAT). Platelet adhesion under flow conditions was studied in heparin anticoagulated blood perfused over fibrillar type I collagen. Thrombus formation *in vivo* was monitored in the cremaster muscle microcirculation after laser injury.

Results: Integrin activation and granule secretion were virtually abolished in CDGI/clopidogrel platelets activated with convulxin (Cvx) or PAR4p. Both responses, however, were only partially reduced in *Rap1b−/−* platelets and no dose response shift was observed. Consistent with these findings, Rap1B-deficient platelets adhered significantly better to collagen under flow conditions than CDGI/clopidogrel cells. In contrast, coated platelet formation, induced by simultaneous stimulation with Cvx and PAR4p, was almost completely abolished in both CDGI/clopidogrel and *Rap1b−/−* platelets. This defect in PS exposure and microvesiculation was not due to impaired calcium mobilization in *Rap1b−/−* platelets. Coated platelet formation was also significantly reduced in platelets treated with inhibitors to Rac1 and Erk MAP kinases, two downstream targets of Rap1b. Consistent with these studies, platelet-dependent thrombin generation was significantly reduced in *platelet-rich-plasma* from *Rap1b−/−*and CDGI/clopidogrel mice. *In vivo*, thrombus formation was observed in Rap1b−/− but not CDGI/clopidogrel mice. In ongoing studies, we will measure thrombus size/stability and fibrin formation at sites of vascular injury to determine if impaired platelet procoagulant activity contributes to the protection of Rap1b−/− mice from thrombosis.

Conclusions: We here demonstrate a critical role for Rap1b signaling in coated platelet formation and platelet-dependent thrombin generation *in vitro*. Our studies further suggest that other Rap isoforms compensate for the pro-adhesive, but not the pro-coagulant signaling provided by Rap1B.

OC 24.3

Contribution of intrinsic and extrinsic coagulation pathways to whole blood clot and thrombus formation under flow

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Background: The formation and composition of platelet-fibrin clots under flow is a dynamic process, tightly controlled by the vascular surface and the local rheological conditions, both of which are different for the arterial and venous systems. Arterial thrombi are enriched in platelets, while venous thrombi are more enriched in fibrin. This implies a different way of control of the thrombus-forming process by thrombin at arterial or venous conditions. Thrombin can be generated via the extrinsic coagulation pathway via factor (F)VIIa (triggered by tissue factor, TF) or the intrinsic pathway via FXIIa (collagen, polyphosphates). How these two pathways contribute to thrombus formation under various flow conditions is largely unknown.

Aim: To study the contribution of thrombin formed via the extrinsic and intrinsic pathways to the formation of platelet-fibrin clots under flow conditions, mimicking those in veins and arteries.

Methods: Blood was used from healthy donors, patients and coagulation factor-deficient mice. Platelet-fibrin clot formation was measured by perfusion of recalcified whole blood over microspotted surfaces, triggering the extrinsic (TF) and/or intrinsic (collagen) coagulation pathways. Employed shear rate were 150, 1000 and 1500/s. Microspots contained low or high contents of TF (1–10 pg/spot) and collagen (5–25 ng/spot). Line-scanning confocal microscopy gave time traces of focal accumulation of fluorescently-labeled platelets ($DiOC_6$), fibrin (AF647-fibrinogen) and coagulant platelet activation (FITC-annexin A5).

Results: Perfusion of human blood over TF alone failed to produce platelet adhesion or fibrin formation. Perfusion over a surface trigger-

ing the extrinsic and intrinsic pathways (TF/collagen) resulted in platelet-enriched thrombi at high shear rate and fibrin-enriched thrombi at low shear rate. This was attributed to a reduced platelet accumulation at low shear (mean covered area 16 ± 2%), compared to the higher shear conditions (41 ± 4% and 47 ± 5%). The time of onset and extent of fibrin formation were comparable for all shear rates. Lowering of the TF content delayed the time to fibrin formation, and FXIIa inhibition caused a further delay, but was without effect on early platelet deposition. Lowering of the collagen content produced the formation of fewer but larger thrombi, with more activated platelets. Lowering of collagen also delayed fibrin formation from 3 ± 1 to 5 ± 1 min, an effect that was mimicked by FXII deficiency. Interestingly, regardless of the surface type and shear rate, thrombin inhibition by hirudin decreased the number and size of platelet thrombi, while fibrin formation was abolished. At various shear rates, deficiency of murine FVIII or FIX, but to a lesser extent FXII, resulted in smaller size thrombi with fewer activated platelets and in little fibrin formation. These effects were maintained in the absence of TF activity, and were normalized upon addition of recombinant FVIII or FIX, respectively.

Conclusion: Thrombin formed by both intrinsic as well as extrinsic pathways stimulates the formation of large multi-platelet thrombi at all shear rates, but most strongly contributes to platelet-fibrin formation at low shears and low collagen. Under flow, TF accelerates this effect of thrombin via FVIII and FIX activation.

OC 24.4

The polyphosphate-binding proteins in the human platelet secretome

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Background: Inorganic polyphosphates (polyP) are negatively charged, linear phosphate polymers that are secreted from dense granules of activated human platelets. We and others have shown that polyP has potent prohemostatic, prothrombotic and proinflammatory properties. In particular, we have shown that polyP binds to several blood clotting proteins and modulates certain steps of the clotting cascade. Given the extensive platelet secretome, we hypothesized that polyP released from platelet dense granules may interact with proteins secreted following platelet activation. Identification of these proteins could provide important clues regarding new biological functions for polyP, beyond its previously documented roles in modulating the plasma clotting cascade.

Aim: To identify the major polyP-binding proteins in the platelet secretome.

Methods: Purified polyP was covalently end-labeled with biotin, which in turn was bound to streptavidin-conjugated magnetic beads (Dynabeads). The resulting polyP-Dynabeads were incubated with releasates obtained from human platelets that had been activated with TRAP (thrombin-receptor agonist peptide). PolyP-Dynabeads were then washed repeatedly and the polyP-binding proteins were eluted with 1 M NaCl. The eluted proteins were identified by MudPIT (Multidimensional Protein Identification Technology) using 2D liquid chromatography and mass spectrometry.

Results: Using MudPIT, we unambiguously identified a total of 157 candidate polyP-binding proteins from the platelet secretome. These proteins fell into several functional groups, including: *Hemostasis-related proteins* (prekallikrien, fibrinogen, plasminogen, multimerin, protein S, and clotting factors V, XIII, and XI); *Serpins* (protease nexin-1, protein C inhibitor, α1-antitrypsin, plasminogen activator inhibitor 1); *Prolyl-isomerases* (parvulin, cyclophilin, and FKBP3 peptidyl-prolyl cis-trans isomerase); *Complement proteins* (factors B, H, C1q, C3, C4A, and C5); *Growth factors* (VEGF, PDGF, and TGFbeta-binding protein); *Adhesive receptors and ligands* (CD42c, CD42d, and fibronectin); *Apolipoproteins* (apoB and apoJ); and *Others* (thrombospondin, beta-thromboglobulin, platelet factor 4, and heparanase). In addition, several proteins with no known function were also identified.

Conclusions: We have now identified a number of new candidate polyP-binding proteins in the human platelet secretome, including proteins known to participate a multitude of cellular processes. We previously reported that polyP triggers the contact pathway of blood clotting, accelerates certain downstream clotting reactions, enhances fibrin clot structure and delays fibrinolysis. Our new findings indicate that polyP may bind to certain serpins and other known regulators of the clotting system, suggesting additional sites at which polyP may modulate hemostasis and thrombosis. Furthermore, given the nature of additional candidate polyP-binding proteins in the platelet secretome, polyP may also influence platelet-mediated inflammatory responses, cell-cell interactions, angiogenesis, intercellular communication, and apoptosis. The many roles of polyP in platelet biology are likely to expand, an area that clearly warrants further investigation.

OC 25 – Platelets and Cancer

OC 25.1

A novel and selective proteasome inhibitor modulate expression of molecules linked to coagulation and angiogenesis independent of NF-kB activation in tumor cells

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Amblyomin-X is a Kunitz-like recombinant protein which has shown inhibitory properties on factor Xa and tenase complex and also inhibits proteasome in human tumor cell lines but not in normal human fibroblasts. Proteasome inhibition generates aggresomes transported by dynein in which K48 and K63-linkage of ubiquitin signaling is involved. This signaling is also important to NF-kB pathway and studies have been reported that recruitment of dynein translocate p65/p50 heterodimer to act as an activator of a large number of genes like tissue factor (TF), TFPI and VEGF. The p50 subunit is generated by its precursor molecule p105 that is processed by the proteasome. TFPI not only inhibits thrombin generation by inhibiting FXa which then inactivates TF/FVIIa complex but also can act as adhesive ligand for cancer cells to extracellular matrices. Additionally, TF can enhance tumor metastasis and VEGF contributes to angiogenesis. The present study intends to evaluate the pathway that links coagulation and cancer in the antitumoral mechanism of action of Amblyomin-X.

Cell culture: Normal human fibroblasts, Mia-PaCa-2 and SK-Mel-28 tumor cell lines.

Real-Time PCR: SYBR® green based reaction.

Aggresome formation: Commercial kit for flow cytometry and fluorescence microscopy. Ubiquitin signaling: Flow cytometry with specific K63 and K48 antibodies.

Western blotting: Cell lysis and immunoblotting after SDS-PAGE with specific antibodies. NF-kB, dynein, Ubc13, VEGF and TF gene expression were upregulated in Mia-PaCa-2 after 24 h of Amblyomin-X treatment. Only NF-kB, Ubc13 and dynein genes were upregulated in SK-Mel-28. Fibroblasts have normalized the expression levels of the targets analyzed in 24 h. Protein expression of some targets has been modified in tumor cells while only p50 has been modulated in fibroblasts. Amblyomin-X induced aggresome formation only in tumor cells. The K63 linkage signal was increased in all cell lines and corroborates with Ubc13 upregulation while K48 linkage has decreased only in tumor cells. Amblyomin-X inhibits proteasome and induces aggresome formation only in tumor cells. The molecule seems to trigger NF-kB signaling as a cell response in fibroblasts but do not target proteasome and cell death. Our data indicates that NF-kB, although high expressed, can be inactive to transcript its targets like TFPI, TF and VEGF, since proteasome inhibition can prevent degradation of IKB, a natural inhibitor of NFkB, which in turns, could remain sequestered and inactive. Moreover, protein expression of p50 subunit has also

decreased in tumor cells and its precursor p105 has increased indicating that proteasome inhibition had affected its proteolysis. Additionally, K63 signaling for aggresome formation and decreased K48 signaling as a cell response, contributes to inactivate NF-kB pathway. Therefore, increased mRNA levels of TF and VEGF in Mia-PaCa-2 indicates that these targets may have its gene expression activated by another pathway independent of NFkB activation, as a tumor response to Amblyomin-X treatment. Aggresome formation and increased TFPI expression may contribute respectively to cell apoptosis and metastasis reduction, effects already observed *in vivo* in previous data, in the mechanism of action of Amblyomin-X.
Financial support: FAPESP.

OC 25.2

Prostate cancer cells signal through Syk-PKC intracellular molecules to induce platelet secretion

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The critical role played by platelets in cancer metastasis is mostly related to their function as a dynamic reservoir of effector molecules which can facilitate tumour vascularisation, growth and metastasis. However, very little information is available about the mechanism of tumour cell induced platelet secretion (TCIPS) or the molecular machinery by which this mechanism is elicited. In the current study, we demonstrate that tumour cells directly induce platelet exocytosis. The molecular mechanisms involved in this response were explored. Platelet secretion was assessed by employing a luminometric assay capable of detecting ATP/ADP released by platelet dense granules following activation. Pre-incubation of platelets with increasing doses (10^2–10^5 cells/mL) of human colon cancer cells (Caco-2) or prostate cancer cells (PC3M-luc) for up to 60 min resulted in a marked dose-dependent secretion from dense granules. Importantly, tumour cell induced platelet secretion (TCIPS) preceded aggregation and was related to the malignancy of the tumour cell line used. Because tumour cell induced platelet aggregation (TCIPA) can be modulated by pharmacological inhibitors of ADP, we first investigated the effect of ADP receptor antagonists, MRS2395 ($P2Y_{12}$) and MRS2179 ($P2Y_1$), on TCIPS mediated by PC3M-luc. In contrast to aggregation, inhibition of platelet ADP receptors enhanced tumour cell mediated platelet secretion, reinforcing the hypothesis that TCIPA is secondary to secretion. We next examined the role of TxA_2receptors (TP) and molecular mediators of TxA_2 synthesis (PLA2 and COX-1), PAR-1 Thrombin receptor, the integrin cell adhesion molecule, GpIIbIIIa and the intracellular signalling molecules Src and PI3K by examining the effects of pharmacological inhibitors. Whereas blockade of TP, PAR-1, PLA2, COX-1, Src and PI3K did not reduced platelet secretion in response to PC3M-luc cells, inhibition of integrin GpIIbIIIa, with either Abciximab or CD41/SZ22 monoclonal antibodies, significantly decreased TCIPS (maximum secretion reduced to $74.24 \pm 9.6\%$; $^*P < 0.05$). Finally, in order to gain specific insights into the molecular machinery of TCIPS we next chose to target Protein kinase (PKC) and upstream intracellular molecules, Syk and PLC, whose role in agonist induced-platelet secretion has been well established. In the presence of GF109203X, PKC broad-spectrum inhibitor, platelet ATP/ADP release was completely abolished in response to PC3M cells (100 vs 28.05 ± 7.4; $^{***}P < 0.0001$) suggesting an essential role for PKC. Importantly, TCIPS was also dramatically inhibited after platelet pre-treatment with either Bay 61-3606 (100 vs 59.09 ± 9.36; $^{**}P < 0.01$) or Piceatannol (100 vs 20.57 ± 5.88; $^{***}P < 0.0001$), both potent Syk inhibitors. In contrast, the broad spectrum PLC inhibitor U73122, had no effect on platelet secretion induced by cancer cells. In conclusion, this study showed for the first time, the capability of cancer cells to directly promote platelet secretion and has identified the major molecular mediators involved in this response, providing initial guidelines for developing promising anti-metastatic therapies.

OC 25.3

A role of CLEC-2 in tumor growth and metastasis

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Background: A platelet activation receptor CLEC-2 binds to podoplanin on the surface of tumor cells and facilitates hematogenous tumor metastasis. It has been reported that anti-podoplanin antibody inhibited hematogenous tumor metastasis in experimental lung metastasis model in mice. However, a role of CLEC-2 in tumor growth and lymphogenous metastasis has not been elucidated to date.
Aims: The aim of the study is to investigate roles of CLEC-2 in tumor growth and lymphogenous metastasis. We also investigate whether CLEC-2 blockade inhibits hematogesous metastasis.
Methods: CLEC-2 chimeras and wild type (WT) chimeras were generated by transplantation of fetal liver cells from CLEC-2−/− or CLEC-2+/+ embryos. B16F10, a podoplanin-positive melanoma cell line, and 3LL, a podoplanin-negative lung carcinoma cell line, were inoculated intravenously into the tail vein of CLEC-2-chemeras or WT-chimeras. After 20 days, the mice were killed and the surface lung metastatic foci were counted. Podoplanin-positive B16F10 cells and podoplanin-negative B16 cells were incubated with or without washed CLEC-2−/− or CLEC-2+/+ platelets and cell numbers were counted after 2 days. B16F10 and 3LL were inoculated into foot pads of CLEC-2- or WT-chimeras. After 20 days, metastasis to the lymph nodes and tumor growth were analyzed by flow cytometry and gravimetry, respectively.
Results: The number of B16F10 metastatic foci in the lung of CLEC-2 chimera was significantly inhibited compared with that in the lung of WT chimeras, whereas no difference was observed in the case of 3LL. Co-culture with WT platelets significantly increased the cell number of B16F10, but not that of 3LL. On the other hand, the numbers of both cell lines did not significantly increase in the presence of CLEC-2−/− platelets. To our surprise, tumor growth and lymphatic metastasis is rather facilitated in CLEC-2-chimera compared with WT chimera.
Conclusions: CLEC-2 in platelets binds to podoplanin in tumor cells and facilitates hematogenous tumor metastasis *in vivo* and tumor growth *in vitro*. However, lymphatic metastasis and tumor growth *in vivo* are facilitated in the absence of CLEC-2. This difference may be due to effects of CLEC-2 on tumor angiogenesis and/or tumor lymphangiogenesis, which is now under investigation.

OC 25.4

Observational study of alternative platelet parameters to predict bleeding risk in patients with hematological malignancies (ATHENA study)

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Background: Previous studies have shown that the platelet count is a poor predictor of bleeding in severely thrombocytopenic patients with hematological malignancies.
Aims: The aim of this study was to evaluate whether other routinely available platelet parameters (mean platelet volume (MPV), platelet mass, immature platelet fraction (IPF), absolute immature platelet number (AIPN), platelet function analyzer-100 (PFA-100)) were better at predicting bleeding than the platelet count.
Methods: A prospective cohort study of adults with a haematological disorder undergoing intensive chemotherapy or stem cell transplant (ISRCTN81226121) at two UK centers (Bristol and Oxford) between September 2010 and September 2012. The study inclusion criterion

was development of thrombocytopenia (platelet count $\leq 50 \times 10^9$/L. From the start of thrombocytopenia, the participants underwent daily formalized bleeding assessments until platelet count recovery, hospital discharge, death, or for up to 30 days. Alongside routine tests, additional venous blood samples were collected into EDTA collection tubes thrice weekly for detailed analysis of platelet parameters. Analysis of the data was performed using a generalized linear model with binomial family and logit link function clustered on patient identity to account for repeated measures.

Results: All 50 participants were followed up until study completion. The baseline characteristics were: mean age 51.0 years; male (33/50); diagnosis (leukemia 16/50; lymphoma 14/50; myeloma 9/50; other 11/50); treatment (chemotherapy 4/50; allograft 33/50; autograft 13/50). Bleeding symptom data were available for 99.7% of study days. The participants had median 3 days of bleeding (any severity) (interquartile range (IQR) 0–6); and median 11 days (IQR 8–16) with platelet count $\leq 50 \times 10^9$/L.

The unadjusted odds ratio (OR) for bleeding the following day when the platelet count was $\leq 50 \times 10^9$/L was 0.98 (95% confidence interval (CI) 0.97–1.00; $P = 0.03$) for the total platelet count; 0.60 (95% CI 0.41–0.88; $P = 0.008$) for the AIPN; and 0.97 (95% CI 0.90–1.03; $P = 0.346$) for the IPF. The AIPN result remained statistically significant when adjusted to account for the total platelet count [OR 0.68 (95% CI 0.52–0.90; $P = 0.007$)]. The IPF result remained statistically non-significant when adjusted to account for the total platelet count [OR 0.94 (95% CI 0.89–1.01; $P = 0.079$)]. The total platelet count result was no longer statistically significant when adjusted to account for the AIPN [OR 1.00 (95% CI 0.99–1.02; $P = 0.442$)] or IPF [OR 0.99 (95% CI 0.97–1.01; $P = 0.246$)]. The full blood count analyzer was unable to report MPV in 36.2% of samples when the platelet count was $\leq 50 \times 10^9$/L, precluding further analysis of MPV and platelet mass. In the PFA-100 test, firm closure occurred in only 9.3% of samples using the PFA-ADP cartridge and 22.4% of samples using the PFA-Innovance cartridge when the platelet count was $\leq 50 \times 10^9$/L.

Conclusions: This preliminary study suggests that the AIPN is a better predictor of bleeding than the total platelet count. The MPV, platelet mass and PFA-100 closure times were not useful because these variables were frequently not estimable during thrombocytopenia.

Funding: NHS Blood and Transplant, UK

OC 26 – Vessel Wall

OC 26.1

Ang-(1-7) and Mas decrease thrombosis in Bdkrb2−/− mice by increasing NO and prostacyclin to reduce platelet spreading and GPVI activation

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Background: Little is known how the plasma kallikrein-kinin (KKS) and renin angiotensin systems (RAS) influence thrombosis risk *in vivo*. We reported (Blood 2006;108:192) that bradykinin B2 receptor deleted mice (*Bdkrb2−/−*) have delayed thrombosis and prolonged bleeding times due to elevation of angiotensin II (AngII) and angiotensin receptor 2 (AT2R) producing increased plasma nitric oxide (NO) and prostacyclin (PGI_2). New investigations in *Bdkrb2−/−* show that the angiotensin receptor 1 antagonist losartan lowers AngII levels but does not reduce thrombosis time. These data indicate that lowering of AngII alone is insufficient to change thrombosis risk. Further, the significance of the long bleeding time in *Bdkrb2−/−*needs characterization.

Aims: Identify additional mechanism(s) for thromboprotection and the precise influence of increased plasma NO and PGI_2 on platelet function in *Bdkrb2−/−*.

Methods: Real-Time PCR was used to measure expression of ACE and Mas. AngII, angiotensin-(1-7) (Ang-(1-7)), NO, PGI_2, cGMP and cAMP were examined by ELISA. Mas, AT2R, GPVI and pSyk were examined by immunoblot. Thrombosis times were assayed using Rose Bengal and Ferric Chloride on carotid arteries. Bleeding time was by tail incision. Platelet function was examined on aggregation, adhesion, spreading, and flow cytometry with JON/A antibody for murine activated integrin $\alpha_{2b}\beta_3$ and WugE9 antibody for P-selectin. JAQ-1 characterized murine GPVI. Platelet spreading was on collagen, GFOGER, and fibrinogen. Platelet flow cytometry was after α- or γ-thrombin, CRP, or convulxin stimulation. Fibrinogen binding was used to characterize ADP stimulation. Bone marrow (BM) transplantation determined if the spreading and thrombosis defects were due to host factors.

Results: New investigations show that *Bdkrb2−/−* have elevated Ang-(1-7), the breakdown product of AngII. Renal mRNA and protein for the receptor of Ang-(1-7), Mas, is also increased. Blockade of Mas with its antagonist A-779 in *Bdkrb2−/−* shortens thrombosis times (58 ± 4 min to 38 ± 4 min), bleeding times (170 ± 13 s to 88 ± 8 s) and lowers plasma nitrate (22 ± 4 mM to 15 ± 5 mM), and 6-keto-$PGF_{1\alpha}$ (259 ± 103 pg/mL to 132 ± 58 pg/mL) ($P < 0.01$ in all cases). *Bdkrb2−/−* platelets have normal α- and γ-thrombin-induced activation and ADP-induced fibrinogen binding. *Bdkrb2−/−* platelets express increased NO, cGMP and cAMP with reduced spreading on collagen, GFOGER, or fibrinogen. *In vivo* treatment with A-779 or combined L-NAME and nimesulide corrects the spreading defect on collagen. *Bdkrb2−/−* platelets have decreased convulxin- or CRP-induced JON/A and P-selectin expression that are partially corrected by *in vivo* A-779, nimesulide, or L-NAME treatment. *Bdkrb2−/−* platelets have reduced ligation-dependent Syk phosphorylation. *In vitro* pretreatment of normal platelets with carbaprostacyclin reduced spreading on collagen and GFOGER, and attenuated Syk phosphorylation and CRP activation. Transplantation experiments show that normal BM in *Bdkrb2−/−* hosts produced platelets with a spreading defect and delayed thrombosis. Transplantation of *Bdkrb2−/−*BM into normal hosts produced platelets with normal spreading and normal occlusion times.

Conclusion: In *Bdkrb2−/−*, combined AT2R and Mas over-expression produce elevated plasma PGI_2 and NO leading to acquired platelet defects in spreading on integrins and attenuated CRP activation, that contribute to thromboprotection. In addition to contact activation, the KKS influences thrombosis risk through the RAS in the vessel wall.

OC 26.2

GRP/Ucma: a novel member of the class of vitamin K-dependent proteins is involved in osteochondrogenic transdifferentiation of vascular smooth muscle cells

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Background: Vitamin K-dependent carboxylation of coagulation factors is essential to proper blood coagulation. Warfarin, a frequently prescribed vitamin K-antagonist, impairs blood coagulation by inhibiting carboxylation. Warfarin also inhibits functionality of vitamin K-dependent proteins which are not participating in blood coagulation but which are involved in maintaining homeostasis of the blood vessel wall. Gla Rich Protein (GRP/Ucma) is a recently discovered novel member of the class of vitamin K-dependent proteins and is potentially a target of warfarin. Its (patho)physiological function, however, is still unknown. It has been shown that GRP/Ucma suppresses osteogenic differentiation of pre-osteoblasts *in vitro*. We and others demonstrated that GRP/Ucma is expressed by vascular smooth muscle cells (VSMCs) and that atherosclerotic plaques, especially calcified ones, contain GRP/Ucma. Since VSMCs can transdifferentiate towards an osteochondrogenic phenotype, we hypothesized that GRP/Ucma sup-

presses osteochondrogenic differentiation of VSMCs and, thereby, contributes to preventing mineralization of the blood vessel wall.
Aims: To investigate the potential role of GRP/Ucma in osteogenic differentiation of VSMCs *in vitro*.
Methods: Human VSMCs were cultured in M199 medium containing 20% fetal bovine serum (FBS). Mouse VSMCs were isolated from aortas dissected from wild type (WT) and $GRP^{-/-}$ C57BL6 mice and maintained in DMEM containing 10% FBS. Cells were pretreated with control medium, heparin (200 U/mL), PDGF-BB (20 ng/mL) or Pi (2 mM) in control medium. After pretreatment, cells were incubated with medium supplemented with 5.4 mM calcium. Mineralization was quantified and normalized to total protein content. Aortic sections from $ApoE^{-/-}$ mice on warfarin were stained immunohistochemically for GRP/Ucma.
Results: In the control, limited mineral depositions were observed whereas in PDGF-BB treated VSMCs, disperse mineralization throughout the entire extracellular matrix was present. Pi treatment of cells resulted in severe mineral depositions, concentrated at focal areas in the extracellular matrix. We observed that VSMCs treated with Pi underwent morphological changes in addition to nodule formation. These nodules co-localized with the focal areas of mineral deposition. Cells treated with heparin underwent morphological changes as well, but not to the extent of nodule formation or mineral deposition. Additionally, heparin treated VSMCs stopped proliferating. In mice VSMCs, mineralization was significantly increased in $GRP^{-/-}$ VSMCs as compared to WT VSMCs. The mechanism for this difference in mineralization remains to be determined. As compared to human VSMCs, initial experiments in which mouse VSMCs were pretreated with Pi, showed no noticeable effect on further increasing mineralization or nodule formation. Immunohistochemical staining of GRP mainly localized to osteochondrogenic cells, present in atherosclerotic plaques of $ApoE^{-/-}$ mice.
Summary: Osteochondrogenic differentiation of VSMCs is a key feature of the vulnerable atherosclerotic plaque. Pi pretreatment of primary VSMCs can mimic this transdifferentiation *in vitro*. Preliminary data in $GRP^{-/-}$ VSMC suggests a role for GRP/Ucma in suppressing osteochondrogenic differentiation of VSMCs *in vitro* thereby attenuating mineralization. In atherosclerotic plaques, osteochondrogenic cells were positive for GRP/Ucma. Future experiments aim at identifying mechanisms through which molecular pathways GRP/Ucma exerts this effect.

OC 26.3
Accelerated senescence of cord blood endothelial progenitor cells in premature neonates is driven by SIRT1 decreased expression

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Background: Since their discovery, endothelial progenitor cells have emerged as an important biological marker for a variety of cardiovascular disease. Both their numbers and their angiogenic potential are decreased in diseases associated with a cardiovascular risk (diabetes, atherosclerosis, …). Among them a specific subtype called Endothelial Colony Forming Cells (ECFC) have been recently characterize both in cord and peripheral blood. These cells emerged as adherent colonies after culture of mononuclear cells under endothelial proceedings conditions and developed as typical endothelial monolayer. They have proliferative potential, express endothelial markers, have the capacity to incorporate into neo-vessels and participle in maintaining of vascular homeostasis *in vivo*. Preterm birth is known to critically increase cardiovascular risk at aduldhood. Epidemiological and experimental studies indicate that early vascular dysfunction occurs in preterm neonates with a low birth weight (PT) and is associated with an impairment of angiogenic properties of ECFC.
Aims: We hypothesized here that ECFC dysfunction might result from premature senescence and investigated the underlying mechanisms.
Methods: We used a cohort of ECFC isolated from cord blood of PT ($n = 29$) or term ($n = 18$) neonates. This research was approved by local ethic committee, and all the parents have provided written informed consent for the use of cord blood in accordance with the Declaration of Helsinki. Senescence of ECFC was judged by senescence-associated ß-galactosidase assay (SA-ß-Gal), morphological appearance and cell cycle analysis.
Results: PT-ECFC displayed an accelerated senescence inversely correlated with gestational age, sustained by growth arrest and increased SA-ß-Gal activity. Increased p16*INK4A* expression together with reduced p21*WAF* level, in the absence of telomere shortening, indicated that premature PT-ECFC ageing results from stress-induced senescence rather than replicative senescence. Interestingly, we found that expression of SIRT1, a NAD-dependent deacetylase possessing anti-aging activities, was dramatically decreased at the RNA and protein levels in PT-ECFC. SIRT1 deficiency was subsequent to epigenetic silencing of its promoter and triggered accelerated senescence of PT-ECFC. Transient *SIRT1* overexpression or chemical-induction by resveratrol treatment led to a significant reduction of SA-ß-Gal staining and senescence in PT-ECFC, accompanied by increased cell proliferation. Furthermore, resveratrol treatment reversed senescence and rescued PT-ECFC angiogenic defect in a SIRT1-dependent manner.
Conclusions: We demonstrate that SIRT1 decreased expression drives accelerated senescence of PT-ECFC, and acts as a critical determinant of PT-ECFC angiogenic defect. Moreover, our findings lay news grounds for understanding the increased risk of cardiovascular diseases of individuals born prematurely and open new perspectives for a targeted therapeutic strategy.

OC 26.4
Endothelial progenitor outgrowth cells on ePTFE grafts respond to hemodynamic preconditioning

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Background: Endothelial progenitor outgrowth cells (EOCs), isolated from whole blood, are a promising cell source for the endothelialization of small diameter vascular graft materials. The ability to incorporate a native endothelium onto a graft may reduce the thrombosis and intimal hyperplasia, the role of hemodynamics on the ability of EOCs to regulate thrombosis and intimal hyperplasia is unknown. This work was performed using well-established, non-human primate models for testing EOC-coated ePTFE grafts *ex vivo* and *in vivo*. The utilization of EOCs and ePTFE vascular grafts represent a clinically-relevant cell source and biomaterial for determining the effects of hemodynamic preconditioning on graft performance.
Aims: This study examined the hypothesis that hemodynamic preconditioning of EOCs reduces their *in vitro* markers of thrombosis and inflammation, reduces platelet and fibrin accumulation on an *ex vivo* shunt, and reduces initial hyperplasia in an *in vivo* graft implant.
Methods: EOCs were seeded on collagen-coated ePTFE grafts and stimulated with 15 dynes/cm^2 steady fluid shear stress. All assays were performed on paired static and hemodynamic stimulated EOC-seeded grafts. EOC expression of key markers of thrombosis and inflammation [CD39, endothelial protein C receptor (EPCR), thrombomodulin (TM), tissue factor (TF), tissue factor pathway inhibitor (TFPI), endothelial nitric oxide synthase (eNOS), intracellular adhesion molecule (ICAM), vascular cell adhesion molecule (VCAM), and platelet endothelial cell adhesion molecule (PECAM)] were quantified using quantitative RT-PCR. EOC production of activated protein C (APC) and factor Xa (FXa) was quantified. Using a well-established arteriovenous shunt model, platelet accumulation and fibrin deposition during 60 min of non-anti-coagulated blood flow were quantified. Additionally, EOC-seeded grafts were implanted with bilateral controls as aorto-iliac bypass grafts for 4 weeks. After explant, histology sections of

the grafts were analyzed for the degree of intimal hyperplasia at each anastamosis.

Results: Hemodynamic stimulation of EOC altered their expression of thrombosis and inflammation-related genes. The hemodynamic stimulation increased EOC expression of genes for CD39 (1.13 ± 0.72, fold change compared to 0 for static), and EPCR (1.18 ± 0.73), while there was a trend for increased expression of TF and TM. Additionally, EOC expression of TFPI (−0.53 ± 0.70) and ICAM (−1.25 ± 1.27) decreased significantly. Hemodynamic stimulation increased EOC production of both FXa ($P = 0.05$) and APC ($P = 0.091$). Interestingly, in *ex vivo* studies, there was no significant difference in platelet accumulation between the hemodynamically stimulated EOCs and the static samples, yet the hemodynamic stimulation did significantly decrease fibrin deposition on the EOC-coated grafts ($P = 0.05$). Initial *in vivo* studies suggest that ePTFE grafts coated with hemodynamically stimulated EOCs decrease the intimal hyperplasia area of 1.49 ± 0.32 mm^2 compared to static cell-seeded grafts with 2.64 ± 0.65 mm^2.

Summary/Conclusion: In a clinically relevant vascular graft model, hemodynamic stimulation of EOCs induced significant changes in gene expression and production of FXa, yet platelet adhesion to the grafts were not significantly altered. Experiments with oscillatory flow-stimulated EOCs in the arteriovenous shunt model are currently underway. Interestingly, *in vivo* implantation of the EOC seeded grafts indicated the hemodynamic stimulation altered the vascular healing response and decreased the extent of anastomotic intimal hyperplasia at 1 month.

OC 27 – Antiphospholipid Syndrome

OC 27.1

Antiphospholipid antibodies induce NF-kB activation exclusively from endosomal compartments of human monocytes

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Background: Antiphospholipid antibody syndrome (APS), caused by pathogenic antiphospholipid antibodies (aPLA), is an auto-immune disease associated with thrombosis and/or recurrent fetal loss. We recently demonstrated TLR2 as one of the TLRs involved in the recognition of aPLA by monocytes. While TLR4 is known to transmit signals from both plasma membrane and endosomal compartments, less is known about the function of endosomal trafficking on TLR2 signaling.

Methods: Primary human monocytes and HEK-Blue2[TRADEMARK] cells were treated with aPLA or with TLR2 ligands, <i*e*. LTA and Pam_3CSK_4, in the presence of endocytosis inhibitors. Expression of TNF and tissue factor (TF) as well as NF-B activation was assessed. In order to evaluate the importance of receptor internalization, clathrin expression was reduced by siRNA and NF-kB activation assessed after treatment with aPLA and TLR2 ligands. Endocytosis of biotinylated LTA and Pam_3CSK_4 or aPLA was evaluated by flow cytometry.

Results: We demonstrate that blocking antibodies to TLR2, TLR1 or TLR6, as well as antibodies to the TLR co-receptor CD14, significantly decreased TNF and TF responses to aPLA, Pam_3CSK_4 and LTA. TLR2 ligands internalization is observed in primary human monocytes and HEK-Blue2[TRADEMARK] cells and we show that NF-kB-mediated cell activation is dependent on ligand internalization. Pharmacological blockade and siRNA approaches revealed the importance of the clathrin/dynamin-dependent endocytic pathway in ligand-induced NF-kB activation. In addition, aPLA, Pam_3CSK_4 and LTA immobilized on large beads, preventing their internalization, are unable to activate NF-kB. Furthermore, in a similar manner as TLR4, we demonstrate that TLR2 ligand uptake is regulated by CD14. Unlike LPS, TLR2 ligands do not induce phosphorylation of IRF-3 or IFNβ expression. Finally, NF-kB activation induced by aPLA, Pam_3CSK_4 and LTA in HEK-Blue2[TRADEMARK] cells or primary human monocytes is regulated by CD14-dependent endocytosis.

Conclusions: Our results reveal that TLR2-ligand complexes are internalized via clathrin- and CD14-dependent endocytosis into primary human monocytes and HEK-Blue2[TRADEMARK] cells; endocytosis of these complexes is required for NF-kB activation. This highlights a new important aspect of the regulation of TLR2-dependent signaling. Although present at the cell surface, TLR2-ligands induce NF-kB activation exclusively from an endosomal compartment. This study provides new insight that may be used for the development of novel therapies through the control of endocytosis and/ or the blockade of CD14 with monoclonal antibodies. In addition, the evidence that TLR1 and TLR6 play also a role in the pathogenesis of aPLA contributes to a better understanding of the antiphospholipid syndrome.

OC 27.2

Beta2-glycoprotein I selectively inhibits the procoagulant functions of thrombin

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Background: β2-glycoprotein I (b2GpI) is an abundant plasma protein has been identified as the major antigen in the antiphospholipid syndrome (APS), a severe thrombotic autoimmune disease. Notwithstanding, the physiological role of β2GpI is still elusive. β2GpI is composed of five domains and displays remarkable structural heterogeneity, varying from a circular closed conformation to an open J-shaped conformation. Thrombin exerts either procoagulant and anticoagulant functions in haemostasis. The procoagulant functions mainly entail conversion of fibrinogen into fibrin and activation of platelets through cleavage of type-1 Protease Activated Receptor (PAR1), whereas the anticoagulant functions are related to its ability to activate the anticoagulant protein-C (PC) in the presence of thrombomodulin (TM). Thrombin accomplishes most of its activities through its hydrolytic active site and two positively charged exosites.

Aim: Quantify and mapping β2GpI-thrombin interaction; study the effect of physiological concentrations of β2GpI (4 μM) on the procoagulant (fibrin generation and platelet aggregation) and anticoagulant (active protein C generation) functions of thrombin.

Methods: Binding of β2GpI to thrombin was studied by Surface Plasmon Resonance on a Biacore X100 instrument. The effect of β2GpI on thrombin-mediated fibrin generation and platelet aggregation was investigated on gel-filtered platelets by turbidimetric and immunocytofluorimetric measurements. The activity of β2GpI on platelet aggregation was also evaluated on healthy donors whole blood by Multiple Electrode Aggregometry. PC activation by thrombin was monitored by measuring the rate of S2366 hydrolysis. Thrombin mutants were expressed in E. coli and *in vitro* refolded. The structural model of β2GpI-thrombin complex was obtained by HEX-6.3 software.

Results: Biacore analysis of β2GpI-thrombin interaction yields $K_d = 34$ nM. Displacement experiments, carried out with specific binders of thrombin exosite-1 (hirugen and HD1 aptamer) or exosite-2 (fibrinogen γ'-peptide and HD22 aptamer) and with thrombin mutants (Arg73Ala and Arg101Ala), having one of the two exosites selectively compromised, indicate that both exosites are involved in β2GpI binding. β2GpI does not affect the affinity of the enzyme for some active-site inhibitors, like p-aminobenzamidine and the N-terminal domain 1–47 of hirudin. β2GpI significantly prolong the clotting time in fibrin generation assay and hinders platelets aggregation ($IC_{50} = 0.36$ mM) by inhibiting cleavage of PAR1 on intact platelets ($IC_{50} = 0.32$ mM). Finally, β2GpI does not alter the ability of thrombin to generate the active protein C, in the presence or in the absence of TM. The docking model indicates that domains III to V of β2GpI interact with thrombin exosites, without masking the protease active pocket.

Conclusion: β2GpI binds to thrombin at both exosites, while leaving the protease active site accessible. β2GpI inhibits the key procoagulant properties of thrombin, without affecting its unique anticoagulant function. These results allow us to speculate that under physiological conditions β2GpI may function as a plasma-soluble anticoagulant protein acting in those vascular compartments where the more potent TM-thrombin pathway poorly functions, as in the large vessels, or is even absent, as in the brain vasculature. Our results can also explain the positive correlation existing between the presence of neutralising auto-antibodies against β2GpI and thrombotic manifestations in APS patients.

OC 27.3

High levels of β2-glycoprotein I protects against secondary major adverse cardiovascular events after PTCA

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Background: We have recently published that high circulating levels of beta2-glycoprotein I is associated with a reduced risk of myocardial infarction in elderly men (Blood 2009; 114: 3656–61).
Aim: Here we studied whether high levels of plasma beta2-Glycoprotein I has also a predictive value for secondary cardiovascular events after percutaneous transluminal coronary angioplasty (PTCA).
Methods: Four hundred andfifty-seven patients with angina pectoris and scheduled for PTCA were included in four different Dutch hospitals (Catharina Hospital, Eindhoven, University Medical Centre, Maastricht, University Medical Center, Leiden and University Medical Center, Utrecht). Informed consent from all patients was obtained. Approval from local ethics committees was obtained. Patients were followed for 60 and 90 days after invention for major adverse cardiovascular events (MACE) being myocardial infarction, PTCA, coronary artery bypass graft or death by cardiovascular disease. Receiver operating characteristic (ROC) curves were used to determine cut-off values for beta2-glycoprotein I.
Results: Of the 457 patients included, 312 were male. The median age was 62 years. At 60 days follow up, 13 patients suffered from MACE, at 90 days 15 individuals suffered from MACE. The mean level of beta2-glycoprotein I in patients without MACE was 444 ± 10.6 μg/mL, the mean level of beta2-glycoprotein I in patients with MACE was 355 ± 50.6 μg/mL. At 60 days of follow-up, a HR of 0.10 (95% ci: 0.03–0.32) for levels above 156 μg/mL beta2-glycoprotein was found. At 90 days of follow-up, a HR of 0.30 (95%ci: 0.11–0.92) for above 368 μg/mL was found. No correlation was found between plasma levels on von Willebrand factor and the risk of MACE after 60 or 90 days. The protection for MACE associated with increased levels of beta2-glycoprotein I did not attenuate after adjusting for age and gender and HS-CRP for both follow-up periods (60 days: HR 0.11, 95% ci 0.03–0.35; 90 days HR 0.32, 95% ci 0.11–0.95), but after adjusting for NT-proBNP the association at 90 days disappeared.
Conclusion: High circulating levels of beta2-glycoprotein I is associated with a reduced risk of recurrence of cardiovascular events after PTCA.

OC 27.4

Anti-β2-glycoprotein I antibodies inhibit the prothrombinase complex in a phospholipid-independent manner

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Background: Antiphospholipid syndrome (APS) is diagnosed in patients with thrombosis or pregnancy morbidity who have persistent antiphospholipid antibodies. Of all antiphospholipid antibody subtypes, the presence of the phospholipid-dependent coagulation inhibitor known as lupus anticoagulant (LA) correlates best with thrombosis. The current paradigm is that LA is caused by competition between antibody-β2-glycoprotein I (β2-GPI) complexes and vitamin K-dependent coagulation factors for binding sites on negatively charged phospholipids. Different observations we have made challenge this axiom.
Aim: To study the effects of LA-inducing antibodies on coagulation:
Methods: We analyzed the effects of LA-inducing monoclonal anti-β2GPI antibody 3B7 and different variants of β2GPI on contact activation and thrombin generation. Moreover, we studied the effects of 3B7 and β2-GPI on intrinsic tenase and prothrombinase complexes.
Results: Lowering the phospholipid concentration in thrombin generation experiments results in both an increased lag time and a decrease in endogenous thrombin potential (ETP). In contrast, addition of 3B7 induced an increase in lag time and had no effect on ETP, suggesting phospholipid-independent effects on coagulation. We further analyzed the effects of 3B7-β2GPI complexes on coagulation with purified coagulation factors. β2GPI in complex with 3B7, but not β2GPI or 3B7 alone, inhibited the prothrombinase complex for 30% and the intrinsic tenase complex for 70%. Substitution of wild-type β2GPI for a mutant that does not bind to phospholipids (β2GPI W316S) abolished the inhibitory effects of antibody-β2GPI complexes on the intrinsic tenase complex, whereas inhibition of the prothrombinase complex remained unaltered. Surface plasmon resonance analysis showed that complexes of 3B7 and wild-type β2GPI or the W316S mutant, but not β2GPI itself, directly interact with activated factor V (K_D: 25 nM), but not with factors VIII, IX, X, and II. To determine the contribution of inhibition of either the intrinsic tenase complex or the prothrombinase complex to lupus anticoagulant activity, we performed an APTT in the presence or absence of the thrombin inhibitor argatroban. As expected, 3B7 prolonged the APTT. Addition of argatroban and analysis of factor Xa generation with a fluorescent substrate showed that 3B7 did not inhibit factor Xa formation in plasma.
Conclusion: Anti-β2-glycoprotein I antibodies differentially interfere with the coagulation reaction. Whereas β2GPI-antibody complexes compete with the intrinsic tenase complex for phospholipid binding, prothrombinase inhibition is independent of phospholipid binding. In plasma, inhibition of the prothrombinase complex seems to be the determining step in anti-β2GPI antibody-induced lupus anticoagulant. We hypothesize that this inhibition is mediated by a direct inhibition of factor Va.

OC 27.5

Pathological mechanisms of antiphospholipid antibodies in trophoblastic cell fusion

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Background: Antiphospholipid Syndrome (APS) is an acquired thrombophilia associated with arterio-venous thrombosis and/or obstetrical complications, and the presence of antiphospholipid antibodies (aPL) in the plasma of patients. It has been suggested that aPL could affect directly invasion and fusion of trophoblastic cells. However, their

pathogenicity is poorly understood. Toll-like Receptors (TLR) have been implicated in pathological activation of aPL on endothelial cells, monocytes and platelets.
Although inflammation has been incriminated in this process, it has been hypothesized that imbalance between autophagy and apoptosis could be related to trophoblastic fusion impairment. Moreover, TLR4 has been implicated in autophagy signalling cascade.
Hydroxychloroquine (HCQ) is an antimalarial molecule safely used during pregnancy and associated with a reduction in fetal losses in lupic patients with aPL.
Aims: The aim of our study was to evaluate the effects and pathological mechanisms of aPL on trophoblastic cell fusion and whether they could be reversed by HCQ treatment.
Methods: The human choriocarcinoma cell line BeWo is regularly used as a cell culture model to mimic trophoblastic cell fusion, triggered by forskolin. Cells were treated or not with forskolin in presence or not of antiβ2GP1 antibodies (antiβ2GP1-Ab) for 48 h. Fusion index (FI) was then determined by immunocytochemistry (ICC) and biochemical differentiation was determined by ELISA measuring hCG secretion. Antiβ2GP1-Ab toxicity was determined by MTT assay.
To assess the role of TLR in the pathological mechanisms of antiβ2GP1-Ab on BeWo cells, TLR expression was determined by qPCR and Western Blot (WB) in the presence or not of antiβ2GP1-Ab. Effects of membranous TLR (respectively 1, 2, 4 and 6) on BeWo cell fusion were then evaluated by ICC, using specific blocking molecules such as anti-TLR2 and 4 antibodies and peptide binding for TLR1/6, 2 and 4, or decreased expression of TLR4 (shRNA) in the presence or not of antiβ2GP1-Ab.
After determining a potent role of TLR4 in trophoblastic cell fusion impairment by antiβ2GP1-Ab, we investigated the expression of some target genes of TLR4 signalling cascades.
Interactions between HCQ and TLR4 were then determined by FI by ICC, hCG secretion by ELISA, qPCR and WB.
Results: FI and hCG secretion are decreased by addition of antiβ2GP1-Ab in a dose-dependent way, independently of antibodies toxicity. In the presence of antiβ2GP1-Ab and anti-TLR4 blocking antibodies, BeWo cell fusion and hCG secretion were restored.
In BeWo cells transfected with shRNA TLR4, antiβ2GP1-Ab do not affect cellular differentiation. Increase in autophagy gene expression (Beclin-1, GABARAPL1 and LC3B) was observed in BeWo cells stimulated by antiβ2GP1-Ab, but not in BeWo cells transfected with shRNA TLR4 plasmid and stimulated by antiβ2GP1-Ab, suggesting that antiβ2GP1-Ab treatment triggers autophagy and that autophagy was at least partly regulated by the TLR4 pathway.
Treating BeWo cells with HCQ decreased autophagy gene and TLR4 expression, and restored BeWo cell differentiation.
Summary/Conclusion: Antiphospholipid antibodies interfere with fusion process and biochemical differentiation of BeWo cells *via* TLR4. HCQ decreases TLR4 and autophagy gene expression and restores differentiation of BeWo cells suggesting its therapeutic interest for aPL affected pregnancies.

OC 27.6

MicroRNA expression in monocytes and neutrophils from primary antiphospholipid syndrome and systemic lupus erythematosus patients. Potential value as biomarkers of atherothrombotic disease

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Background: miRNAs are key players in a wide range of molecular and pathophysiological processes. Recently, several studies have examined their involvement in the pathogenesis of systemic lupus erythematosus (SLE) and antiphospholipid syndrome (APS). However, no study has evaluated the expression profile of miRNAs associated with the cardiovascular and the atherothrombotic risks observed in these autoimmune diseases.
Aims: To identify the miRNAs involved in the regulation of pro-inflammatory, prothrombotic and oxidative status in SLE and APS patients.
Patients and Methods: *In silico* search was performed to find putative binding sites of miRNAs in various mRNA of proinflammatory factors (MCP-1, MIP-1a, IL-1b, -2, -6, -8, -17, -23, VEGF and tPA) prothrombotic proteins (TF and PAR2) and oxidative stress markers (peroxides production, antioxidant enzymes and mitochondrial activity) was performed. Neutrophils and monocytes were isolated from 11 APS patients, 17 SLE patients and 26 healthy donors. Selected miRNAs were quantified by RT-PCR. The expression of proinflammatory proteins was evaluated by flow cytometry and flow cytomix. Antioxidant enzymatic activity was also quantified. C*arotid intima*-media *thickness*(CIMT) was measured as a marker of early atherosclerosis.
Results: *In silico* search reported miR-124a, -125a, -125b, -146a, -155, and -222 as candidates to regulate the expression of several proinflammatory proteins and oxidative status observed in SLE and APS patients. The expression levels of these miRNAs appeared significantly decreased in neutrophils from SLE and APS patients compared to healthy donors. However, only the miR-124a was found reduced in monocytes from SLE and APS patients, while miR-146a was increased. The expression levels of the miRNAs analyzed in SLE patients negatively correlated with the disease activity (SLEDAI) and the anti-dsDNA titers. In addition, an inverse correlation between anticardiolipin antibody titers and miRNAs expression was found in APS patients. Moreover, significant negative correlations between miRNAs expression and molecules related to mitochondrial dysfunction and oxidative stress were observed in APS monocytes and neutrophils. Inflammatory proteins showed specificity in their association with miRNAs depending on the disease analyzed, so that in APS inverse correlations were found with VEGF-R1, IL-8 and PAR2, while in LES significant correlations related to IL-2, IL-6, IL-10 and MCP.1 Low levels of miR-146a in APS and SLE neutrophils, and miR-155 in APS neutrophils, were associated with pathological CIMT. The presence of thrombotic events in APS and SLE was associated with low levels of miR-146a and miR-155 in neutrophils and monocytes.
Conclusions: miRNAs differentially expressed in monocytes and neutrophils from APS and SLE patients correlate with markers of autoimmunity, inflammation, thrombosis and oxidative stress, and are associated with atherothrombotic processes. Therefore, these miRNAs might be considered potential biomarkers of pro-inflammatory pathology in both atherosclerosis and autoimmune diseases.

OC 28 – Coagulation Factor VIII

OC 28.1

Factor VIII: where is it synthesized?

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Background: The precise source(s) of coagulation factor VIII (F8) synthesis has remained controversial. Production of coagulation-related proteins in liver is well documented, but this and other recent studies challenge historical paradigms attributing coagulation F8 expression specifically to hepatocytes. Tissue surveys confirm significant hepatic as well as renal transcription of F8 mRNA, with much lower levels in spleen and many other organs. *In situ* localization of synthesis has not clarified our understanding. At the cellular level, endothelial cells (EC) or sinusoidal EC and a number of other alternative cell types throughout the body have been implicated in F8 synthesis. This prompted our study of F8 synthesis in the whole animal by genetic techniques.

Aims: Defining the site of F8 synthesis using cell-type specific F8 gene inactivation in the mouse.
Methods: We developed a conditional knockout of the murine F8 gene. 'Floxed' ($F8^F$) mice, which express normal levels of plasma F8, were generated by inserting LoxP sites flanking exons 17/18. When $F8^F$ mice are crossbred with various Cre-expressing mouse strains, excision of exons 17/18 occurs, causing F8 gene inactivation in Cre-expressing cells and their progeny. We utilized six strains of mice with differing tissue-specific synthesis of Cre recombinase. Plasma F8 activity was measured by chromogenic assay. Gene inactivation and expression were assessed by PCR and RT-PCR analysis.
Results: Germline (Meox2)-Cre expression results in an inherited, severe hemophilic F8 knockout ($F8^{KO}$) phenotype, while tissue-specific Cre's cause gene inactivation only in specific cell types, depending on the promoter controlling Cre expression. While Alb-Cre drives F8 gene inactivation with high efficiency and specificity in hepatocytes, affected mice are phenotypically indistinguishable from normal controls. In contrast, marked reduction in plasma F8 was seen in all EC-expressing Cre strains tested. Tek-Cre and Cdh5-$Cre^{(Spe)}$ resulted in mice with nearly undetectable plasma F8, while a less efficient Cdh5-$Cre^{(Mlia)}$ model results in a 30–40% reduction in F8. Tek-Cre is expressed with high efficiency in EC generating a functional F8-knockout phenotype, typically with a complete absence of plasma F8. Since each of these 'endothelial' Cre strains express Cre in both hematopoietic and endothelial compartments, we studied a hematopoietic cell-expressed Vav1-Cre model which resulted in modest, but significant F8 reduction. Although plasma F8 activity is undetectable in germline $F8^{KO}$ and affected Tek-Cre mice, low level transcription of exon 17/18-deleted $F8^{KO}$ message continues in liver as two alternatively-spliced mRNA variants expressed at roughly equivalent levels. There is no evidence that these transcripts result in measureable F8 (similar transcripts are identified in current hemophilic mice).
Summary/Conclusion: The completely normal phenotype following F8 gene inactivation by Alb-Cre suggests no significant F8 synthesis in hepatocytes. In contrast, gene inactivation in EC causes reduced plasma F8 activity ranging from moderate to a severe hemophilia phenotype dependent on endothelial Cre efficiency. All currently available EC-specific and hematopoietic-specific Cre models exhibit variable penetrance into both endothelial and hematopoietic compartments that is presumably due to their differentiation from a common hemangioblast progenitor. Altogether our data indicates that F8 synthesis is primarily a function of endothelial cells and not hepatocytes.

OC 28.2

Factor VIII A2 domain stabilization enhances potency and efficacy in hemophilia A mice

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Background: An important negative regulator of activated factor VIII (FVIIIa) cofactor activity is subunit dissociation. FVIII molecules with stabilized activity have been generated by elimination of charged residues at the A1-A2 and A2-A3 interfaces. These molecules exhibited reduced decay rates as part of the factor Xase complex and retained their activities under thermal and chemical denaturing conditions (Wakabayashi H, et al. *J Thromb Haemost*. 2009;7[3]:438–444).
Aim: We describe the potency and efficacy of one such variant, D519VE665V, a mutant of B-domain–deleted FVIII (BDD-FVIII).
Methods: FVIII activity was measured using the two-stage chromogenic assay and the one-stage activated partial thromboplastin time (aPTT) assay. Hemostasis parameters were measured using the thrombin generation assay (TGA) and rotational thromboelastometry (ROTEM). Efficacy was assessed in Hemophilia A (HemA) mice subjected to various models of vascular injury (tail clip, laser injury, and tail vein transection).
Results: D519VE665V potency was increased two-fold by the two-stage chromogenic assay relative to BDD-FVIII. The potency was equivalent to BDD-FVIII by the one-stage aPTT assay. Compared with BDD-FVIII, D519VE665V demonstrated enhanced TGA and ROTEM responses: four-fold by peak thrombin and two-fold by clot initiation time, respectively. Efficacy testing in HemA mice demonstrated enhanced efficacy for D519VE665V vs BDD-FVIII. By both the tail clip and tail vein transection models, D519VE665V had a two-fold increase in efficacy, whereas by laser injury, fibrin-thrombi accumulation was greater (four-fold increase in fibrin and eight-fold increase in platelet accumulation, respectively) compared with BDD-FVIII.
Conclusion: These results demonstrate that stabilizing A2 subunit association of FVIII can prolong its cofactor activity and lead to enhanced protection against vascular injury in an animal model of hemophilia.

OC 28.3

Activated blood coagulation factor VIII interacts with cluster III complement-type repeats of the low-density lipoprotein receptor-related protein

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Background: It was previously found that the clearance of coagulation factor VIII (FVIII) is mediated by the low-density lipoprotein receptor-related protein (LRP) (Lenting P., et al, 1999 and Saenko E., et al, 1999). Within LRP, FVIII was shown to interact with the complement-type repeats (CRs) of clusters II and IV, but not with cluster III. In LRP, clusters II and IV bind a majority of its known ligands, which exceeds 30. At the same time, cluster III is considered to serve a minor role, since it was shown to interact only with alpha-2-macroglobulin receptor-associated protein (RAP), an intracellular LRP chaperon, and lipoprotein ApoE, constituting beta-VLDL. Previously, we found that upon activation, FVIII (FVIIIa) has some changes in regard to its binding with LRP; in particular, it exposes a high-affinity site in the A2 domain for the clusters II and IV (Sarafanov A., et al, 2007).
Aims: In the present work, we tested in more detail how activation of FVIII affects its interaction with LRP, specifically with its cluster III.
Methods: Recombinant fragments of LRP and their mutant forms were expressed in a baculovirus system. The proteins were purified using combination of affinity and size exclusion chromatography and assessed for the binding with FVIII in a surface plasmon resonance assay.
Results: We found that FVIII and FVIIIa each bind to both clusters II and IV, while FVIIIa produced considerably higher signals. Strikingly, FVIIIa was found to bind cluster III in the same fashion, whereas FVIII remained essentially inactive towards this cluster. The FVIIIa binding to the cluster III was strongly inhibited by an anti-FVIII antibody fragment, iKM33, known to have an epitope within the C1 domain of FVIII light chain. Next, we found that a heterodimer A1/A3-C1-C2, known to be a physiological product of FVIIIa dissociation, also interacted with cluster III and this process was inhibited by iKM33. These data demonstrate that thrombin cleavage of FVIII results in exposure of a binding site on its light chain for LRP cluster III. To map the interactive site on this cluster, we tested binding of FVIIIa with nine CR doublets that spanned cluster III. We identified a number of positive binding CR doublets overlapping an extended region of adjacent CRs within the cluster; specificity of these interactions was confirmed by the inhibitory effect of iKM33 as above. Finally, we tested interaction of FVIIIa and cluster III with a mutated conservative residue within each active binding CR. We observed a significant decrease in the binding that also confirmed the cluster III mapping data.
Summary/Conclusion: We demonstrated that cluster III of LRP binds activated FVIII, and characterized the interactive sites within these

molecules. We also showed that FVIIIa binds LRP clusters II and IV, and is more active for the binding to all three clusters than non-activated FVIII. Altogether, our data indicate that LRP serves a role in clearance of activated FVIII *in vivo*, and cluster III is involved in this process.

OC 28.4

Identification of monoclonal antibodies protecting activated Factor VIII against spontaneous inactivation by A2 subunit dissociation

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Background: Factor VIII (FVIII) is an essential cofactor of the coagulation system. Conversion of FVIII to the active cofactor (FVIIIa) occurs by distinct cleavages that convert it into a heterotrimer consisting of A1 and A2 subunits non-covalently associated with the activated light chain. FVIIIa is inherently unstable and inactivates with a half-life of 2 min as a consequence of spontaneous A2 subunit dissociation. The physiological importance of this mechanism for down-regulation of the coagulation response is suggested by the association of mild haemophilia with point mutations in FVIII that accelerate A2 dissociation.
Aim: Based on the hypothesis that regulation of FVIIIa activity is limiting the generation of thrombin and consequently clot formation under conditions of mild or moderate haemophilia, a screen for antibodies protecting FVIIIa against A2 subunit dissociation was undertaken.
Methods: BALB/c mice were immunized with FVIII and derivatives, and hybridomas prepared by standard methods.
Results: Screening of hybridoma supernatants in a FVIIIa decay-dependent FXa generation assay identified two non-competing classes of antibodies with a hit rate of about 1%. To map their recognition sites on FVIII, hydrogen-deuterium exchange mass spectrometry was employed which identified non-overlapping epitopes in the A2 and A3 domains in agreement with distinct functional properties of the two classes. In particular, antibodies targeting the A3 domain were found to interfere with FVIII activation, while antibodies specific for A2 (represented by 4F143) bound with high affinity to FVIII/FVIIIa and neither affected VWF binding nor activation by thrombin. In a FXa generation assay, 4F143 reduced the apparent rate of FVIIIa decay up to four-fold and normalized the accelerated inactivation of the FVIII S289L variant associated with mild haemophilia A. Interestingly, the protective effect was strictly dependent on the bivalency of 4F143 and also on the presence of a phospholipid membrane surface. Titration studies demonstrated that the membrane dependency was bell-shaped with a progressively declining effect of 4F143 as the vesicle concentration was increased above a peak concentration of 5 μM. Taken together, these data are consistent with a mechanism by which 4F143 simultaneously engages two FVIIIa molecules on the membrane surface. This configuration would promote re-assembly of dissociated FVIIIa by retaining the A2 domain in close proximity to the membrane and thus to any surface-bound A1-light chain fragment available for reassociation.
To explore whether such mechanism would promote sustained thrombin generation in the presence of a physiological surface, 4F143 was tested in congenital haemophilia A plasma supplemented with washed human platelets. In this system, 4F143 was found to restore thrombin generation in the presence of 0.3% FVIII to the level obtained with 1–2% FVIII in the absence of antibody.
Conclusion: In conclusion, the present work demonstrates a novel antibody-based approach to intercept a regulatory pathway of relevance in mild haemophilia A. *In vivo* studies are undertaken to investigate the impact of this pathway on the bleeding phenotype in general haemophilia.

OC 28.5

Clearance of FVIII in a rat perfused liver model and isolated primary liver cells in the presence and absence of VWF

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Background: FVIII is predominantly cleared in the liver. Members of the low density lipoprotein receptor family including the low density lipoprotein-related protein, LRP, have been previously reported to mediate clearance of FVIII either directly[1] or indirectly via VWF[2]. Other potential receptors include macrophage mannose receptor (MMR)[3]. In a genome-wide association study, SNPs in the region of stabilin-2 (Stab2, scavenger-like receptor) were found to correlate with VWF and FVIII levels[4].
Aims: To analyse the relative importance of a selection of clearance receptors in FVIII elimination using an isolated perfused liver model and primary liver cell cultures in the absence and presence of VWF.
Methods: Rat livers were isolated and cannulated via the portal vein and vena cava to generate an *ex vivo* recirculating model. Recombinant human FVIII (2–5 nM) was added to a Krebs Henseleit/BSA perfusate buffer with or without either plasma-derived VWF (20 nM), a VWF fragment containing the FVIII binding site (VWF-D'D3A1) (35 nM), ligands for LRP (receptor associated protein, RAP, 0.7 or 1.2 μM) or MMR (Man6Lys5, 500 nM). Samples from the perfusate were analysed for FVIII and VWF concentrations by activity and antigen assays. For primary liver cell studies, cells were isolated via collagenase perfusion of rat livers. Our previous hepatocellular studies identified hepatocytes (parenchymal cells, PCs) and liver sinusoidal endothelial cells (LSECs) as having a major role in FVIII elimination; therefore, these cells were isolated and separately plated in tissue culture plates. Iodine (^{125}I) or fluorescently labelled FVIII was incubated with cells for up to 2 h with or without VWF, VWF-D'D3A1, RAP, different MMR ligands, as well as, an antibody against Stab2. Membrane bound and internalized radioactivity was subsequently quantified.
Results: Both FVIII and VWF disappeared from the perfusate over time. Almost 80% of FVIII dosed was cleared after 60 min, and approximately 25% of VWF dosed was cleared in the presence and absence of FVIII. As expected, VWF and VWF-D'D3A1 reduced FVIII uptake in the rat livers and in primary liver cells. In contrast, FVIII clearance was not affected by RAP neither when added as a single bolus dose together with FVIII nor when constantly infused during the perfusion. RAP was, however, able to decrease t-PA clearance. Man6Lys5 also had no effect on FVIII liver clearance. In cell studies, an excess concentration of RAP and MMR ligands did not reduce FVIII binding or internalization. Interestingly, an anti-Stab2 antibody inhibited FVIII internalization in LSECs.
Summary/Conclusion: VWF and VWF-D'D3A1 reduced FVIII clearance in isolated rat livers and blocked uptake in primary liver cells, while an excess concentration of ligands to either LRP or MMR did not. In addition, Stab2 may play a role in FVIII uptake in LSECs. These data suggest that other major clearance pathways may be involved in FVIII clearance when LRP and MMR are blocked and that more than one receptor is probably involved in liver clearance of FVIII.
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OC 28.6

Difference in the membrane-bound organization of human and porcine factor VIII

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Factor VIII (FVIII) is a multidomain blood plasma glycoprotein, which causes hemophilia A when defective or deficient. FVIII biological function is a co-factor to the serine protease Factor IXa (FIXa) within the membrane-bound tenase complex assembled on the activated platelet surface during the propagation phase of coagulation. Recombinant human FVIII (hFVIII) concentrate is the most efficient cure for Hemophilia A. Recombinant porcine FVIII (pFVIII) is used in patients developing inhibitory antibodies against the human form. Both hFVIII and pFVIII large single chain glycoproteins composed of six domains: A1-A2-B-A3-C1-C2. Recombinant human and porcine FVIII are usually expressed without the B domain and consist of two non-covalently bound polypeptide chains: the light chain (LC) of the A3-C1-C2 domains holding the membrane interaction sites and the heavy chain (HC) of the A2-A1 domains holding the main FIXa interaction sites. The sequence identity between the two proteins is 84%. pFVIII also binds to human FIXa *in vivo*, forming functional tenase complexes. Cryo-EM is unique in giving direct structural information at closest to physiological conditions and subnanometer resolution. Cryo-EM can study the macromolecular structure of proteins and protein complexes in a lipid environment, since the samples are fully hydrated and the lipid bilayer well resolved.

We have combined cryo-EM and image analysis to study the membrane-bound structure of two recombinant Factor VIII forms, human and porcine helically organized on functionalized single lipid bilayer nanotubes (LNT) resembling the activated platelet surface.

Our results demonstrate unambiguously that human and porcine FVIII, differ significantly in their organization when assembled helically onto LNT at the same conditions (lipids, protein to lipid ration, buffer, salt concentration and temperature). The cryo-EM structures of both proteins at intermediate resolution (~12–14 Å) show that the pFVIII-LNT are tightly packed with a shallow twist angle, resulting in wider tubes. The hFVIII-LNT has a more pronounced twist, extending the protein-protein interaction in the plane of the membrane. Fitting of the hFVIII-BDD structure within the cryo-EM maps confirms a reorganization of the LC domains for both human and porcine FVIII forms, such as the C2 domain interacts directly with the membrane. The C1 domain does not interact directly with the membrane forming an extended interface with the A1 and A3 domain supporting the A2 domain. This organization of the FVIII-LC domains has been previously observed for helically organized hFVIII-LC on LNT, as well as for hFVIII organized in membrane-bound 2D crystals.

Resolving the FVIII membrane-bound organization for different FVIII forms (human and porcine) by cryo-EM reveals the structural role of the significant Hemophilia A mutations and completes our knowledge for FVIII function in hemostasis. Understanding the implication of the difference in sequence for the FVIII membrane-bound organization will help design better drugs and therapies against Hemophilia A.

OC 29 – Fibrinolysis – I

OC 29.1

Pharmacological inhibition of PAI-1 activity prolongs the lifespan of klotho mice, a murine model of accelerated aging

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Background: Plasminogen activator inhibitor type-1 (PAI-1) expression is stimulated by biologic stressors that promote cellular senescence and its induction is a validated senescence indicator *in vitro* and *in vivo*. Furthermore, PAI-1 expression levels are inversely proportional to telomere length. Senescent cells accumulate in aging tissues, which likely contributes to physiological aging. In humans, age-dependent increases in PAI-1 levels have been observed in plasma and in a variety of clinical conditions including obesity, insulin resistance, and inflammation. Additionally, PAI-1 levels increase in animals in plasma and in the heart as a function of age. Interestingly, *klotho* (*kl*/*kl*) mice, a murine model of accelerated aging, develop age-dependent increases in PAI-1 levels in plasma, and in tissues, including the kidney, aorta, and heart. Klotho protein regulates fibroblast growth factor-23 (FGF23) signaling and phosphate excretion in the kidney. *kl*/*kl* mice are deficient in the Klotho protein and display numerous phenotypic abnormalities resembling human aging, including emphysema, osteoporosis, arteriosclerosis, and ectopic calcification associated with a markedly shortened average lifespan (64 days).

Aims: To test the hypothesis that PAI-1 is a critical determinant of the aging phenotype in *kl*/*kl* mice. We aimed to investigate the effects of pharmacological inhibition of PAI-1 activity on senescence and survival in a murine model of aging.

Methods: *kl*/*kl* mice were administered a novel PAI-1 antagonist, TM5441 (100 mg/kg/day) mixed in the standard chow diet. Plasma samples were collected at baseline and at 2 and 4 months of treatment. Body weights of mice were monitored weekly. Plasma levels of FGF23 were measured using a commercially available ELISA kit.

Results: While no *kl*/*kl* mice lived longer than 120 days, 100% of *kl*/*kl* mice receiving TM5441 were alive beyond that time point. PAI-1 inhibition prolonged the survival of *kl*/*kl* mice by 4.6-fold with 3/5 of mice receiving TM5441 alive at age 265 days ($P = 0.0004$ by Log-rank test). TM5441 treatment also improved the overall health of *kl*/*kl* mice in terms of weight gain and activity levels. We observed that after 4 months of TM5441 treatment, plasma levels of FGF23 were reduced by 65% in *kl*/*kl* mice ($P = 0.018$).

Conclusion: These results confirm the hypothesis that PAI-1 is a critical determinant of the aging phenotype in *kl*/*kl* mice and clearly demonstrate that pharmacologic inhibition of PAI-1 activity may serve as a novel therapeutic approach to prevent senescence and aging-associated diseases.

OC 29.2

Global gene expression profiling in PAI-1 knockout murine heart and kidney: molecular basis of cardiac-selective fibrosis

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Background: Fibrosis is due to excessive accumulation of extracellular matrix proteins during wound healing. Fibrosis can affect every organ in the body and is one of the major contributors to morbidity and mortality in humans. At present, there is no effective therapy for organ fibrosis. Although extensive studies have been done to understand the molecular basis of fibrosis, there is a lack of fundamental information on the initiators of fibrogenesis. Previous studies demonstrated that aged plasminogen activator inhibitor-1 (PAI-1) knockout mice develop spontaneous cardiac-selective fibrosis without affecting any other organs including kidney, lung and liver. Therefore, the PAI-1 knockout model of cardiac fibrosis provides an excellent opportunity to find novel contributors to cardiac fibrosis.

Aims: We hypothesized that differential expressions of profibrotic and antifibrotic genes in PAI-1 knockout hearts and unaffected organs lead to cardiac-selective fibrosis. The aim of this study was to identify the initiator(s) of fibrosis in PAI-1 knockout hearts and to determine the status of those initiator(s) in PAI-1 knockout kidneys.

Methods: Hearts and kidneys explanted from aged PAI-1 knockout and wildtype mice were subjected to histological study. Levels of collagen were measured by Masson's trichrome staining. To test our

hypothesis, we performed genome-wide gene expression profiling of transcripts derived from aged PAI-1 knockout hearts and kidneys. Total RNA from wildtype and PAI-1 knockout hearts and kidneys were isolated. The variations of global gene expression profiling were compared within four groups: wildtype heart vs. knockout heart; wildtype kidney vs. knockout kidney; knockout heart vs. knockout kidney and wildtype heart vs. wildtype kidney. Array data was validated in wildtype and PAI-1 knockout hearts and kidneys by quantitative PCR analysis and by Western blotting in endothelial cells undergoing endothelial to mesenchymal transition (EndMT).

Results: Results revealed that while myocardial tissues derived from aged PAI-1 knockout mice showed significantly elevated levels of collagen accumulation compared to age- and sex-matched wildtype controls, collagen accumulation in kidneys derived from aged PAI-1 knockout mice were insignificant and comparable with age- and sex-matched wildtype controls. This suggests that PAI-1 deficiency is associated with age-dependent cardiac-selective fibrosis. Analysis of Illumina-based microarray data revealed that several genes involved in different biological processes such as immune system processing, response to stress, cytokine signaling, cell proliferation, adhesion, migration, matrix organization and transcriptional regulation are affected in hearts and kidneys by the absence of PAI-1. Importantly, while the expression of several genes, involved in profibrotic pathways including Ankrd1, Dbp, Egr1, Scx, Klf6, IGFBP6, Timp1/2 and Loxl1, are upregulated in PAI-1 knockout hearts, the expression of those genes is downregulated in PAI-1 knockout kidneys. The quantitative PCR analysis data of a few genes including Ankrd1, Egr1, Dbp and Timp1 further confirmed the microarray data. The protein levels of a few genes including Ankrd1 and Timp1 were elevated during EndMT.

Conclusion: To our knowledge, this is the first comprehensive report on the influence of PAI-1 on global gene expression profiling in heart and kidney. These results have identified new molecular targets for the prevention and treatment of fibrotic disorders.

OC 29.3

Plasmin induces *in vivo* monocyte recruitment through protease-activated receptor-1, MEK/ERK and CCR2 mediated signaling

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Background: The Plasminogen/Plasmin (Plg/Pla) system is associated with a variety of biological activities beyond the classical dissolution of fibrin clots, including cell migration, tissue repair and inflammation. Although the capacity of Plg to induce cell migration is well defined, the mechanism *in vivo* underlying this process is elusive.

Aims: To investigate the effect of Pla on cell migration *in vitro* and *in vivo* and the role of mitogenactivated protein kinase (MAPK) ERK1/2, protease activated receptor-1 (PAR-1) and CCL2/CCR2 axis in this process.

Methods: We performed *in vitro* migration assay (wound healing assay) in culture of MEFs (Mouse Embryonic Fibroblasts) or mouse leukemia monocyte macrophage (RAW 264.7). The cells were treated with Pla (2 μg/mL) at different times, or pretreated with the MEK1/2 inhibitor U0126 (15 μM), a serine protease inhibitor Leupetin (25 μg/mL) or the lysine analog tranexamic acid (0.01 M) 60 min. prior to and throughout Pla treatment and processed for microscopic counting of migrating cells or western blot analysis for phosphorylated ERK1/2. BALB/C mice were challenged by i.pl. (intrapleural) injection of Pla (2 μg/cavity) and the cells present in the pleural cavity harvested at different times or 48 h after pre-treatment with specific inhibitors (U0126 60 μg/cavity, i.pl.; leupeptin 100 μg/mouse, i.p.; SCH79797, 5 mg/kg, i.p.) 1 hour before Pla. Cells were processed for total and differential leukocyte counts and western blot analysis for P-ERK1/2 and IkB-a. Pleural levels of cytokines IL-1β, IL-6 and TNF-α and the chemokine monocyte chemoattractant protein 1 (MCP-1/CCL2) were analyzed by ELISA. CCR2 knockout mice and wild-type littermates were injected with plasmin and the cells present in the pleural cavity were harvested at 48 h after and processed for total and mononuclear cell count. All procedures described here had prior approval from the Animal Ethics Committee of Universidade Federal de Minas Gerais (CETEA/UFMG, Protocol number: 19/2011).

Results: Pla induced *in vitro* migration of murine fibroblast and macrophages dependent on MEK/ERK pathway and by requiring its proteolytic activity and lysine binding sites on cell surfaces.

Plasmin injection into the pleural cavity of mice induced a time-dependent influx of mononuclear cells that was associated with augmented ERK1/2 and IkB-α phosphorylation and increased levels of CCL2 and IL-6 in pleural exudates. *In vivo* inhibition of protease activity by using leupeptin or a PAR-1 antagonist (SCH79797) prior to Pla injection abolished Pla-induced mononuclear recruitment and ERK1/2 and IkB-α phosphorylation. Interestingly, inhibition of MEK/ERK pathway abolished Pla-induced CCL2 upregulation and mononuclear cell influx. In agreement with a requirement for CCL2 to Pla-induced cell trafficking, CCR2−/− mice were not responsive to Plainduced mononuclear recruitment.

Conclusion: Pla-induced mononuclear cell recruitment *in vivo* was dependent on PAR-1 activation of the MEK/ERK/NF-kB pathway which led to the release of CCL2 and activation of CCR2.

Financial Support: FAPEMIG, CNPq and PRPq-UFMG.

OC 29.4

Metalloproteinase-9 higher increase after thrombolysis is associated with hemorrhagic transformation of lesion and with poor stroke outcome

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Background: In experimental animals metalloproteinases (MMPs) play a detrimental role related to severity and hemorrhagic transformation of the ischemic lesion. Tissue plasminogen activator (tPA) enhances and MMPs inhibitors (TIMPs) antagonize such effects.

Aims: This study aimed to expand clinical evidence in this connection.

Methods: Blood was taken at baseline and 24 h after tPA thrombolysis from 327 patients [mean age = 68 years, mean National Institutes of Health Stroke Scale (NIHSS) = 11.9] with acute ischemic stroke, all treated with i.v. tPA thrombolysis within 4.5 h. MMP-1, -2, -3, -7, -8, -9, and TIMP-1, -2, -4 were measured (in ng/mL) using Bio-Plex suspension array system and R&D kits. Delta median values [(post tPA-baseline)/baseline] of each MMP or TIMP were analyzed related to symptomatic hemorrhage (SICH), death, 3 month modified Rankin Scale (mRS) (3–6 vs. 0–2), and correlated with baseline, 24 h and 7 days NIHSS.

Results: Delta increases: 1) SICH vs. non SICH: significant for MMP-9 and TIMP-4 [MMP-9: 0.38 vs. −0.18, $P = 0.006$; TIMP-4: 0.67 vs. 0.22, $P = 0.015$]; 2) Death vs. survival: significant for MMPs-1, -8, -9 and TIMP-1 [MMP-1: 0.01 vs. −0.14, $P = 0.048$; MMP-8: 0.46 vs. −0.09, $P = 0.011$; MMP-9: 0.37 vs. −0.17, $P = 0.007$; TIMP-1: 0.17 vs. −0.02, $P = 0.011$]; 3) mRS 3–6 vs. 0–2: significant for MMP-8, -9, and TIMP-4 [MMP-8: 0.12 vs. −0.17, $P < 0.001$; MMP-9: 0.11 vs. −0.21, $P < 0.001$; TIMP-4: 0.50 vs 0.16, $P < 0.001$]. Both MMP-9 and TIMP-4 deltas were strongly correlated with baseline, 24 h, and 7 days NIHSS (Spearman P ranging from 0.003 to 0.001). Adjusting for demographics, baseline glucose or NIHSS, infection and atrial fibrillation, only MMP-9 remained an independent determinant of death

[OR = 1.55 (95% CI 1.08–2.21)], SICH [OR = 1.38 (95% CI 1.01–1.90)], or mRS 3–6 [OR = 1.35 (95% CI 1.01–1.80)].

Conclusions: Our data add substantial clinical evidence about MMP-9 as detrimental factor in ischemic stroke patients treated with thrombolysis, and may prompt randomized controlled trials (RCTs) testing the treatment of acute ischemic stroke patients with drugs able to inhibit MMPs in combination with tPA thrombolysis.

OC 29.5

Mechanistic studies on a fibrin specific streptokinase from *Streptococcus pyogenes*

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Background: Haemolytic streptococci (Groups A, C and G) are common human pathogens responsible for a range of diseases. As an early defence mechanism bacteria are trapped in fibrin networks at the site of infection, with factor XIII (FXIII) cross-linked fibrin reported to be more effective. In response streptococci secrete streptokinase (SK), a non-proteolytic activator of human plasminogen, to degrade fibrin and aid dissemination of the bacteria throughout the body. Most of our understanding of the mechanism of action of SK is based on SK from the H46a strain of Group C *Streptococcus equisimilis,* because of its long-term use as a thrombolytic drug. Importantly plasminogen activation by H46a-SK is independent of fibrin. SK however contains polymorphic sequence variations, for example SK from M1 type Group A Streptococcus (GAS, *Streptococcus pyogenes*) shares 88% sequence identity with H46a-SK, although the significance of these sequence variations in relation to mechanism of action and pathogenicity is not known.

Aims: The aim of this study was to investigate mechanistic differences between H46a-SK and GAS-SK relating to plasminogen activation, plasmin-SK activity, and in response to FXIII cross-linked fibrin.

Methods: GAS-SK and H46a-SK were cloned and expressed in *E. coli*. Plasminogen activation rates were measured in solution, and at the surface of pre-formed fibrin clots in microtitre plates, against the chromogenic substrate for plasmin S-2251. Amidolytic activity of plasmin and the SK-plasmin complexes was determined against S-2251. Fibrinolytic activity (±FXIII) was measured in two different assay systems: a microtitre plate based method monitoring clot formation and lysis through absorbance change, and a physical method where lysis of a pre-formed clot is determined by collapse of clot structure.

Results: Plasminogen activation and fibrinolysis activity of recombinant H46a-SK was, like native H46a-SK, independent of fibrin. For GAS-SK however plasminogen activation rates in solution were 18-fold lower than H46a-SK, and required fibrin to increase activity to the level of H46a-SK. In the microplate assay fibrinolysis activity of GAS-SK was two-fold higher than H46a-SK, irrespective of cross-linking by FXIII. Both H46a-SK and GAS-SK in complex with plasmin increased plasmin enzyme activity (k_{cat}/K_M) by two-fold against S-2251 compared with plasmin alone, although for H46a-SK the k_{cat} was two-fold higher and for GAS-SK the K_M was two-fold lower relative to plasmin alone. In the physical clot lysis assay the rate of lysis was dose-dependent, with faster fibrinolysis rates at low concentrations for GAS- and H46a-SK-plasmin compared to plasmin alone.

Conclusions: We have identified an SK from GAS with fibrin-targeted plasminogen activation and increased fibrinolytic activity over H46a-SK. In complex with plasmin both SKs increase amidolytic activity, but through a different underlying mechanism. At low concentrations SK increases plasmin fibrinolytic activity, and our results suggest this could relate to differences in substrate specificity or fibrin binding. GAS is generally regarded as the more important human pathogen, and this difference in mechanism of action may contribute to the difference in pathogenicity. An understanding of the structural interactions critical to the fibrin-targeted activity may also provide a rationale for the design of new thrombolytic drugs.

OC 29.6

Platelet factor XIII stabilises thrombi via cross-linking of a2antiplasmin to fibrin

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Background: We have shown that the antifibrinolytic function of plasma-derived factor XIII (FXIII) is exclusively mediated via cross-linking of $\alpha_2$antiplasmin(α_2AP) to fibrin[1]. Platelets contain three distinct pools of FXIII; cellular FXIII (FXIII-A) in the cytoplasm and plasma derived FXIII within the α-granules and on their membrane surface.

Aim: To investigate the contribution of platelet FXIII to thrombus stability.

Method: Platelet-poor plasma (PPP) or platelet-rich plasma (PRP) was prepared by centrifugation of whole blood. Washed isolates of platelets were also prepared. Model thrombi were generated from PPP, PRP, FXIII-depleted plasma or α_2-AP depleted plasma ± isolated platelets. In some cases a TG inhibitor (1 mM) or neutralising antibody to α_2-AP (150 μg/mL) was included. Incorporation of FITC-labelled fibrinogen allowed thrombus lysis to be quantified as release of fluorescence. FXIII activity in plasma, platelet releasates and platelet fractions was analysed by an in-house activity assay and detected on Western blots with monoclonal antibodies to FXIII-A and FXIII-B subunits.

Results: FXIII activity was considerably higher in PRP (1.5 ± 0.05 IU/mL) than PPP (0.9 ± 0.12 IU/mL; $P < 0.005$, $n = 4$) and was below the limit of detection in FXIII-depleted plasma. Lysis of thrombi formed with FXIII-depleted plasma was increased 11.8-fold relative to normal plasma (184 ± 6.87 FU/min compared to 15.6 ± 6.29 FU/min). Addition of different concentrations of platelets stabilised model thrombi formed from FXIII-depleted plasma to increasing extents; 0.5×10^8 platelets/mL (1.8-fold; 104 ± 22.8 FU/min); 1×10^8 platelets/mL (2.6-fold; 71 ± 25.5 FU/min); 2.5×10^8 platelets/mL (6.8-fold; 27 ± 10.6 FU/min); and 5×10^8 platelets/mL (15.5-fold;11 ± 4.2 FU/min). Stabilisation of thrombi formed from FXIII-depleted plasma was evident whether freshly isolated or lysed platelet preparations were used, indicating that the source of FXIII was present on the platelet surface or was actively released during platelet activation. Inclusion of an antibody to α_2-AP in thrombi formed from FXIII-depleted plasma had no effect in the absence of platelets, but increased lysis 11.6-fold in the presence of 5×10^8 platelets/mL (11.7 ± 1.4 FU/min vs 136 ± 23.1 FU/min). Thrombi formed from α_2-AP-depleted plasma lysed at a rapid rate (120 ± 11.4 FU/min) and were not stabilised by addition of 5×10^8 platelets/mL. No change in lysis was noted on inclusion of a TG inhibitor in thrombi formed from α_2-AP-depleted plasma ± 5×10^8 platelets/mL, indicating that in the absence of plasma α_2-AP, platelet FXIII was unable to stabilise thrombi. Western blotting of lysed preparations of platelets showed a band at 83 kDa, consistent with the presence of FXIII-A subunit. No FXIII-B subunit could be detected, suggesting that cellular FXIII-A from the cytoplasm contributes to thrombus stabilisation.

Conclusions: We have shown that platelets supply a pool of FXIII-A that confers antifibrinolytic function by cross-linking plasma α_2-AP to fibrin. The magnitude of the effect is concentration dependent, with normal circulating concentrations necessary for maximal thrombus stabilisation.

OC 30 – Inherited Risk Factors for Venous Thrombosis – I

OC 30.1

Towards identification of novel inherited genetic risk factors for venous thromboembolism

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Background: Venous thromboembolism (VTE) is a multicausal disease that can be attributed to both genetic and environmental risk factors. Nowadays, genetic risk factors can be identified in approximately half of the patients with VTE, even if stringent criteria are applied, such as the absence of clinical risk factors or old age. This suggests that our knowledge about the genetic basis of venous thrombosis is incomplete, and novel thrombophilic defects remain to be found. To address this issue the GENES study was initiated. We collected Dutch families with unexplained thrombophilia, in which deficiencies of protein C, protein S and antithrombin, the presence of factor V Leiden and prothrombin G20210A, and persistently elevated levels of factors VIII, IX and XI were excluded.
Methods: In order to identify new hereditary risk factors for VTE, we selected two large GENES families (Family D and Family K), in which 5 or more individuals had had objectively confirmed VTE.
Whole exome sequencing data was generated for 5 affected individuals from each family. With the assumption of a dominant trait, all heterozygous single nucleotide variants (SNVs), insertions and deletions (INDELs) being present in all affected relatives, unique for each family, as well as shared by both families, were selected. The list of candidate variants was further down-sized by excluding non-coding variant without any potential splice site prediction and involving a low conserved base pair, as well as synonymous variants without any potential splice site prediction. In a first approach we restricted our focus to all candidate variants located in the established or possibly related genes to thrombosis ($N = 125$).
Results: None of the candidate variants were shared by all ten affected individuals. In family D, two variants located in genes associated with thrombosis were identified, in STX2 (c.94T>G, p.Phe32Val) and in ITGB3 (c.176T>C, Leu59Pro). In family K, three variants located in genes associated with thrombosis were identified, in APOH (c.796G>T, p.Val226Leu), KLK8 (c.595G>A, p.Val199Ile) and in KLK11 (c.146G>A, Gly49Glu). None of these variants have a low minor allele frequency (MAF): respectively 2.5%, 16.2%, 23.8%, 5.2% and 7.5% for the European-American population. Only the STX2 variant is predicted to be deleterious by four different algorithms for predicting pathogenicity (SIFT, PolyPhen2, LRT and MutationTaster).
Conclusion: Although all variants, with exception of the STX2 variant, show relatively high MAF, co-segregation analysis and assessment of the presence and frequency of these mutations in case-control populations is necessary to determine their role in VTE. The STX2 gene, reported to be associated with von Willebrand factor levels, seems to be the most interesting candidate. Additionally, variants in other genes than the 125 included in this analysis could explain the phenotype in our families, and further evaluation is ongoing.

OC 30.2

Hidden antithrombin deficiencies

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Background and Aims: The key anticoagulant role of antithrombin explains the high risk of thrombosis associated to even minor reductions. Thus, functional assays to detect antithrombin deficiency are included in thrombophilic tests despite the low incidence of this disorder (1%). Molecular analysis of *SERPINC1*, the gene encoding antithrombin, is restricted to patients with evident deficiency (< 70%) and shows a high mutation detection rate (85–95%). However, no molecular analysis is routinely done in cases with anticoagulant activity within the normal range.
Methods and Results: During the last 10 years, we received 137 samples from patients with potential antithrombin deficiency, all identified with functional methods (anti-FXa) performed at least 6 months after the thrombotic event. We confirmed the deficiency in 88 cases, but 49 cases had no deficiency in the sample delivered to our centre. We also sequenced *SERPINC1* in these 49 cases. We pointed out the high prevalence of the Cambridge II variant (Ala384Ser): 6/49 patients carried this mutation in heterozygosis (12%). A borderline deficiency (70%) was detected at origin in five cases, but 1 reported severe deficiency (50%). This mutation, relatively common in Spain (0.3%), moderately increases the risk of thrombosis (3/5-fold) since it only mildly impairs the anti-FIIa activity without effect on the anti-FXa activity. The reduced levels identified in one determination are explained, according to *in vitro* results, by a moderate antithrombin consumption when the thrombin generated under some specific conditions is not fully controlled by the Cambridge II variant. The second mutation was identified in the asymptomatic mother of a 2-year old child with deficiency (30–35%) who died after a cerebral vein thrombosis despite anticoagulant therapy. This case had conflictive anti-FXa results at origin (two determinations of 40% and one of 100%). Interestingly, heating the plasma delivered to our centre (93%) at 42 °C for 15 min resulted in a 45% loss of activity. The proband had a mutation in heterozygosis not previously described: Val105Ala, that affects a conserved residue located at the end of the C-helix. Recombinant expression in HEK-EBNA cells produced a highly unstable protein. The third mutation identified in a 38-year old patient who suffered a pulmonary embolism, affected the signal peptide, three positions before the proteolitic cleavage (Val-3Glu), resulting in a smaller variant lacking the first two residues of the mature molecule (Antithrombin Dublin). The pathological relevance of this variation, which is a low prevalent polymorphism (0.2%) has been suggested but no demonstrated, as this variant has normal activity and heparin affinity. The mechanism responsible for the reduced activity identified at one moment in this patient (68%) has to be characterized.
Conclusion: Our study suggests that the prevalence of antithrombin deficiency in venous thrombosis may be underestimated. We identified three *SERPINC1* variations, two polymorphisms and a new mutation that are not detected using functional tests, but that under specific conditions caused a reduced anticoagulant capacity potentially increasing the risk of thrombosis. Our data stimulate the molecular analysis of *SERPINC1*, particularly the genotyping of Ala384Ser, in cases with one positive determination of antithrombin deficiency.

OC 30.3

The risk of venous thrombosis varies in different ethnic groups

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Background: Ethnic differences in the incidence of venous thrombosis have been appreciated for many years. However, with few exceptions, most of the studies are based on administrative databases from North America and China.
Aim: The aim of this study was to investigate the risk of venous thrombosis in different first and second generation immigrant groups.
Methods: The MEGA (Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis) study is a population-based case-control study on risk factors for venous thrombosis from the Netherlands. Inclusion criteria consisted of patients and controls

of whom information was available on the country of birth. For the analysis related to immigration background, patients were compared with random digit dialing (RDD) controls. First generation immigrants were classified as those who were born outside the Netherlands. Second generation immigrants were similarly defined as first generation immigrants, except that second immigrants were born in the Netherlands, while both parents were born in the same country except Netherlands. In total, 6899 participants were included, of whom 4300 patients and 2599 RDD controls. Odds ratios (ORs) with 95% confidence intervals (95% CIs) were calculated as estimates of the relative risk, and were adjusted for age, sex, body mass index, smoking, hormonal factors, alcohol consumption, physical activity and malignancy by unconditional logistic regression.

Results: The risk of venous thrombosis varied according to the region of birth. When compared with the Dutch, Eastern Europeans reached the highest and East/Southeast Asians the lowest risk of venous thrombosis with OR of 2.35, (95% CI, 1.09–4.59) and 0.44 (95% CI, 0.29–0.68), respectively after multivariate adjustments. Caribeans showed an intermediate lower risk of 0.69 (95% CI, 0.36–1.30) after multivariate adjustments. We did not observe a major difference on the risk for VT between first and second generation immigrants, although the number of second generation immigrants was small for some groups. Subgroup analysis did not show major differences according to immigration groups, except for Eastern Europeans, who had a higher risk for unprovoked event with an OR of 3.79 (95% CI, 1.44–9.97). In comparison with Dutch controls, East/Southeast Asians controls had lower prevalence of factor V Leiden (6% and 1%, respectively) and the prothrombin G20210A mutation (2% and 1%, respectively) but higher blood group non-O (54% and 62%, respectively). Risk of VT in East/Southeast Asians adjusted for age, sex, factor V Leiden and blood group non-O was 0.53 (95% CI, 0.35–0.80). Analysis of a panel of procoagulant, anticoagulant, profibrinolytic and genetic factors are underway and is expected to be available before the ISTH 2013 congress.

Conclusion: The risk of VT varies in different populations. The risk of VT in East/Southeast Asians was the lowest and was virtually unchanged after adjustment for several environmental and genetic known risk factors for VT.

OC 30.4

Genetic influence on risk of venous thromboembolism in women

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Background: Several studies have suggested an important genetic contribution to the risk of developing venous thromboemblism (VTE), with an estimated heritability of around 50–60%. Single nucleotide polymorphisms (SNPs) in the *F5*, *F2*, *FGG* and *F11 genes*, together with ABO blood group genotype, are firmly established as risk factors. A number of additional SNPs have emerged in the literature as being associated with the risk of VTE, but with less supportive replication data.

Aims: To perform a comprehensive analysis of both established and emerging genetic risk variants for VTE in a case-control study of women with a first VTE.

Method: We studied 2775 women from a national-wide case-control study, the ThromboEmbolism Hormone Study (TEHS). TEHS was conducted in Sweden in 2002–2008 and focused on women taking hormone compounds (mean age 46 (18–65) years). The study was approved by the regional medical ethics committee, and all women signed an informed consent. Forty selected SNPs were genotyped using the Illumina Goldengate platform, Pyrosequencing[TRADEMARK] technology or Taqman[TRADEMARK] technology. Associations were assessed by logistic regression. Adjustment in different models was made for age, body-mass index (BMI), and estrogen intake. In addition, interaction between SNPs and estrogen intake was also evaluated. Finally, weighted genetic risk scores were computed and examined in relation to disease.

Results: Association with VTE could be confirmed ($P \leq 0.001$) for *F5* (rs6025, odds ratio (OR) 3.51 (CI 2.77–4.45)), *F2* (1799963, OR = 1.86 (CI 1.27–2.73)), *ABO* (rs579459 (used as proxy for rs2519093), OR = 1.57 (Cl 1.39–1.78)), *ABO* (rs514659, OR = 1.51 (Cl 1.35–1.69)), *FGG* (rs2066865, OR = 1.38 (Cl 1.22–1.55)), *FGB* (rs1800788, OR = 1.28 (Cl 1.13–1.45)) and *F11* (rs2289252, OR = 1.19 (Cl 1.19–1.32 $P = 0.002$)) after adjustment for age. Further adjustments for BMI and estrogen intake influenced the results marginally. Even if both covariates are strongly associated with VTE, no significant interaction was demonstrated with the selected SNPs. A genetic risk score, based on significantly associated SNPs in TEHS, was compared with the top 5 VTE SNPs used in an earlier publication. Both models explained approximately the same proportion of the clinical phenotype, with the area under the ROC curve attaining 0.65. When age, BMI and estrogen intake were added to the model, the area under the ROC curve increased to 0.73

Summary/Conclusion: TEHS confirms the role of established genetic risk variants for VTE in the setting of women with a first thrombosis. With the exception of *ABO* rs579459, used as proxy for rs2519093, it failed to replicate less established SNPs selected from the literature. A genetic risk score based on the most robustly associated risk SNPs and established clinical risk factors may improve the prediction of VTE.

OC 30.5

Candidate genetic polymorphisms and their associations with incident and recurrent venous thrombosis

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Background: The prediction of recurrent venous thrombosis (VT) remains a challenge and needs to be improved to guide treatment options. Multiple genetic variants have been associated with incident VT, but their associations with recurrent VT are mostly unknown.

Aims: The aim of this study was to estimate associations between candidate single nucleotide polymorphisms (SNPs) and incident/recurrent VT. SNPs were selected if we found prior published evidence for validated associations with incident VT.

Methods: We studied 22 candidate SNPs in the following genes: ABO, F2, F5, F11, F13, SERPINE1, PROC, FGG, SERPINC1, GP6, HIVEP1, STXBP5, VWF, KNG1, KLKB1, TC2N, and PROCR. SNPs were either measured or imputed using information from several genotyping panels. Participants were of European ancestry and part of a large, population-based study of VT in Washington State, USA. In a case-control study of incident VT (women only), we compared cases with a first validated VT (deep vein thrombosis and/or pulmonary embolism [PE]) from 1995 to 2010 with controls matched on age and study design variables using logistic regression adjusted for matching factors and the genotype panel used. In an inception cohort, men and women with incident VT were followed for a second VT and associations with recurrent VT were estimated using a Cox proportional hazards model adjusted for sex, age and the genotype panel used.

Results: For incident VT, 897 cases were compared with 2644 controls (mean age 66.6 years, BMI 28.9 kg/m2). Forty percent of cases were idiopathic and 34% presented with PE. A total of 662 cases (89% women, 51% idiopathic, 58% PE) with a mean age of 65.8 years at the first VT were followed for a median of 5.1 years for the development of recurrent VT ($n = 100$ recurrent events). Factor V Leiden (rs6025) and prothrombin mutation G20210A (rs1799963) were strongly associated with both incident VT (OR 3.8, 95%CI 2.8–5.1 and OR 1.7,

95%CI 1.1–2.7, respectively) and recurrent VT (HR 1.8, 95%CI 1.1–3.1 and HR 2.4, 95%CI 1.1–5.3, respectively). SERPINE1 rs2227631 was also associated with both incident (OR 1.2, 95%CI 1.0–1.3) and recurrent VT (HR 1.3, 95%CI 1.0–1.8). For ABO polymorphisms, associations with recurrent VT were slightly weaker than associations with incident VT: rs8176719 HR 1.2 (95%CI 0.9–1.7) vs. OR 1.5 (95% CI 1.3–1.7); rs2519093 HR 1.3 (95%CI 0.9–1.9) vs. OR 1.4 (95%CI 1.2–1.7). We found associations in opposite directions between FGG rs2066865 and incident VT (OR 1.3, 95%CI 1.1–1.5) and recurrent VT (HR 0.8, 95%CI 0.5–1.0, $P = 0.08$). No other associations with recurrent VT were observed.

Summary/Conclusion: Among 22 SNPs with previously demonstrated associations with incident VT, 3 showed associations with recurrent VT in this population-based inception cohort. Although power was limited for the recurrence analyses, we identified genetic prediction for recurrent VT that included a novel finding for SERPINE1 rs2227631; this needs replication in an independent cohort. Further, the predictive value for recurrent VT of Factor V Leiden and G20210A was higher than reported previously.

OC 30.6

Sex difference in incidence of first venous thrombosis: a higher risk in men than in women

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Background: Previous analyses reported a higher risk of recurrent venous thrombosis in men than in women, while no contrast in the frequency of first venous thrombosis was apparent. A possible explanation could be that, for a first event, a risk difference between the sexes is masked by female exposure to hormonal risk factors.

Aims: The aim of our study was to assess the risk of a first venous thrombosis in men compared with women once hormonal risk factors (defined as oral contraception use, postmenopausal hormone therapy use or pregnancy/ puerperium) have been accounted for.

Methods: From the MEGA study, a large case-control study on risk factors for first venous thrombosis, consecutive patients with a first episode of venous thrombosis were included. Partners of patients were invited to participate as controls if they had no history of venous thrombosis. For the current analyses all complete couples in whom the woman was not exposed to hormonal risk factors were analyzed: 1868 in total. As nearly all partner controls (98%) were of the opposite sex to the patient, if men and women experience first venous thrombosis at a similar rate, one would expect the male/ female ratio in the MEGA study to be approximately 1:1. To assess whether this was the case, conditional logistic regression was used to calculate odds ratios (OR) and 95% confidence intervals (95% CI) for venous thrombosis in men compared with women without hormonal risk factors. Analyses were stratified in 10-year age categories and adjusted for body mass index and smoking. The study was approved by the Medical Ethics Committee of the Leiden University Medical Center and all participants provided written informed consent. Funding: Netherlands Heart Foundation (grant NHS 98.113), Netherlands Organisation for Scientific Research (grant 912-03-033|2003), Dutch Cancer Foundation (grant RUL 99/1992).

Results: Overall, there were more couples in whom the patient was male and the partner was female (67%) than couples in whom the patient was female and the partner was male (33%). Men had a 2.2-fold (95% CI, 1.9–2.5) higher risk of first venous thrombosis than women without hormonal risk factors. When 10-year age categories were viewed separately, risk estimates ranged from 4.8 (95%CI, 1.1–21.6) for the age group 18–30 years, to 2.1 (95%CI, 1.6–2.7) for the age group 60–70 years. Adjustment for body mass index and smoking did not materially affect the results.

Summary/Conclusions: Men have a higher risk of first venous thrombosis than women when exposure to hormonal risk factors is accounted for. This shows that the sex-difference found for recurrent venous thrombosis also exists for a first event. As risk estimates were highest in the youngest age categories, one of the genetic differences between men and women may explain our findings.

OC 31 – Mechanisms in Cancer and Haemostasis

OC 31.1

Association of mean platelet volume with cancer-associated venous thromboembolism: results from the vienna cancer and thrombosis study (CATS)

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Background: Cancer patients are at increased risk of venous thromboembolism (VTE). Recently, several parameters have been identified to predict VTE in cancer patients. High platelet counts were consistently shown to be associated with increased risk of VTE. Mean platelet volume (MPV), which has been proposed to reflect platelet activity, was reported to be associated with arterial and venous thrombosis in patients without cancer. Data on the role of MPV in cancer patients are scarce and its association with risk of cancer-associated VTE is not known.

Aims: The aim of the study was to analyse MPV in patients with different types of cancer compared to healthy individuals and to investigate the association of MPV with risk of occurrence of cancer-associated VTE.

Methods: MPV were routinely determined in EDTA blood samples using a Sysmex XE-2100 hematology analyzer in the Vienna Cancer and Thrombosis Study, which is an ongoing prospective, observational cohort study that started in 2003. Patients with newly diagnosed cancer or progressive disease after remission were included and followed for a maximum of 2 years. Primary endpoint was occurrence of symptomatic VTE. Sixty-five age- and sex-matched healthy subjects served as controls.

Results: A total of 1544 patients (700 women; median age 62 years) with tumours of the lung ($n = 250$), breast ($n = 225$), brain ($n = 200$), colon ($n = 159$), prostate ($n = 148$), pancreas ($n = 99$), stomach ($n = 52$); lymphoma ($n = 217$) and other tumour entities ($n = 194$) were included in the study. Cancer patients had lower median MPV levels (fL) compared to healthy controls (10.2, [25th–75th percentile: 9.6–10.8] vs. 10.3 [10.0–11.0]; $P = 0.022$). During a median observation time of 506 days, 114 (7.4%) cancer patients developed VTE. MPV was inversely correlated with platelet count ($r = -0.29$; $P < 0.001$). The hazard ratio (HR) of MPV per 1 fL increase for VTE was 0.86 ([95% CI: 0.70–1.05], $P = 0.136$). High MPV ($\geq$ 75th percentile of all cancer patients; $\geq$ 10.8 fL) was associated with a statistically significantly decreased risk of VTE compared to MPV below the 75th percentile (HR [95% CI]: 0.58 [0.36–0.93], $P = 0.024$). In multivariable analysis, including platelet count and soluble P-selectin, this association remained statistically significant (0.56 [0.35–0.91], $P = 0.018$). In Kaplan-Meier analysis, the cumulative probability of VTE after 1 year was 4.7% in patients with high MPV and 8.3% in those with lower MPV (Log-rank test: $P < 0.001$). In subgroup analysis, the association of high MPV ($\geq$ 10.8 fL) with decreased VTE-risk was most pronounced in pancreatic cancer (HR [95% CI]: 0.16 [0.04–0.71], $P = 0.016$). After one year, the probability of VTE was 5.1% in those with high MPV compared to 27% in those with lower MPV ($P = 0.006$).

Summary/Conclusions: MPV was lower in cancer patients compared to controls. Interestingly, high MPV was associated with decreased risk of VTE in cancer patients and might have a favourable effect on development of cancer-associated VTE. However, this finding is contrary to results observed in patients without cancer patients. Further studies

are needed to confirm our results and to elucidate the underlying mechanisms, which influence platelet count, platelet size and VTE in cancer.

OC 31.2

Protease-activated receptor-1 in the pancreatic tumor microenvironment favors cancer progression and chemoresistance

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Background: Pancreatic cancer is often accompanied by ongoing coagulation activation as evident from elevated markers of thrombin generation like thrombin-anti-thrombin and prothrombin activation fragment F1.2. In line with the emerging role of thrombin-PAR-1 signalling in pathophysiology, this axis is suggested to favor cancer progression. Indeed, PAR-1 is expressed in several invasive cancers of epithelial origin, and tumor cell PAR-1 seems to directly promote invasion and tumorigenesis. Interestingly, we observed that PAR-1 is mostly expressed in the microenvironment of pancreatic tumors and not in tumor cells themselves. The importance of PAR-1 in the tumor microenvironment of pancreatic cancer remains unexplored however.
Aim: Considering the apparent importance of coagulation in pancreatic cancer progression and the presence of PAR-1 in the stromal compartment of this tumor type, we challenge the hypothesis that thrombin-PAR-1 signalling in the tumor microenvironment drives pancreatic cancer progression and drug resistance.
Methods: 4×10^5 PANC02 cells were orthotopically injected into the pancreas of wildtype and PAR-1 deficient mice. From day 7, mice were treated with gemcitabine or saline twice weekly. Four weeks after cancer cell inoculation, mice were sacrificed to assess tumor weight and volume and to perform histopathological and immunohistochemical analyses of primary tumors and metastatic sites. Moreover, *in vitro* experiments were performed in order to determine the role of PAR-1 in macrophage recruitment and chemokine production.
Results: PAR-1 deficient animals presented smaller primary tumors (weight of 0.95 ± 0.14 g and volume of 0.94 ± 0.15 cm^3) than wildtype controls (weight of 1.96 ± 0.36 g and volume of 1.95 ± 0.43 cm^3) and only 1 mouse (1/8) presented metastasis as compared to all wildtype controls. Interestingly, the number of CD31 positive vessels in tumors from wildtype animals (42.3 ± 1.5) was around 1.5-fold higher than the number in those from PAR-1 deficient animals (27.5 ± 0.8) suggesting PAR-1-dependent angiogenesis to drive pancreatic cancer growth.
Intriguingly, the combination of routine gemcitabine chemotherapy with the absence of PAR-1 in the microenvironment almost completely blocked pancreatic cancer growth and only two out of eight animals still presented very small tumors. Immunohistochemical examination of these tumors showed a remarkable (three-fold) reduction in the number of macrophages in the tumor microenvironment of PAR-1 deficient mice as compared to wildtype controls. In line, thrombin stimulation of monocyte/macrophage cells (RAW264.7) potentiated their migration towards MCP-1 by around four-fold. Importantly, thrombin-driven migration was inhibited by pre-treatment with the specific PAR-1 inhibitor P1pal-12. In addition, we observed that fibroblasts secrete MCP-1 in a PAR-1-dependent manner and that fibroblast conditioned medium acts as a strong chemoattractant for RAW264.7 cells. Interestingly, fibroblast conditioned medium prepared in the presence of thrombin further enhanced migration. Moreover, we show that once monocytes have migrated into the tumor microenvironment, they enhance subsequent monocyte migration by secreting MCP-1 (in part) in a PAR-1-dependent manner. The migrated monocytes/macrophages subsequently induce drug resistance as evident from experiments in which monocyte conditioned medium reduced gemcitabine-induced pancreatic cancer cell death.
Conclusion: We identify a novel role of PAR-1 in the pancreatic tumor microenvironment and pinpoint PAR-1 as a potential target to combat drug resistance of pancreatic cancer.

OC 31.3

Kinome profiling of coagulation factors VIIa, Xa and thrombin-dependent signaling reveals common and coagulation factor-unique signaling pathways

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Rationale: It is well established that, beyond their role in hemostasis, coagulation factors(F)VIIa, FXa and thrombin are involved in the pathogenesis of a broad range of diseases, among which cancer, via signaling through their cellular receptors, the Protease-activated receptors (PARs). These G protein-coupled receptors (GPCRs) are irreversibly activated through proteolytic cleavage by coagulation proteases. Among the four currently known PARs, PAR-1, -3 and -4 are cleaved by thrombinFVIIa signals through PAR-2 whereas FXa can activate both PAR-1 and PAR-2. PAR activation in turn induces transmembrane signalling to G proteins, thereby impacting on a substantial network of signalling pathways. So far, conclusions drawn about the molecular mechanisms that govern cellular effects induced by coagulation factors are based mainly on measurement of phosphorylation of a few signalling pathways, leaving an incomplete picture of the underlying intracellular events. Additionally, PARs display functional selectivity or 'biased agonism', a process known for GPCRs by which distinct ligands acting on the same receptor can elicit different signaling responses.
Aims: To generate a comprehensive overview of the total complement of cellular kinase activity, thereby enabling analysis of coagulation factors signaling effects without *a priori* assumptions as to signaling pathways affected by these proteases.
Methods: The highly metastatic breast carcinoma cell line MDA-MB-231, which constituvely express PAR-1 and -2, were stimulated for 10 min with FVIIa, FXa or thrombin. Subsequently, we used kinomics peptide arrays, containing 1024 specific kinase substrate sequences, to produce a systems biology analysis of the cellular kinase activity in lysates of cancer cells stimulated with the different coagulation factors. The results were validated by western blots
Results: FVIIa, Xa and thrombin induced substantial signaling events. Single data analysis of arrays with cell lysates resulted in significant differences in the phosphorylation of 44 kinases between cells stimulated with FVIIa and vehicle, 25 kinases between FXa and vehicle, and 34 kinases between thrombin and vehicle. Phosphoproteome profiles also revealed common and divergent coagulation factor-dependent kinase activity. FVIIa modulated specifically the activity of 14 kinases, FXa of 4 kinases and thrombin of 5 kinases. FVIIa and FXa shared a modulation in the activity of 5 kinases, FXa and thrombin of 4 kinases, while FVIIa and thrombin had in common the modulation of the activity of 13 kinases. Finally, the activity of 11 kinases was found to be commonly regulated by all three coagulations factors. Gene ontology analyzis revealed that FVIIa triggered an early induction of metabolism and MAPKinase cascades, while FXa regulates signaling pathways classically associated with inflammation. Thrombin, on the other hand, governs pathways regulating cell cycle and apoptosis.
Conclusion: Overall, we generated a comprehensive overview of individual coagulation-induced signalling pathways in cancer cells. These data might be of importance for a better understanding for the role of coagulation factor in pathophysiology and thus provide potential therapeutical targets for therapy.

OC 31.4

Elevated aPC levels reduce cancer metastasis independent of aPC's cytoprotective effect

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Background: Recent studies have suggested that the activated protein C (aPC) pathway plays a role in limiting tumor metastasis, with data supporting the hypothesis that this is reliant upon the cytoprotective effects of endogenous aPC mediated through the S1P1 receptor downstream of protease activated receptor 1, PAR1. While convincing work has been done to explore this mechanism by blocking endogenous aPC, it is not known if elevated aPC levels protect against cancer progression via the same mechanisms.

Aim: This study aimed to determine the relevant function(s) of overexpressed aPC in reducing tumor metastasis.

Methods: A liver gene transfer model using viral vectors was utilized to achieve a wide range of sustained expression of wildtype (WT) or mutant murine aPCs. C57BL/6 experimental mice expressing stable levels of aPCs and age and gender matched control mice receiving PBS were injected intravenously with 2.5×10^5 murine melanoma B16F10 cells, which metastasize to the lungs. After 3 weeks the number of pulmonary tumors was determined.

Results: Overexpression of WT aPC in C57BL/6s decreased the rates of metastases in a dose-dependent manner compared to PBS controls. WT aPC expression levels 4× normal (7.3 ± 1.5 ng/mL) did not reduce the number of pulmonary tumor foci compared to PBS controls (aPC < 3 ng/mL). However, mid-dose expression 7× normal (25.6 ± 4.8 ng/mL) and high-dose expression 28× normal (118 ± 6 ng/mL) led to increasing reduction in tumor rates ($P < 0.05$ and < 0.01, respectively). Modified aPCs were then tested to identify the critical functions responsible for protection with aPC overexpression. Two mutants with reduced anticoagulant function but intact cytoprotective function, L38D and 5A, were generated. aPC-L38D cannot interact with its cofactor, protein S, and has ~two-fold reduced anticoagulant function, though at the high doses used there was no prolongation of the aPTT. Compared to PBS controls, aPC-L38D expression significantly reduced the number of tumor foci ($P < 0.01$), similar to WT aPC. Conversely, aPC-5A, which consists of five mutations in the protease domain and leads to ~10-fold reduction of anticoagulant function, was unable to protect against metastasis, even at high doses and with intact cytoprotective effect. An additional mutant, aPC-E149A, which is unable to interact with the main aPC cytoprotective receptor, PAR1, was generated to further explore the involvement of the aPC cytoprotective effect. Mice expressing high levels of aPC-E149A were equally protected against metastases as those expressing WT aPC compared to PBS controls ($P < 0.01$).

Conclusion: In contrast to early findings that endogenous aPC helps control cancer progression in a PAR1-mediated, cytoprotective effect-dependent fashion, our findings suggest that the large reductions seen in tumor metastasis with overexpression of aPC are independent of the cytoprotective effects of the aPC pathway.

OC 31.5

Synergistic anti-cancer effects with dabigatran etexilate and cisplatin or cyclophosphamide

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Background: Coagulation proteases and the generation of thrombin are increased in tumors. In addition, chemotherapeutic agents commonly used to treat malignant cancers have been shown to increase cancer-associated thromboses. Thrombin can modify tumor cell behavior directly through the activation of protease-activated receptors (PAR) or indirectly by generating fibrin matrices.

Aims: To investigate the extent to which treatment with the oral thrombin inhibitor, dabigatran etexilate (Pradaxa®), will act synergistically with chemotherapeutic agents to block tumor growth and metastasis in murine tumor models.

Methods: The effect of combined treatment with standard chemotherapeutic agents (i.e. cyclophosphamide or cisplatin) and dabigatran etexilate was evaluated following (i) orthotopic injection of 4T1 mammary adenocarcinoma cells in the mammary fat pad of Balb/c mice, and (ii) i.p. injection of ID8 ovarian adenocarcinoma cells in C57BL6 mice. 4T1 tumor growth was monitored by caliper measurements. Peritoneal tumor spread of ID8 tumor cells expressing the luciferase reporter gene was monitored by bioluminescence imaging. Treatment was initiated when 4T1 primary tumors were palpable (30–80 mm^3) at 2 weeks following 4T1 tumor cell injection or when ID8 bioluminescence was elevated at 5 weeks past ID8 tumor cell injection. Mice were treated once a week with cyclophosphamide (50 mg/kg, i.p.) or cisplatin (2 or 4 mg/kg, i.p.) with or without dabigatran etexilate (oral gavage, 80 mg/kg, bid).

Results: Dabigatran treatment alone had no significant effect on the growth of the primary 4T1 tumor, but it decreased the number of lung metastases. Dabigatran treatment resulted in no weight loss in mice. Cisplatin (4 mg/kg) alone inhibited both primary tumor growth and the number of lung metastases. Whereas treatment with only cyclophosphamide had no significant inhibitory effect on the growth of the primary tumor, treatment with both cyclophosphamide and dabigatran etexilate significantly inhibited growth of primary 4T1 tumors. Compared with treatment with dabigatran alone, there were fewer 4T1 lung metastases in mice treated with both 1) dabigatran and cyclophosphamide or 2) dabigatran and cisplatin. In the ID8 tumor model, single treatment with dabigatran etexilate, cisplatin, or cyclophosphamide exerted a transitory inhibition of tumor spread, but only co-treatment with both dabigatran etexilate and cisplatin (2 mg/kg) significantly decreased ID8 tumor spread as measured by bioluminescence.

Conclusion: Co-treatment with dabigatran etexilate and low doses of either cisplatin or cyclophosphamide synergistically inhibits tumor growth and metastasis. These results suggest that dabigatran may be beneficial in not only preventing thrombotic events in cancer patients, but also as adjunct therapy to treat malignant tumors.

Supported by funds from Boehringer Ingelheim Pharma.

OC 31.6

Protease-activated receptor 2 in the tumor microenvironment inhibits lymphangiogenesis and subsequent lymph node metastasis in a murine pancreatic cancer model

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Background: ancreatic cancer is among the most common malignancies associated with thromboembolic events and thromboembolic events confer a significantly worse prognosis in pancreatic cancer. The underlying mechanisms are poorly understood but it is well accepted that protease activated receptors (PARs) may be the key receptors at the interface between coagulation and cellular process involved in cancer progression. PAR-2 specifically functions as a cell-surface sensor for coagulation factors VIIa and Xa and the proteolytic activation of PAR-2 on tumor cells is suggested to induce angiogenesis and breast cancer development. The importance of PAR-2 in the tumor microenvironment in general and in pancreatic cancer specifically remains unexplored however.

Aims: To elucidate the importance of PAR-2 in the tumor microenvironment during pancreatic cancer development. We specifically aimed to determine whether PAR-2 would drive tumor growth and/or metastasis, and to elucidate the underlying mechanism.

Methods: Wildtype murine pancreatic cancer cells (PANC02; 4×10^5 cells in 50 μL PBS) were orthotopically injected into the pancreas of 6 week old wildtype C57BL/6 mice and/or PAR-2 deficient mice (eight mice per group). Five weeks after cancer cell inoculation, all mice were sacrificed after which pancreatic tumors and lymph nodes were collected for further analysis. Tumour cell proliferation were tested by ki67 staining (*in vivo*) and BrdU cell proliferation assay (*in vitro*). Tumour angiogenesis was analyzed by CD31 and lyve-1 staining (*in vivo*) and tube formation assay (*in vitro*).

Results: At the moment of sacrifice, tumors in wildtype animals had a mean weight of 1.82 ± 0.46 g and a mean volume of 2.75 ± 0.67 cm^3. Interestingly, tumors in PAR-2 deficient mice were significantly smaller (1.10 ± 0.42 cm^3) and lighter (0.91 ± 0.44 g) than in wildtype untreated animals. Immunohistochemical analysis of primary tumours for ki67 (proliferation marker) showed that the remaining tumours in PAR-2 deficient mice are less proliferative. Ongoing *in vitro* BrdU assays suggest that cells in the tumor microenvironment indeed drive pancreatic cancer cell proliferation in a PAR-2 dependent manner.

Interestingly, in contrast to the smaller primary tumors, the size and number of metastasis to the lymph nodes was dramatically increased in PAR-2 deficient animals compared to wildtype mice. Immunohistochemical analysis of primary tumors for CD31 (endothelial cell marker) and lyve-1 (specific for lymph endothelial cells) showed that the number of blood vessels did not differ between wildtype and PAR-2 deficient animals, whereas the number of lymphatic vessels within the tumor was significantly increased in mice lacking PAR-2 in their microenvironment. *In vitro* tube formation assay show that PAR-2 does not inhibit the intrinsic tube forming capacity of human lymphatic endothelial cells. Interestingly however, blocking PAR-2 signaling on these human lymphatic endothelial cells potentiates cancer cell induced tube formation.

Conclusion: PAR-2 expression in the tumor microenvironment plays a dual role during pancreatic cancer progression. On one hand it facilitates primary tumor growth whereas on the other hand it limits lymph angiogenesis and subsequent metastasis to the lymph nodes.

OC 32 – Mechanisms of Atherosclerosis

OC 32.1

Role of platelet-specific junctional adhesion molecule. A (JAM-A) in atherosclerosis progression

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Background: Junctional adhesion molecule A (JAM-A) is a transmembrane adhesion glycoprotein from the IgG superfamily expressed by endothelial and epithelial cells as well as by leukocytes and platelets. JAM-A contributes to the organization of endothelial tight junctions and participates in platelet adhesion and leukocyte transmigration by homo- and heterophilic interactions.

Aims: This study sought to characterize the molecular mechanism that controls JAM-A on platelets (trJAM-A) and to identify the role of JAM-A in the pathology of atherosclerosis.

Methods: In our studies we used mice with a specific trJAM-A-deletion after cross-breeding of JAM-$A^{flox/flox}$ mice and mice that express cre-recombinase under control of the platelet factor 4 promoter. For the atherosclerosis experiments we used double knock-out mice in an apolipoprotein E-deficient (ApoE$^{-/-}$) background (trJAM-$A^{-/-}$ and $^{+/+}$ ApoE$^{-/-}$).

Results: Our results revealed that expression of JAM-A was almost completely absent on platelets of trJAM-$A^{-/-}$ mice, based on western blot, flow cytometry and quantitative immunofluorescence microscopy experiments. JAM-A expression on leukocytes, endothelial- and smooth muscle cells in different tissues did not show any difference between trJAM-$A^{-/-}$ and JAM-$A^{+/+}$ mice, as shown by western blotting and double immunofluorescence staining against JAM-A and von Willebrand factor (endothelium cells) and α-smooth muscle actin (smooth muscle cells). Furthermore, Multiplate® functional analysis *in vitro* showed that lack of trJAM-A resulted in an increased platelet aggregation in response to agonists like adenosine diphosphate (ADP), indicating for platelet hyperresponsivity, consistent with a recent previous study (Naik et al., Blood 2012). To answer the question whether trJAM-A is involved in atherosclerosis progression, we fed double knock-out mice (trJAM-$A^{-/-}$ and $^{+/+}$ ApoE $^{-/-}$) a high fat diet for 12 weeks, harvested aorta and aortic root for plaque quantification. *En face* lipid staining on aorta revealed that lack of trJAM-A significantly increased plaque formation in the whole aorta (10.6 ± 2.35 vs. $24.6 \pm 4.50\%$, $n = 7–9$) and in thoraco-abdominal part (9.70 ± 2.53 vs. $21.3 \pm 4.46\%$, $n = 7–9$) but did not show any change neither in the aortic arch nor in the aortic root. These observations supported the hypothesis that trJAM-A may play role in early atherosclerosis. In addition, investigating the plaque composition in the aortic root did not show any significant difference, neither in macrophage- (MAC-2 staining), smooth muscle cells-, endothelium cells content nor in CD3-positive T cell recruitment into the plaque. Nevertheless, assessment of plaque phenotype revealed that deletion of trJAM-$A^{-/-}$ resulted in significantly more plaques with an advanced phenotype. This indicates that lack of trJAM-A, besides accelerating plaque development during early stages, may also play a role in macrophage polarization and differentiation during the progress of atherosclerosis.

Summary/Conclusion: Our results identify JAM-A as an endogenous inhibitor of platelet function and of early atherosclerosis progression. These results will advance our knowledge about JAM-A in platelet function and the molecular mechanisms of JAM-A and of platelets in the pathophysiology of atherosclerosis.

OC 32.2

The role of vessel wall P2Y12 in early atherogenesis is not blocked by ticagrelor or clopidogrel

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Background: The pivotal role of platelets in atherothrombosis is well defined but the extent of their role in early atherogenesis is less clear. The $P2Y_{12}$ receptor is responsible for amplifying and sustaining platelet activation and inhibition of $P2Y_{12}$ modulates the vessel wall response to injury. $P2Y_{12}$ inhibitors, including ticagrelor and clopidogrel, perform a crucial role in the treatment and prevention of thrombotic events. Ticagrelor is also an inhibitor of adenosine reuptake by erythrocytes and has been shown to block ADP-induced vasoconstriction in isolated arteries. Ticagrelor, compared to clopidogrel, reduces mortality risk in patients with acute coronary syndromes but the mechanisms for this mortality reduction remain to be established.
Aims: Our aims were to investigate the role of $P2Y_{12}$ in atherogenesis and determine any $P2Y_{12}$-mediated or non-$P2Y_{12}$-mediated effects of ticagrelor and clopidogrel on this process.
Methods: $ApoE^{-/-}$ and $ApoE^{-/-}P2Y_{12}{}^{-/-}$ male mice were fed either chow or western diet for 12 weeks and atherosclerotic burden was assessed by *en face* oil red O (ORO) staining of whole aortae and histological analysis of the aortic sinus and brachiocephalic artery. Bone marrow transplants were performed to determine the roles of platelet versus vessel wall $P2Y_{12}$ in early atherogenesis following 4 weeks of western diet. The effects of pharmacological inhibition of $P2Y_{12}$ on atherosclerotic burden were investigated using $ApoE^{-/-}$ and $ApoE^{-/-}P2Y_{12}{}^{-/-}$ mice fed western diet and either twice-daily doses of ticagrelor (100 mg/kg), daily clopidogrel (20 mg/kg) or mannitol (control) for 4 weeks. Platelet $P2Y_{12}$ inhibition was assessed by PAR4-mediated P-selectin expression.
Results: ORO staining showed no difference in total lesion area between $ApoE^{-/-}$ and $ApoE^{-/-}P2Y_{12}{}^{-/-}$ mice for either diet when assessing the aorta as a whole. However, further analysis of the aortic arch alone uncovered a significant reduction in atheroma in $ApoE^{-/-}P2Y_{12}{}^{-/-}$ mice fed a western diet ($P < 0.0001$). Similarly histological analysis revealed lesion area was attenuated in the brachiocephalic artery ($P < 0.05$) but not in the aortic sinus. Bone marrow transplants demonstrated that mice deficient in vessel wall $P2Y_{12}$, regardless of platelet $P2Y_{12}$ expression, had significantly reduced lesion area in both the aortic sinus and brachiocephalic artery ($P < 0.001$).
$ApoE^{-/-}$ and $ApoE^{-/-}P2Y_{12}{}^{-/-}$ mice fed either ticagrelor or clopidogrel exhibited no reduction in whole aortae lesion area compared to controls despite effective $P2Y_{12}$ inhibition. Separate analysis of the aortic arch and descending aorta, demonstrated no region-specific differences in lesion formation. Histological analysis of the aortic sinus confirmed these results.
Summary/Conclusion: Vessel wall $P2Y_{12}$ plays an important role in promoting atherosclerotic lesion development. Despite the proven role of platelets in atherothrombosis, we found no role of platelet $P2Y_{12}$ in early atherogenesis. Ticagrelor and clopidogrel were unable to inhibit lesion development in $ApoE^{-/-}$ or $ApoE^{-/-}P2Y_{12}{}^{-/-}$ mice, indicating these $P2Y_{12}$ inhibitors have no effect on early atherogenesis via $P2Y_{12}$- or non-$P2Y_{12}$-mediated routes.

OC 32.3

Deficiency of the anticoagulant annexin A5 attenuates atherosclerotic plaque development in ApoE−/− mice

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Background: Annexin A5 (anxA5) is a human anticoagulant that inhibits the formation of thrombin by shielding procoagulant surfaces containing phosphatidylserine (PS). Thrombin is implicated as key regulator of atherosclerotic plaque formation. Inhibition of thrombin has been shown to attenuate atherosclerotic plaque progression.
Aims: The aim of this project is to explore the role of anxA5 in atherosclerotic plaque development in a murine model for atherosclerosis.
Methods: AnxA5 deficient mice were created by homologues recombination by replacing intron 3 and 4 with the β-galactosidase gene. $ApoE^{-/-}$:$anxA5^{-/-}$ double knock-out mice were generated by backcrossing $anxA5^{-/-}$ with $ApoE^{-/-}$ for at least five generations (both C57Bl6 background). Both $apoE^{-/-}$ and $apoE^{-/-}$:$anxA5^{-/-}$ mice were fed normal chow diet and sacrificed at 14, 20 and 26 weeks of age. The aortic arch was dissected and used to assess plaque size and phenotype by (immuno)-histological and mRNA analysis.
Results: Baseline characteristics including cholesterol, triglycerides and blood- and bone marrow cell composition were not significantly different between $apoE^{-/-}$ and $apoE^{-/-}$:$anxA5^{-/-}$ mice. However, $ApoE^{-/-}$:$anxA5^{-/-}$ deficient mice had less circulating B-cells as compared to $apoE^{-/-}$ mice.
At 14 weeks of age no neo-intima formation was present in either single or double knock-out mice, which is in agreement with data published on the $apoE^{-/-}$ model. At 20 and 26 weeks of age however, in the lesser curvature of the arch a significantly smaller plaque was formed in the $apoE^{-/-}$:$anxA5^{-/-}$ compared to the $apoE^{-/-}$. Additionally, $apoE^{-/-}$:$anxA5^{-/-}$ mice displayed a more stable phenotype with significantly more collagen (at 20 weeks) and less apoptotic cells (at 26 weeks).
Summary/Conclusion: Our data indicate that the anticoagulant protein anxA5 is involved in plaque development and stability in the aortic arch and in the brachiocephalic artery. Recent data show reduced phagocytosis by anxA5, indicating that the absence of anxA5 *in vivo* might have increased phagocytosis and thus a decreased plaque development. The effect of anxA5 on angiogenesis, vascular leakage and smooth muscle cell migration is currently under investigation.

OC 32.4

Calcium, cholesterol and TLT-1 in atherosclerosis

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Background: Vascular injury and inflammation represent a vicious cycle that involves activation of platelets, leukocytes, and endothelial cells. Platelet activation not only leads to subsequent deposition of cytokines in the arterial wall but also to the expression of platelet a-granule receptors; P-selectin, and the Triggering Receptor Expressed in Myeloid cells (TREM)-like transcript (TLT-1). Previous studies removing p-selectin in either an APOE or LDLR-deficient genetic background have demonstrated that p-selectin deficiency leads to a reduction of fatty lesions compared to $apoE^{-/-}$ or $LDLR^{-/-}$ mice. These results suggest that p-selectin promotes atherosclerotic lesion progression. TLT-1 is found exclusively on megakaryocytes and platelets and like p-selectin, is stored in the a-granules of resting platelets.
Aims: Studies with *treml1*−/− mice demonstrated seemingly overlapping phenotypes with p-selectin−/− including prolonged bleeding times, delayed neutrophil migration, higher basal neutrophil counts, and hemorrhage in response to the Shwartzman reaction. The similar phenotypes upon removal of P-selectin and TLT-1 lead us to hypothesize that removal of TLT-1 would also delay the formation of atherosclerotic lesions in an *apoE* deficient mice model
Methods: In this novel atherosclerosis mouse model, we evaluated lesion development and cholesterol levels in mice on an atherogenic diet at different time points.
Results: Lesion evaluation demonstrated three surprising revelations. First, an unexpected trend where the lesions in the *apoE*/*treml1* double null mice have intensified compared to the *apoE*−/− or *treml1*−/− mice. Second, this trend is reversed after 20 weeks of high fat diet

where although both mice progressed to an advance fibrous plaque stage and the differences in lesion sizes were not significantly different, however a significantly higher number of apoE mice display calcification compared to the DN mice. Third, and perhaps the most intriguing finding was that the DN mice have significantly higher cholesterol levels when compared to apoE−/− mice. The increased cholesterol levels extend to the *treml1*−/− mouse when compared to wild type mice at 4 weeks on HFD, this difference, however, gradually subsides as wild type mice cholesterol levels increase over 20 weeks. Interestingly, cholesterol levels in 50 week old mice on chow diet revealed minimal differences between test and control mice suggesting the higher cholesterol levels are related to increased dietary intake

Summary: Our work defines a surprising role for TLT-1 in the progressing of atherosclerosis and in the regulation of serum cholesterol levels during atherogenesis. Collectively, our data suggest that the HFD may induce the expression of TLT-1 at secondary sites outside of platelets and has prompted a search for other cell types that may produce TLT-1. The current status of our investigation will be presented in this poster.

OC 32.5

Sphingosine-1-phosphate induced TNF-a expression in natural killer T (NKT) cell hybridomas through S1P2/Gq/PLC/PKC pathway and mediated migration of NKT cell hybridomas

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Background: Natural killer T (NKT) cells are unique lymphocytes that recognize glycolipid antigen and produce various cytokines through T cell antigen receptor. NKT cells contribute to metabolic disorders such as glucose intolerance and cardiovascular diseases. Sphingosine-1-phosphate (S1P) is a bioactive sphingolipid released from red blood cells or activated platelets. S1P regulates cell proliferation, inflammation and migration via S1P receptors. There are five subtypes of S1P receptors ($S1P_1$-$S1P_5$) and each of them is coupled with G proteins. NKT cells express $S1P_1$, $S1P_2$ and $S1P_4$. We have previously shown that S1P increased the expression of tumor necrosis factor-α (TNF-α) in NKT cell hybridomas via $S1P_2$ receptor. TNF-α is a representative pro-inflammatory cytokine and a risk factor of insulin resistance. TNF-α produced by NKT cell could exacerbate atherosclerosis and diabetes. High-fat-diet induces infiltration of NKT cells in mouse adipose tissue and S1P receptor is also reported to mediate the migration of T lymphocytes.

Aims: We aimed to clarify the mechanism of S1P-induced TNF-α expression in NKT cell hybridomas, and to determine whether S1P induces the migration of NKT cell hybridomas.

Methods: NKT cell hybridoma, 1B6 cells and 2E10 cells, were established by fusing sorted mouse NKT cells with BW1100 thymoma cells. These cells were used as a model of NKT cells. Hybridomas were treated with Rho kinase inhibitor (Y-27632), MAP kinase kinase inhibitor (U0126), uncoupler of Gi and Go proteins from the S1P receptors (pertussis *toxin)* or protein kinase C (PKC) inhibitor (Ro-31-8220), and then stimulated with 1 μM S1P. TNF-α mRNA expression and TNF-α protein production were determined by real-time PCR and ELISA, respectively. Cell migration assay was performed with modified Boyden chamber system (Chemotaxcell).

Results: S1P receptor antagonists decreased the TNF-α mRNA expression induced by S1P in NKT cell hybridomas. Y-27632 and pertussis toxin did not inhibit the increased expression of TNF-α induced by S1P. U0126 and Ro-31-8220 inhibited the S1P-inducible TNF-α expression. The migration of NKT cell hybridomas was increased by S1P. FTY720, an immunosuppressive S1P receptor modulator, reduced their migration induced by S1P.

Conclusions: These results suggest that $S1P_2$/Gq/PLC/PKC pathway could mediate the increased TNF-α expression in NKT cells induced by S1P. Overexpression of TNF-α by S1P may induce glucose intolerance. S1P also induced the migration of NTK cell hybridomas, potentially provoking inflammatory response. S1P and S1P receptor might be attractive therapeutic targets for metabolic syndromes and atherothrombotic disorders.

OC 32.6

Effect of immunization of C5a and C3a released from complement component C5 and C3 respectively on early atherosclerotic lesion in Apobtm2SgyLdlrtm1Her/J mouse model

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Background: C5a and C3a are protein fragments released from complement component C5 and C3 containing 74 and 77 amino acids in human, respectively. Complement is a central effector system within the immune system and is implicated in a range of inflammatory disorders and complement components have been detected in atherosclerotic lesions, from the fatty streak through fibrous to complicated plaques. Binding of both C3a and C5a to their receptors lead to important biological functions. Both receptors are expressed in human atherosclerotic coronary plaques. Therefore, investigation of the mechanisms of the effect of C5a or C3a may provide some important information for the treatment of atherosclerosis. In the present study we have therefore studied the role of C5a and C3a in atherosclerotic lesion development through immunization of $Apob^{tm2Sgy}Ldlr^{tm1Her}$/J mice with these proteins.

Aim: To assess whether immunizing $Apob^{tm2Sgy}Ldlr^{tm1Her}$/J mice with C5a and C3a is effective in reducing atherosclerotic lesions.

Results: Immunization with C5a and C3a elicited high level of antibodies against C5a and C3a in $Apob^{tm2Sgy}Ldlr^{tm1Her}$/J mice. In addition, mice immunized with the C5a (GST-tagged) and C3a (GST-tagged) showed a greater reduction in lesion size by 24% ($P = 0.007$) and 19% ($P = 0.042$), respectively compared to the control mice immunized with GST only and the immunization was also associated with a significant decrease in lesion area in descending aortas compared with that in controls, showing 74.5% ($P = 0.003$) and 57.1% ($P = 0.003$) reduction for C5a and C3a-immunized mice, respectively. The reduction in the lesion size in aorta sinus and in descending aortas correlated with a change in cellular composition of the plaques, and an altered cytokine/chemokine secretion in serum or in stimulated spleen cells as well as specific cellular immune responses when compared with controls. Conclusions

Immunization of mice with C5a and C3a was effective in reducing early atherosclerotic lesions.

OC 33 – Megakaryocytes and Thrombopoiesis

OC 33.1

The hepatic Ashwell-Morell receptor regulates thrombopoietin production

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Introduction: The highly conserved hepatic Ashwell-Morell (asialoglcyoprotein) receptor (AMR) can bind and remove blood asialoglycoproteins although the identity of endogenous ligands has

been elusive. We previously showed that desialylated cold-stored platelets are ingested by hepatic AMR. We now hypothesized that desialylated platelets serve as communicators between the AMR to stimulate thrombopoietin (Tpo) production and to regulate bone marrow homeostasis.

Results: (i) Desialylated human platelet uptake by AMR stimulates hepatic Tpo mRNA translation *in vitro*. Human platelets were desialylated using a2-3, -6, -8 sialidase from *Clostridium perfringens* or left untreated and incubated with HepG2 cells *in vitro*. The relative ratio of Tpo mRNA/CycloA mRNA in the human hepatic cell line HepG2 was determined and compared. Tpo mRNA expression increased 30 min after addition of desialylated platelets, and further increased by 2.2 and 2.9-fold after 4 and 6 h, respectively. In marked contrast, Tpo mRNA translation in HepG2 cells incubated with control platelets was only slightly increased. (ii) Transfusion of desialylated (using a2-3, -6, -8 sialidase) mouse platelets into wild type (WT) mice stimulates hepatic Tpo mRNA expression *in situ*. Hepatic Tpo mRNA levels increased by ~50% after 12 h of platelet infusion into WT mice, and plasma Tpo levels increased by 36% (24 h) to 58% (48 h) following infusion of desialylated platelets. (iii) Endogenously desialylated platelets were isolated from *St3gal4*$^{-/-}$ or AMR null mice (*Asgr2−/−)* mice. These mice lack either the critical sialyltransferase ST3GalIV, which adds sialic acid to glycoproteins (*St3gal4*$^{-/-}$) or the ability to remove senile, desialylated platelets (Asgpr2$^{-/-}$). Desialylated platelets isolated from the *St3gal4−/−* or *Asgr2−/−* mice and infused into WT mice caused an increase in hepatic Tpo mRNA levels at 12 h post-transfusion. Plasma Tpo concentrations increased in parallel with Tpo mRNA levels, peaking by day two post-infusion. Desialylated platelets given to *Asgr2−/−* mice had no effect on Tpo mRNA synthesis or on Tpo plasma levels. WT, but not mice receiving desialylated platelets rapidly (within 24 h) released new platelets into blood and on day 10 post-treatment, corresponding to the Tpo plasma increase. Accordingly, following desialylated platelet transfusion, megakaryocyte numbers increase in the bone marrow of WT but not of *Asgr2−/− mice*. (iv) Desialylated platelet uptake by AMR regulates hepatic Tpo mRNA expression *in situ*, as evidenced by reduced hepatic Tpo mRNA levels were reduced by ~50% in *Asgr2−/−* mice which lack the ability to take up desialylated platelets. In contrast, livers isolated from *St3gal4−/−* mice in which platelet turnover is accelerated due to inefficient membrane glycoprotein sialyation and the uptake by the AMR receptor, exhibit increased Tpo mRNA levels relative to WT livers. The difference in the Tpo mRNA levels between the two mouse genotypes reveals that platelet uptake stimulates a 1.9-fold increase in Tpo mRNA expression.

Conclusion: Our data support the hypothesis that uptake of desialylated platelets via AMR stimulates Tpo mRNA expression. We conclude, that platelets desialylate as they circulate and become the primary AMR ligand in blood, thus providing a novel physiological feedback mechanism to regulate plasma Tpo levels and platelet production.

OC 33.2

Microthrombocytopenia and impaired platelet integrin function in conditional profilin1-deficient mice

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Background: Blood platelets are small anucleated cell fragments generated from bone marrow megakaryocytes (MKs) by a cytoskeleton–driven process. Thereby, mature MKs form long cytoplasmic protrusions, so-called pro-platelets, which extend into the sinusoids within the bone marrow and are finally released through a shear-dependent mechanism. Profilin1 is a small actin-binding protein catalyzing the exchange of nucleotides on G-actin monomers. Moreover, it was shown to incorporate ATP-G-actin into growing actin filaments, therefore directly interfering with actin assembly. However, the overall significance of profilin1 in platelet formation and function is unknown.

Aim: We aimed to investigate the function of profilin1 in platelet production and function.

Methods: Platelet formation and function was analysed in mice lacking profilin1. To delete profilin1 exclusively in MKs and platelets, mice with a floxed profilin1 gene were crossed with PF4 (platelet factor 4)-Cre mice.

Results: Mice lacking profilin1 in MKs and platelets suffered from a microthrombocytopenia with a platelet count reduction of 30–40%. The circulating small platelet population was discoid with well distributed granules and displayed markedly impaired integrin inside–out activation and outside-in signalling. Interestingly, profilin1–deficiency had no effect on F-actin assembly in a flow cytometry–based assay but resulted in significantly increased platelet microtubule content as revealed by Western blot analysis and transmission electron microscopy. Furthermore, the microtubules were severely misorientated in resting and spread profilin1–deficient platelets as shown by confocal fluorescence microscopy. The mutant platelets displayed delayed spreading on a fibrinogen-coated surface and were unable to form filopodia on a von Willebrand factor matrix.

In vivo, profilin1 deficiency did not affect MK numbers in the bone marrow but the cells displayed an altered ultrastructure with an increased formation of empty membranes and microparticles. Confocal microscopy on cryo-sections of whole femora revealed pre-platelet release within the bone marrow, which indicates an accelerated pro–platelet production. The premature platelet release, in combination with an increased clearance of the circulating platelets, contributed to the observed thrombocytopenia.

Conclusion: The profilin1 knockout mouse constitutes a rare model of thrombocytopenia with small platelet size. Further, these data demonstrate that profilin1 is crucial for proper platelet formation and sizing but also in the regulation of the tubulin cytoskeleton and integrin function in these cells.

OC 33.3

Functional characterisation of novel regulators of haematopoiesis: from GWAS to function

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Background: Platelet count and volume are independent risk factors for heart attacks and ischaemic strokes. We therefore used meta-analyses studies (GWAS) to identify genes encoding novel regulators of megakaryopoiesis and platelet formation (HaemGen Consortium, Nature, 2011:480, 201). For about three-quarters of the 68 genetic signals associated with platelet count and volume credible gene candidates could be inferred. Two-third of these encode proteins hitherto unknown to be implicated in megakaryopoiesis and platelet formation. We hypothesise and have preliminary evidence that these new proteins are important rate-limiting factors of megakaryopoiesis and platelet formation, making them prime targets for further functional characterisation.

Aim: To delineate the function of genes identified by a platelet GWAS by gene knockdown in a relevant model organism

Methods: To this end, we performed a high-throughput reverse genetic screen in zebrafish using morpholino (MO) knock down approach. This was followed by in-depth functional analysis of selected genes using a wide panel of different haematopoietic markers with the main aim to further our understanding of the function of the novel regulators of blood formation.

Results: We included 16 genes in the screen to identify novel pathways essential in thrombopoiesis and haematopoiesis in general. Knock down of all but four resulted in 50–90% reduction in the number of thrombocytes in a CD41-transgenic zebrafish. To further investigate lineage-specific effects of the candidate genes we assessed the status of definitive erythropoiesis, myelopoiesis and lymphopoiesis in MO injected embryos. The information gleaned from the initial knock-down/phenotyping was used to generate heat-map of gene expression profiles and to cluster genes with similar phenotypes. Consequently, we were able to hierarchically position candidate genes on the haematopoietic tree and to assign them a potential role during haematopoietic differentiation.

Conclusion: Using the from-GWA study-to-function strategy we have not only identified a series of genes that represent novel regulators of thrombopoiesis and haematopoiesis, but this work also represents, to our knowledge, the first example of a functional genetic screening strategy that is a critical step toward obtaining biologically relevant functional data from GWA study for blood cell traits. The results of these studies are now informing our next step in exploring the relationship between rare sequence variants in platelet GWAS-genes and the count and volume of platelets.

OC 33.4

PEAR1 attenuates megakaryopoiesis via regulation of the PTEN/PI3K pathway

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Background: Platelet endothelial aggregation receptor-1 (PEAR1) is expressed on platelets and endothelial cells. We recently reported that interactions between PEAR1, C-src, Fyn and the p85/Phosphoinosite 3-kinase (PI3K) subunit constitute a signaling complex, causing sustained aIIbb3 activation via Akt phosphorylation. The overexpression of PEAR1 in mouse bone marrow cells decreased the number of myeloid progenitors during *in vitro* clonogenic assays, suggesting that PEAR1 regulates the early stages of hematopoietic differentiation. Whether PEAR1 is a determinant of megakaryocyte (MK) differentiation is presently unclear.

Methods and Results: To investigate the role of PEAR1 in platelet formation, we assessed and manipulated PEAR1 expression *in vitro* in differentiating human $CD34^+$ hematopoietic progenitor cells and *in vivo in* zebrafish embryos. We abolished expression of PEAR1, via lentiviral vectors encoding shPEAR1 in human $CD34^+$ cells and via morpholino injection in zebrafish embryos.

PEAR1 expression increased during $CD34^+$ cell differentiation up to the megakaryocyte stage. Lentiviral-mediated PEAR1 knockdown in $CD34^+$ cells enhanced cell proliferation three-fold and the formation of CFU-MK, without substantially modifying progenitor maturation and the number of CFU-erythrocytes. To characterize PEAR1 target genes in MKs grown from cytapheresis isolates, we compared control shDsRed- and shPEAR1-transduced MKs on day 12 by profiler qRT-PCR analysis. Interestingly, we found that a target of the Notch pathway, *HES1*, was upregulated without expression modification of Notch receptors. *PTEN*, a phosphatase regulating the PI3K pathway, and *CCND1* (cyclin D1) were both downregulated. Although GATA-1 and PU-1 transcription factors are implicated in megakaryopoiesis, their expression was not affected in the absence of PEAR1. To study the role of PEAR1 *in vivo*, CD41:eGFP zebrafish were chosen, expressing GFP under control of the CD41 promotor. In these zebrafish, *Pear1* expression increased progressively the first 3 days of embryo development. Correspondingly, low concentrations (50 mM) of ATG- and splice-blocking PEAR1 morpholinos enhanced thrombopoiesis in the zebrafish, without affecting erythropoiesis at day 2 or 3, confirmed by western blotting of GFP. The injection of 50 mM PEAR1 MO resulted in elevated Akt phosphorylation, as revealed by western blots of 3-day-old *Pear1* knockdown zebrafish, coupled to transcriptional downregulation of the phosphatase PTEN isoform *Ptena*. Neutralization by morpholinos of *Ptena,* but not of *Ptenb,* phenocopied the *Pear1* zebrafish knockdown and triggered a rise in Akt phosphorylation and thrombocyte numbers. At higher MO concentrations, the thrombocyte count dropped, in relation to developmental problems, which worsened dose-dependently, upon injection with more PEAR1 MO. Up to 50 mM PEAR1 MO, no defects were observed, but at 100 mM PEAR1 MO, 42% of the fish presented a mild (23%) to severe (19%) morphological phenotype. Abnormalities ranged from defective development of the nervous system (lack of eyes and head) to severe phenotypic abnormalities, motility limitations and lethality at 500 mM.

Conclusions: In summary, the present study reports that PEAR1 is progressively upregulated during human megakaryopoiesis. PEAR1 is a negative regulator of cell proliferation, but not of MK maturation. This work demonstrates for the first time that PEAR1 modulates the PI3K/PTEN pathway, a critical determinant of Akt phosphorylation, megakaryopoiesis and thrombopoiesis.

OC 33.5

Tropomyosin 4 is a novel regulator of platelet production in mice and humans

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Background: Alterations in platelet count (PLT) and size (MPV) are associated with an elevated risk of cardiovascular disease. To elucidate the molecular mechanisms controlling PLT and MPV, genome-wide association studies (GWAS) have been undertaken to detect common genetic variants associated with these traits. A recent GWAS meta-analysis led to the identification of single nucleotide polymorphisms (SNPs) in two novel loci encoding the actin cytoskeletal regulators *Tropomyosin (TPM) 1* and *TPM4* (Gieger et al. Nature 2011). Both common SNPs were associated with platelet volume and count in healthy individuals. Silencing of the gene homologues in zebrafish abolished the formation of thrombocytes. Together, these findings suggest an important role for tropomyosins in platelet production. The physiological functions of tropomyosins in hematopoiesis have yet to be elucidated.

Aim: Define the roles of tropomyosins in hematopoiesis, with a particular focus on the megakaryocyte lineage.

Methods: We identified a mouse model of *Tpm4* dysfunction in a mouse mutagenesis screen. Platelet and megakaryocyte function were assessed *in vitro* and *in vivo* employing cell biology, biochemistry and microscopy. Results were complemented with bioinformatic analysis of human blood cells.

Results: In a mouse mutagenesis screen for novel regulators of platelet production in mice we identified a pedigree harbouring a splice site mutation in the *Tpm4* gene. The mutant allele appears to be hypomorphic, expressing almost undetectable levels of the major Tpm4 protein isoform. Importantly, consistent with the phenotype associated with the SNP identified in humans (Gieger et al. Nature 2011), *Tpm4* dysfunction leads to macrothrombocytopenia in mice. Bone marrow transplants demonstrated that this phenotype is intrinsic to hematopoietic cells. Erythroid, myeloid and lymphoid cell numbers are normal indicating a highly specific role of *Tpm4* in platelet production.

Notably, *Tpm4* dysfunction did not affect the life span or *in vitro* function of mutant platelets. Likewise, megakaryocyte numbers and maturation appeared normal. However, mutant megakaryocytes displayed

markedly decreased proplatelet formation *in vitro*, and increased premature fragmentation in the bone marrow.
Together, these results indicate a crucial role of *Tpm4* specifically in the terminal stages of platelet production. Ongoing studies suggest that *Tpm4* dysfunction affects the activity of cofilin, thus linking *Tpm4* to several cytoskeletal signaling pathways implicated in platelet production in mice and humans.
Summary: We identified a mouse line with *Tpm4* dysfunction, which exhibits a platelet phenotype that corroborates the observed effect of the common SNP rs8109288 in the human *TPM4* gene on platelet volume and count. Our results provide evidence that *TPM4* is a direct regulator of platelet production in mice and humans and specifically required for the terminal stages of platelet production. They also suggest that tropomyosins might play an important role in hematopoiesis more broadly.

OC 33.6

Redundant functions of RhoA and Cdc42 in platelet biogenesis

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Background: The Rho GTPase family members RhoA, Cdc42 and Rac1 play major roles in cytoskeletal reorganisation that occurs during platelet production from megakaryocytes (MK) and their activation at sites of vascular injury. We have shown that a MK-specific RhoA-deficiency results in a moderate macrothrombocytopenia and impaired platelet responses downstream of G_{13}- and G_q-coupled receptors. On the other hand, mice with a MK-specific Cdc42-deficiency also display a moderate thrombocytopenia but increased agonist-induced secretion, whereas mice lacking Rac1 in MKs display normal platelet counts but defective phospholipase C γ2-mediated platelet activation. Although the signalling pathways downstream of RhoA, Cdc42, and Rac1 partially overlap, possible redundant functions of these Rho GTPases in regulating platelet biogenesis and function have remained elusive.
Aims: The aim of this study was to investigate functional redundancies of RhoA and Cdc42 or Rac1 in platelet production and function.
Methods: Mice with MK-/platelet-specific (PF4-Cre/loxP) double-deficiencies in RhoA and Cdc42 (referred to as *RhoA/Cdc42−/−*) as well as RhoA and Rac1 (referred to as *RhoA/Rac1−/−*) were generated by intercrossing the respective single knockout mice. Platelet function was assessed by biochemical methods. Platelet and MK morphology, and proplatelet formation were investigated *in vitro* and *in situ* by confocal and transmission electron microscopy (TEM).
Results: In contrast to single deficiency of either RhoA or Cdc42, which displayed only moderately reduced platelet counts, double-deficiency in RhoA and Cdc42 resulted in a severe macrothrombocytopenia with platelet counts below 25% of control mice. The size of the remaining platelets was very heterogeneous with some platelets of enlarged size and detailed analysis by TEM revealed a highly abnormal ultrastructure concerning shape, vacuoles, paucity in granules, and abnormal distribution of microtubules resulting in a pronounced reduced platelet life span. Furthermore, *RhoA/Cdc42−/−* mice displayed impaired integrin activation upon stimulation with G protein coupled receptor- and (hem) immunoreceptor tyrosine-based activation motif-agonists. Histological analyses revealed only a slight increase in MK number in bone marrow but a five-fold increase in the spleen that did not correlate with signs of splenomegaly. Interestingly, RhoA/Cdc42 double-deficiency did not impair *in vitro* MK differentiation and quantitative proplatelet formation as assessed by light microscopy. However, MKs showed a severely altered ultrastructure *in situ*. In contrast to this phenotype, RhoA/Rac1 double-deficiency phenocopies the respective single knockouts without any additional effects in the double-knockout animals.
Conclusion: These results demonstrate for the first time a functional redundancy of RhoA and Cdc42, but not RhoA and Rac1, in platelet biogenesis.

OC 34 – Platelet Disorders – I

OC 34.1

Functional studies and proteomics in platelets and fibroblasts reveal a lysosomal defect with increased cathepsin-dependent apoptosis in ATP1A3 defective alternating hemiplegia of childhood

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Background: Alternating hemiplegia of childhood (AHC) is a rare syndrome with repeated hemiplegic episodes, paroxysmal events and global neurological impairment. Symptoms start early and typically disappear during sleep. Only very recently, heterozygous *de novo* missense mutations in the *ATP1A3* gene, coding for the isoform a3 of the sodium/potassium ATPase alpha-3 subunit, have been identified in AHC patients. However, the underlying pathogenesis mechanism remains unknown.
Aims: The aim of this work was to elucidate the biological mechanisms at the basis of *ATP1A3* defective AHC.
Methods: As platelets are relatively easy available cells and represent a good cellular model to study defects in neuropathologies with a subclinical platelet defect, morphological and functional experiments were performed in nine unrelated AHC patients.
Results: Mutation analysis of *ATP1A3* revealed mostly D801N or E815K variants. Platelets from the AHC patients presented with structural and functional abnormalities of granules positive for the lysosomal marker CD63. Electron microscopy showed dense granules with an abnormal core often polygonal and irregular with an empty border instead of a round dense core. Platelets spread on fibrinogen and stained for lysosomal marker CD63 showed significantly more centralized and fused platelet granules in the AHC cases compared to control platelets. The epinephrine-induced platelet aggregation lacked the secretion-dependent secondary activation step in the patients, while other aggregations with standard concentrations of ADP and collagen were normal. The patients' fibroblasts also showed abnormal granule structures with a five-fold higher number of autophagic vacuoles compared to controls and again CD63 staining was increased for the patients. Proteomic analysis of platelets and fibroblasts showed a total of 93 differentially expressed proteins in AHC, mainly involved in metabolism. Pathway analysis performed on these proteins showed a major alteration of the network of 'cell function and maintenance' in this disease, a function that can be attributed to lysosomes. Interestingly, 7 of these altered proteins were detected in both platelets and fibroblasts, including the lysosomal protein cathepsin. AHC fibroblasts revealed significantly increased levels of activated cathepsin B and a stronger activation of cathepsin-dependent apoptosis.
Summary/Conclusions: Our study is the first to link *ATP1A3* defects in AHC to a platelet and fibroblast lysosomal defect with evidence of increased apoptosis. However, further studies are needed to define how this lysosomal defect is related to decreased ATPase activity.

OC 34.2

Identification of novel FLI1 mutations in two families with a platelet secretion defect

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Background: Inherited platelet function disorders (PFD) that cause mild mucocutaneous bleeding symptoms are heterogeneous and can seldom be linked to a causative gene defect using clinical and laboratory phenotype alone. We recently developed a strategy for diagnosis of PFDs using in-solution enrichment and next generation sequencing (NGS) of 216 candidate genes (*J Thromb Haemost, 2012;10:306*). In this study, we have adopted this approach to investigate affected members of two families with a heritable bleeding tendency and a diagnosis of storage pool disease.

Aims: To utilise NGS to investigate the genetic basis of a PFD in two families with the laboratory features of storage pool disease.

Methods: Two parent/child pairs from unrelated families, in which both mild bleeding and alopecia segregated with a platelet dense granule release defect were recruited through the UK-Genotyping and Phenotyping of Platelets (UK-GAPP) study. The coding and flanking intronic sequences of 260 genes encoding mediators of platelet function and number were captured from genomic DNA using an Agilent-SureSelect targeted array and sequenced on an ABI-SOLiD 3+ platform. Sequences were mapped to the UCSC hg19 reference sequence using BWA and Bowtie and previously identified population variants were removed from the analysis. Novel single nucleotide variants (SNVs) that were common to each of the parent/child pairs were confirmed by Sanger sequencing. Site-directed mutagenesis was used to introduce two SNVs identified in *FLI1* into the pSG5-FLI1 expression construct. The ability of the recombinant FLI1 mutants to bind to an ETS site in the *GP6* promoter was examined in HEK 293T cells using the dual-luciferase reporter assay to measure *GP6* promoter activity.

Results: NGS of 260 platelet genes identified 4 SNVs in four genes that were common to both affected members of Family-1, including a heterozygous *FLI1* c.1009C>T transition, predicting a p.R337W substitution in the FLI1 DNA binding domain. The two affected members of Family-2 shared only a heterozygous *FLI1* c.1028A>G transition predicting a p.Y343C substitution, also in the FLI1 DNA binding domain. Comparison of the transcriptional activities of wild-type (WT) FLI1 and the R337W and Y343C variants on the *GP6* promoter revealed a complete loss in transcriptional activity of the R337W and Y343C FLI1 variants when compared to WT-FLI1. When either the R337W or the Y343C variant was co-expressed with WT-FLI1, there was a significant reduction in the transcriptional activity of approximately 50% compared to WT-FLI1 alone ($P < 0.05$) demonstrating that these variants result in a loss of function of FLI1.

Conclusions: We have identified two novel *FLI1* variants which cause a loss in transcriptional activity of FLI1 *in vitro* in two families with storage pool disease. Given the role of FLI1 in megakaryopoiesis, and in regulating several haemostasis related genes, these loss of function variants are likely to contribute to the phenotype in these families. Our results support the use of NGS technology in the investigation of patients with inherited PFDs.

OC 34.3

A mutation in β3 integrin causing macrothrombocytopenia displays constitutive active aIIbβ3 and aVβ3 and proplatelet-like structures on immobilized fibrinogen

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Background: Mutations in integrin αIIb or β3 usually cause Glanzmann thrombastenia (GT), an autosomal recessive severe bleeding disorder characterized by absence of platelet aggregation with all physiological agonists, and normal platelet count and morphology. Some rare mutations in the αIIb or β3 genes have been reported and are characterized by macrothrombocytopenia and autosomal dominant inheritance. These entities were termed GT-like syndromes. One such example is a recently described mutation in two unrelated Italian patients. Molecular analysis revealed an in frame deletion of exon 13 of ITGB3 gene causing loss of amino acids 647–686 of the βTD ectodomain (Haematologica 2009, 94:663–9). The patients exhibited macrothrombocytopenia, impaired ADP and epinephrine induced platelet aggregation, slightly decreased surface expression of αIIbβ3 but with slightly increased ligand binding and normal adhesion of platelets to immobilized fibrinogen yet with impaired spreading. Further characterization of the effects of the mutation was however hampered because the patients' platelets harbored one normal allele.

Aim: To examine the influence of the natural mutation on αIIbβ3 and αVβ3 functions.

Methods: We expressed the β3-βTD_del with wild type (WT) αIIb in baby hamster kidney (BHK) cells and compared surface expression and function with cells harboring WT αIIb and β3.

Results: The surface expression of the mutated αIIbβ3 integrin was not significantly different from cells expressing WT-αIIbβ3. However, the mutated cells bound soluble fibrinogen and PAC-1 antibody indicating that the mutant cells were constitutively active unlike WT cells. The integrin chimera αV(hamster)β3 (human)-βTD_del was expressed similarly to WT-αV(hamster)β3 (human) and was also constitutively active. Cells expressing the αIIbβ3-βTD_del displayed increased adhesion and spreading on immobilized fibrinogen. After 2 h of incubation, the spreading was accompanied by multiple cell extensions. Some of the extensions were long, thin and had bulbous tips suggesting cytoskeleton reorganization similarly to pro-platelet structures. Such extensions were hardly observed in cells expressing WT-αIIbβ3.

Conclusions: The thrombocytopenia and platelet size variation in GT-like patients can be explained by the presence of proplatelet-like extensions formed on immobilized fibrinogen which results in constitutively active αIIbβ3. These data and the macrothrombocytopenia in the patients with GT-like syndrome suggest that integrin β3 is involved in platelet formation in addition to its well known role in platelet function.

OC 34.4

Treatment modalities and outcomes in 204 surgical procedures in 96 Glanzmann thrombasthenia (GT) patients: the International Prospective Glanzmann's Thrombasthenia Registry (GTR)

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Background: Standard treatment for GT, a severe autosomal recessive bleeding disorder with defective platelet $\alpha_{IIb}\beta_3$, is platelet transfusion.

Recombinant FVIIa (rFVIIa) has been effective in GT with platelet antibodies and/or platelet refractoriness.

Aims: To assess the efficacy of rFVIIa, compared to other systemic hemostatics in GT with or without antibodies and/or refractoriness for surgical prophylaxis.

Methods: Analysis of data from GTR, an international multicenter observational study on the efficacy and safety of rFVIIa and other treatment modalities for bleeding and surgery in GT patients.

Results: From 2004-2011, GTR enrolled 218 patients from 45 sites (15 countries, 4 continents) with 1073 admissions. Out of these, 96 patients (F/M: 52/44 [54%/46%]; age ≥ 18y, 69 [72%]) were treated for 204 surgical procedures (minor/major, 167/37 [82%/18%]). Out of 55 patients with known disease type, 48 (87%) had type 1 GT. History of antibodies were reported in 43 patients [45%], refractoriness in 23 [24%], and antibodies+refractoriness in 17 [18%], while 47 [49%] had no antibodies/refractoriness. Treatments analyzed include antifibrinolytics (AF), rFVIIa ± AF (r7), platelets ± AF (P) and rFVIIa+platelets ± AF (r7+P). The top minor procedures were dental (n = 132, 79%), endoscopy (n = 11, 6.6%) and nasal (n = 8, 4.8%). The top major procedures were GI (n = 9, 24.3%) and orthopedic (n = 9, 24.3%). Most frequent treatment for minor procedures was r7 (n = 120 [72%]); and for major procedures, r7 and r7+P (13 [35%] each). In GT with no antibodies/refractoriness, r7 was 100% efficacious for both minor (n = 41) and major procedures (n = 7), similar to or better than that for P (minor 11/11 = 100%; major 5/6 = 83%) and r7+P (minor 4/4 = 100%; major 6/9 = 67%). In GT with antibodies and/or refractoriness, the efficacy of r7 (minor procedures 69/79 = 87%; major 4/6 = 67%) was not inferior to that for P (minor 10/14 = 71%; major 2/2 = 100%) and r7+P (minor 7/9 = 78%; major 2/4 = 50%). A few patients were treated with AF alone, with an overall efficacy rate of 56% for nine minor procedures, but 100% for three major procedures. rFVIIa was generally given initially at a median of ~90 μg/kg at ~2 h intervals, for both minor (median 2–3 doses) and major (number of doses: higher and variable) procedures. The median (mean ± SD) number of platelet units used in patients with no antibodies and/or refractoriness, was 1 (2 ± 1) for minor and 5 (8 ± 7) for major procedures. In patients with antibodies and/or refractoriness, the median platelet use was higher and variable, up to 60 U for one major procedure. Only one thromboembolic event occurred in an adult female with refractoriness treated with r7+P for a major procedure. One additional allergic reaction was attributed to platelets.

Summary/Conclusions: (1) The GTR provides the largest experience to date on surgical procedures in GT. (2) GTR results show that the efficacy of rFVIIa ± AF was similar to platelets ± AF and rFVIIa+platelets ± AF in surgical procedures in GT patients, regardless of investigator-reported antibodies/refractoriness status. (3) rFVIIa ± AF was safe for surgical procedures. (4) Platelets were efficacious in GT patients with investigator-reported antibodies and/or refractoriness, but higher doses were reported. (5) The combined use of rFVIIa+platelets could be attributed to the insufficient efficacy of one or the other alone.

OC 34.5

The β-1 tubulin R307H single nucleotide polymorphism is associated with significantly worse thrombocytopenia and distinct platelet physiology in Bernard Soulier monoallelic 'Bolzano' mutation patients

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Background: Single nucleotide polymorphisms (SNPs) in the hematopoietic-specific β-1 tubulin isotype have been correlated with alterations in platelet physiology. We previously showed that the β-1 R307H SNP identified ITP patients with more severe thrombocytopenia, increased risk of failure to immune-modulatory treatments, and unique platelet dynamics compared to non-SNP patients. The diversity of disease pathophysiology in ITP made interpreting these results difficult. Bernard Soulier monoallelic 'Bolzano' mutation (BSS-BM) patients are unique as they have a single mutation (c.515C>T in *GPIBA*) causing macrothrombocytopenia, but a variable clinical severity of unknown etiology. These unique disease characteristics made BSS-BM an ideal disorder to further examine the physiologic relevance of the β-1 R307H SNP.

Aim: To determine if the β-1 tubulin R307H SNP is associated with significant differences in clinical outcomes and platelet physiology in patients with BSS-BM.

Methods: After approval from regulatory boards and informed consent from patient was obtained, we genotyped 92 previously-reported BSS-BM patients for the presence of the β-1 tubulin R307H SNP (rs6070697G>A). Platelet count and mean platelet volume (MPV) were measured by impedance in 92 patients and by optical laser scanning in 20 patients; manual counts from peripheral blood smears were obtained in 23 patients. Platelet biomass was calculated as the product of manual platelet count and optical MPV in 20 patients, as impedance measurement of these parameters has been shown to be unreliable. Means of platelet count, MPV, and platelet biomass were compared between SNP and non-SNP patients; mean WHO bleeding scale scores were also compared. Pearson correlation coefficients for platelet count and MPV were calculated separately for SNP and non-SNP groups.

Results: β-1 R307H SNP carrier frequency (heterozygote SNP + homozygote SNP) was 36% in BSS-BM patients, similar to the general population. BSS-BM patients with the R307H SNP had significantly worse thrombocytopenia compared to non-SNP patients as measured by all three methods (impedance: 70 v 95, $P < 0.001$; optical: 71 v 119, $P = 0.01$; manual: 76 v 124, $P = 0.01$; all reported counts × 10^9/L). There was no significant difference in mean optical MPVs (SNP: 15 fL; non-SNP: 15.1 fL). Mean platelet biomass was 32% lower in the SNP group (0.13% of blood volume) versus the non-SNP group (0.19%; $P = 0.02$). Mean WHO bleeding scores did not significantly differ between the genotype groups. Platelet count and MPV were strongly and inversely correlated in the SNP group ($R = -0.9$, $P = 0.03$) but not the non-SNP group ($R = -0.24$, $P = 0.24$).

Summary/Conclusions: BSS-BM patients with the β-1 tubulin R307H SNP had significantly worse thrombocytopenia and lower platelet biomass compared to non-SNP patients; furthermore, a significant correlation between platelet count and MPV was detected for SNP patients but not non-SNP patients. These associations between the R307H SNP and changes in clinical parameters and platelet physiology are consistent with our findings in ITP. These similar findings in two distinct platelet disorders support a strong relevance for the R307H SNP in platelet physiology. Further investigations of the R307H SNP in more common platelet disorders may have clinical utility, and investigations into the mechanistic basis for our findings are underway.

OC 34.6

The BRIDGE bleeding and platelet disorders exome sequencing project: presentation and first discoveries

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Background: The purpose of the BRIDGE-BPD consortium (www.bridgestudy.org) is through international collaboration to identify the genetic basis of hitherto unresolved rare bleeding and platelet disorders (BPDs) by exome sequencing. Patients with an abnormal platelet count, volume, ultrastructure and/or function and/or a bleeding disorder of unknown etiology and of early onset are enrolled by 34 specialized referral centres in Belgium, France, Germany, the Netherlands, the UK and the USA. Cases with known inherited BPDs are enrolled in a parallel project overseen by the ISTH ThromboGenomics working

party. So far, 748 BPD cases of unknown etiology have been enrolled and clinical and laboratory information has been deposited in the central BRIDGE-BPD database. The challenge of homogenization of clinical phenotype information is supported by the coding of each patient with terms from the Human Phenome Ontology (HPO) application.

Methods: The DNA samples are quality-controlled and sequenced at Cambridge University in the UK on the Illumina HiSeq2000 platform following targeted enrichment with the Roche Nimblegen SeqCap EZ Human Exome v3.0 protocol. Variant calling makes use of the GATK software package together with the SAMtools and DinDel applications. Alleles are removed by filtering against the results of the 1000 Genomes and UK10K projects. The consequences of the variants are interpreted using standard consequence prediction algorithms, our recently developed protein-protein interaction network and ENCODE and BluePrint results on the experimentally-driven functional annotation of the non-coding fraction of the genome. As a next step toward understanding how newly discovered genes contribute to changes in haemostasis, we perform studies in model organisms and in *in vitro* differentiated megakaryocytes obtained from haematopoietic stem cells and patient-derived induced pluripotent stem cells.

Results: The initial analysis of the results of the first 50 cases identified four new genes. First *NBEAL2* and *RBM8A* were discovered as the causative genes for Grey Platelet and Thrombocytopenia with Absent Radius syndromes, respectively. Second in a patient with a rare platelet defect with abnormal alpha granules and severe bleeding, a compound heterozygous mutation was identified in a new gene on 15q15. Finally, homozygosity mapping revealed a homozygous 2-base pair deletion in a gene at 6q22.1 in a child born to healthy but consanguineous parentage and with symptoms of epilepsy, neurodegeneration, hypotonia, hypogonadotropic hypogonadism, and defective platelet secretion. As was the case for the first two, also the latter two genes were not known to be implicated in megakaryocyte and platelet biology. The genes on 15q15.1 and 6q22.1 regulate the structure of the extracellular matrix components dermatan sulphate and collagen and cell cycling, respectively.

Conclusion: The successful proof-of-principle studies prompted the sequencing of further cases and the results of the analysis will be shared. In conclusion, the hypothesis-free surveying of the 64 million bases of coding fraction of the genome results in the identification of novel genes, which are causative of rare inherited BPDs and point to novel biologically relevant molecular pathways in haemostasis.

OC 35 – Platelet Signaling – I

OC 35.1

A recombinant HPA-1a antibody with abrogated Fc? receptor binding for treatment of fetomaternal alloimmune thrombocytopenia: proof of principle in human studies

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Background: Fetomaternal alloimmune thrombocytopenia (FMAIT) caused by the maternal generation of antibodies against the Human Platelet Antigen-1a (HPA-1a) carried by fetal platelets can result in intracranial haemorrhage and intrauterine death. Current treatment options for FMAIT during pregnancy involve either administration to the mother of IV immunoglobulins with or without corticosteroids or intrauterine transfusions of HPA-1a negative platelets which carries a 15% risk of fetal death. We have developed a therapeutic human recombinant high affinity HPA-1a antibody (B2G1Dnab) that competes for binding to the HPA-1a epitope but which carries a modified constant region that does not bind to Fc gamma receptors. Previous *in vitro* competition studies with a range of clinical anti-HPA-1a sera have shown that B2G1Dnab blocks monocyte chemiluminescence by > 75%. Studies in chimaeric mice have confirmed that a non-destructive HPA-1a blocking antibody can prevent *in vivo* platelet destruction by polyclonal HPA-1a antibodies.

Aims: In this first-in-man study we aim to answer two questions. First does the therapeutic B2G1Dnab antibody affect platelet survival in circulation. Second, in competition studies with a destructive anti-HPA-1a does B2G1Dnab demonstrate a gain in platelet survival *in vivo*.

Method: Eighteen HPA-1a1b (matching fetal platelet group) volunteers were recruited and *in vivo* platelet survival studies carried out by means of dual radiolabelling. Two aliquots of autologous platelets were generated from each volunteer and sensitized *ex vivo* with combinations of antibodies. After re-injection to the volunteers, platelet survival was observed by means of serial sampling and gammacounting. In a first phase, we compared survival of unsensitised platelets and platelet sensitized with either B2G1Dnab or the destructive equivalent IgG1 antibody B2G1. In a second phase we looked at platelet survival after sensitization with a combination of B2G1 (as an exemplar of destructive HPA-1a antibody) and B2G1Dnab (as would be the case in fetuses receiving B2G1Dnab as therapy) in proportions shown previously to reduce monocyte response by 50% and 80%. Permission for the study was granted by the Local Research Ethics Committee (LREC), the Administration of Radioactive Substances Advisory Committee (ARSAC) and the Medicines and Healthcare devices Regulatory Agency (MHRA).

Results: Platelets sensitized with B2G1Dnab were shown to have parallel survival kinetic compared to unsensitised platelets (calculated survival 196 vs 189 h respectively) whilst platelets sensitized B2G1 completely disappeared from circulation within the first 2 h (calculated survival 18 min). Platelets sensitized with a combination of B2G1 25%/B2G1Dnab 75% and B2G1 10%/B2G1Dnab 90% had calculated survivals of 44 and 58 min respectively. This two to three-fold gain compared to platelets sensitized with B2G1 alone would translate to a platelet count 2–3 times higher than baseline in the fetus, conferring a major reduction in the risk of intracranial haemorrhage.

Conclusions: in this first in man set of studies we demonstrate the therapeutic potential of B2G1Dnab for treatment of pregnancies affected by HPA-1a antibodies paving the way for further clinical studies. In addition, the efficient clearance of platelets sensitized with B2G1 also opens up the opportunity to carry out studies of prophylaxis to prevent alloimmunisation in HPA-1a negative mothers.

OC 35.2

The P2X1 receptor antagonist NF449 protects mice from experimental transfusion related acute lung injury

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Background: Transfusion Related Acute Lung Injury (TRALI) is one of the first causes of transfusion related mortality. It is defined as a non-cardiogenic pulmonary edema during or within 6 h after blood transfusion. In most cases, two sequential events contribute to the appearance of this syndrome. The first hit is the inflammatory condition of the patient causing sequestration and priming of neutrophils in the pulmonary compartment. The second hit is the transfusion of a blood product containing antibodies that activate the primed neutrophils of the recipient and lead to lung injury. Neutrophils are thus key players in this pathology through their migration into the lungs and their subsequent activation. Among the various signals involved in neutrophil chemotaxis, ATP was reported to play an essential role through activation of the ATP-gated $P2X_1$ cation channel.

Objectives: Our aim was to evaluate the role of the $P2X_1$ receptor in an experimental model of TRALI.
Methodology: TRALI was induced using a 2-hit model in which Balb/C mice are primed with i.p. injection of LPS (0.1 mg/kg) 24 h before i.v. challenge with a cognate MHC I antibody (0.5 mg/kg). TRALI was estimated by: (i) the rate of mortality of mice after 2 h, (ii) the measure of the lung edema assessed by protein content in bronchoalveolar fluids and (iii) the lung inflammation by evaluating the presence of neutrophils using immunohistological analysis. The role of the $P2X_1$ receptor was evaluated using the NF449 $P2X_1$ antagonist (10 mg/kg) administered i.v. either before LPS, or before the MHC I antibody injection, or at both steps.
Results: Within 2 h after MHC I injection, 70% of control mice died, whereas only 30% of NF449-treated mice died ($P < 0.01$, $n = 20$). Accordingly, lung edema was reduced in mice receiving NF449 (1367 ± 971 mg/mL) as compared to control mice (3537 ± 971 mg/mL) ($P < 0.05$, $n = 6$). Immunohistological analysis of lung sections with an anti-neutrophil antibody revealed a lesser amount of neutrophils infiltrated into pulmonary tissues of NF449-treated mice (65311 ± 5614 pixels) as compared to control mice (171253 ± 20264 pixels) ($n = 3$, $P < 0.001$). To delineate the role of the $P2X_1$ receptor in each event leading to TRALI, NF449 was administered either only before LPS sensitization or only before MHC I injection. In both cases, the rate of mortality was only slightly decreased in NF449-treated mice suggesting that the $P2X_1$ receptor is involved both in the recruitment of neutrophils and in their activation.
Conclusion: Our data indicate that the $P2X_1$ receptor plays a key role in TRALI and suggest that its pharmacological inhibition could represent a novel therapeutic approach.

OC 35.3

The mechanical regulation of VWF GPIba dependent platelet triggering on a single cell

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Background: Binding of platelet membrane receptor GPIbα to von Willebrand factor (VWF) A1 domain tethers platelets to disrupted vascular surface during the haemostatic process. The VWF-A1-GPIbα interaction under flow can trigger outside-in signaling cascade, characterized by distinct elevations of cytosolic calcium concentration. GPIbα is hypothesized as an mechano-sensor of platelet, however, its mechano-sensing mechanism still remains obscure.
Aim: Using the single-molecule accumulative force clamp assay with a biomembrane force probe (BFP), we studied force-induced signal initiation by repeated brief contacts of a single platelet with a glass bead coated with VWF-A1 domain in a precisely controlled fashion.
Materials and Methods: Recombinant proteins including wild-type (WT) VWF A1 domain, gain-of-function A1 mutant (R1450E), loss-of-function A1 mutant (G1324S) were prepared. The washed platelets were purified and kept discoid after the venous blood draw. In BFP experiment, VWF-A1 was also covalently linked to the probe bead aspired by a micropipette, which was opposed by another micropipette aspiring a discoid platelet. The force was calculated from the deformation of the red blood cell (RBC). The epi-fluorescence channel was added to allow the simultaneous measurements of force regulation and calcium signals.
Results: Contacting a single platelet with a bead coated VWF-A1 of low density resulted in adhesion kinetics on the single bond level. We observed a catch-bond for the VWF-A1-GPIbα interaction, in which the bond lifetime increased with force to a maximum (~25pN) and then decreased with force. The von Willebrand disease (VWD) mutations in A1 shifted this biphasic pattern either toward higher force (40pN for G1324S, type 2M) or to lower force (10pN for R1450E type 2B). On the other hand, we found that single bond of WT A1-GPIbα interaction could trigger the calcium flux but not enough to induce platelet activation. However, force and accumulative lifetime threshold existed, above which the A1-GPIbα bond could trigger calcium mobilization within ~30 s. The stronger calcium triggering was observed at 40pN than 25pN for WT and G1324S A1. In contrast, the R1450E, described as the gain-of-function mutant, showed little calcium triggering effect while the G1324S, known as the loss-of-function mutant, showed stronger calcium triggering than WT.
Summary: Coupling of force and bond lifetime was both necessary and sufficient for platelet calcium triggering. The structural variation in ligand altered the triggering energy barrier. With the single molecule method, we provided new understandings on the mechano-sensing mechanism of the VWF-A1-GPIbα interaction.

OC 35.4

The Rap guanine nucleotide exchange factor CalDAG-GEFI is phosphorylated and regulated by protein kinase A

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Background: In circulating platelets, the guanine nucleotide exchange factors CalDAG-GEFI mediates the Ca^{2+}-dependent activation of the small GTPase Rap1b, which is required for integrin $\alpha_{IIb}\beta_3$ inside-out activation and platelet aggregation. cAMP is the major intracellular inhibitor of platelet function, and the cAMP-activated protein kinase A (PKA) antagonizes both Rap1b and integrin $\alpha_{IIb}\beta_3$ stimulation. PKA directly phosphorylates Rap1b, but this event regulates subcellular localization of Rap1b, rather than its activation. Therefore, the precise mechanisms for the prevention of agonist-induced Rap1b stimulation by cAMP in platelets remains unclear.
Aims: To investigate whether CalDAG-GEFI is a target for PKA to mediate cAMP-promoted inhibition of Rap1b.
Methods: The phosphorylation of CalDAG-GEFI was investigated *in vitro* and in intact cells as incorporation of radiolabelled phosphate and by immunoblotting. The effect of PKA-mediated phosphorylation on CalDAG-GEFI activity was established both in platelets, and in HEK293 cells transfected with specific serine to alanine mutants of CalDAG-GEFI.
Results: In this work we demonstrate that CalDAG-GEFI is phosphorylated by PKA *in vitro*, as well as in transfected cells treated with the cAMP-increasing agent forskolin. cAMP increase triggers CalDAG-GEFI phosphorylation also in intact platelets, and this effect is associated to the inhibition of Ca^{2+}-induced Rap1b activation and platelet aggregation. By computational sequence analysis and site-directed mutagenesis, the serine residues 116 and 586 have been identified as the PKA phosphorylation sites. The substitution of these two serine residues with alanine was sufficient to completely abolish PKA-mediated CalDAG-GEFI phosphorylation in transfected cells. Two additional potential PKA phosphorylation sites were found to be present on CalDAG-GEFI, serine 117 and serine 147, however these residues did not contribute to CalDAG-GEFI phosphorylation. Forskolin-induced phosphorylation of CalDAG-GEFI also occurred in transfected HEK293, and was associated to the prevention of Rap1b activation by intracellular Ca^{2+} elevation. Moreover, cAMP-mediated inhibition of Rap1b activation was lost in HEK293 cell transfected with a double mutant of CalDAG-GEFI (S116A and S586A), that failed to be phosphorylated by PKA.
Conclusions: These results demonstrate that phosphorylation of CalDAG-GEFI by PKA affects its activity on Rap1b, and represents a novel mechanism for cAMP-mediated inhibition of Rap1b in platelets.

OC 35.5

Genetic deletion of Vaccinia H1-related (VHR) phosphatase inhibits collagen-induced platelet activation and thrombus formation without affecting bleeding time in mice

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Background: A critical limitation of current antiplatelet therapies is their inability to separate a reduction in thrombotic events from an increase in bleeding occurrences. Vaccinia H1-related (VHR) phosphatase is a dual-specific protein phosphatase, the physiological role of which is not known. We used genetic manipulations to generate mice lacking VHR and found that this phosphatase plays a critical role in platelet function and in arterial thrombosis.

Aims: The goal of this project is to investigate the role of VHR in thrombosis and hemostasis, using transgenic mouse model, with a specific focus on deciphering the function of this phosphatase in platelet biology and GPVI signaling pathways.

Methods: VHR deficient mice were generated by homologous recombination. Platelet adhesion, aggregation and secretion assays were performed using VHR deficient and wild type platelets. Intracellular calcium fluxes were studied in fura-2 loaded platelets. Aggregate formation on collagen surface was analyzed under flow conditions. Arterial thrombosis was assessed in a model of pulmonary embolism and upon ferric chloride induced carotid artery injury. Tail bleeding times were measured.

Results: VHR-deficient platelets display impaired collagen-related peptide (CRP) and collagen-induced aggregation and granule secretion compared to platelets from wild-type mice. However, Thrombin- and ADP-induced platelet aggregations were not affected by VHR deficiency. Consistently, aggregate formation and phosphatidylserine exposure of VHR-deficient platelets on collagen under flow was reduced. In addition, VHR-deficient mice were more resistant to collagen- and epinephrine-induced thromboembolism, compared to wild-type mice, and showed impaired thrombus formation upon carotid artery injury. Intriguingly, VHR deficiency did not affect bleeding times compared to wild-type mice. At the molecular levels, we found that VHR deficiency leads to a decrease of Src family kinase activatory phosphorylation upon GPVI triggering with CRP. In addition, convulxin-induced Ca^{2+} flux was greatly reduced (50%) in VHR-deficient platelets compared to wild-type platelets.

Conclusions: All together, our data suggest that VHR plays a selective and essential role in collagen-induced platelet activation and in arterial thrombus formation *in vivo*. Given that VHR-deficient mice remain healthy and do not exhibit any spontaneous phenotype, inhibition of VHR may prove effective as an alternative and safe antiplatelet strategy in the treatment of arterial thrombosis.

OC 35.6

SHIP1 deficiency affects platelet internal contraction and integrin dynamics during the first steps of platelet activation

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SHIP1 is a lipid phosphatase capable to dephosphorylate the PI3-kinase product phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3) into PtdIns(3,4)P_2, and to interact with several signalling proteins as a docking protein. In platelets, previous studies suggest dual effects of this phosphatase as it may negatively regulate platelet adhesion and spreading on a fibrinogen surface and positively regulate thrombus growth *ex vivo* and *in vivo*.

Little is known about its implication during the early phase of platelet activation in suspension, before aggregation. Using SHIP1 knock-out mice we show that the absence of this phosphatase has no significant impact on Akt, ERK or Src family kinases activation following thrombin stimulation in the absence of aggregation. However, using a confocal-based morphometric analysis we found that SHIP1 is involved in the regulation of cytoskeleton organization and platelet internal contractile activity when platelets are stimulated by thrombin in suspension, independently of integrin engagement. Both the RhoA/Rho-kinase pathway activation and myosin IIA relocalization are affected in the absence of SHIP1. Interestingly, SHIP1 interacts with the membrane skeleton and its absence impacts on the maintenance of the association of integrins to this network following thrombin activation. Using super resolution microscopy we show that the SHIP1-regulated contractile cytoskeleton controls $\alpha_{IIb}\beta_3$ integrin dynamics.

Altogether, our data reveal a lipid-independent function for SHIP1 in the regulation of the contractile cytoskeleton and integrin dynamics in the absence of platelet aggregation.

This study complies with the Declaration of Helsinki.

OC 36 – Reversal of Anticoagulant Agents

OC 36.1

Early reversal with prothrombin complex concentrate in vitamin K antagonist-treated patients with severe haemorrhage is associated with decreased mortality

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Background: Vitamin K antagonist (VKA) therapy is associated with a high rate of mortality in patients with severe haemorrhage, in particularly those with intracranial bleeding. French guidelines recommend prothrombin complex concentrate (PCC) and vitamin K infusion for complete reversal of VKA therapy in case of severe haemorrhage.

Aim: This observational cohort study was set-up to test the hypothesis that early appropriate VKA reversal decreases 7-day mortality rate.

Methods: Prospective data from patients on VKA therapy admitted in 44 emergency departments with life-threatening haemorrhage were analysed, with particular attention given to intracranial haemorrhage (ICH), gastrointestinal, thoracic, or deep muscles bleeding. In addition to the type of haemorrhage, the International Normalized Ratio, the treatment and 7-day mortality were recorded. Early reversal was considered appropriate when the guidelines (reversal perfusion of prothrombin complex concentrate (PCC) at a dose superior or equal to 20 IU/kg in equivalent-IX factor associated to, at least, 5 mg of vitamin K) were followed in a predefined delay of 8 h after admission. A multivariate analysis was used to assess the role of early and appropriate reversal in 7-day mortality in all patients and in the ICH group.

Results: Over a 14-month period, data from 822 vitamin K antagonist-treated patients with severe haemorrhage were collected. The following bleeding rates were seen: gastrointestinal (32%), intracranial (32%), muscular (13%), and 'other' (23%). The 7-day mortality was 13% (n = 110) in the whole cohort and 33% (n = 86) in patients with ICH. Appropriate reversal was performed in 38% of all patients and 44% of ICH patients. Multivariate analysis showed a significant two-fold decrease in the 7-day mortality rate in patients with early reversal

(OR = 2·15 [1·20–3·88]; P = 0·011); this mortality reduction was also observed when only ICH was considered (OR = 3·23 [1·53–6·79]; P = 0·002).

Conclusion: When guidelines on VKA reversal are followed, the management of major haemorrhages with PCC and vitamin K infusion within 8 h after hospital admission, is associated with a significant two-fold decrease in the 7-day mortality rate.

OC 36.2

In vitro characterization, pharmacokinetics and reversal of supratherapeutic doses of dabigatran-induced bleeding in rats by a specific antibody fragment antidote to dabigatran

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Background: The new oral anticoagulants have demonstrated efficacy and safety in preventing stroke in patients with atrial fibrillation; however, one feature they all share is the lack of a specific antidote in cases of emergency, such as excess bleeding. A fully humanized monoclonal antibody fragment (Fab) against dabigatran has been shown to effectively reverse anticoagulation and bleeding effects of dabigatran in animal models. Structural studies have also shown the similarity of the Fab binding site for dabigatran to thrombin.

Aim: This study investigated if the structural similarities of the Fab binding site had any enzymatic activity and influenced coagulation by binding to and activating other thrombin substrates.

Methods: The dissociation constant (K_D) and on-rate (k_{on}) of Fab binding to dabigatran were determined experimentally; the off-rate (k_{off}) was calculated. Potential off target binding to other thrombin substrates such as vWf, factors V, VIII and XIII, PAR-1, protein C (both activation peptides and purified compounds) and S-2238 substrate was also tested using surface plasmon resonance (SPR) binding to immobilized Fab. Any prothrombotic activity of the Fab was determined by testing the ability to induce coagulation in various assays in human plasma including endogenous thrombin potential (ETP) and ecarin chromogenic assay (ECA). Human platelet aggregation studies were performed with Fab and specific PAR-1 agonist, SFLLRN, using light transmission aggregometry.

Results: The X-ray crystal structure of dabigatran in complex with the antidote reveals many structural similarities of dabigatran recognition to thrombin. The Fab has a tight binding affinity to dabigatran, with a K_D of 2.1 ± 0.6 pM, ~350-fold more potent than the 0.7 nM K_D of dabigatran binding to thrombin, this is achieved by a tighter network of interactions and a larger buried surface area. This binding has a rapid k_{on} ($3.4 \pm 0.4 \times 10^5$/M/s) and a slow k_{off} ($0.7 \pm 0.2 \times 10^{-6}$/s), resulting in a calculated complex half life of ~275 h. Due to structural similarities to thrombin active site, potential enzymatic activity was investigated. There was no effect on thrombin generation with 3 mg/mL Fab added to plasma (ETP, 0.89-fold of control), in contrast, FEIBA (0.8 U/mL), resulted in 2.06 increase of ETP. ECA was also not changed vs control when adding up to 3 mg/mL Fab. Further studies investigated the binding of Fab to other substrates of thrombin, such as vWf, factors V, VIII and XIII, PAR-1, protein C using SPR. There was no binding seen at substrate concentrations relevant physiologically. The only specific binding of the immobilized Fab was to dabigatran. Platelet aggregation studies showed that conc up to 3 mg/mL Fab did not induce platelet aggregation, while the PAR-1 agonist, SFLLRN, induced maximal platelet aggregation with 2 μM, final concentration. The Fab did not potentiate or antagonise submaximal aggregation induced by PAR-1 activation.

Conclusions: These data show the specificity and selectivity of the Fab for dabigatran, and that despite structural similarities to thrombin in the binding site to dabigatran, there are no direct interactions with fibrinogen or other thrombin substrates. Thus, this agent holds promise for specifically reversing dabigatran-induced anticoagulation and bleeding effects.

OC 36.3

Active site-mutated thrombin S195A but not active site-blocked thrombin counteracts the anticoagulant activity of dabigatran in plasma

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Background: Dabigatran etexilate (DE) is an oral anticoagulant in clinical use. DE is activated to its active form, dabigatran, after administration and dabigatran inhibits thrombin by active-site binding. No specific antidote for DE is currently available, if reversal becomes necessary. We previously found no ability of candidate reversal agents such as prothrombin complex concentrates or recombinant VIIa in a mouse model of DE-associated coagulopathy and bleeding diathesis (Lambourne MD et al., J Thromb Haemostasis 2012; 10: 1830–40).

Aim: To test the hypothesis that forms of thrombin with mutationally or chemically modified active sites would antagonize dabigatran in plasma, sparing active thrombin from inhibition.

Methods: Recombinant prethrombin-1 S195A was activated to S195A thrombin using prothrombinase and purified to homogeneity using SP-Sepharose, and further processed to γ_T-S195A thrombin using immobilized trypsin. Active (α-) thrombin was reacted with FPR-chloromethylketone, yielding FPR-thrombin. Restoration of endogenous thrombin function by these exogenous thrombin derivatives was measured using diluted thrombin times (TTs) of human plasma samples containing dabigatran (provided as controls in the HemoClot assay) or murine plasma samples from CD1 mice treated with 60 mg/kg DE by oral gavage 90 min prior to blood collection. Mice were separately injected intravenously with 0.2 mg γ_T-S195A thrombin 75 min following DE administration, tails were transected, shed blood quantified, and plasma obtained from blood collected by cardiac puncture 105 min post-DE. ^{125}I-labelled S195A thrombin was separately injected into untreated mice and acid-precipitable plasma radioactivity was followed over time.

Results: Introducing 0.1 mg/mL S195 A or γ_T-S195A thrombin into human plasma containing 0.11 μg dabigatran/mL was equally effective at reducing the TT (from 78 ± 13 to 20 ± 1 s in both cases, mean ± SD, n = 3–12). At 0.4 mg/mL S195 A but not γ_T-S195A thrombin showed reduced ability to antagonize dabigatran (TT values of 56 ± 3 and 17 ± 1 s, respectively). In mouse plasma containing 0.14 μg dabigatran/mL, 0.1 mg/mL γ_T-S195A thrombin reduced the TT from 67 ± 1 to 20.1 ± 0.3 s (mean ± SD, n = 3–6); in contrast, adding 0.1 mg/mL S195A thrombin or FPR-thrombin elevated the TT (to 79 ± 5 and 111 ± 22 s, respectively). In mice injected with both DE and γ_T-S195A thrombin, TT values declined by 40 ± 10% (mean ± SD, n = 3) after γ_T-S195A thrombin administration compared to post-DE, pre-thrombin values. No difference in blood loss between DE-treated mice was noted with or without γ_T-S195A thrombin injection. One hour post-injection, only 1.2% of the total dose of radiolabeled S195A thrombin remained in the murine circulation.

Summary/Conclusions: γ_T-S195A thrombin partially restored coagulation in murine and human plasma samples containing dabigatran, and in plasma samples from mice treated with DE and γ_T-S195A thrombin. The superior dabigatran antagonism of γ_T-S195A thrombin over other thrombin derivatives may relate to its not competing with thrombin for exosite 1 ligands. S195A thrombin seems to maintain a minimally modified active site capable of binding dabigatran. Further modifications to γ_T-S195A thrombin to slow its clearance may enhance its antagonism of DE *in vivo*.

OC 36.4

Reversal of direct factor Xa inhibitors using factor Xa zymogen-like variants

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Oral direct factor Xa (FXa) inhibitors are emerging anticoagulants that have the potential to simplify dosing schemes and hemostatic monitoring in patients with prothrombotic diseases when compared to warfarin. The lack of a specific countermeasure to their effects, however, is a critical unmet clinical need that could limit the widespread adoption of these agents due to fears of unmanageable bleeding. We hypothesized that the effects of these inhibitors could be overcome by increasing the effective concentration of the inhibited enzyme, FXa. Administration of wild type FXa to achieve this is theoretically problematic due to its short half-life in plasma and the potential for thrombosis. Through mutagenesis to alter the equilibrium of the zymogen-to-protease transition, our group has developed long half-life variants of FXa that exist in a zymogen-like (inactive) conformation. These variants can be thermodynamically rescued to the protease (active) conformation by binding to the cofactor FVa. We have shown that these variants are potent, effective procoagulant agents in the setting of hemophilia. We evaluated whether one of these variants, FXa^{I16L}, could overcome the effects of rivaroxaban, a direct FXa inhibitor, in thrombin generation assays (TGA) and whole blood rotational thromboelastography (ROTEM). In TGAs, 500 nM rivaroxaban, the typical therapeutic plasma concentration, decreased peak thrombin generation to 22.5% of that observed in pooled normal human plasma (NHP). Addition of 1 nM or 3 nM FXa^{I16L} to 500 nM rivaroxaban-inhibited plasma restored peak thrombin generation to 84.1% and 102.1% of NHP, respectively. Endogenous thrombin potential (ETP) was also reduced by this dose of rivaroxaban to 49.8% of NHP, and addition 1 nM or 3 nM FXa^{I16L} yielded ETP values of 90.1% and 94.3%, respectively. At a higher concentration of rivaroxaban, 7.5 μM, peak thrombin was 5.2% of NHP and 3 nM FXa^{I16L} restored this value to 67.8% of NHP. In ROTEM experiments, 500 nM rivaroxaban prolonged clotting time to 154% of that of untreated blood. 0.3 nM and 3 nM FXa^{I16L} shortened clot time to 122% and 70% of normal blood, respectively. 2.5 μM rivaroxaban lengthened clot time to 263% of untreated blood, and addition of 0.3 nM and 3 nM FXa^{I16L} shortened clotting time to 156% and 86% of normal. These data suggest that a FXa zymogen-like variant can effectively reverse the anticoagulant effect of a direct FXa inhibitor in plasma-based and whole-blood coagulation assays at both therapeutic and supratherapeutic concentrations of the inhibitor. A thorough *in vivo* hemostasis study is necessary to determine the full potential of this variant as an emergency procoagulant to reverse bleeding due to overanticoagulation.

OC 36.5

Effects of three-factor and four-factor prothrombin complex concentrates on the pharmacodynamics of rivaroxaban

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Background: Rivaroxaban is an oral Factor Xa inhibitor that has been developed as an alternative to warfarin. Although there is no specific antidote for rivaroxaban, four-factor prothrombin complex concentrate (PCC) rapidly corrects the effect of rivaroxaban on prothrombin time (PT) and thrombin generation in healthy adults. However, the specific constituent inPCCthat drives this effect is unknown, and this may be clinically important because differentPCCformulations have different concentrations of coagulation factors.

Aims: The aim of this study was to examine the effects of Beriplex P/N, a four-factorPCC, and Profilnine SD, a three-factorPCCthat lacks FactorVII, on PT and thrombin generation in adult volunteers receiving rivaroxaban.

Methods: In this open-label, single-centre, parallel-group study, 35 healthy adults were treated with supra-therapeutic doses of rivaroxaban 20 mg twice daily for 4 days to attain steady-state concentrations. On Day 5, 4 h after rivaroxaban administration, subjects were randomized to receive either: three-factor PCC (Profilnine SD) single bolus dose of 50 IU/kg, four-factor PCC (Beriplex P/N) single bolus dose of 50 IU/kg or a control saline single bolus dose of 100 mL. The PT and endogenous thrombin potential were measured before and serially afterPCCor saline administration. The two PT reagents used were Neoplastin Plus and Thromborel S.

Results: Thirty-four subjects (mean age 46 years, mean body mass index 25.8 kg/m^2) completed the study. Mean pharmacokinetic parameters for rivaroxaban were consistent across all three treatment groups. PT prolongation with rivaroxaban varied depending on the PT reagent; however, the effects of the PCCs were consistent regardless of the reagent used. Within 30 min, four-factor PCC reduced the mean PT by 2.5–3.5 s, whereas three-factor PCC produced only a 0.6–1.0 s reduction. In contrast to their effects on the PT, three-factor PCC more effectively reversed rivaroxaban-induced changes in endogenous thrombin generation (area under the concentration-time curve, peak and time-to-peak values) than four-factor PCC. Changes in lag-time values did not differ greatly.

Treatment-emergent adverse events (TEAEs) occurred in 51.4% of subjects. The most common TEAEs were abdominal discomfort, gingival bleeding, back pain and headache (8.6% each). Most TEAEs occurred during the rivaroxaban 20 mg twice-daily lead-in period. There were no clinically evident thromboembolic events, nor were there any significant changes in clinical laboratory values or vital signs.

Summary/Conclusions: Although four- and three-factor PCCs shorten the PT in rivaroxaban-treated subjects, four-factorPCChas a greater effect on reducing the mean PT than three-factorPCC. In contrast, three-factorPCChad a greater effect on reversing rivaroxaban-induced changes in endogenous thrombin potential than four-factorPCC. The explanation for the discrepant results on PT and thrombin generation are unclear but may reflect the absence of Factor VII in three-factor PCC Profilnine and the presence of heparin in four-factor PCC Beriplex. Ongoing studies will address these possibilities. Administration of PCCs in the presence of rivaroxaban was reported as safe and well tolerated, with no signs of prothrombotic response.

OC 36.6

Reversal of dabigatran effects by factor VIIa in a cell-based model of coagulation

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Background: Dabigatran etexilate (Pradaxa®) is a prodrug that is metabolized to a small molecule direct thrombin inhibitor. As with all of the new oral anticoagulants, there is no specific reversal agent available. Several studies have suggested that recombinant factor VIIa (FVIIa), or prothrombin complex concentrates (PCCs) might reverse the effects of dabigatran on laboratory assays. Both have been used to reverse the effects of dabigatran in isolated patient case reports, though with mixed results. A better understanding of reversal strategies is badly needed.

Aims: We have previously reported that a 4-component PCC reverses some, but not all, of the effects of dabigatran on thrombin generation

in a physiologically relevant cell-based model. The goal of the current study was to similarly assess the effects of rFVIIa on parameters of thrombin generation.

Methods: We employed a modification of our well-characterised, cell-based model for these studies. We used adherent LPS-treated human monocytes as the tissue factor source (1 pM). Freshly isolated human platelets and different concentrations of dabigatran were added to citrated normal pooled plasma. Thrombin generation was monitored continuously in a fluorometric plate reader using the fluorogenic thrombin substrate from the calibrated automated thrombogram assay (CAT®; Stago). rFVIIa (NovoSeven®) was purchased from the hospital pharmacy.

Results: As others have reported for conventional CAT® assays, we found that dabigatran prolonged the lag before onset of thrombin generation. It also progressively depressed the rate and peak level of thrombin production. rFVIIa shortened the lag, but did not return it to normal. rFVIIa also increased the rate and peak level of thrombin generated, though it did not chnge the total amount of thrombin generated (AUC). Strikingly, the effectiveness of rFVIIa in improving parameters of thrombin generation was highly dependent on the level of dabigatran present. At dabigatran approximating therapeutic levels rFVIIa was effective, but at supratherapeutic dabigatran levels rFVIIa was not able to overcome its effects on rate or peak thrombin. This was true even at levels of rFVIIa above those used clinically. The magnitude of the response to FVIIa varied somewhat depending on the blood donor. However, the pattern of response to FVIIa was similar with all donors.

Conclusion: Our results suggest that rFVIIa can reverse the effects of dabigatran on the rate of thrombin generation and peak thrombin level, but this is restricted to levels of dabigatran approximating the therapeutic range. At higher levels of dabigatran rFVIIa did not reverse its effects on thrombin generation. The dependence of rFVIIa effects on the dabigatran level may explain, at least in part, its inconsistent ability to reverse dabigatran in clinical practice.

OC 37 – Therapy in Haemophilia A

OC 37.1

Targeting factor VIII expression to platelets by intraosseous delivery of lentiviral vectors into bone marrow corrects murine hemophilia A with and without pre-existing inhibitors

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Platelets may comprise an ideal vehicle for delivering factor VIII (FVIII) in hemophilia A (Hem A) as FVIII stored in platelet α-granules is protected from neutralization by inhibitory antibodies and, during bleeding, activated platelets locally excrete their contents to promote clot formation. Previous reports showed platelet-stored FVIII (in transgenic mice or following *ex vivo* gene therapy) can partially correct the HemA phenotype. In order to avoid specific challenges posed by *ex vivo* gene delivery including, in particular, the requirement to pre-condition the subject, we aimed at limiting transgene expression to the megakaryocyte lineages using intraosseous (IO) delivery of 20 μL self-inactivating lentiviral vectors (LV) containing either GFP (G-GFP-LV) or a B-domain deleted FVIII (G-FVIII-LV) gene under the control of glycoprotein 1bα promoter. Firstly, we evaluated IO delivery of LV for *in situ* gene transfer into bone marrow (BM) cells. we confirmed that GFP expressed in 6.4% of hematopoietic stem cells (HSCs), 3.4% of $B220^+$, and 9.0% of $CD11c^+$ BM cells on day 29 after IO administration of 20 μL M-GFP-LV driven by a MND promoter. Secondly, in G-GFP-LV (6.0E+08 TU/mL) treated mice, GFP was undetectable in BM HSC or B220+, CD11c+ or CD11b+ cells, but was detectable in a small population of CD42d+ platelets, consistent with previous observations that only limited amounts of GFP can be stored in platelet α-granules during thrombopoiesis. The percentage of GFP^+CD42d^+ platelets was highest on day 7 after treatment (0.119%) and then decreased to 0.025% by day 35 and stabilized thereafter (0.025% on day 91 and 0.028% on day 161), suggesting that platelets containing the transgene products did not elicit transgene-specific immune responses. In G-FVIII-LV (6.0 E+07 TU/mL) treated HemA mice, FVIII was also undetectable by intracellular flow cytometry in BM HSCs, or B220+, CD11c+ or CD11b+ cells on day 92, whereas up to 2% of CD42d+ platelets were FVIII positive in recipients. Next, we induced anti-FVIII inhibitors in HemA mice by IP infusion of FVIII (2U/mouse, three times per week for 3 weeks) and then treated these mice with G-FVIII-LV. FVIII was detected in 0.32% of CD42d+ platelets ($n = 4$) on day 56 and more platelets (0.60%) contained FVIII in repeated LV-treated mice ($n = 4$). Finally, we evaluated the treated HemA animals with high (20 μL) vs. low dose (2 μL LV) G-FVIII-LV for phenotypic correction of bleeding diathesis by tail clip assay. In the high dosage group, the blood loss was 41% ($n = 7$), 48% ($n = 5$) and 33% ($n = 5$) on days 35, 118 and 160, respectively compared with control HemA (normalized to 100%) and wild-type (2.5%) mice. In the low dosage group, the percentage of the blood loss was 43% ($n = 7$), 54% ($n = 4$) and 42% ($n = 4$) on days 35, 118 and 160, respectively. Partial phenotypic correction of HemA was achieved over a 5 month experimental period following single-dose IO delivery of G-FVIII-LV without pre-conditioning. These results indicate that direct transduction of bone marrow cells targeting platelet-specific FVIII expression may provide an effective therapy to treat hemophilia A in the absence and presence of pre-existing inhibitors.

OC 37.2

A-LONG: results from a Phase 3 study of safety, efficacy, and pharmacokinetics of long-lasting recombinant factor VIII Fc fusion protein (rFVIIIFc)

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Background: Prophylaxis is the standard of care for prevention of bleeding episodes and arthropathy in severe haemophilia A. However, frequent injections (3–4 times/week) are required, potentially impacting adherence, quality of life, leading to increased morbidity. To reduce frequent injections, a long-lasting recombinant FVIII Fc fusion protein (rFVIIIFc), consisting of a single molecule of recombinant FVIII covalently linked to the Fc domain of immunoglobulin G1 with no intervening sequence, was engineered. Fc fusion is an established

technology that utilises the endogenous IgG cycling pathway to prolong the half-life of therapeutic proteins.

Aims: This phase 3 study evaluated the safety, efficacy, and PK of rFVIIIFc given as prophylaxis, for treatment of acute bleeding, and for perioperative management in previously-treated patients (PTPs) with severe haemophilia A.

Methods: Eligible male subjects ≥ 12 years old, with severe haemophilia A (< 1 IU/dL [1%] endogenous FVIII), ≥ 150 prior exposure days (ED) to FVIII, and no history of FVIII inhibitor, received individualised prophylaxis (Arm 1; 25–65 IU/kg every 3–5 days), weekly prophylaxis (Arm 2; 65 IU/kg), or episodic (Arm 3; 10–50 IU/kg) treatment. Subjects from each arm were eligible to enter a surgery subgroup. PK properties of rFVIIIFc were compared with rFVIII (Advate®; sequential PK subgroup). Overall study duration was ≤ 75 weeks. The primary efficacy endpoint was annualised bleeding rate (ABR; Arm 1 vs. Arm 3). ABR in Arm 2 vs. Arm 3, prophylaxis dose and interval, number of injections required for treatment of bleeding episodes, and perioperative haemostasis were evaluated. The primary safety endpoints included the incidence of adverse events (AEs) and inhibitor development.

Results: One hundred and sixty-five subjects were enrolled at 60 centres; 93% completed the study. Median age was 30 years (range, 12–65). Geometric mean (95% CI) terminal half-life was 19.0 (17.0–21.1) hours for rFVIIIFc vs. 12.4 (11.1–13.9) hours for rFVIII. Geometric mean time to 1% FVIII activity following 50 IU/kg of rFVIIIFc was approximately 5 days. Additionally, area under the concentration curve, geometric mean residence time, and time to 1% and 3% FVIII activity were significantly increased with a decrease in clearance for rFVIIIFc versus rFVIII, $P < 0.001$. Comparable PK profiles of rFVIIIFc were observed at Week 14. Median ABR for individualised and weekly prophylaxis were 1.6 and 3.6, respectively, vs. 33.6 for episodic treatment. The median dose and dosing interval with individualised prophylaxis were 78 IU/kg and 3.5 days, respectively; approximately 30% of subjects achieved a mean dosing interval of 5 days over the last 3 months on study. Ninety-eight percent of bleeding episodes were controlled with 1–2 injections. Perioperative haemostasis with rFVIIIFc was rated as excellent or good for all nine major surgeries. rFVIIIFc was well tolerated. No rFVIIIFc-related serious adverse events were reported. No inhibitors were detected.

Summary/Conclusions: Current data from A-LONG, the largest registrational study in haemophilia A, demonstrate that rFVIIIFc was efficacious, safe, and well-tolerated showing an improved PK profile vs. rFVIII concentrate. These data suggest that rFVIIIFc may significantly reduce injection frequency for prevention and treatment of bleeding in severe haemophilia A, potentially improving adherence and patient outcomes.

OC 37.3

A novel bispecific antibody (ACE910) against coagulation factors IXa and X improves procoagulant activity of patients with hemophilia A *ex vivo* to hemostatic level

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Background/Aims: Requirement of frequent infusions of factor VIII (FVIII) concentrates for patients with hemophilia A (HA) and bleeding control in the patients with anti-FVIII inhibitor are the critical issues. Recently, we generated a novel bispecific antibody against FIXa and FX, ACE910, which mimics the cofactor function of FVIII to exert *in vivo* hemostatic activity (Nat Med 2012, ASH2012), and started a phase I clinical study. Since ACE910 possesses a different antigenicity from FVIII, it can improve the intrinsic pathway coagulation even in the presence of inhibitor. Therefore, ACE910 will be used for the HA patients without fear of developing inhibitor, as well as for the patients who have already developed the inhibitor. Other important advantage in HA treatment is that subcutaneous infusion is possible and long-acting over 1–2 weeks can be expected. In the present study, we examined the *ex vivo* hemostatic effect of ACE910 on HA patients.

Methods: Twelve HA patients (3 without inhibitor and 9 with inhibitor) were enrolled. The effect of ACE910 on the parameters of ROTEM [clotting time (CT), clot formation time (CFT)], which was triggered by recalcification, was examined. Next, we compared the effect of ACE910 with that of FVIII concentrates on commercially available FVIII-deficient plasma by using APTT-clot waveform analysis (CWA). Each value of CWA parameters (APTT, maximum velocity and acceleration) was converted to FVIII activity (IU/dL).

Results: In ROTEM, when ACE910 were added to the whole blood at 0, 1, 5, 10, 20 μg/mL, the median values of CT were 4714*, 2048*, 1119, 1056, 792 s, respectively [*P < 0.01 vs normal control, 936 s (761–1128)], showing normalized value at 5 μg/mL or more dose. CFT [2896*, 432, 326, 216, 151 s at respective dose, *P < 0.01 vs normal control, 313 s (202–514)] were also normalized at 1 μg/mL or more dose. The presence of inhibitor showed no significant difference. In CWA, APTT was normalized (FVIII activity > 30 IU/dL) by addition of 1.25 μg/mL or more ACE910. However, the maximum velocity and acceleration were just improved equally to mild HA (FVIII activity 5–30 IU/dL) by addition of 5–40 μg/mL ACE910. When the FVIII-deficient plasma was mixed with anti-FVIII inhibitor (10 BU), the effect of ACE910 was quite similar. We also confirmed the activity of ACE910 in plasma taken from the HA patients.

Discussion: It is noteworthy that FVIII requires additional time to be activated by thrombin, while ACE910 does not require the process. Therefore, in the mode of action, ACE910 is different from wild type FVIII. Our findings indicated that improvement of parameters such as coagulation velocity and acceleration seemed to be useful for assessment and monitoring of the effect of the antibody on FVIII function *in vivo*. Nevertheless, we concluded that spiked ACE910 exerted its cofactor activity in the blood of HA patients dose-dependently even in the presence of inhibitor.

Conclusion: Our findings strongly suggested that clinically-achievable concentration of ACE910 could exert hemostatic effect for HA patients regardless of the presence of inhibitor.

OC 37.4

An RNAi therapeutic targeting antithrombin increases thrombin generation and improves hemostasis in hemophilia mice

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Background: The hemostatic system balances the need to control blood loss with the need to prevent thrombosis. In hemophilia, the loss of certain procoagulant factors (Factor VIII (FVIII) and Factor IX (FIX), in the case of hemophilia A and B, respectively) results in an imbalance of the hemostatic system toward a bleeding phenotype. Interestingly, there have been reports suggesting that coinheritance of prothrombotic mutations (e.g. Factor V Leiden, protein C deficiency, protein S deficiency, antithrombin deficiency, prothrombin G20210A) may ameliorate the clinical phenotype in hemophilia. We are currently investigating the use of RNA interference (RNAi) to target the natural anticoagulant antithrombin (AT) as strategy to rebalance the hemostatic system and improve thrombin generation, and therefore hemostasis, in hemophilia. Previously, a short interfering RNA (siRNA), ALN-AT3, was developed against AT and demonstrated potent activity in both wild-type and hemophilia mice after single subcutaneous (SC) administration (ED50 ~ 1 mg/kg).

Aims: There are two main aims in this work. First, we investigate the ability of ALN-AT3 to silence AT, and consequently, increase throm-

bin generation in a dose-dependent manner in hemophilia mice. Second, we explore whether AT reduction is able to improve hemostasis in a dose-dependent manner in hemophilia mice.
Methods: Hemophilia mice (HA and HB) received single subcutaneous injections of ALN-AT3 at doses ranging from 1 to 30 mg/kg. Plasma samples were collected from animals and analyzed from thrombin generation using a Calibrated Automated Thrombinoscope (CAT). Hemostasis was assessed using intravital microscopy in a laser injury model of the microvasculature of the cremaster muscle.
Results: Dose-dependent increases in thrombin generation were observed, with full normalization of thrombin generation back to wild-type control levels in hemophilia mice treated with the highest dose of ALN-AT3. Similarly, improvement in hemostasis was observed after laser injury, with dose-dependent increases in platelet accumulation and fibrin deposition rates. While no stable clot formation was observed after laser injury in hemophilia mice treated control vehicle, a stable clot was observed at all laser injury sites in the ALN-AT3 treated groups.
Conclusions: These data suggest that the use of a novel RNAi therapeutic targeting AT is a promising approach for the treatment of hemophilia, and potentially, other bleeding disorders. Further, the SC route of administration, long duration of action, and applicability to persons with hemophilia who have inhibitors, make this a particularly

OC 37.5

Engineering a novel rFVIII-VWF D'D3 fusion protein to enhance stability and improve pharmacokinetic properties of FVIII

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Background: More than 95% of circulating FVIII exists in a non-covalent complex with von Willebrand Factor (VWF). While VWF stabilizes and protects FVIII from clearance, it also subjects FVIII to VWF-dependent clearance, thus imposing a limit on the magnitude of half-life extension of rFVIII that is achievable by current technologies, which correlates with an approximately two-fold half-life extension compared to rFVIII in animal models. Engineering a stabilized FVIII molecule that does not bind to circulating VWF may enable circumvention of this VWF clearance pathway and provide a strategy for further extending the half-life of rFVIII molecules.
Aims: Develop a novel fusion protein consisting of FVIII and VWF domains that provides the protection and stability of endogenous VWF while evading the limitation imposed by VWF.
Methods: Truncated VWF variants were screened by hydrodynamic injection in VWF-deficient mice to identify the minimal domains that could stabilize endogenous FVIII. Selected VWF domains were then fused to FVIII using Fc as a scaffold. A thrombin-cleavable linker was inserted in between the VWF and Fc domain to facilitate the release of the VWF domains upon FVIII activation. The *in vitro* plasma stability of fusion proteins were determined by FVIII activity assay. The molecular integrity and circulating half-lives of rFVIII-VWF domain fusion proteins were evaluated in FVIII deficient (HemA) and FVIII/VWF deficient (DKO) mice.
Results: The 477 amino acid D'D3 domain of VWF (linked to the Fc monomer through a 98 amino acid thrombin cleavable linker) was identified as the optimal configuration for providing maximum stabilization and protection to the FVIII molecule. To prevent multimerization, two cysteine residues in the D'D3 domain that lead to covalent dimerization of native VWF were mutated in the fusion protein. VWF D1D2 region was required for proper function of the D'D3 domains produced in the cell culture. rFVIII-VWF D'D3 fusion proteins displayed enhanced plasma stability compared to rFVIIIFc and stability was further enhanced by substituting the FVIII moiety with a single chain FVIII isoform. *In vitro* the D'D3 domain prevented association of rFVIII with endogenous VWF, a finding that was corroborated by similar pharmacokinetic profiles for the rFVIII-VWF D'D3 fusion protein in both HemA and DKO mice. A terminal half-life of 13 h in DKO mice was achieved by the lead rFVIII-VWF D'D3 fusion protein, representing an approximately 54-fold improvement over that of BDD-FVIII.
Summary/Conclusions: We have developed a rFVIII-VWF fusion protein with a prolonged *in vivo* half-life that is uncoupled from the fate of VWF but retains the stability ordinarily conferred by endogenous VWF. Liberation of FVIII from VWF represents the fundamental difference between rFVIII-VWF D'D3 fusion protein and other rFVIII molecules that are currently under clinical investigation. This approach provides a platform for incorporation of additional half-life extension technologies that can ultimately surmount the half-life extension ceiling imposed by endogenous VWF.

OC 37.6

Results of haemostatic efficacy, safety, and pharmacokinetics/pharmacodynamics of a plasma-derived factor VIIa and factor X (MC710) in haemophilia patients with inhibitors: phase II clinical trial

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Background: MC710, a mixture of plasma-derived activated factor VII (FVIIa) and factor X (FX) at a protein weight ratio of 1:10, is a novel bypassing agent for haemostasis in haemophilia patients with inhibitors. MC710 is designed to administer FVIIa and its substrate FX concomitantly for greater potency than rFVIIa, and is long acting due to the long half-life of FX. We have already reported the results of a clinical pharmacological study (Phase I trial) wherein MC710 was intravenously administered at a single dose to haemophilia patients with inhibitors in non-bleeding state and the dose-dependency of pharmacokinetic (PK), pharmacodynamic (PD) parameters, and safety from 20 to 120 μg/kg (as FVIIa dose) were confirmed [1, 2].
Aims: In a Phase II trial, we evaluated the haemostatic efficacy and safety of single doses of MC710, and investigated PK and PD parameters in joint bleeding episodes in male haemophilia patients with inhibitors.
Methods: This trial was a multi-centre, open-label, non-randomized study of two doses (60 and 120 μg/kg), allowing for re-administration of different MC710 dosages to the same subjects. All subjects provided written informed consent. This trial was approved by the institutional review board of each participating institute. MC710 was intravenously administered at a single dose to the patients within 3.5 h after the onset of joint bleeding (shoulder, elbow, knee, or ankle) to evaluate haemostatic efficacy, safety, and PK (FVII:C, FX:C, FVII:Ag, and FX:Ag) and PD (APTT clot waveform analysis, PT, and thrombin generation test) in bleeding state. Haemostatic efficacy was determined for each treatment by investigator's evaluation using changes in visual analogue scale (VAS) for pain reduction, girth of joint (only for knee) for decrease in swelling, and range of motion (ROM) of the bleeding joint for improvement of joint mobility.
Results: In this trial, 19 subjects (nine haemophilia A (HA) patients with inhibitors and 10 haemophilia B (HB) patients with inhibitors) were enrolled. MC710 was given to six subjects including two HA patients with inhibitors and four HB patients with inhibitors. The

results of the study showed that in nine bleeding episodes, seven treatments were rated as 'excellent' or 'effective' in haemostatic efficacy at 8 h after administration. PK parameter values were close to those of the non-bleeding state. PD parameters changed drastically after administration and those changes were maintained for up to 24 h. No serious or severe adverse events were observed after administration; furthermore, measurement of several diagnostic markers revealed no signs or symptoms of disseminated intravascular coagulation (DIC).

Summary/Conclusion: The haemostatic potential of MC710 was confirmed at doses of 60 and 120 μg/kg in this trial. MC710 is thus expected to be a safe and efficacious novel bypassing agent for controlling bleeding in haemophilia patients with inhibitors. An expanded study (Phase III trial) in haemophilia patients with inhibitors and haemorrhage is ongoing to investigate the duration of MC710 efficacy, and the safety of repeated administration.

References:

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OC 38 – Tissue Factor

OC 38.1

Heme-induced vascular permeability leads to the extravascular TF-dependent activation of coagulation

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During hemolytic anemia, an excess of free heme in the blood leads to oxidative stress, vascular inflammation, activation of coagulation and thrombosis. Heme has been reported to increase tissue factor (TF) expression on endothelial cells (EC) and macrophages *in vitro*. In this study we investigated the mechanism of heme-induced activation of coagulation *in vivo*. C57Bl/6 mice were given a bolus intravenous injection of 0–35 μmol/kg heme or vehicle. Plasma was collected at 0–6 h after injection. Coagulation activation and inflammation were assessed by plasma thrombin anti-thrombin (TAT) complexes and plasma interleukin (IL)-6 levels, respectively. Administration of 17.5 and 35 μmol/kg of heme resulted in 1.8 ($P < 0.001$) and 2.6 ($P < 0.001$) fold increases, respectively, in plasma TAT levels 6 h after injection. In time course experiment (35 μmol/kg heme), plasma TAT levels increased rapidly at 1 h (2.2-fold, $P < 0.001$) and remained elevated at 3 (1.9-fold, $P < 0.001$) and 6 h (2.5-fold, $P < 0.001$).

Heme induces a rapid release of P-selectin and von Willebrand Factor (vWF) from EC Weibel-Palade bodies. P-selectin anchors ultralarge vWF strings to the endothelium and creates a thrombogenic surface. We observed significant increase in total plasma vWF ($P < 0.05$) and the amount of ultra large vWF multimers 1 h after heme injection (35 μmol/kg). However, inhibition of P-selectin with anti-P-selectin antibody (50 μg/kg) did not attenuate heme-induced activation of coagulation.

Next, we determined the role of TF in heme-induced activation of coagulation and inflammation. Thirty minutes before heme injection, C57Bl/6 mice were pretreated with the rat anti-mouse TF antibody 1H1 (25 mg/kg, single i.p. injection) or rat IgG. Heme (35 μmols/kg) induced leukocytosis (2.3-fold, $P < 0.001$) and increased plasma levels of interleukin-6 (8.5-fold, $P < 0.01$). However, inhibition of TF with 1H1 antibody did not affect any of these parameters. Heme increased plasma TAT levels from 4.6 ± 1.3 to 9.6 ± 1.3 ng/mL ($P < 0.001$); this increase was significantly attenuated by 1H1 treatment (6.8 ± 0.4 ng/L, $P < 0.01$). Importantly, TAT levels were also reduced by 40% ($P < 0.01$) in heme injected low TF mice (expressing 1–2% of normal TF levels) compared to control mice. Interestingly, the total number of phosphatidylserine-positive microparticles (MP) was not increased and there was no detectable TF-positive MP activity in the plasma after heme treatment, suggesting that MPs are not involved in heme-mediated coagulation activation.

To determine the cellular source of TF that contributes to the activation of coagulation, we used $TF^{flox/flox}$, Tie-2 Cre mice (TF deficiency in hematopoietic and ECs). Deletion of TF from these cells did not affect plasma TAT levels in heme injected mice, indicating that some other cellular source of TF activates coagulation. Importantly, we demonstrated that heme rapidly increased vascular permeability in the lung (2.7-fold increase in Evans Blue content compared to controls, $P < 0.05$), indicating EC damage and exposure of the extravascular TF.

Our data indicate that heme-induced activation of coagulation, but not inflammation, is TF-dependent. In contrast to *in vitro* data, TF expressed by extravascular cells rather than leukocytes or ECs is the likely source of TF contributing to heme-mediated activation of coagulation in mice.

OC 38.2

The insulin-like growth factor 1 receptor mediates tissue factor/FVIIa-induced cell survival

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Background: The binding of factor VIIa (FVIIa) to tissue factor (TF) initiates blood coagulation and activates various intracellular signalling pathways. Solid tumors often have high expression of TF and recently, we showed that TF/FVIIa-complex formation protects cancer cells from TNF-related apoptosis inducing ligand (TRAIL)-induced apoptosis. The anti-apoptotic signalling did not involve the engagement of TF co-receptors PAR1 or PAR2 but possibly another cell surface component. The receptor tyrosine kinase (RTK) insulin-like growth factor 1 receptor (IGF-1R) is a major regulator of cell survival, and is implicated in the pathogenesis of several disease states, including cardiovascular disorders and cancer. Although we and others have described transactivation of RTKs by the TF/FVIIa complex, there are no published reports concerning TF and the IGF-1R.

Aim: The aim of the current study was to investigate the involvement of the IGF-1R in TF/FVIIa-mediated protection against TRAIL-induced apoptosis in breast and prostate cancer cells.

Methods: MDA-MB-231 breast and PC3 prostate cancer cells were incubated with 100 ng/mL TRAIL and 100 nM FVIIa, either alone or in combination, while the IGF-1R was blocked using specific inhibitors (PPP; AG1024) or siRNA. The caspase-8 and -3 levels, nuclear alterations, and cellular size were then monitored for 6 h by the ArrayScan VTI microscope. Human monocytes were isolated from whole blood and induced to express TF by stimulation with 10 ng/mL LPS for 3 h. The phosphorylation status of the IGF-1R and its associations to the 14-3-3 family of adaptor proteins were determined using the Duolink *In Situ* proximity ligation assay. The phosphorylation of AKT was determined by western blot.

Results: Both in the IGF-1R inhibitor-treated and in the gene silenced MDA-MB-231 cells, the antiapoptotic signalling mediated by TF/FVIIa was significantly reduced to levels comparable to TRAIL-treatment alone. The phosphorylation of AKT, whose activation is mandatory for the survival signalling of TF/FVIIa, was also sensitive to IGF-1R inhibition. Our findings were verified in PC3 cells and the possibility that AG1024, PPP, and the siRNA treatment evoke apoptosis on their own was clearly ruled out.

TF/FVIIa furthermore induced a tyrosine phosphorylation of the IGF-1R in MDA-MB-231 cells as well as in human monocytes. This led to an association of the IGF-1R and members of the 14-3-3-family, an event previously known to initiate pro-survival signalling pathways.

Summary/Conclusions: We propose that the IGF-1R is a key mediator of TF/FVIIa antiapoptotic effects. We also demonstrate, for the first time, that FVIIa-treatment leads to IGF-1R transactivation in several

cell types, and association of the IGF-1R to the 14-3-3 family of adaptor proteins. Our novel findings thus couple the coagulation system to the IGF-system, and shed new light on both TF/FVIIa and IGF-1R signalling.

OC 38.3

Red blood cells enhance LPS-induced tissue factor and TNFalpha in monocytes

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Introduction: We have previously shown that the expression of tissue factor (TF) activity in LPS-stimulated human monocytes is enhanced two fold by blood platelets in a granulocyte dependent reaction. Although red blood cells (RBCs) have been reported to accelerate thrombin generation by their surfaces, a possible interaction between RBCs and monocytes in the expression of TF has not been shown. RBC transfusion has been demonstrated to increase platelet activation and aggregation *in vitro* in healthy individuals. In some old data not reported we found a positive correlation between RBC and LPS-induced in whole blood and TNFα, IL-6 and P-selectin when screening a large population. This prompted a further search for a possible interaction between RBC and activated monocytes.

Methods: Whole blood was collected from healthy individuals with Fragmin as anticoagulant. RBC, granulocytes and mononuclear cells were isolated by centrifugation of the blood on top of Lymphoprep/Polymorphprep as described earlier. The granulocytes, mononuclear cells and platelets were after washing re-suspended in plasma (Fragmin) and added increasing volumes of RBC and correcting volume by adding saline.

Test aliquots were prepared by combining mononuclear cells, granulocytes and platelets from 1 mL blood that was re-suspended in 0.5 mL plasma (Fragmin) and RBCs as indicated below. These samples were added 5 ng/mL LPS and incubated for 2 h at 37 °C in a rotary incubator. EDTA was added to stop the reaction and mononuclear cells isolated and frozen and thawed before measurements of TF in a specific TF activity assay. In separate experiments the incubation samples were centrifuged for collection of plasma to quantify TNFα that was measured in an ELISA assay.

Results: RBC enhanced LPS-induced TF activity in a dose dependent manner with an optimal effect of 8.8×10^9/mL (final concentration) RBCs in the sample (49.7 ± 19.0 mU/10^6 to 80.4×8.6 mU/10^6, $n = 6$). At higher concentration of RBCs, there was a less effect of them.

TNFα was measured in plasma samples, and a significant rise was observed between 1.1×10^9/mL (1.53 ± 0.40 ng/mL) and 17.6×10^9/mL (2.74 ± 0.75 ng/mL, $n = 8$) RBCs added to the white cells and platelets.

When screening blood of healthy individuals ($n = 143$), it was found a positive correlation coefficient between RBCs and LPS-induced TNFα, IL-6 and P-selectin of respectively 0.22 ($P < 0.01$), 0.19 ($P < 0.05$) and 0.33 ($P < 0.001$).

Discussion: The enhancement of LPS-induced TF activity as well as TNFα in monocytes by RBCs shows that RBCs has a pro-coagulant and a pro-inflammatory effect. This is in agreement with our observation that P-selectin and the production of TNFα in LPS-stimulated blood of a large population correlated positively with RBCs. Our results may at least partially account for the higher risk of thrombosis at high RBC counts.

OC 38.4

Endothelial cells uptake and recycle microparticle-derived tissue factor to the cell surface with augmented procoagulant activity

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Background: Endothelial cells may express TF in response to inflammatory cytokines. However, there is also *in vitro* and *in vivo* evidence that TF-positive microparticles transfer TF to vascular endothelial cells. Recent evidence suggests that microparticles bind to the surface of cells and are then internalised by endocytosis. However, it is not known whether TF associated with microparticles is internalised by an endocytotic process and whether this TF is then recycled back to the cell surface or degraded.

Aims: In this study, we have demonstrated the uptake of TF from microparticles by microvascular endothelial cells and have examined the mechanisms involved in the recycling of TF together with the exposure of phosphatidylserine, resulting in increased TF activity on the surface of endothelial cells.

Methods: Human dermal blood endothelial cells (HDBEC) were incubated with TF-positive and TF-negative microparticles isolated from the media of MDA-MB-231 and MCF-7 cell lines respectively. Cell surface TF antigen and activity were measured over 6 h using an anti-TF antibody, and a thrombin generation assay, respectively. The exposure of phosphatidylserine was also analysed by annexin V-labelling. TF expression was also monitored using quantitative RT-PCR. In addition, samples of cells were incubated with cyclohexamide to prevent protein synthesis, or a dynamin inhibitor (Dynasore) to prevent endocytosis, prior to incubating with microparticles. Finally, microparticles were isolated from the media of MDA-MB-231 cells expressing a TF-tGFP hybrid protein. Endothelial cells were incubated with the microparticles and the co-localisation of TF with the Rab family of proteins analysed by confocal microscopy.

Results: Measurement of TF antigen on the surface of HDBEC revealed two distinct peaks at 30 and 180–240 min post-incubation of the cells with TF-positive, but not TF-deficient microparticles. However, only the second peak was concurrent with high TF activity as determined by a chromogenic thrombin-generation assay. Annexin V-labelling of the HDBEC-surface showed the exposure of phosphatidylserine following 90 min incubation with microparticles, which explains the high TF activity associated with the second antigen peak. Analysis of TF mRNA levels revealed no *de novo* expression of TF mRNA in response to microparticles, and pre-incubation of the cells with cyclohexamide did not prevent the appearance of TF. In contrast, blocking endocytosis with Dynasore prolonged the disappearance and prevented the re-appearance of TF antigen on the cell surface. Incubation of HDBEC with microparticles containing TF-tGFP revealed the early co-localisation of TF with Rab4 and Rab5, which was followed by co-localisation with the late endosomal/trans-Golgi network marker Rab9, and the recycling endosome marker Rab11.

Conclusions: These data suggest a mechanism by which TF-containing microparticles are internalised by endothelial cells and the TF moiety recycled to the cell surface. Together with the exposure of phosphatidylserine, this is capable of inducing a substantial increase in the procoagulant potential of the surface of endothelial cells.

OC 38.5

Tissue factor expression and signalling in aortic valve interstitial cells: insights into fibro-calcific aortic valve disease

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Background: Aortic valve stenosis (AVS) is an atherosclerosis-like process characterized by valve interstitial cell (VIC) proliferation and commitment to fibrocalcification and pro-inflammatory cytokines. To date, no effective medical treatment is able to improve the clinical course of the disease. Tissue factor (TF) and thrombin expressions were previously shown to be significantly associated with fibrocalcification in human stenotic aortic valves.
Aims: We investigated differential TF expression in normal and pathological valves and VIC and further describe its downstream signalling pathways in normal VIC and its role in their profibrotic commitment.
Methods: Fibrocalcific and normal human aortic valves were collected ($n = 50$ and $n = 6$ respectively) from which tissue lysates and valve interstitial cells (VIC) were isolated. VIC phenotype was analysed by flow cytometry. VIC obtained from normal aortic valves were stimulated or not with IL-1beta (major inflammatory cytokine involved in AVS at 1 ng/mL). In valve lysates and in VIC, TF and PAR-2 were quantified by qPCR for relative mRNA expression, and by flow cytometry/ELISA for protein and activity quantification.
Downstream signalling pathways ERK (cell activation and proliferation) and Smad2 (pro-fibrosis transcription factor) of VIIa/TF in stimulated VIC were assessed by westernblotting after incubation of TF-overexpressing VIC with VIIa (50 ng/mL for 30 min) in the presence or in the absence of anti-TF antibody. Results are expressed as median (interquartile range).
Results: VIC were defined by flow cytometry as being CD31 and CD68 negative and and stemness marker (CD90). Relative mRNA expressions of TF and PAR-2 were significantly higher in fibrocalcific vs normal valve lysates (3.2 (1.5-.8) vs 0.18 (0.02–0.29), $P < 0.05$; 1.15 (0.48–1.62) vs 0.16 (0.02–0.22), $P < 0.05$; respectively). Normal VIC constitutively expressed active TF mRNA and protein (relative mRNA 4.1 (2.1–21.2); activity 36.2 (13.2–101.3) pg/mL) as well as PAR-2 receptor (mRNA and protein by flow cytometry). TF expression was significantly increased in fibrocalcific VIC (relative mRNA 22.8 (9.8–33.8), $P = 0.05$; activity 153 (71.5–247.0) pg/mL, $P < 0.05$) when compared to normal VIC. Following IL-1beta stimulation of VIC, TF expression was significantly upregulated when compared to unstimulated cells (relative mRNA 15.4 (12.9–28.2) fold increase, $P < 0.05$) and was associated with an increase of TF activity (2.7 (2.2–5.5) fold increase, $P < 0.05$). The VIIa stimulation of TF-overexpressing VIC entailed an upregulation of ERK and Smad2 signalling pathways (pERK/ERK activation ratio 3.5 (2.9–4.8) fold increase; pSmad2/Smad2/3 activation ratio 1.8 (1.2–1.8) fold increase). This upregulation was blunted by preincubation of VIC with a specific anti-TF antibody.
Summary/Conclusions: Our results demonstrate the implication of TF/VIIa/PAR-2 axis in VIC commitment to fibrocalcific aortic valve disease. Thus, modulation of this pathway may represent a new therapeutic target for the early medical treatment of AVS.

OC 38.6

Increased expression of tissue factor in intra-abdominal adipose tissue in a metabolic syndrome model: a new insight ?

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Background: Tissue factor (TF), the principal initiator of blood coagulation, plays an important role in atherosclerosis. TF is considered to be the main contributor to plaque instability by favoring thrombogenicity and angiogenesis. Metabolic syndrome (MetS), a major risk factor of atherosclerotic cardiovascular diseases, is characterized by the accumulation of dysfunctional intra-abdominal adipose tissue secreting adipokines and other proinflammatory factors.
Aims: We aimed at studying adipose tissue TF expression and its modulation in relation to MetS.
Methods: Eighty five male Sprague Dawley rats were placed on a fructose-enriched (60%) diet or a normal diet during 6 weeks. Development of MetS was confirmed via blood pressure measurement, blood sampling and MRI study of fat distribution. TF expression (antigen, activity and mRNA) was assessed in adipose tissue (subcutaneous, intra-abdominal and perivascular), adipose tissue conditioned medium and in cultured pre-and mature adipocytes.
Results: We confirmed that a fructose-enriched diet induced an early stage of MetS with arterial hypertension, insulin resistance, increased plasma cholesterol and triglycerides levels, and body fat redistribution, as identified by MRI ($P < 0.01$). TF expression occurred in all adipose tissues in both groups of rats (activity, antigen and mRNA), significantly more so in the MetS group. In particular, TF expression was significantly higher in intra-abdominal adipose tissue of MetS rats compared to control rats (activity: 1286 versus 812 mU/g of protein respectively, $P < 0.01$) with similar results regarding TF antigen. Cultured fat cells (adipocytes and stromal fraction which contains pre-adipocytes) produced TF which activity increased with the degree of cell differentiation, more in the MetS group compared to control rats (5424 versus 2570 mU/g of protein respectively, $P < 0.005$). When cultured, intra-abdominal adipose tissue secreted functionally active TF (69 mU/mg protein).
Conclusions: This study shows for the first time the expression of functionally active TF in various adipose tissues of an early nutritional acquired MetS rat model. TF expression was significantly higher in intra-abdominal adipose tissue and could accordingly, via a paracrine effect, contribute to MetS related atherosclerotic complications.

OC 39 – Von Willebrand Disease – Basic Aspects

OC 39.1

The type 2B p.R1306W von Willebrand factor mutant shows a large enhancement of sensitivity to shear stress that favors its interaction with the platelet receptor

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Background: Shear stress triggers conformational changes of von Willebrand factor (VWF) from a globular to a stretched status, responsible for both exposure of the binding site for the platelet receptor GpIb and its self-association. This mechanism supports the formation of platelet plug under high shear stress in flowing blood. Natural VWF mutants of type 2B VW disease (2B-VWD) are generally considered having an increased affinity for platelet GpIb, in turn responsible also for accelerated hydrolysis by ADAMTS-13.

Aims: This study was aimed at assessing whether the type 2B p.R1306W mutant has an intrinsic higher affinity in the minimal binding domain for GpIb present in the A1-A2-A3 domain or rather the increased interaction with the platelet receptors stems from an increased sensitivity to shear stress that exposes the interaction sites for GpIb upon the conformational changes induced by shear forces.

Methods: Surface plasmon resonance spectroscopy was used to measure the interaction between WT and mutant p.R1306W A1-A2-A3 domain with the recombinant GpIb part containing the VWF binding domain. The analysis of VWF self-association was performed under controlled shear stress conditions ranging from 0 to 60 dyn/cm^2 by atomic force microscopy and dynamic light scattering spectroscopy. The pro-aggregating properties of full length recombinant WT and p.R1306W VWF forms were studied by light aggregation and platelet adhesion studies in flow chamber.

Results: Measurements on the interaction between WT and mutant p.R1306W A1-A2-A3 domain with GpIb, showed that binding to GpIb is characterized by similar K_d values equal to about 20 nM. On the other hand, full length WT-VWF does not significantly interact with GpIb under static conditions, whereas the p.R1306W mutant at 1.9 μg/mL already showed a significant interaction with the receptor. At values of shear stress < 10 dyn/cm^2, the full length p.R1306W mutant already unfolds and undergoes an initial self-aggregation with formation of a network characterized by increased roughness compared to WT-VWF. Dynamic light scattering spectroscopy showed that the hydrodynamic radius of WT VWF in the absence of shear is about 2.5-fold lower than that of the 2B VWF mutant (87 ± 22 nm vs 210 ± 60 nm). Mechanical stretching experiments showed that full length p.R1306W mutant needs 30% less energy to be reversibly strained compared to WT protein. The difference of ~ 30% is related to the energy/length ratio and in the simplest picture of a homogeneous solid subject to a shear stress, the work done per unit length during the strain (equal to the strain energy stored per unit length) depends on the square of the average shear stress applied. Hence, even little differences in energy can reflect much bigger differences in shear stress to be applied.

Summary/Conclusion: These findings showed that then p.R1306W mutation enhances the avidity of this type 2B VWF mostly by increasing the sensitivity to shear stress that facilitates exposure of GpIb binding sites and proteolytic attack by ADAMTS-13. This effect contributes to loss of high molecular weight VWF multimers and to platelet consumption, often observed in this disease

OC 39.2

The von Willebrand disease type 2B p.V1316M mutation inhibits platelet functions by interfering with Ca^{2+} mobilization and integrin αIIbβ3 activation pathways

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Background: Von Willebrand disease type 2B (VWD2B) is characterized by gain-of-function mutations in von Willebrand factor (VWF) which exhibits increased binding to platelet glycoprotein (GP)Ibα. The bleeding tendency observed in VWD2B is currently explained by the absence of high molecular weight-VWF multimers and by thrombocytopenia.

Aims: We postulate that constitutive binding of VWF may alter platelet functions, and thus participate in the bleeding tendency of VWD2B patients. To test this hypothesis we analyzed platelet functions using three models: (i) a murine model of VWD2B due to p.V1316M mVWF expression and associated with a severe phenotype; (ii) human control platelets pretreated with recombinant hVWF/p.V1316M and (iii) platelets from a VWD2B patient with the severe hVWF/p.V1316M mutation.

Methods: Spreading, aggregation, secretion, and αIIbβ3 activation were examined in static conditions in washed platelets stimulated with various agonists. Thrombus formation was performed on collagen under flow at 300/s and 1500/s. Platelet signaling including Rap1 activity, calcium release induced by G-proteins-coupled receptors (GPCRs) or by collagen receptors and GPVI signaling was examined.

Results: Platelets from mice expressing mVWF/p.V1316M or from a VWD2B patient bearing the hVWF p.V1316M mutation exhibited inhibition of integrin αIIbβ3 activation and platelet aggregation, secretion, or spreading, as induced by various agonists (collagen, convulxin, ADP and PAR4-AP). Consistent with inhibition of integrin αIIbβ3 activation, alteration of thrombus growth under flow on collagen, was also noted in both cases. Interestingly, Rap1 activation and calcium signaling as induced by various agonists were markedly diminished both in human control platelets pretreated with recombinant hVWF/p.V1316M and in platelets from the patient exhibiting the p.V1316M hVWF mutation. Interestingly, upon stimulation of GPVI signaling by convulxin, Ca^{2+} influx was drastically diminished, but Ca^{2+} store release and phospholipase (PLC)γ activation were normal (or slightly increased). This suggests a block between Ca^{2+} store release and Store Operated Calcium Entry (SOCE), possibly at the level of the SOC channel Orai1-Stim1 complex. In contrast, PLCβ-dependent Ca^{2+} store release triggered by GPCRs signaling pathways (P2Y and PAR receptors for ADP and thrombin, respectively) and Ca^{2+} influx were both reduced to comparable levels. This suggests a block upstream Ca^{2+} store release and SOCE, thus pointing to the GPCR-Gq-PLCβ pathway.

Conclusion: We conclude that V1316M VWF alters platelet aggregation, spreading and thrombus growth, via inhibition of αIIbβ3 activation, by interfering with Rap1 activation and with the cytosolic Ca^{2+} rise, by an upstream or downstream block of Ca^{2+} store release depending on agonists. While the mechanism by which VWF/p.V1316M acts still remains to be elucidated, our data strongly argue in favor of a role for thrombopathy in the bleeding tendency of V1316M VWD2B patients.

OC 39.3

Defective angiopoietin-2 release from von Willebrand disease patients' blood outgrowth endothelial cells

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Von Willebrand factor (VWF) mediates platelet adhesion to damaged endothelium and stabilises coagulation factor VIII. VWF is stored within and directs the formation of Weibel-Palade bodies (WPB) in endothelial cells (EC). WPB contain many proteins including the angiogenic regulator Angiopoietin-2 (Ang-2). Von Willebrand disease (VWD) is the most common inherited bleeding disorder caused by decrease or dysfunction of VWF. The cellular basis of VWD is poorly understood. VWD can be associated with angiodyplasia, describing the presence of vascular lesions which can cause intractable bleeding. We demonstrated a role for VWF in angiogenesis and hypothesized that angiodysplasia might be linked to dysregulated angiogenesis through several pathways. We have used Blood Outgrowth EC (BOEC) to study the cellular basis of VWD and angiogenesis. Mononuclear cells were isolated from peripheral blood of four type 1, four type 2 VWD patients and nine healthy controls and cultured to obtain BOEC. Various EC markers confirmed the identity of the BOEC. In this study confocal microscopy confirmed VWF and Ang-2 co-staining within BOEC WPB. We discovered a heterogeneous basal release of Ang-2 from patient and control cells over a 75 min time-course with no significant difference between the groups. We previously demonstrated decreased VWF levels in type 1 VWD patients' BOEC. mRNA levels were predictive of plasma VWF levels in these patients confirming a defect in VWF synthesis (Pearson $R = 0.9976$, $P = 0.024$, $r^2 = 0.9952$). BOEC from type 1 VWD patients also showed defects in processing, storage and/or secretion of VWF with reduced VWF staining of WPB by confocal microscopy. Two patients with type 1 VWD showed increased levels of pro-peptide containing VWF. In this study we discovered that three type 1 patients showed no Ang-2 release post-cellular stimulation, consistent with the inability of these BOEC to correctly package WPB. One patient showed significant release of Ang-2 ($P < 0.05$); interestingly this patient showed the highest levels of VWF within the type 1 group with more WPB and VWF strings released upon stimulation. Our previous study showed that levels of VWF were normal in BOEC from three type 2 VWD patients, supporting the dysfunctional VWF model. However, one type two patient showed decreased VWF synthesis and storage and increased levels of pro-peptide containing VWF, indicating a complex cellular defect. We now show that all type two patients' BOEC show a significant release of Ang-2 upon stimulation, confirming correct packaging of WPB. Together our data suggests that the angiogenesis defect in type 1 VWD patients is due to dysregulation of Ang-2 storage and release, but that other mechanisms may be more relevant in type 2 VWD. Ongoing studies will define Ang-2 mRNA expression and long-term constitutive release from EC from VWD patients. These results demonstrate that isolation of endothelial cells from VWD patients provides novel insight into the cellular mechanisms of the disease and may aid in developing treatment for this common inherited bleeding disorder.

OC 39.4

Applying *in silico* analysis to the historically reported VWF exon 42 deletion reveals a probable L1 non-autonomous retrotransposition mediated deletion pathomechanism

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Introduction: Partial deletions of the von Willebrand factor gene (*VWF*) have been recognised as contributing to the pathogenesis of types 1, 2 and 3 von Willebrand disease (VWD). Peake et al (1990):75 654 reported an out-of-frame homozygous deletion of exon 42 (ex42del) in a type 3 VWD patient, proposed to introduce a premature stop codon into the *VWF* sequence within exon 43. A novel 182 bp insertion at the breakpoint junction was shown to be derived from an unknown site within the genome. Aim

To undertake *in silico* analysis to derive the full sequence around the breakpoints and determine the mechanism by which the deletion arose.

Methods: To assess the extent of repetitive element involvement at the breakpoint junctions and of the genomic insert, RepeatMasker was used. Nucleotide BLAST was used to find information on the genomic insertion and DNA sequence motif analysis was performed based on published information.

Results: Peake et al. could not identify repetitive sequences directly adjacent to the breakpoints and this remains the case with current methods. Nucleotide BLAST of the 182 bp insert against the human genome revealed that sequence matched *VWF* intron 38, with 96% homology (MegaBLAST). Analysis of 5′ and 3′ breakpoint regions identified topoisomerase cleavage sites directly adjacent to the breakpoints. Repetitive element analysis of the insert using RepeatMasker revealed presence of a 3′ truncated long interspersed nuclear element (LINE, L1 class) in the first 124 bp with the remainder comprising non-repetitive sequence, derived from intron 38. The mutation can be described as c.7081+86_7287+1044delins182; p.Ala2361Gly*fs*45*.

Conclusion: Previously reported L1 retrotransposition events within *F8* and *F9* contain inserts larger than reported here. These L1 inserts are associated with transposition of L1 DNA only and contain long poly A tails, capable of integrating into the genome at complementary sites, through target-site primed reverse transcription, whereby L1 endonucleases nick complementary sites and integrate reverse transcribed L1 DNA into the genome. The L1 insertion causes the disease phenotype, disrupting the reading frame and reducing gene expression. In this instance, the likely mechanism of ex42del is through a non-classical L1 retrotransposition mediated deletion event; with disease phenotype resulting from the out-of-frame ex42del. In the proposed mechanism, the 182 bp sequence is copied from intron 38 of *VWF* by another full length L1 element (non-autonomously). Topoisomerase cleavage at the sites directly adjacent to the breakpoints result in a DNA strand break, removing exon 42 sequence and flanking intron. Breakpoints are processed and the 182 bp mRNA template anneals to microhomologous sites at the 3' breakpoint. Finally, mRNA is converted to cDNA via reverse transcription and the strand break repaired by DNA synthesis. The reason for not finding 100% homology with the intron 38 inserted sequence was possibly due to the differences between the historical *VWF* sequence data and reference sequence information now available. This first report of a possible *VWF* deletion arising through non-classical L1-retrotransposition extends understanding of mechanisms responsible for *VWF* deletion mutations.

OC 39.5

Investigation of the contribution of von Willebrand factor (VWF) propeptide mutations to type 3 VWD using *in vitro* cellular studies and patient-derived blood outgrowth endothelial cells (BOEC)

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Background: In the Canadian type 3 VWD study, 42% of mutations identified were found in the VWF propeptide, and index cases (IC) with mutations in this region had higher bleeding scores (BS) than IC with mutations elsewhere in VWF (median BS = 22 vs.13, $P = 0.012$).

Aims: To investigate the molecular pathogenesis of VWF propeptide (VWFpp) mutations identified in the Canadian type 3 VWD population using a heterologous mammalian cell system and patient-derived blood outgrowth endothelial cells (BOEC). As well, to investigate the potential to restore normal VWF biosynthesis by co-transfections with VWFpp.

Methods: Two mutations located in the VWFpp were investigated; the recurrent in-frame deletion of exons 4 and 5 (ex4-5del), and a novel missense mutation in exon 15, c.1897T>C (p.Cys633Arg). VWF secretion, multimerization, storage, stimulated release, and confocal immunofluorescence (IF) microscopy were examined *in vitro* in HEK293T cells and in BOEC. To investigate the potential to restore normal VWF function, HEK293T cells were co-transfected with each mutation and a human wild-type (WT) VWFpp plasmid. VWF secretion, and multimerization were investigated.

Results: Previous *in vitro* expression of homozygous ex4-5del showed markedly reduced secretion (2% of WT), levels of VWF in the cell lysates comparable to WT, loss of multimerization and absence of pseudo-Weibel-Palade bodies (WPBs). Heterozygous ex4-5del expression (1:1 WT:variant) resulted in reduced VWF secretion (14% of WT), cellular VWF comparable to WT, and normal multimers. Homozygous expression of p.Cys633Arg in HEK293T cells showed markedly reduced secretion (< 1%), intracellular retention (20% more than WT), loss of multimerization and absence of pseudo-WPBs. Heterozygous expression of p.Cys633Arg resulted in reduced VWF secretion (21% of WT), a 10% increase in the cell lysates compared to WT, and normal multimerization.

BOEC were obtained from a homozygous and heterozygous ex4-5del patient and from two homozygous and one heterozygous p.Cys633Arg patient. Compared to normal BOEC, both heterozygotes showed quantitative abnormalities in WPB formation with 50% and 62% fewer WPBs in the ex4-5del and p.Cys633Arg patients, respectively. WPBs were qualitatively different in the heterzoygotes, being more round than the typical cigar-shaped observed in normal BOEC. Associated with these WPB defects, heterozygous ex4-5del showed a minimal response to stimulation with PMA (increase in%VWF secretion was 55% that of normal) whereas the increase in p.Cys633Arg BOEC was comparable to normal (93%). A complete absence of WPBs in the three homozygotes was observed, with only diffuse VWF staining. This diffuse VWF staining co-localized with the ER marker, calnexin, indicating ER retention. Other proteins found in WPBs – P-selectin, IL-8 and CD63 did not co-localize with the diffuse VWF staining. Co-transfections of the mutant plasmids with the WT VWFpp did not restore VWF secretion, or multimerization.

Summary/Conclusions: We have shown that two different *VWF* mutations, a complete in-frame deletion of two exons and a missense mutation, which are both located in the VWF propeptide have similar effects on VWF biosynthesis and secretion. This further highlights the importance of VWF propeptide mutations in regulating VWF production and highlights the value of continued work using BOEC to study VWD.

OC 39.6

Novel variations in platelet GPCRs identified in patients with a historical diagnosis of Type 1 von Willebrand disease

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Background: Data from the Molecular and Clinical Markers for the Diagnosis and Management of Type 1 von Willebrand Disease (MCMDM-1VWD), and other studies support a role for genes outside of the von Willebrand factor locus in contributing to the expression of type 1 von Willebrand disease (VWD). We recently identified two mutations in the platelet $P2Y_{12}$ ADP receptor gene (*P2RY12*) in patients with type 1 VWD recruited through the MCMDM-1VWD study and showed that they could contribute to the bleeding phenotype of these patients (Blood 2009;113:4110–4113, Blood. 2011;118:5641–5651).

Aims: To investigate whether variations in platelet G-protein coupled receptor (GPCR) genes other than *P2RY12* contribute to the bleeding tendency in type-1 VWD patients.

Methods: The exonic and flanking intronic sequences of the platelet GPCR genes *P2RY1, F2R, F2RL3, TBXA2R* and *PTGIR* were amplified from the genomic DNA of 146 index cases diagnosed with type 1 VWD and recruited through the MCMDM-1VWD study, and then sequenced on an automated ABI 3730 DNA capillary sequencer.

Results: Seven candidate heterozygous GPCR gene single nucleotide variations (SNVs) were identified in eight index cases. Two *F2R* SNVs were detected in two index cases, a missense mutation in the 7th transmembrane domain of PAR-1 (c.1063 C>T, p.L355F) and a single nucleotide transition in the 5' UTR (c.-67 G>C). One index case was heterozygous for a missense *F2RL3* SNV (c.65 C>A) predicting a p.T22N substitution in the PAR-4 propeptide which was co-inherited with a synonymous SNV (c.6680C>T, p.S218=) in *TBXA2R* which encodes the Thromboxane A2 receptor. Two synonymous *F2RL3* mutations were identified in two further index cases (c.402 C>G, p.A134=; c.1028 G>C p.V343=). None of these SNVs were present among at least 80 control subjects recruited through the same centres as the cases. A missense mutation in the gene encoding the prostacyclin receptor *PTGIR* (c.44 T>C, p.V15A), was identified in three index cases from two centres. However this SNV also occurred in two control subjects (two alleles of 326 examined). None of the candidate mutations that were identified had previously been reported on dbSNP.

Conclusions: We have identified seven novel GPCR mutations in patients with a historical diagnosis of type 1 VWD, comprising three non-synonymous and three synonymous SNVs and 1 promoter change. Therefore, of the 146 index cases examined, 10 are heterozygous for candidate mutations in platelet GPCR genes (6.8%). These results further support the potential contribution of other loci to the bleeding phenotype of patients with type 1 VWD, highlighting the heterogeneity of the disease.

OC 40 – Angiogenesis and Arteriogenesis – II

OC 40.1

The ability of cleaved high molecular weight kininogen (HKa) to induce endothelial cell apoptosis and inhibit angiogenesis is not dependent on the urokinase-type plasminogen activator receptor (uPAR)

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Background: Endogenous anti-angiogenic polypeptides derived from proteolysis of parent proteins play an important role in regulating angiogenesis in normal and pathological conditions. High molecular weight kininogen (HK), a plasma protein and an important member of intrinsic coagulation pathway, undergoes proteolysis by kallikrein and other proteases to release the mitogenic nonapeptide bradykinin, leaving behind cleaved high molecular weight kininogen (HKa), which expresses potent antiangiogenic activity. Previous studies from our laboratory and others have demonstrated that HKa induces DNA fragmentation and selective apoptosis of proliferating endothelial cells, and inhibits angiogenesis *in vivo*; however, the mechanisms by which HKa inhibits angiogenesis remain controversial as does the role of the urokinase receptor (uPAR), which has been reported in several studies to mediate the antiangiogenic effects of HKa.
Aims: The goal of this study was to directly determine the role of the urokinase receptor in the anti-endothelial and anti-angiogenic activity of HKa.
Methods: We directly examined the role of uPAR in HKa-induced endothelial cell apoptosis by examining the effects of HKa on endothelial cells treated with control or uPAR siRNA. The role of uPAR in inhibition of angiogenesis by HKa *in vivo* was determined by comparing the effects of HKa on angiogenesis in Matrigel plugs in wild type and uPAR-deficient mice. Matrigel plugs that did or did not contain HKa were prepared in uPAR deficient mice and wild-type littermates, and angiogenesis in the Matrigel measured after 9 days.
Results: Cells treated with uPAR siRNA retained full viability and proliferative potential, as well as complete susceptibility to HKa-induced apoptosis as measured by morphology (cell contraction), nuclear fragmentation, chromatin condensation and chromosomal DNA fragmentation. A similar pattern of activation of caspases 3 and 7 was also observed in uPAR and control siRNA treated endothelial cells. *In vivo*, angiogenesis was inhibited equally well in HKa containing plugs that were placed in wild-type or uPAR deficient mice.
Summary/Conclusion: Though HKa binds to uPAR and may influence some uPAR-dependent activities, uPAR is not essential for the anti-endothelial cell effects of HKa *in vitro* or *in vivo*. The antiangiogenic mechanism(s) of HKa remain uncertain.

OC 40.2

Thrombin receptor PAR-1 activation on endothelial progenitor cells enhances chemotaxis associated gene expression and leukocytes recruitment by a COX-2 dependant mechanism

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Background: Endothelial progenitor cells (EPC), and especially endothelial colony forming cells (ECFC), play an important role in neovasculogenesis and could promote therapeutic endothelial repair in patients with cardiovascular disease. However, little is known about their proinflammatory potential. We previously found that *in vitro* PAR-1 activation enhances ECFC angiogenic properties, suggesting the interest of ECFC preconditioning with the hexapeptide SFLLRN for future therapeutic use. We also showed that PAR-1 activation triggers IL-8 synthesis and release by ECFC, acting thus with a paracrine effect to enhance circulating angiogenic cells (CAC, the second EPC population) recruitment *in situ* and cell cooperation during neovascularization. Within an inflammatory context, COX-2 pathway has been also shown to regulate IL-8 in cancer cells.
Aims: To explore inflammation effects on ECFC through PAR-1 activation, and whether these effects modify ECFC angiogenic properties and chemotaxis/recruitment capacity.
Methods and Results: We first screened in ECFC, by real-time qPCR (Taqman® and SyBR Green® chemistry) on the ABI Prism 7900 HT (Applied Biosystems, Courtaboeuf, France), expression of genes known to be associated to inflammation and chemotaxis. Among them, 32 are expressed at a basal level, including CXCL, CCL, CSF, CXCR, CCR, CSF-R, Cell Adhesion Molecule and Interleukin families. Then ECFC PAR-1 was activated with the SFLLRN peptide and we found a significant increase in nine genes associated to chemotaxis expression, including CCL2 and CCL3. PAR-1 was next silenced using a specific small interfering RNA (siRNA, sc-36663, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and expression of those nine genes was abrogated. Furthermore, COX-2 expression was found to be dramatically up-regulated consequently to PAR-1 activation. COX-2 silencing with the specific COX-2-siRNA (sc-29279, Santa Cruz Biotechnology, Santa Cruz, CA, USA) also triggered the nine previous genes down-regulation. CCL2 and CCL3 were afterward tested on ECFC through classical angiogenic *in vitro* assays, and were found to stimulate ECFC migration and tubulogenesis. Conditioned media (c.m.) from control-siRNA- (Allstars Neg. control siRNA, Qiagen, Cambridge, MA, USA) and COX-2-siRNA-transfected ECFC, stimulated or not with SFLLRN, were produced to explore ECFC paracrine recruitment toward leukocytes with the hindlimb ischeamia model. Analysis with either flow cytometry for muscles lysates or Laser Doppler Perfusion Imaging, showed that the capacity to recruit leucocytes of the c.m. from ECFCs stimulated with SFLLRN, was abrogated when COX-2 was silenced.
Conclusions: ECFC PAR-1 activation induces CCL2 and CCL3 mRNA expression. Those chemotactic ligands enhance ECFC angiogenic properties and could increase leukocytes recruitment and cell cooperation through a COX-2 dependant mechanism.

OC 40.3

Peritoneal fluid reduces angiogenesis-related microRNA expression in cell cultures of endometrial and endometriotic tissues from women with endometriosis

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Background: Endometriosis, defined as the presence of endometrium outside the uterus, is one of the most frequent gynecological diseases. It has been suggested that modifications of both endometrial and peritoneal factors could be implicated in this disease. Endometriosis is a multifactorial disease in which angiogenesis and proteolysis are dysregulated. microRNAs (miRNAs) are small non-coding RNAs that regulate the protein expression and may be the main regulators of angiogenesis. Our hypothesis is that peritoneal fluid from women with endometriosis could modify the expression of miRNAs that regulate angiogenesis and proteolysis in the endometriosis development.
Aim: The aim of this study was to evaluate the influence of peritoneal fluid from patients on the miRNA expression profile and to correlate

this profile with several angiogenic and proteolytic factors in endometrial and endometriotic cell cultures from women with endometriosis compared with women without endometriosis.
Methods: Endometrial and endometriotic cell cultures were treated with peritoneal fluid pools from patients and controls. miRNAs expression arrays were performed in Affymetrix platform. Results were analyzed with Partek Genomic Suite software. To validate results from arrays, we studied the expression of six miRNAs by RT-PCR as well as protein and mRNA levels of vascular endothelial growth factor-A (VEGF-A), thrombospondin-1 (TSP-1), urokinase plasminogen activator (uPA) and plasminogen activator inhibitor-1 (PAI-1) by ELISA and qRT-PCR respectively. Informed consent was obtained from all subjects of the study and it was approved by the medical ethics committee of our Institution.
Results: GeneChip miRNA 2.0 Array contains 1105 human probes for mature miRNAs. Profiling of these miRNAs was completed for control endometrial and patient endometrial and endometriotic cell cultures treated with peritoneal fluids from patients and controls. Pathological peritoneal fluids induced a significant dysregulation of miRNAs profile: 85 mature miRNAs were differentially expressed ($P < 0.05$) (75 down-regulated and 10 up-regulated) in patient endometrial cell cultures in comparison to cell cultures from the same patient samples without peritoneal fluid exposition. After the 'in silico' study of the target genes for those miRNAs differentially expressed we selected three miRNAs related to angiogenesis (miR-17-5p, -20a, -221). Additionally, we selected three miRNAs that did not show significant differential expression but are related to angiogenesis and endometriosis (miR-16, -125a, -222). Patient peritoneal fluid pools induced a significant reduction of all studied miRNAs levels in endometrial and endometriotic cell cultures. Moreover, both peritoneal fluids induced a significant increase in VEGF-A, uPA and PAI-1 protein levels in all cell cultures without significant increase in mRNA levels. Endometrial cell cultures from patients treated with patient peritoneal fluid showed lower expression of miRNAs ($P < 0.05$) and higher expression of VEGF-A protein ($P < 0.01$) than cultures from controls ($P < 0.01$). Furthermore, a significant inverse correlation was observed between changes in VEGF-A protein and miR-17-5p ($r = -0.739$, $P = 0.001$), miR -20a ($r = -0.676$, $P = 0.001$), miR-125 ($r = -0.567$, $P = 0.01$) and miR-222 ($r = -0.494$, $P = 0.037$) levels in endometriotic cell cultures after treatment with peritoneal fluid.
Conclusion: In conclusion, this '*in vitro*' study indicates that peritoneal fluid from women with endometriosis modulates the expression of miRNAs that could contribute to the angiogenic and proteolytic disequilibrium observed in this disease.

OC 40.4
MicroRNAs expression profile in endometriosis: its relation with angiogenesis and fibrinolytic factors

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Background: Endometriosis, one of the most frequent gynecological diseases, is defined as the presence of endometrial tissue in extrauterine locations. It is a multifactorial disease in which the angiogenesis may be involved. Similarly to tumor metastases, endometriotic implants require neovascularization to proliferate, invade the extracellular matrix and establish an endometriotic lesion. microRNAs (miRNAs) are non-coding RNAs that regulate the translation of their mRNAs target. Recently, miRNAs have been described as the main regulators of angiogenesis and different expression of several miRNAs has been found in endometriosis.
Aim: The aim of this study was to analyze the miRNA expression profile in endometriosis and to correlate this profile with several angiogenesis (VEGF-A, TSP-1) and fibrinolytic factors (uPA, PAI-1) in endometriotic lesions in comparison with control and patient endometrium.
Material and Methods: miRNAs expression arrays were performed in Affymetrix platform and results were analyzed with Partek Genomic Suite software. To validate results from arrays, nine miRNAs were validated employing miRCURY LNA Universal RT microRNA PCR from EXIQON. VEGF-A, TSP-1, uPA and PAI-1 protein levels were quantified by ELISA. Fifty-three women with endometriosis and thirty-eight women without the disease (controls) were studied. Informed consent was obtained from all subjects of the study and it was approved by the medical ethics committee of our Institution.
Results: GeneChip miRNA 2.0 Array contains 1105 human probes for mature miRNAs. Profiling of these non-coding RNAs was completed in control and patient endometrium and endometriotic lesions (ovarian endometrioma, peritoneal implants and rectovaginal nodules). The PCA of the results obtained from Affymetrix GeneChip miRNA 2.0 arrays confirmed that endometriotic tissues clustered separately from control endometrial tissues. When the endometriotic tissue samples were compared to the endometrial control samples, 145 mature miRNAs were found to be differentially expressed ($P < 0.05$) (69 up-regulated and 76 down-regulated). The 'in silico' study of the target genes for those miRNAs differentially expressed enabled us to select nine miRNAs related to angiogenesis and proteolysis (miR-16, -29c, -202, -411, -411*, -424, -449b, -636, 935) with a significant different expression ($P < 0.05$) in endometriotic tissues in comparison with the control endometrial tissue. Patient endometrium showed higher VEGF-A levels ($P < 0.01$) and lower expression of miR-202 and miR-449b ($P < 0.05$) in comparison to control endometrium. However, ovarian endometrioma showed significantly higher expression of the angiogenic inhibitor TSP-1 ($P < 0.01$) and higher expression of miR-29c and miR-202 than control and patient endometrium ($P < 0.05$). A significant inverse correlation between miR-449b and VEGF-A protein levels ($r = -335$, $P < 0.05$) was obtained in patient endometrium and a direct correlation between miR-202 and TSP-1 protein levels was observed in ovarian endometrioma ($r = -380$, $P < 0.05$). Peritoneal implants showed a significant increase in VEGF-A in comparison to ovarian endometrioma ($P < 0.01$).
Conclusions: The higher angiogenic activity observed in patient endometrium might contribute to the capability of implantation of endometrial cells at ectopic sites. The different expression of miRNAs could modulate the expression of angiogenic factors which may play an important role in the pathogenesis of endometriosis. Functional studies are needed to confirm the VEGF-A targeting by the miRNAs found in the present study.

OC 41 – Animal Models of Antiphospholipid Syndrome

OC 41.1
Adenosine generation protects in a murine model of antiphospholipid antibody-induced miscarriages

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Background: Autoantibodies (aPL-ab) generated in Antiphospholipid Syndrome (APS) cause arterial and venous thrombosis, and miscarriages. Antiphospholipid antibodies stimulate cytokine release leading to tissue factor (TF) expression, inflammation and complement activation, all of which have been implicated in the pathogenesis of APS-related foetal loss. ATP and ADP are extracellular purines and important signaling molecules that activate inflammation and thrombosis,

respectively. ATP and ADP are hydrolysed by the cell surface enzyme CD39 (NTPDase) to AMP and AMP in turn is subsequently hydrolysed to adenosine by the action of another enzyme, CD73. In contrast to the effect of ATP and ADP, adenosine signals via A2 receptors to inhibit inflammation and suppress TF expression on endothelial cells and monocytes. We have previously shown that CD39 over expression is antithrombotic and protective against complement-mediated endothelial activation (Dwyer et al., J Clin Invest 2004; 113: 1440–46) and suppresses TF expression on pancreatic islet cells (Dwyer et al, Transplantation 2006; 82 (3):428–32). Moreover, we have also shown that adenosine generated by the action of CD39 is vascular-protective in a model of ischaemia reperfusion injury (Crikis et al., Am J Trans-plant 2010; 10: 2586–95).

Aims: To analyse the role of purineregic nucleotides in APS miscarriages.

Methods: We have established an aPL-ab-induced model of miscarriages by administration of aPL-ab (purified from patients with APS) to pregnant mice (10 mg on day 8 and 12 of pregnancy followed by analysis on day 15).

Results: We applied this model to mice with modifications of several of the purinergic pathway enzymes:

1 CD39-Transgenic (CD39-TG on a BALB/c strain) mice with increased hydrolysis of ATP and ADP to AMP and adenosine: demonstrate reduction in aPL-ab-induced miscarriages. Resorption frequency in wild-type (WT-BALB/c) treated with non-immune IgG, 21% ±6 (SEM); WT treated with aPL-ab, 40% ±5; CD39-TG treated with aPL-ab, 14% ± 3; ($P = 0.0008, n = 7$/group).
2 CD39-null (CD39−/−, on a C57Bl/6 strain, which is more resistant to miscarriages than BALB/c) mice with decreased hydrolysis of ATP and ADP: demonstrate higher frequency of miscarriages. Resorption frequency with aPL-ab: 3 ± 2% in WT-C57BL/6 and 15 ± 4% in CD39−/−, $P = 0.036, n = 7$/group).
3 CD73−/− (C57BL/6 strain) mice cannot hydrolyse AMP further to adenosine: demonstrate higher frequency of miscarriages. Resorption frequency with aPL-ab: 2 ± 2% in WT-C57BL/6 and 11 ± 2% in CD73−/−, $P < 0.05, n = 7$/group).
4 Adenosine receptor $A_2AR-/-$ show an increased trend to miscarriages (P = ns, as yet).

We have initiated studies to discriminate between the effects of CD39 on the uterus versus placenta.

We further demonstrated that TF mRNA expression is more (> 2-fold, $P < 0.05$) in cohorts with increased miscarriages. Also, complement activation (detected by immunohistochemistry) and TNF expression (TNF mRNA as a marker of inflammatory cytokine release) is reduced in the placentae of CD39-TG mice that have fewer miscarriages as compared with WT after aPL-ab administration.

Conclusions: Hydrolysis of ATP and ADP and adenosine generation is protective in APS miscarriages by reducing inflammation, TF expression and complement activation.

OC 41.2

In vivo thrombus formation fostered by a human cofactor independent monoclonal anticardiolipin antibody

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Background: Antiphospholipid syndrome (APS) is an autoimmune disease characterized by arterial and/or venous thrombosis and thromboembolic events associated with the presence of antiphospholipid antibodies (aPL). APLs are a heterogeneous family of autoantibodies that bind negatively charged phospholipids such as cardiolipin, phosphatidylserine, and lysobisphosphatidic acid and phospholipid binding proteins such as β2-glycoprotein I (β2GPI). Currently, it is postulated that only aPL binding to β2GPI are able to induce thrombosis *in vivo*. We have previously cloned monoclonal aPL IgG from patients with the APS. One of them, HL5B, binds to cardiolipin and phosphatidylserine in a cofactor independent manner. It does not bind to β2GPI. *In vitro* HL5B induces prothrombotic and proinflammatory pathways in monocytes and endothelial cells.

Aim: We intended to investigate the potential of HL5B to induce thrombus formation *in vivo*.

Methods: Thrombus formation in living mice has been estimated using high speed real-time intravital fluorescence microscopy. In anesthetized mice a median laparotomy was performed and the inferior vena cava (IVC) was exposed by atraumatic surgery. A space holder was placed on the outside of the vessel and a permanent narrowing ligature was performed exactly below the left renal vein. Subsequently, the wire was removed to avoid complete vessel occlusion. Thus, flow reduction was induced in IVC. Platelets and leukocytes were visualised by labelling them respectively with rhodamine B and acridine orange. Fibrin was visualised with labelled anti-fibrin antibody. Rotem thrombelastography has been performed to evaluate the fibrin formation in whole blood at physiological temperatures (37 °C). To estimate tissue factor (TF) dependent fibrin formation corn trypsin inhibitor (CTI) was added to inhibit FXIIa and thus to block the contact pathway of blood coagulation.

Results: In IVC of anesthetized mice thrombus formation was dramatically enhanced 3 h after intravenous injection of HL5B but not by control IgG. Thrombus formation was abolished by preincubation of HL5B with a synthetic peptide which blocks the antigen binding site of HL5B and prevents binding of HL5B to cardiolipin and other phospholipids. Moreover, an inhibitory anti-TF antibody prevented thrombus formation induced by HL5B. In addition, HL5B increased fibrin formation in mouse carotid artery model of thrombosis. Clotting time of whole blood drawn from mice injected with HL5B was shortened in comparison to mice injected with IgG. A similar observation was made with human blood preincubated with HL5B.

Conclusions: Our results show that the human monoclonal aPL HL5B promotes arterial and venous thrombus and fibrin formation via TF pathway in living mice. To our knowledge, this is the first description of *in vivo* thrombus induction by a cofactor independent anticardiolipin aPL which has significant implications for our understanding of the APS.

OC 41.3

IgA anti-β2glycoprotein I antibodies are pathogenic in a mouse model of antiphospholipid syndrome

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Background: Recently exclusive IgA anti-β_2GlycoproteinI ($a\beta_2GPI$) seropositivity – in the absence of any other antiphospholipid (aPL) antibodies- has been reported, particularly in SLE patients. A significant proportion of those patients (70–80%) were found to have Antiphospholipid Syndrome (APS)-clinical manifestations (e.g. thrombosis and/or pregnancy losses). APL antibodies of the IgG and IgM isotypes have been shown to be pathogenic *in vivo*, but there is limited data demonstrating the thrombogenicity of IgA $a\beta_2GPI$ antibodies *in vivo*.

Aims: Here we examined the effects of affinity purified IgA $a\beta_2GPI$ antibodies isolated from patients with exclusive IgA $a\beta_2GPI$ positivity on thrombus formation and tissue factor (TF) upregulation in mice.

Methods: IgA was isolated from pooled sera of four APS patients (IgA-APS) with isolated IgA $a\beta_2GPI$ titers (≥ 80 SAU) – two had strokes, one had a confirmed deep vein thrombosis and one had two pregnancy losses – and from normal human serum (IgA-NHS) using

an Immobilized Jacalin column (Pierce Biotechnology). IgA aβ_2GPI in the IgA-APS and in the IgA-NHS preparations was determined by ELISA (INOVA Diagnostics), the protein concentration by the Bradford method and the lupus anticoagulant by using a modified silica clotting time (SCT) assay. CD1 mice were inoculated with 500 μg of either IgA-APS or IgA-NHS on two occasions at a 48 h interval. Seventy-two hours after the first injection, the size of induced thrombi in the femoral vein was determined as described previously (Circulation 1996; 94: 1746–1751). Tissue factor activity was determined in homogenates of pooled carotid arteries and in peritoneal macrophages using a chromogenic assay. Student's t test was used to determine differences between IgA-APS and IgA-NHS treated mice.

Results: IgA-APS and IgA-NHS were rendered endotoxin free by the Limulus amebocyte lysate assay, and did not have detectable levels of IgG or IgM. The IgA-APS preparation but not the IgA-NHS was positive for IgA aβ_2GPI (103.7 SAU) and LA [SCT ratio IgA-APS/IgA-NHS = 2; normal < 1.2]. Thrombus size (1.7-fold increase, $P = 0.02$), TF activity in carotids (2.9-fold increase, $P = 0.004$) and TF activity in peritoneal macrophages (3.5-fold increase, $P = 0.0003$) were significantly elevated in IgA-APS treated mice when compared to mice treated with IgA-NHS.

Conclusion: These data show for the first time that IgA aβ_2GPI antibodies are thrombogenic and upregulate TF in mice. Detection of IgA aβ_2GPI antibodies – currently not included in the classification criteria for APS – may further identify a group of patients with APS-associated clinical manifestations that otherwise would have been missed with tests used routinely in the clinical laboratory to confirm APS.

OC 41.4

Affinity purified antibodies directed against domain I of β2GPI are pathogenic in a mouse model of thrombosis

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Background: Circulating IgG antiphospholipid antibodies (aPL) against β2glycoprotein I (a2GPI) are a serological hallmark for diagnosis of the antiphospholipid syndrome (APS). We and other groups have shown that aPL targeting domain I (DI) of β2GPI (aDI) are APS-specific, predominantly correlating with (venous) thrombosis. We have also demonstrated that recombinant DI inhibits aPL-induced thrombosis in a mouse microcirculation model. To date however, no study has confirmed a direct pathogenic link between aDI and features of the APS.

Aims: We have now employed the same mouse model to determine the thrombogenic potential of affinity purified polyclonal aDI IgG derived from APS sera.

Methods: Serum from one female APS patient was incubated with His-tagged DI coupled to nickel beads to adsorb aDI antibodies. The bead-serum mix was then centrifuged, serum re-collected and antibodies bound to DI-coupled beads were eluted. IgG from re-collected serum (aDI-poor) and eluted fractions (aDI-rich) was then obtained by protein G purification. Serum and IgG fractions (100 μg/mL) were tested for aCL (GPLU), aβ2GPI (GBU, in-house calibrator) and aDI (GDIU, in-house calibrator) activity. For *in vivo* experiments, CD1 mice were inoculated (on two occasions at a 48 h interval) with 100 μg/mL of either aDI-poor IgG, aDI-rich IgG, or IgG from healthy volunteers (NHS-IgG) as a control (five animals per group). Seventy-two hour after the first injection, the size (μm^2) of induced thrombi in the femoral vein was determined (Circulation 1996;94:1746–1751). Tissue factor (TF) activity (pM/mg/mL protein) was determined in homogenates of pooled carotid arteries and peritoneal macrophages using a chromogenic assay. Mouse serum was obtained on the day of surgery and tested for the presence of circulating whole human IgG.

Results: Purified aDI-rich IgG displayed high aCL (90GPLU), aβ2GPI (95GBU) and aDI (50GDIU) activity whilst aDI-poor IgG displayed high aCL (90GPLU) but reduced aβ2GPI (47GBU) and aDI (17GDIU) activity. NHS-IgG was negative in all assays. aDI-rich IgG induced significantly larger thrombi compared to aDI-poor and NHS-IgG ($P < 0.001$). In addition, aDI-rich IgG induced the greatest increase in TF activity in carotids (1.4-fold) and peritoneal macrophages (3.3-fold) compared to NHS-IgG. In contrast, aDI-poor IgG induced smaller thrombi and less macrophage TF activity compared to aDI-rich IgG and did not increase carotid TF activity above that of NHS-IgG

Conclusion: This is the first study to directly demonstrate the thrombogenic potential of affinity-purified aDI IgG *in vivo*. Despite aDI-poor IgG retaining aCL and, to a lesser extent, aβ2GPI activity, significantly larger thrombi and elevated TF activity were induced with aDI-rich IgG. Our findings support the concept that although circulating aPL recognizing different domains of β2GPI can be pathogenic, the major population that drive thrombosis are directed against DI.

OC 42 – Bleeding and Anticoagulants

OC 42.1

Comparison of four scores for the prediction of major bleeding in patients with acute venous thromboembolism. Findings from the RIETE registry

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Background: Stratification of the individual bleeding risk prior to initiation of anticoagulation in patients with acute venous thromboembolism (VTE) has the potential to assist clinicians for decisions about the proper intensity and duration of antithrombotic therapy. It is unclear which of the four properly validated and internationally accepted scores that have been recommended for the achievement of this important task has the best predictive value.

Aims: We sought to compare the predictive value of four scores (by Landefeld, Beyth, Kuijer and Ruiz-Gimenez, respectively) for the development of major bleeding complications (defined as widely accepted) occurring in the first 3 months in patients with acute VTE treated with conventional anticoagulation.

Methods: Based on patients demographics and baseline clinical characteristics available for the population of RIETE Registry (an ongoing international, multicenter, prospective registry of consecutive patients with VTE who were all followed-up for at least 3 months), we identified the subgroup of patients presenting all the prognostic variables required for the determination of the four predictive scores, and then calculated the ability of each score for predicting the bleeding risk.

Results: Of 40 265 patients enrolled in this registry, we identified 8717 patients (21.6% of RIETE population) meeting the eligibility criteria. Overall, 82 patients (0.9%) experienced at least one episode of major bleeding, which was fatal in 20 (24.4%), within 90 days of the index VTE event. The proportion of patients classified as having a low risk varied between 1.2 and 3.7%, that of patients having an intermediate risk between 76 and 93%, and that of patients classified as having a high risk between 6.1% for the Kuijer score and 18% for the Ruiz-Gimenez score. The area under the receiver operating characteristic

ranged between 0.55 and 0.60, the positive predictive value between 1.5 and 3.2, and the likelihood ratio between 0.72 and 1.59.
Conclusions: All the existing validated scores show a very low ability for prediction of the bleeding risk in patients with acute VTE undergoing conventional anticoagulation

OC 42.2

Recurrence of intracranial hemorrhage after resumption of anticoagulation in patients who had a first episode occurred during Vitamin K Antagonists anticoagulation. Results of a collaborative study

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Background: Vitamin K antagonists (VKA) treatment is associated with an increased risk of intracranial hemorrhage (ICH). However, the risk of recurrent ICH after a first episode is still uncertain. Some patients carry a so high thromboembolic risk, requiring to start or restart VKA treatment after ICH.
Aim: Aim of our study was to evaluate the risk of recurrent ICH in patients on VKA after a first episode.
Methods: We collected data of patients eligible for the study from the database of 27 Centres affiliated to the Italian Federation of Anticoagulation Clinics.
Results: We enrolled 267 patients (males 163, 61%, median age 73.9 years). Eighty-eight patients (33%) were on treatment for mechanical heart valves, 121 (45.3%) for atrial fibrillation and 45 (16.8%) for venous thromboembolism. The index event was spontaneous in 99 patients (38.5%); the site was Intraparenchimal in 86 patients (32.8%), subdural hematoma in 131 (4a 9.0%) and subaracnoid in 45 (17.2%). The total period of follow-up after resumption of treatment was 778 pt-years. An ICH recurred in 20 patients (7.5%; rate 2.56 × 100 pt-years) at median time of 16.5 months and was fatal in five patients (25%; rate 0.4 × 100 pt-years). Male sex, hypertension, prosthetic valves, previous ischemic stroke, renal failure, cancer and spontaneous events were associated to the risk of recurrence, even if none of them reached the statistical significance.
Conclusions: In our study patients with history of ICH and who need anticoagulation carry a significant risk of recurrent ICH. These patients require a careful evaluation of thromboembolic risk to estimate the net clinical benefit of treatment.

OC 42.3

The predictive ability of bleeding risk stratification models in very old patients on VKA treatment for venous thromboembolism. Results of the prospective collaborative EPICA study

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Background: The optimal duration of anticoagulant treatment after venous thromboembolism (VTE) should be evaluated in relation to bleeding risk. This assessment is particularly difficult with elderly patients, because of their increased risk of both recurrences and hemorrhages. Bleeding risk stratification models have been proposed, but their predictive ability in very elderly patients is unknown.
Aim: We aimed to assess six bleeding stratification models in this setting, by using information available in our dataset.
Methods: Patients aged ≥ 80 years receiving vitamin K antagonists (VKA) for the secondary prevention of VTE were eligible for this prospective cohort study. All patients were followed at Italian Anticoagulation Clinics for the monitoring of VKA treatment. Risk factors for bleeding were collected and major bleeding events and mortality were documented during follow-up. The association of bleeding events with the available risk factors was tested by means of Cox regression analysis; the *c* statistic was used to quantify the predictive validity of the classification schemes.
Results: One thousand and seventy-eight patients (37.2% males, mean age 84 years) were enrolled in the study, for a total observation period of 1981 patient-years. The rate of major bleeding was 2.4 × 100 patient-years (47 events, 1 was fatal). Mortality rate was 5.2 per 100 patient-years. None of the considered risk factors resulted to be significantly associated with bleeding events. The predictive validity of the risk stratification models was low and the most accurate model was not specifically developed for VTE patients ($HEMORR_2HAGES$, c-statistic 0.60, 95% CI 0.49–0.70).
Conclusions: Bleeding risk stratification models appear to have little accuracy in very elderly VTE patients.

OC 42.4

Statin use and bleeding complications during treatment with vitamin K antagonists: a cohort study in 8188 patients with atrial fibrillation

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Background: Statins decrease atherosclerosis which could lead to improved vessel wall health. Theoretically, this could result in fewer bleeding complications in patients who are treated with vitamin K antagonists (VKAs). Additionally, atherosclerosis is known to increase with age, possibly resulting in different effects of statins along strata of age.
Aims: To determine the effect of statins on bleeding complications during VKA treatment, overall and for different age categories.
Methods: All patients with atrial fibrillation who started treatment with VKAs at the Anticoagulation Clinic Leiden between 2003 and 2009 were selected. Information on medication use and bleeding complications were obtained from patient records. Hazard ratios (HRs) and 95% confidence intervals (95%CI) were calculated for minor and major bleeding by means of a Cox regression model with time-varying determinants. HRs were also computed for three strata of age: 50–65 years ('young'), 65–75 years ('middle aged'), > 75 years ('elderly') and adjusted for sex, INR-target range, diabetes, hypertension, and use of anti-platelet drugs. Analyses were stratified by incident (patients who started using statins during follow-up) and prevalent statin use (patients who used statins at baseline) compared with non-use. This avoids the overoptimistic effects obtained from analyses in prevalent users, as they have already 'survived' a period of statin treatment before starting VKA treatment (Danaei *et al*., Am J Epidemiol, 2012). Furthermore, they are generally healthier than subjects who started treatment but discontinued after some time. Neither informed consent nor approval by a medical ethics committee is, according to Dutch law, required for studies in which data are collected from the records by the treating physician.
Results: Eight thousand one hundred and eighty-eight Patients were treated with VKAs for a mean period of 2.1 years. Four hundred and fifty-two Major and 1787 minor bleeding complications occurred during the follow up of 18 105 patient-years. Statin use was associated with slightly reduced risks of minor bleeding (HR 0.88 95%CI 0.79–

0.98) and major bleeding (HR 0.83 95%CI 0.67–1.02). After stratification on age and restriction to incident statin use, all HRs in the various age-categories increased to estimates indicative of absence of effects or increased risks (above unity), ranging from 0.98 to 3.22. One exception occurred for major bleeding in the elderly where the HR in incident users remained below unity (HR 0.66 95%CI 0.40–1.07). Apart from chance variation as an explanation, this may also be the result of additional selection bias, since, according to the guidelines, statins should only be prescribed to elderly with a reasonable life expectancy.

Conclusions: In overall analyses, statins appear to protect against bleeding complications in patients receiving anticoagulant treatment. After stratification on age and restriction to incident use, in an effort to reduce selection bias, these protective effects largely disappeared. This bias giving overoptimistic views on the complementary effects of statins is similar to what was found in earlier studies on hormone replacement therapy – suggesting protection against coronary heart disease which was not borne out in randomised trials – and may also be present in other observational studies on statins.

OC 43 – Clinical Aspects of Atherosclerosis

OC 43.1

Reduced propagation of blood outgrowth endothelial cells in subjects with advanced subclinical atherosclerosis

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Background: Atherosclerotic coronary artery disease (CAD) represents one of the principal causes of mortality in developed countries. Clinical manifestations of CAD consist of acute or chronic myocardial damage induced by a decrease in supply of oxygen-rich blood. This sequel of events is due to the formation of atherosclerotic plaques resulting in the tightening of the lumen in the affected coronary vessels. Several cellular components, including endothelial cells from the arterial wall are involved in this process.

Aims: In order to better understand the pathophysiology of CAD, we have established cultures of blood outgrowth endothelial cells (BOECs) from peripheral blood of patients with premature cardiovascular disease (CVD) and their first degree relatives.

Methods: Patients with premature CVD (men < 51 year, women < 56 year) and their apparently healthy first degree relatives were included. From 70 patients and 157 first degree relatives, blood was drawn for cultures of blood outgrowth endothelial cells. Furthermore, all first degree relatives underwent multi-detector computed tomography to establish coronary calcification (CAC) as a marker of subclinical CVD. On average, from 14 to 21 days after isolation and establishment of cultures, BOEC colonies were observed. These cultures were further propagated, establishing their proliferative capacity and subsequently cryopreserved.

Results: As for the relatives: 99 (63%) had a CAC score of 0 and were considered relatives without subclinical CVD. The other 58 (37%) relatives displayed a positive CAC score, and were considered as subjects with subclinical CVD. We were able to isolate BOEC colonies in 52 (74%) of the patients, in 79 (80%) of the relatives without subclinical CVD and in 42 (72%) of the relatives with subclinical CVD. In relation to the ability of the isolated colonies to proliferate, we were capable to expand colonies in 43 (61%) of the patients, in 31 (53%) of the relatives with subclinical CVD and in 69 (70%) of the relatives without subclinical CVD. Differences between patients and relatives without subclinical CVD were not significant ($P = 0.272$). Differences between relatives with and without subclinical CVD were significant ($P = 0.039$; adjusted for age, sex and smoking).

Conclusion: These observations demonstrate that the number of circulating BOEC precursors in relatives with subclinical CVD is diminished when compared to relatives without subclinical CVD and patients. Additionally, the proliferative capacity of the isolated endothelial cells was similar between relatives without subclinical CVD and patients. However, it was significantly lower in relatives with subclinical CVD. The mechanism underlying the reduced frequency of BOEC precursors in the circulation of these subjects is currently under investigation.

This clinical study has been performed under patient's informed consent and approved by a recognised medical ethics committee, complying as well the Declaration of Helsinki .

OC 43.2

Plasma levels of matrix metalloproteinases and circulating endothelial cells in patients with peripheral arterial disease: relationship with disease severity and effect of treatment with prostanoids

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Background: Peripheral Arterial Disease (PAD) is one of the most common manifestations of atherosclerosis and is associated with inflammatory and endothelial changes. The presence of PAD is a powerful and independent predictor of cardiac and cerebral ischemic events. Matrix Metalloproteinases (MMPs), a family of zinc-dependent endopeptidases able to degrade the extracellular matrix, are involved in atherosclerosis development and complications. Iloprost, a stable prostacyclin analogue, promotes local perfusion and relieves symptoms in patients with Critical Limb Ischaemia (CLI), often with an effect lasting for weeks or months after the end of the infusion. Our aim was to investigate circulating MMPs plasma levels and CECs in patients with PAD, their association with disease severity and the effects of treatment with iloprost infusion.

Materials and Methods: Eighty-eight patients suffering from PAD were enrolled in the study. According to Leriche-Fontaine classification, seven patients were asymptomatic (class I); 28 patients had intermittent claudication (class II), 14 patients presented rest pain (class III) and 39 had trophic lesions (class IV). Forty-one age- and sex-matched healthy subjects were enrolled as controls. Patients with CLI (class III and IV) were treated with daily intravenous infusions of Iloprost for 14–28 days. Blood samples were collected immediately before and at the end of the daily iloprost infusion on the first and last day of treatment for the measurement of plasma MMPs by zymography (ng/mL), CECs (cells/microl) by flow cytometry. Plasma vWF (%-Ag), MPO (ng/mL) and elastase (ng/mL) were also measured by ELISA. Circulating endothelial cells (CECs) and von Willebrand Factor (vWF) represent markers of endothelial damage (ED). Mieloperoxidase is a marker of inflammation and leukocyte activation.

Results: Plasma levels of MMP-9, but not of MMP-2, were significantly higher in PAD patients compared with controls (583.9 ± 78.8 vs 164.3 ± 25.6 ng/mL, $P < 0.001$) and more precisely in stage II (475.3 ± 104.0 ng/mL, $P < 0.005$), stage III (835.6 ± 186.8 ng/mL, $P < 0.001$); stage IV (570.3 ± 123.4 ng/mL, $P < 0.001$).

CECs were also significantly higher in PAD patients than in controls (1.83 ± 0.23 vs 0.32 ± 0.05 cells/microl) and with a progressive increase with the advancement of the disease stage (stage I: 0.067 ± 0.11 cells/microl, $P < 0.05$; stage II 2.36 ± 0.55 cells/microl, $P < 0.001$; stage III 3.03 ± 1.89 cells/microl, $P < 0.001$; stage IV 3.08 ± 0.91 cells/microl, $P < 0.001$). vWF and MPO were also significantly increased in PAD patients (170.8 ± 7.59 vs 95.87 ± 4.20 Ag%, $P < 0.001$; 32.20 ± 4.22 vs 15.91 ± 2.98 ng/mL, $P < 0.005$, respectively). Treatment with iloprost decreased significantly plasma levels of MMP-9, both at the first and last day of infusion (day 1 pre =

699.6 ± 98.3 post = 407.9 ± 44.4 ng/mL, $P < 0.001$; day 21–28 pre = 566.2 ± 112 post = 406 ± 75.5 ng/mL, $P < 0.05$), while it decreased MMP-2 only after the last infusion (day 1 pre = 780.9 ± 66.1; day 21–28 post = 643.3 ± 55.0 ng/mL, $P < 0.05$). Moreover, Iloprost decreased CECs (day 1 pre = 2.71 ± 0.65 post = 1.78 ± 0.48, $P < 0.05$; day 21–28 pre = 1.33 ± 0.92 post = 1.08 ± 0.57 cell/microl).
Conclusions: MMP-9, CECs, vWF and MPO are significantly enhanced in PAD. Treatment with Iloprost significantly reduces MMPs and CECs but not vWF and MPO. The prognostic value of MMP-9 and CECs in CLI and of their changes induced by iloprost and the potential for pharmacologic targeting of MMPs in PAD deserve further evaluation.

OC 43.3

Human coronary thrombus formation is associated with degree of plaque disruption and expression of tissue factor and hexokinase II in atherosclerotic plaques

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Background: Although coronary atherosclerotic plaque disruption is a trigger of acute myocardial infarction (AMI), it does not always result in complete luminal occlusion and AMI. Therefore, the formation of a large thrombus is critical to the onset of AMI, and determinants of the size of coronary thrombus after plaque disruption remain unknown.
Aims: This study of autopsy cases aimed to determine relationships between vascular factors and thrombus size in human coronary arteries in AMI and non-cardiac death.
Methods: We examined 43 coronary arteries with thrombi from autopsy cases of AMI (plaque rupture, $n = 15$; plaque erosion, $n = 8$) and non-cardiac death (plaque rupture, $n = 5$; plaque erosion, $n = 15$). Culprit lesions of coronary arteries were examined histologically and immunohistochemically using antibodies against CD68, CD163, smooth muscle actin, tissue factor (TF), glucose transporter (Glut)-1 and hexokinase (HK)-II. We used image-analysis software to measure thrombus size, ratio of thrombus area to luminal area, ratio of intima to media (I/M ratio), stenotic ratio, degree of plaque disruption and the numbers of nucleated cells that were immunopositive for Glut-1 and HK-II. We defined the degree of plaque rupture as the extent of disrupted fibrous cap over a lipid core, and plaque erosion as the extent of damage beneath mural thrombus. Immunopositive areas for CD68, CD163, SMA and TF were assessed using color imaging morphometry.
Results: Thrombus size, ratio of thrombus area to luminal area, and the I/M ratio were significantly larger in coronary arteries with AMI than in those with asymptomatic thrombus. Immunopositive areas for TF were abundant in ruptured plaques of AMI. Macrophages (immunopositive for CD68 or CD163) surrounded the lipid core of ruptured plaques and the eroded portions of plaques of AMI. Staining for HK-II and Glut-1 was somewhat positive in macrophages and abundant in ruptured plaques in AMI. The size of coronary thrombus positively correlated with the degree of plaque disruption ($r = 0.82$), I/M ratio ($r = 0.36$) and with immunopositive areas for CD68 ($r = 0.32$) and TF ($r = 0.44$). The ratio of thrombus area to luminal area also positively correlated with the degree of plaque disruption ($r = 0.79$), I/M ratio ($r = 0.57$), stenotic ratio ($r = 0.42$) and immunopositive areas for TF ($r = 0.47$) and HK-II ($r = 0.37$).
Conclusions: Degree of plaque disruption, large plaque with stenosis, and TF content in atherosclerotic plaque would be important factors to the onset of AMI, and HK-II expression in macrophages might reflect thrombogenicity of coronary plaques.

OC 43.4

Vitamin K-antagonists: a two edged sword. Bi-phasic effect of VKA on atherosclerotic plaque development

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Background: Vitamin K-antagonists (VKA) are treatment of choice and standard care for patients with venous thrombosis and thromboembolic risk. Both in experimental animals and humans, VKA have shown to promote medial and intimal vascular calcification. Previously we have shown that VKA administration induce plaque vulnerability in atherosclerotic plaques. However, clinically it is not known which patients on VKA develop severe vascular calcification. Current studies demonstrate a role for coagulation in atherogenesis and novel oral anticoagulants (NOAC) seem to inhibit atherogenesis.
Aim: In this study we investigated the time-dependent effects of VKA on atherogenesis.
Methods: Sixty-six male apoE (−/−) (8 weeks) mice were put on a vitamin K deficient western type diet mixed with 100 mg/kg vitamin K1 (control group) or 1.5 g/kg vitamin K1 and 3.0 g/kg warfarin (warfarin group). Mice were sacrificed after 7, 13 or 19 weeks and histochemical plaque analyses were performed.
Results: De novo plaque development was significantly reduced in warfarin treated mice, indicating a beneficial effect of anticoagulation on atherogenesis. *In vitro*, warfarin significantly reduced VSMC (vascular smooth muscle cell) migration and nodule formation. In the last timepoint, severe atherosclerosis developed in both control and warfarin groups. However, warfarin treatment was detrimental for both plaque development and plaque phenotype. Immunohistochemical analyses showed a significant increase in macrophage content, increased elastin breaks and an increase in both medial and intimal calcification. On the contrary, control mice displayed an increase in plaque collagen and VSMC content, indicative for a stable plaque phenotype. *In vitro*, ox-LDL increased calcification of VSMCs, which was reinforced by co-treatment with warfarin.
Conclusion: Our data indicate that anticoagulation with VKA can be beneficial for both lowering thrombotic risk and inhibiting atherogenesis, but might be detrimental when given to patients suffering from arterial disease. Detailed knowledge about effects of different anticoagulants on atherogenesis will help to improve treatment of patients in need of anticoagulant therapies and support personalized medicine.

OC 44 – Coagulation – II

OC 44.1

TALEN-mediated vitamin K epoxide reductase knockout in human cells for vitamin K cycle study

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Vitamin K epoxide reductase (VKOR), an endoplasmic reticulum (ER) membrane protein, is responsible for regeneration of reduced vitamin K (vitamin K hydroquinone, KH_2) from its oxidized form (vitamin K epoxide, KO) in the vitamin K cycle. KH_2 is a cofactor of gamma-glutamyl carboxylase that post-translational modifies vitamin K-dependent proteins involved in several biological functions including blood coagulation. Structure-Function studies of VKOR and its homologues have led to different proposals for mechanisms for the action of VKOR. Furthermore, although VKOR polymorphisms have been successfully used as pharmacogenetic markers for warfarin dosage predictions, enzymatic studies of VKOR mutants found in patients do not well correlate with their clinical resistance phenotype. These discrepancies presumably arise from the *in vitro* activity assay of VKOR performed in an artificial environment using the non-physiological reductant dithiothreitol.

To overcome these problems, we report here the establishment of a transcription activator-like effector nucleases (TALENs)-mediated VKOR knockout human cell line for VKOR *in vivo* activity assay. We designed TALEN pairs to target human VKOR in HEK293 cells stably expressing a reporter protein (FIXgla-PC) (Tie, JK *et al.* Blood, 2011, 117:2967–74). Transfected cells were functionally screened by determining the efficiency of reporter protein carboxylation when the cell colonies were first fed with 5 μM KO and then with 11 μM vitamin K. Our results show that it is necessary to knock out both VKOR and VKOR-like enzyme in HEK 293 cells in order to eliminate KO reductase activity. Using the established double gene knockout cell line, we further confirmed that the conserved loop cysteines (43 and 51) in VKOR are not required for intra-molecular electron transfer. In addition, we tested warfarin resistance of VKOR mutants found in individuals resistant to warfarin treatment. Our results show that 10 of the VKOR mutants that exhibit no activity in *in vitro* assays are active in our *in vivo* assay and shows different warfarin resistances.
In conclusion, we established a human cell line with both VKOR and VKOR-like enzymes ablated by TALENS for the functional study of the vitamin K cycle. We further demonstrated that this cell line can be used for VKOR function and warfarin resistance studies and will be a useful tool to understand the vitamin K cycle.

OC 44.2
Recombinant FVIIa acts independently of tissue factor to restore haemostasis and clot dynamics

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Background: Recombinant factor seven (rFVIIa, NovoSeven ®) is a by-passing agent for the treatment of haemophilia patients with inhibitors. Despite several lines of circumstantial evidence no direct *in-vivo* studies have been performed to show that rFVIIa acts independently of tissue factor (TF) at pharmacological doses.
Aims: To test the hypothesis that rFVIIa acts in a TF-independent manner after laser induced vascular injury and after transection of the tail tip.
Methods: TF-low ($mTF^{-/-}$, hTF^{+}, expressing 1% hTF) mice in which human TF was abrogated by anti hTF-pAb, and heterozygotes controls ($mTF^{-/+}$, hTF^{+}) were used. Recombinant FVIIa was administered at a dose level 10 mg/kg in both experiments through a jugular catheter. Ten mg/kg has previously been shown to normalize bleeding haemophilia A mice. The catheter was also used for the dosing of anti hTF-pAb (or vehicle) and any fluorophores.
Laser induced vascular injury: The aggregating platelets (labelled with Dylight fluorophore conjugated to rat anti mouse GP1bβ mAb) and degranulating platelets (labelled with Alexa 633 fluorophore conjugated to anti-P-selectin mAb) were quantified after intra vital fluorescence microscopy for 5 min following laser induced vascular injury to arterioles of the cremaster muscle. Injuries for which 5–10 RBCs were found exterior to the lamina elastic interna were included. The maximum fluorescence and the integrated fluorescence over time were calculated for each of the two fluorophores, giving a total of four endpoints/parameters. The data were analysed by a mixed model, parameter = group, where group was a fixed effect and the individual animal was a random effect.
Tail bleeding: Five and 10 min prior to transection of 4 mm of the tail tip the fully anesthetised mice were dosed with anti hTF-pAb (or vehicle) and rFVIIa, respectively, through a jugular catheter. The tail was immersed in saline (37 °C). Bleeding time was recorded for 30 min and blood loss determined by measuring the haemoglobin concentration. Kruskal-Wallis test and Dunn's post test were applied.
Results: The laser induced vascular injury model showed that platelet aggregation and degranulation were significantly compromised in TF-low mice receiving ahTF-Ab relative to the control mice (all parameters: $P < 0.001$). The maximum as well as the integrated fluorescence for aggregating and for degranulating platelets were significantly increased (all parameters: $P < 0.005$) and these were not significantly different from those obtained from the control group after rFVIIa was administered.
In the mouse tail bleeding model, the bleeding time and blood loss increased significantly in the TF-low mice receiving ahTF-Ab compared to controls ($P < 0.001$). Administration of 10 mg/kg rFVIIa significantly decreased the bleeding time and blood loss as compared to vehicle administration ($P < 0.01$ and $P < 0.001$) and to levels similar to those of the control group (no statistically significant difference).
Conclusion: Haemostasis and clot formation in the two models were greatly reduced in TF-low ($mTF^{-/-}$, hTF^{+}) mice given anti hTF-pAb. A pharmacological dose of 10 mg/kg rFVIIa corrected haemostasis and clot formation showing that rFVIIa acts independently of tissue factor.

OC 44.3
The extended reactive centre loop of protein C inhibitor balances its pro- and anti-coagulant functions

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Protein C inhibitor (PCI) is a member of the superfamily of serine protease inhibitors (serpins). Initially identified as the main plasma inhibitor of activated protein C (APC), it is now known to have a wide range of functions, both inside and outside of haemostasis. PCI inhibits the coagulation proteases thrombin, APC, fXa, fXIa and the tissue factor:fVIIa complex. PCI has the potential to serve both pro- and anticoagulant roles in the blood, and its inhibitory properties can be modulated by interactions with cofactors, such as thrombomodulin and glycosaminoglycans. Our previous crystal structure of native PCI showed that PCI shares the canonical serpin fold, but also has some unusual features, including an extra long and flexible reactive centre loop (RCL) that contains a 3-residue C-terminal extension. Based on our subsequent structure of the thrombin:PCI:heparin Michaelis complex, several implications for this extension were proposed. The increased RCL flexibility caused by the C-terminal RCL extension was hypothesised to reduce the rate of complex formation as well as reduce Michaelis complex stability due to the entropic costs of restraining a highly flexible region. It was also proposed that the increased length of the C-terminal portion of the RCL would allow proteases to engage a larger range of surface exosites. To test these hypotheses, we generated a range of loop truncation variants of PCI and evaluated their inhibition of thrombin, APC and fXa in the presence and absence of cofactors. We found that truncation of the RCL increases the rate of inhibition of pro-coagulant proteases, without affecting the rate of APC inhibition. These studies reveal that the increased length of the RCL of PCI helps to balance its pro- and anticoagulant roles.

OC 44.4
Contribution of red cells to thrombin generation in sickle cell disease (SCD)

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The sickle mutation in the hemoglobin A gene (Hb S) is carried by 7–8% of the African-American population and provides a selective advantage by conferring resistance to *plasmodium falciparum*. One in 625 African Americans are born with homozygous sickle-cell disease (HbSS) that is associated with vaso-occlusive manifestations (pain crises) of varying severity. Crises are generally ascribed to obstruction of the microvasculature secondary to decreased deformability of hypoxia-induced sickling of red blood cells (RBCs) and ensuing activation of coagulation and inflammatory pathways. We recently demon-

strated that RBCs contribute a significant fraction (40%) of the thrombin generating potential of blood, and unlike platelets (60%), this thrombin generation proceeds through the meizothrombin (mIIa) intermediate. Due to the markedly enhanced phosphatidylserine (PS) expression by sickled RBCs, we hypothesized that mIIa production in HbSS patients would be significantly increased compared to a healthy control group. To test this hypothesis, we recruited seven outpatients with HbSS, in their non-crisis, 'steady states,' and six healthy African-American controls. Five pM tissue factor was added to their whole blood samples in the presence of corn trypsin inhibitor. Thrombin generation was assessed over time using mIIa-antithrombin (mTAT) or α-thrombin-antithrombin (αTAT) complex ELISAs. Prothrombin consumption was measured by Western blotting, and PS expression (measured by bovine lactadherin binding to RBCs) by flow cytometry. RBC counts were significantly lower in HbSS individuals ($2.5 \pm 0.7 \times 10^6/\mu L$) compared to controls ($4.9 \pm 0.6 \times 10^6/\mu L$), but platelet numbers were not statistically different ($274 \pm 87 \times 10^3/\mu L$ for HbSS *vs.* $235 \pm 70 \times 10^3/\mu L$ for controls). The rate of αTAT generation in the HbSS cohort was slightly faster (15%) (74 ± 8 nM/min; mean ± SEM) than the control group (63 ± 4 nM/min), however both groups reached a similar maximum level (515 ± 49 nM *vs.* 502 ± 14 nM, respectively). The rate of mTAT generation in the HbSS cohort was significantly faster (50%)(1.5 ± 0.1 nM/min) than that observed in the control group (1.0 ± 1.1 nM/min) and displayed a significantly higher (36%) maximum mTAT level (10.1 ± 0.4 *vs.* 6.5 ± 0.5 nM respectively). Prothrombin consumption in the HbSS cohort was double that seen in the control group (9.3% *vs.* 4.7% per min). PS expression was observed in 6.67% of erythrocytes in HbSS (range 1.8–19.4%) *vs.* 0.59% (range 0.48–0.91%) in the control group. When the rates of mTAT formation were plotted against the relative PS concentration, correlation coefficients of 0.51 and 0.72 were observed for the HbSS and control groups, respectively. These data support the hypothesis that the increase in PS expression observed in HbSS red cells leads to increased thrombin generation through the mIIa intermediate. Although there was no statistical difference in the absolute αTAT levels between the two groups, the higher rate of mIIa generation and prothrombin consumption observed in the HbSS patients, together with their much lower RBC numbers, suggests that a much higher proportion of thrombin is generated on RBC surfaces. These findings may be relevant to the chronic hyper-activation of coagulation in SCD that may in turn contribute to vaso-occlusive complications of the disease.

OC 45 – Glycoprotein Ib Functions

OC 45.1

Platelet interaction with von Willebrand factor is enhanced by shear-induced clustering of glycoprotein Iba

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Background: Initial platelet arrest at the damaged arterial vessel wall is mediated through the interaction of platelet Glycoprotein (GP) Ibα with the A1 domain of surface-bound von Willebrand factor (VWF). This interaction occurs at sites of elevated shear force and strengthens upon increasing hydrodynamic drag. Although the improved interaction requires shear-dependent exposure of the VWF A1 domain, the contribution of GPIbα remains incompletely understood. We have previously found that GPIbα clusters upon platelet cooling and hypothesized that a similar mechanism enhances binding to VWF under physiological conditions.

Methods: We analyzed the surface distribution of GPIbα in platelets exposed to VWF or shear force with Förster Resonance Energy Transfer (FRET) using time-gated Fluorescence Lifetime Imaging Microscopy. Platelet interaction with VWF was analyzed with light transmission aggregometry and real-time video microscopy.

Results: GPIbα was dispersed on the surface of resting platelets (FRET efficiency of $0.9 \pm 0.3\%$). Both platelet adhesion to VWF at a shear rate of 1600/s or exposure to a high shear rate of 10 000/s induced GPIbα clusters (FRET efficiencies of $10.3 \pm 1.1\%$ and $9.10 \pm 0.8\%$, respectively). Clustering induced by high shear force was reversible and not accompanied by granule release or αIIbβ3 activation. It did not require VWF contact, as clustering also occurred in von Willebrand disease type 3 platelets. GPIbα-dependent platelet interaction with VWF improved upon clustering, as demonstrated by a 2.5-fold increased ristocetin induced agglutination and a 28% decreased rolling velocity when perfused over VWF. Shear exposure induced both GPIbα translocation to lipid rafts and p38 MAP kinase-mediated arachidonic acid release. Liberated arachidonic acid triggered 14-3-3ζ-induced clustering of GPIbα.

Conclusions: GPIbα clusters upon exposure to shear force, which enhances the interaction between platelets and VWF. These findings emphasize the role of GPIbα as a sensitive mechanoreceptor and give a new perspective on the molecular mechanism of platelet adhesion.

OC 45.2

Distinct roles for platelet GPIba, PAR4 and fibrin in regulating thrombin-dependent recruitment of leukocytes to sites of vascular injury

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Background: Thrombin is a pleotropic protease with a broad range of pathophysiologic functions linked to fibrin generation and the regulation of coagulation as well as through cleavage of protease-activated receptors (PARs) on the surface of vascular and haemopoietic cells. Although PARs are the major catalytic target for thrombin on platelets, platelet GPIbα is the major thrombin binding site. Despite extensive investigation the physiological relevance of this interaction remains elusive. Although clinical and experimental evidence supports a major regulatory role for thrombin in thromboinflammatory responses the relative contribution of fibrin, PAR cleavage and GPIbα in this process remains ill-defined.

Aim: To investigate the relative contribution of fibrin, PAR4 and GPIbα in regulating the thrombin-dependent recruitment of leukocytes to sites of vascular injury.

Methods: An intravital murine model of microvascular thrombosis induced by focal mechanical injury with microinjector needles in the mesenteric venous circulation was employed to monitor leukocyte-thrombus interactions by epifluorescence and confocal microscopy in wild-type, $PAR4^{-/-}$ and transgenic mice expressing a mutant form of GPIbα (GPIbα-D277N) that has a selective defect in GPIbα-thrombin binding. Thrombotic responses were modulated via localised microinjection of platelet agonists or systemic hirudin injection in some experiments.

Results: Mechanical endothelial perturbation resulted in a rapid and reproducible platelet thrombotic response that was highly efficient at promoting leukocyte recruitment and migration to sites of vascular injury. The thrombotic and leukocyte recruitment responses were thrombin dependent as they were eliminated by systemic injection of hirudin, whereas localised microinjection of α-thrombin markedly enhanced both responses. Notably, thrombin injection and the corresponding increase in fibrin generation limited leukocyte migration from the margins of thrombi to the site of vascular injury. Conversely, eliminating fibrin generation with hirudin greatly enhanced leukocyte migration when platelet activation and thrombus formation was induced by microinjected CRP and PAR4 activating peptide. To investigate the role of thrombin binding to GPIbα in regulating leukocyte-

thrombus interactions we developed a transgenic mouse model expressing a mutant form of human GPIbα (replacement of the aspartic acid at position 277 with asparagine D277N) on the surface of GPIbα$^{-/-}$ mouse platelets. α-thrombin and VWF binding studies confirmed a selective defect in thrombin binding to the mutant receptor. As compared to control mice (human GPIbα-WT), both thrombotic and leukocyte migratory responses were enhanced in GPIbα-D277N mice. Studies using PAR4$^{-/-}$ mice to analyse the role of thrombin cleavage of PAR4 in regulating platelet activation and leukocyte recruitment studies revealed a critical role for thrombin activation of platelets in promoting stable thrombotic responses and leukocyte recruitment and migration to vascular injury sites.

Conclusions: These results demonstrate that thrombin activation of platelets through PAR4 is critical for leukocyte-thrombus interactions at sites of endothelial perturbation, independent of fibrin generation. Paradoxically fibrin appears to play a negative regulatory role in limiting leukocyte migration. Disrupting the binding of thrombin to GPIbα enhanced platelet activation, leukocyte recruitment and fibrin generation. Thus thrombin binding to surface GPIbα appears to play a major role in negatively regulating the proteases' catalytic function towards fibrinogen and PAR4, thereby modulating thrombin's thromboinflammatory function.

OC 45.3

Platelet adhesion and activation by the oral colonizer *Streptococcus oralis*: key roles for platelet receptors GPIba, FcgRIIa and P2Y12

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Background: Infective endocarditis (IE) is considered the 4th leading cause of life threatening infectious disease. It results from colonisation by microorganisms of the inner surfaces of the heart and is characterised by the formation of septic thrombi on cardiac valves. Current treatment requires aggressive multi-antibiotic therapy often coupled with surgery to remove septic vegetations and/or replace the damaged heart valve thus preventing congestive heart failure. The oral bacterium, *Streptococcus oralis* is recognised for its ability to colonise damaged heart valves and is frequently isolated from patients with IE. Previous studies have shown that bacteria can support platelet adhesion and induce activation, steps critical in the pathogenesis of IE. However, the mechanism of platelet recruitment and activation by *S. oralis* is poorly understood.

Objective: To identify the mechanism of platelet adhesion to *S. oralis* under physiological shear conditions and to investigate the molecular mechanism(s) leading to platelet activation.

Methods: Platelet interactions with immobilized bacteria were assessed using a parallel flow chamber. Whole blood was perfused at venous and arterial shear rates and recorded in real-time using epi-fluorescent microscopy. Platelet activation was determined by light transmission aggregometry. Dense granule secretion was measured by luminometry using a luciferin/luciferase assay. Molecular pathways of platelet adhesion and activation were investigated using antibodies and pharmacological inhibitors. This was coupled with immunoprecipitation, protein pull down assay and western blotting for key platelet signalling proteins.

Results: In whole blood *S. oralis* supported platelet adhesion under venous (50–200/s) and arterial shear rates (800/s). Platelets rolled along immobilised *S. oralis* through an interaction with GPIba as shown by inhibition of platelet adhesion by pre-incubation of platelets with anti-GPIba MAb MB45 ($P < 0.001$) or perfusion of whole blood from a patient lacking GPIba (Bernard Soulier Syndrome). Following rolling, platelets firmly adhered to *S. oralis* and platelet microaggregate formation was observed at 200/s and 800/s. Platelet activation required IgG binding to the bacterium and crosslinking to platelet FcgRIIA as demonstrated by inhibition of platelet aggregation after removal of IgG ($P < 0.01$) or upon blockade of FcgRIIA by the MAb IV.3 ($P < 0.01$). This interaction led to phosphorylation of the ITAM domain on FcgRIIa and both IgG and FcgRIIA dependent dense granule secretion. Resultant secretion of ADP amplified the platelet response via $P2Y_{12}$, triggering RAP1 activation. This was shown by inhibition of aggregation by apyrase ($P < 0.05$) or P2Y12 blockade by AR-C69931MX ($P < 0.01$) and $P2Y_{12}$ dependent activation of RAP1 to its GTP bound form by pre-incubation of platelets with AR-C69931MX and RAP1 pull down assay and western blot analysis.

Conclusions: These results propose a model of interaction between *S. oralis* and platelets that leads to the formation of a septic platelet-bacterial vegetation on a damaged heart valve. Furthermore, this paper contributes to growing evidence that FcgRIIA is a novel drug target for development of treatments for IE.

OC 45.4

Macrothrombocytopenias with abnormalities of the VWF/GPIb-IX/filamin A/myosin 2A axis correspond to defects in megakaryocyte membrane formation and abnormal granule repartition.

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Background: Platelet inherited disorders constitute a growing group of syndromes with an increasing number of genes causatively linked to their development. For a long time, the majority of thrombocytopenias have been considered to result from immunological processes, now an increasing number are being shown to be congenital in origin. Platelet size and morphology changes are frequent abnormalities resulting from changes in megakaryocyte (MK) maturation and proplatelet formation.

Aim: To further characterize platelet and MK morphology for patients with von Willebrand disease Type 2B (VWD2B), Bernard-Soulier Syndrome (BSS), Filamin A (FLNA) and MYH9-Related syndromes (MYH9 -RD) that all affect the VWF-GPIb-IX-cytoskeletal axis.

Methods: Platelets and MKs from 18 patients with macrothrombocytopenia and identified mutations corresponding to VWD2B, BSS, FLNA and MYH9-RD were examined by transmission electron microscopy (TEM) and morphometric analysis was used to quantify changes in cell morphology. MKs were cultured from $CD34^+$ enriched peripheral blood cells. Immunofluorescence (IF) localization of membrane glycoproteins, VWF, FlnA and myosin 2A was also performed by confocal microscopy.

Results: For patients with VWD2B, FLNA and MYH9-RD morphometric analysis all showed varying numbers of enlarged round platelets many of which contained large zones enriched in internal membranes (previously designated as membrane complexes) and devoid of granules. Only for VWD2B were platelet agglutinates seen, a process induced by the mutated VWF. In X-linked FlnA deficiency, FlnA was abnormally distributed within the cytoplasm with a subpopulation of FlnA-negative platelets that tended to be larger. For MYH9-RD, abnormal internal membranes were frequent and occasionally platelets truly giant. In contrast, in BSS, for the large majority of platelets, the internal membrane was poorly developed. Again there is a defect in granule distribution; occasional giant granules were seen for all disorders. TEM of MKs highlighted for all disorders an early abnormal repartition of the demarcation membrane system (DMS) and an

abnormal granule distribution; membranes were often regrouped in ball-like structures or in specific zones devoid of granules more often seen for MYH9-RD. For FLNA, images of disintegrated structures and signs of apoptosis were apparent. The DMS was quantitatively reduced for BSS. In all situations, decreased amounts of membrane available for proplatelet development result in enlarged platelets.
Conclusions: GPIb-IX, FlnA and myosin 2A all contribute to the organization of DMS during MK maturation underlining the importance of the link between GPIb-IX and the cytoskeleton for membrane repartition. Decreased amounts of internal membranes or a decreased potential of membrane availability due to the aberrant presence of ball-like reserves, affect the formation of proplatelets and subsequently the size and shape of platelets. Furthermore, the observation that VWF abnormally pre-bound to GPIb can itself produce abnormalities implies that the process of driving membranes within the cytoplasm has to be free of constraints. It can also be deduced that when the GPIb-IX-cytoskeleton axis is abnormal, the granules and the membranes are not free to move regularly from the MK cytoplasm into proplatelets as shown by the persistance of ball-structures and abnormal granule distribution inside the platelets.

OC 46 – Infection and Coagulation

OC 46.1
Platelets control CVB3 infection *in vivo*

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Background: It has been recently demonstrated that mice profoundly depleted of platelets (> 95% depletion) and infected with the LCMV Armstrong strain developed hemorrhagic spots in several organs along with high viral titers and increased mortality. Interestingly, the presence of 15% of platelets (partial depletion) was sufficient to prevent vascular damage but not viral replication. These observations not only confirm the novel notion that platelets are necessary to protect vascular integrity and are critical mediators of viral clearance, but also underscore an underappreciated relationship between platelet-mediated hemostasis, and viral infection.
Aim: To examine whether this novel function of platelets is specifically related to a murine pathogen as LCMV, or if it is also present during other viral infections and using a human virus.
Methods: C57BL/6 male mice 5 weeks old were inoculated intraperitonally (IP) with PBS or with 30 μL of a specific polyclonal antiserum against platelets diluted 1:4 in PBS every 48 h. One day after the first injection, groups of mice (4–8) were infected IP with 1×10^4 UFP of a myocarditic variant of Coxsackievirus B3 (CVB3), or 1×10^4 UFP of the LCMV, Armstrong strain used as positive control. Platelet-depleted and non-depleted non-infected animals were used as negative controls. After 14 days post infection (dpi) survived animals were sacrificed and heart or spleen tissues from CVB3 or LCMV-infected animals respectively, tested for the presence for infectivity of virus by plaque assay as well as for histopathology.
Results: The platelet antiserum treatment produced 92% of platelet depletion at 4 h, 86% at 24 h and 78% at 48 h post-treatment. At 6 dpi, mortality was 25% in CVB3-infected and 50% in CVB3-infected and depleted mice. No further deaths were observed after 6 dpi or in depleted uninfected animals. Infectivity assays of homogenates of heart tissues from CVB3-infected mice were 25% positive (1/4) and the only positive show approximately 10 UFP/mg of tissue. In contrast, in CVB3-infected and platelet depleted mice showed 100% (4/4) infectivity ranging 1×10–10^2 UFP/mg of heart tissues. Analysis of histopathology showed typical multifocal acute myocarditis in all infected animals. However, the number and the extent of the lesions were approximately double in hearts of infected and depleted animals. In agreement with previously published results, infectious LCMV was detected only in the spleen of LCMV-infected and depleted animals. Bleeding was not observed in any group.
Summary/Conclusions: Our results indicate that murine platelets have a role in controlling viral infection not only for the murine pathogen LCMV but also for a human pathogen as CVB3. This uncontrolled viral infection results in a more severe pathology.

OC 46.2
ProCPB2 (TAFI) deficiency protects against polymicrobial sepsis

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Background: CPB2 (TAFI) is a basic carboxypeptidase for which fibrin and complement C5a have been shown to be physiological CPB2 substrates. However, the role of CPB2 in infection is unclear. We hypothesized that in polymicrobial sepsis proCPB2 deficient (KO) mice would have enhanced C5a generation, leading to increased inflammation and perhaps mortality.
Methods: Polymicrobial sepsis was induced in wild type (WT) and proCPB2 KO mice by cecal ligation and puncture (CLP). Blood was collected by cardiac puncture, and organs harvested. Plasma was assayed for CBC, ALT, AST, BUN and creatinine. Lung edema was determined as lung wet/dry weight ratio. Peritoneal lavage protein and cell content was measured. In some experiments mice were treated with a fibrinolysis inhibitor, tranexamic acid (TA) or a complement C5a receptor antagonist (C5aRA).
Results: To our surprise, proCPB2 KO mice have a significant improvement in survival compared to WT mice ($n = 30$, $P = 0.021$). The median survival time of 3 days in WT was extended to 3.5 days in proCPB2 KO mice ($P = 0.015$). Lung edema was decreased from a wet/dry weight ratio of 4.3 ± 0.52 (WT) to 2.7 ± 0.58 (proCPB2 KO) ($n = 11$, $P = 0.0001$) at 48 h after CLP. Peritoneal lavage protein levels and cell counts at 48 h were lower in proCPB2 KO than in WT with CLP. Liver damage assessed by AST and ALT in plasma was increased in the WT mice compared to proCPB2 KO mice (ALT: 211 ± 162 vs. 53 ± 34 U/L $P = 0.027$, AST: 344 ± 261 vs. 136 ± 83 U/L $P = 0.03$ for WT vs. proCPB2 KO at 48 h, $n = 10$). Similarly kidney damage as assessed by BUN and creatinine was less in the proCPB2 KO (BUN: 34 ± 49 vs. 19 ± 4 mg/dL, creatinine: 0.39 ± 0.67 vs. 0.1 ± 0.06 mg/dL for WT vs. proCPB2 KO at 48 h, $n = 10$). There was a greater decrease in the white blood cell count of WT mice than proCPB2 KO mice. Inflammatory mediators such as IL-6 and total C5a were lower in plasma from proCPB2 KO mice at 6 h after CLP than in WT plasma.
Treatment of both WT and proCPB2 KO animals with TA caused a decrease in survival and worsening of other markers showing that fibrinolysis was important to survival in CLP. However, the proCPB2 KO animals still showed an improvement in survival indicating that the survival advantage was not solely due to enhanced fibrinolysis in the proCPB2 deficiency state. If enhanced C5a generation was the reason for the improved survival of proCPB2 KO mice, then blockade of C5a receptors with C5aRA would decrease survival. However, both WT and CPB2 animals treated with C5aRA had improved survival and other surrogate markers were better, demonstrating that enhanced C5a was not responsible for the amelioration of CLP in proCPB2 KO mice. The possibility that osteopontin (OPN) was the CPB2 substrate was disproved as CLP was similar in proCPB2 KO and proCPB2/OPN double KO mice.
Conclusions: These data show that deficiency of proCPB2 protects against polymicrobial sepsis with a concomitant reduction in inflammation and damage to multiple end organs. Fibrin, OPN and C5a were not the dominant mechanism for the protection observed in proCPB2 KO animals.

OC 46.3

Influenza and dengue viruses upregulate interferon-induced transmembrane (IFITM) proteins in human platelets: novel immune sensing of viral pathogens

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Background: IFITM proteins (IFITM-1, -2, and -3) mediate cellular resistance to influenza, dengue, and other viruses. IFITM expression in human platelets at baseline and during conditions of influenza and dengue viral infectivity has not previously been reported.
Aims: We investigated *IFITM* mRNA and protein expression in human platelets, hypothesizing responses to inflammatory signaling in viral infections are a key component of the platelet immune response.
Methods: We examined freshly-isolated platelets from prospectively enrolled patients with acute influenza A/H1N1 or dengue infection ($n \geq 20$) and, for comparison, age- and gender-matched healthy controls ($n \geq 20$). IFITM mRNA and protein expression in isolated platelets was quantified via qRT-PCR and western blots, respectively. We also studied platelets isolated from healthy subjects ($n = 8$) before (day 0) and after (day 10 ± 3) administration of the 2012–2013 trivalent inactivated influenza vaccine (TIV), which contains influenza A/H1N1 hemagglutinin. In separate experiments, CD34-derived megakaryocytes or freshly isolated platelets from healthy subjects were stimulated with IFN-g at various concentrations or infected directly with dengue virus (MOI 10, day 3). IFITM expression was measured as above and ICC detected dengue virus in CD34-derived megakaryocytes. While we examined all three *IFITM* gene products, we focused on detailed characterization of IFITM-3, which has the greatest anti-viral effects.
Results: Platelet *IFITM-3* mRNA is present basally in healthy subjects and increases markedly (~100-fold higher, $P > 0.0001$) during acute influenza and dengue infection. IFITM-3 protein in infected patients is significantly upregulated (> 20-fold higher, $P > 0.0001$) and correlates with *IFITM-3* mRNA levels ($r^2 = 0.33$, $P > 0.05$). Plasma IFN-g levels (which induces IFITMs) were higher in infected patients compared to healthy controls (10.6 vs. 1.5 pg/mL, $P > 0.05$). Influenza vaccination upregulated platelet *IFITM* protein expression (threefold increase, $P > 0.05$), although to a lesser degree than in infected patients. Plasma levels of IL-6 and IL-8 did not increase following vaccination, suggesting that vaccination did not induce a sustained systemic inflammatory response. In CD34-derived megakaryocytes, infection with dengue virus or, in separate experiments, stimulation with IFN-g, upregulated *IFITM-3* mRNA and protein expression. IFN-g did not increase *IFITM-3* in isolated human platelets. Other IFITM family members showed similar patterns of regulation although the magnitude of responses varied.
Summary/Conclusions: These findings provide novel biological evidence that platelets undergo dynamic changes in their molecular signature during acute viral syndromes. Specifically, IFITM proteins are markedly upregulated in platelets from infected patients and in healthy human subjects following vaccination with inactivated influenza virus. As pro-inflammatory cytokines did not increase following administration of the TIV, these findings suggest that increased platelet IFITM expression is not merely due to systemic inflammatory responses. Rather, megakaryocytes appear to receive signals that alter *IFITM* expression in response to immune stimulation in the absence of acute infection. We postulate that platelet IFITM induction may occur in a signal-dependent mechanism mediated through megakaryocytes, perhaps during interactions with viruses or viral particles in the lung or via systemic inflammatory pathways.

OC 46.4

Thrombin-mediated fibrin generation supports innate defense against pulmonary plague in mice

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Background: The gram-negative bacterium *Yersinia pestis* causes plague, a highly contagious, rapidly progressing, and often fatal disease. Immunization of mice with the *Y. pestis*-derived peptide $YopE_{69\text{-}77}$ improves their survival. The *Y. pestis* Pla protein promotes fibrinolysis by activating host plasminogen while inactivating alpha-2-antiplasmin, plasminogen activator inhibitor 1 (PAI-1) and thrombin-activatable fibrinolysis inhibitor (TAFI). Pla mutant *Y. pestis* has reduced virulence, suggesting a protective role of fibrin during experimental plague.
Aims: We evaluated functional roles for thrombin-mediated fibrin formation in an established mouse model of pulmonary plague.
Methods: Wild type D27 and attenuated D27-pLpxL *Y. pestis* strains were used in controlled pulmonary infection studies in mice. The *Y. pestis* strain D27-pLpxL has reduced virulence compared to D27, allowing for survival studies and opportunity to explore mechanisms of pathogenesis. Infected mice were monitored daily and euthanized when they became unresponsive or recumbent. The number of viable bacteria in lung, spleen, and liver were measured by homogenizing tissues in saline, plating serial dilutions on BHI agar, and counting CFU after 2 days. Wild type (WT) C57BL/6, warfarin-treated or untreated, $YopE_{69\text{-}77}$-immunized, and transgenic mice with deficiencies in fibrinogen, tissue factor (TF), PAI-1, TAFI, or factor XI (FXI) were used in the experiments.
Results: All fibrinogen-deficient mice succumbed to D27 sepsis significantly faster than littermate control fibrinogen-heterozygous mice ($P > 0.0001$). The bacterial burden measured at day 4 after challenge revealed an increase in numbers of bacteria in the lung and liver tissues of fibrinogen-deficient mice. All WT and FXI deficient mice survived D27-pLpxL infection. In contrast, about half of the warfarin treated mice, and most (> 90%) of the fibrinogen deficient mice and mice with very low levels of TF, died of sepsis following pulmonary D27-pLpxL infection ($P = 0.007$). Interestingly, immunization against plague with $YopE_{69\text{-}77}$ further enhanced the mortality of D27 infection during warfarin-treatment, in the low TF mice, and in the PAI1 and TAFI knockout mice. These observations suggest that infection by bacteria that cause plague induces a TF-pathway-dependent thrombin generation response that leads to formation of protective fibrin. Further, this fibrin appears to be an integral part of and critical to the host's innate and T-cell mediated immunity against invasion by *Y. pestis*. The approaches used herein to prevent fibrin formation support the hypothesis that fibrin provides an innate defense system against *Y. pestis* infection. TF-initiated and other coagulation-dependent defense systems may serve as natural countermeasures of mammalian organisms to fight off certain infections by bacteria that target the coagulation system for virulence.
Conclusions: Antithrombotic therapies that inhibit blood coagulation cascade components *downstream* of FXI and the contact activation complex may worsen sepsis outcomes by inadvertently increasing the virulence of pathogens that invade the host by targeting fibrin. This could help explain, in part, why several antithrombotic treatments, from heparin to activated protein C, have failed to improve the outcome of sepsis in large clinical studies. Our studies suggest that if antithrombotic treatments are needed during undefined sepsis, using modalities that do not interfere with natural TF-dependent hemostatic and antibacterial fibrin generation would be safer.

OC 47 – Inherited Risk Factors for Venous Thrombosis – II

OC 47.1

A sensitized whole genome ENU mutagenesis screen identifies an Arp2 missense mutation as a novel suppressor of lethal thrombosis in the factor V Leiden mouse

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Background: Factor V Leiden (FVL) is the most common known VTE risk factor, but has only 10% penetrance. This is governed largely by genetic factors called modifier genes that alter VTE disease development. Identification of these genetic factors could provide novel insights into the development and management of VTE.
Aims: A sensitized dominant whole genome ENU mutagenesis approach in a perinatal lethal mouse model of homozygous FVL (FVQ/Q), tissue factor pathway inhibitor deficiency (Tfpi+/−) was used to identify FVL modifier genes.
Methods: The ENU screen consisted of crossing ENU-treated male FVQ/Q mice with FVQ/+ Tfpi+/− females. Surviving G1 offspring were analyzed to identify survivors with the lethal FVQ/Q Tfpi+/− genotype. Whole exome capture and sequencing was performed using the Agilent SureSelect mouse whole exome capture kit and Illumina HiSeq sequencing. Sequence data were analyzed bioinformatically using SAM tools and GATK. Complete blood counts were performed using an Advia 2120.
Results: Analysis of 7128 G1 offspring (~2X genome coverage) identified 98 FVQ/Q Tfpi+/− mice that survived to weaning. Fourteen FVQ/Q Tfpi+/− G1 mice exhibited transmission of a putative suppressor mutation to two or more FVQ/Q Tfpi+/− G2 offspring. Exome sequencing and variant analysis on a progeny tested member of eight of the 14 lines resulted in 100-fold sequence coverage and revealed a small number of high confidence novel heterozygous (dominant) single nucleotide variants (SNVs) in each sample. Sanger re-sequencing of all 11 SNVs in a cohort of mice from line 1 confirmed that a G to C mutation (chromosome 11 base 19,977,300) in the *Actr2* gene is the dominant FVL modifier in this line. FVQ/Q Tfpi+/− mice carrying this mutation had significantly enhanced lifespan (Kaplan-Meier, $N = 31$ $P > 0.0001$). This mutation resulted in an R286G substitution in a highly conserved amino acid in the Arp2 protein, part of the Arp2/3 complex. Arp2/3 regulates cell shape by controlling intracellular actin branching and polymerization. Analysis of 63 progeny from an Arp2 R/G x Arp2 R/G cross revealed only three live Arp2 G/G mice ($P > 0.001$), suggesting that R286G is a loss of function mutation and that significant lethality is associated with the homozygous genotype. Complete blood counts (Advia 2120) performed on 36 *Actr2* heterozygous and 22 wildtype littermates revealed no significant differences in platelet count, red and white blood cell counts, hematocrit or hemoglobin. However, measurements of mean platelet dry mass were significantly altered in *Actr2* heterozygous mutant mice (1.25 vs. 1.30 for wildtype, $P > 0.005$).
Summary/Conclusions: Partial deficiency of Arp2 appears to alter platelet structure/function, resulting in a shift in hemostatic balance which restores survival to the otherwise lethal FVQ/Q Tfpi+/− phenotype. These results suggest that variation in Arp2 or related genes could modify FVL dependent VTE risk in humans and may also function as independent VTE risk factors. These genes also represent novel therapeutic targets for thrombotic disease.

OC 47.2

Genetic variants in cell adhesion molecule 1 (CADM1): a validation study of a novel endothelial cell venous thrombosis risk factor

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Introduction: In a large family with protein C deficiency we recently identified a candidate gene, *CADM1*, which apparently interacted with protein C deficiency in increasing the risk of venous thrombosis.
Aim: The aim of this study was to determine whether *CADM1* gene variants also interact with prothrombotic protein C pathway abnormalities in increasing the risk of venous thrombosis outside this single protein C deficient family.
Methods: We genotyped over 300 *CADM1* gene variants in the population-based MEGA case-control study (consent and ethical approval obtained). For this analysis, we included only individuals of North- and West-European origin. We compared venous thrombosis risks between cases with low protein C activity ($n = 194$), low protein S levels ($n = 23$), high factor VIII levels ($n = 165$) or factor V Leiden carrier ($n = 580$), and all 4004 controls. Low protein C activity was defined by taking the lower limit of normal protein C activity (67% of normal in our laboratory) as cut-off point. Similarly, for low protein S levels, we used the lower limit of normal protein S antigen levels (67% of normal in our laboratory) as a cut-off point. When individuals were on oral anticoagulant therapy at time of blood draw, we calculated the expected protein C activity relative to factor VII activity and the expected protein S levels relative to factor II activity by linear regression. The observed levels were classified as 'low' when the observed/expected ratio was below the geometric mean minus 2 standard deviations as calculated among control subjects. High factor VIII levels were defined as factor VIII activity higher than the geometric mean plus two standard deviations among controls. Positive associations were repeated in all 3496 cases and 4004 controls.
Results: We found 21 *CADM1* variants which were associated with venous thrombosis in one of the protein C pathway risk groups. After mutual adjustment, six *CADM1* variants remained associated with venous thrombosis. The strongest evidence was found for rs220842 and rs11215504. For rs220842, the odds ratio for venous thrombosis was 3.1 (95% CI 1.1–8.1) for cases with high factor VIII levels compared to controls. In addition, this variant was associated with an increased risk of venous thrombosis in the overall study population with an odds ratio of 1.5 (95% CI 1.0–2.2). For rs11215504, the odds ratio for venous thrombosis was 1.8 (95% CI 1.2–2.7) among individuals with high factor VIII levels. In the overall study population the odds ratio for rs11215504 was 1.1 (95% CI 1.0–1.3).
Conclusions: In a population-based association study, we confirm a role for *CADM1* variants in increasing the risk of venous thrombosis by interaction with protein C pathway abnormalities.

OC 47.3

Exacerbated venous thromboembolism in mice with the protein S Tokushima mutation

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Background: Protein S (PS) is a regulator of blood coagulation that acts as a cofactor for activated protein C (APC). Homozygous PS deficiency in humans causes life-threatening purpura fulminans at birth and PS-null mice show embryonic lethality. Heterozygous PS defi-

ciency is milder but firmly associated with an increased risk of thrombosis in both humans and mice. The K196E mutation in PS, also known as PS Tokushima, causes decreased APC cofactor activity *in vitro* and is a common genetic risk factor for deep vein thrombosis in Japanese (odds ratio = 4.7–5.6). Approximately 2% of the general Japanese population is estimated to carry the mutant E-allele, whereas the mutation is not observed in Caucasian populations. We have recently generated PS-K196E knock-in mice for investigating effects of the PS-K196E mutation *in vivo*.

Aims: In this study, we analyzed thrombotic state of PS-K196E mice while comparing with mice carrying the factor V Leiden (FV-R504Q) mutation, the common genetic risk for thrombosis in Caucasian.

Methods and Results: A deep vein thrombosis model of electrolytic inferior vena cava injury was applied in wild-type, heterozygous PS-K196E ($PS^{+/E}$), homozygous PS-K196E ($PS^{E/E}$) and homozygous FV-R504Q ($FV^{Q/Q}$) mice with a direct current of 200 μA for 10 min. At 48 h after the injury, $PS^{E/E}$ mice as well as $FV^{Q/Q}$ mice showed increased thrombus weight accompanied by a decrease in peripheral platelets as compared to wild-type mice. Peripheral platelet counts after the injury were also lower in $PS^{+/E}$ mice than in wild-type mice. Following the induction of pulmonary embolism by intravenous infusion of tissue factor or long-chain polyphosphate (a natural activator of the contact pathway of coagulation), $PS^{+/E}$, $PS^{E/E}$ and $FV^{Q/Q}$ mice showed increased degree of vascular occlusion in lungs and decreased survival compared with wild-type mice. These results support a direct causal relationship between the PS-K196E mutation and increased susceptibility to venous thrombosis in Japanese. To examine effects of the mutation on arterial ischemic diseases, temporary focal cerebral ischemia was applied in mice by cauterization of a middle cerebral artery with transient occlusion of bilateral common carotid arteries. $FV^{Q/Q}$ mice showed larger infarct volumes 24 h after the ischemia-reperfusion and lower 7-day survival than wild-type mice. In contrast, infarct volumes in $PS^{+/E}$ and $PS^{E/E}$ mice were comparable to those in wild-type mice, suggesting that the PS-K196E mutation does not cause aggravation of ischemic stroke. Consistent with these findings, the FV Leiden mutation has been reported as a risk factor for ischemic stroke in children and young adults, whereas there are no epidemiological data to suggest significant association between the PS-K196E mutation and stroke.

Conclusions: The PS-K196E mice represent an *in vivo* evaluation system to help uncovering ethnic differences in pathophysiological features and drug responsiveness for thrombosis.

OC 47.4

Functional analysis of the thrombomodulin gene c.1418C>T polymorphism. Its association with venous thrombosis

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Background: Thrombomodulin (TM) is an endothelial cell membrane protein that acts as a cofactor for thrombin in the activation of protein C (PC). A common SNP in the coding region of the thrombomodulin gen (*THBD*), c.1418C>T (rs1042579), which results in the replacement of Ala455 by Val, has been described. This dimorphism is located in the TM region responsible for thrombin binding and PC activation, suggesting a potential role in the modulation of TM function. However, its association with venous and arterial thrombosis is not consistent.

Aims: To investigate the association of this polymorphism with VTE.

Methods: We genotyped this polymorphism and measured, with specific ELISAs, soluble (s) TM and circulating activated PC (APC) levels in 1276 VTE patients and 1262 control subjects, genotyped human umbilical vein endothelial cells (HUVEC) from 100 umbilical cords, and quantified TM levels in HUVEC-conditioned media (CM) and cell lysates. We also analyzed the relation between this polymorphism and *THBD* mRNA and TM protein expression. Informed consent was obtained from all subjects of the study and it was approved by the medical ethics committee of our Institution.

Results: The presence of the 1418T allele was associated with a reduced VTE risk in the subgroup of subjects with > 45 years of age (OR = 0.58; 0.47–0.72) but not in those ≥ 45 years of age, and there was an inverse correlation between sTM and the number of 1418T alleles ($P > 0.001$). In both patients and controls, there was a trend to a positive correlation between APC levels and the number of the 1418T alleles, while a significant inverse correlation was observed between APC and sTM levels among controls ($r = -0.161$, $P = 0.001$). The sTM level in HUVEC-CM significantly decreased ($P > 0.001$), but the TM level in HUVEC lysates increased ($P > 0.001$) when the number of 1418T alleles increased. Western blot analysis of HUVEC lysates confirmed the results obtained with the TM ELISA. Finally, the expression of *THBD* in HUVEC was not significantly affected by the rs1042579 genotype.

Conclusions: These results show that the presence of the *THBD* 1418T allele is associated with a decrease in sTM, both in plasma and in HUVEC-CM, and with an increase in the amount of membrane-bound TM in HUVEC, which could explain the reduced risk of VTE in those individuals carrying this allele. As there were no significant differences in *THBD* mRNA levels according to the c.1418C>T polymorphism in HUVEC, together these results suggest that the membrane-bound TM 455Val variant (1418T allele) might be more stable and less prone to shedding. sTM represents cleaved forms of membrane-bound TM with loss of part of the serine-threonine rich region, the transmembrane domain, and the cytoplasmic tail. The 455Val residue is located not far from the presumed cleavage site and may induce a protection from TM cleavage by proteases. The protective effect of the 1418T allele against VTE was only manifested in younger people, which may explain the differences with previous studies where older people were analyzed.

OC 48 – Inhibitors in Haemophilia A – II

OC 48.1

Prediction of the extent and duration of desmopressin response in moderate and mild hemophilia A

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Background: Desmopressin (1-deamino-8D-arginine vasopressin) is used in patients with hemophilia A and von Willebrand disease to improve hemostasis. Inter-individual variation in desmopressin response necessitates testing in individuals prior to treatment. To date, several modifiers of this response have been identified, such as FVIII:C baseline level, age and type of *F8* gene mutation. As Area Under the Curve (AUC) is used to evaluate bioavailability and clearance of drugs, it may also be a valuable tool to evaluate FVIII:C response after desmopressin. This, as AUC not only expresses maximum FVIII:C levels, but also duration of the effect.

Aim: To investigate if AUC is an adequate tool to measure the maximum effect and the duration of desmopressin response in patients with hemophilia A.

Methods: Mild and moderate hemophilia A patients in whom a desmopressin test was performed (intravenously with 0.3 μg/kg; intranasally with 300 μg) at our Hemophilia Treatment Center were included. Patients with an inhibitor against FVIII at desmopressin use were excluded. We collected FVIII:C and VWF:Ag levels before and at 1, 3

and 6 h after desmopressin administration. Patient characteristics included: age, blood type, body weight (adjusted in obese patients), *F8* gene mutation and familial desmopressin response. We calculated the AUC of FVIII:C between baseline and at 6 h post-infusion as a measure of desmopressin response (AUC0-6). Univariate and multivariate regression analyses were performed to identify predictors of desmopressin response as depicted by the AUC0-6. The beta-coefficient (β) displays the increase of the outcome per unit increase in predictor in these analyses. The study was not subject to the Medical Research Involving Human Subjects Act and was approved by the Medical Ethics Committee.

Results: In total, 115 patients were included; 106 received desmopressin intravenously, nine intranasally. Five had moderate and 110 mild hemophilia A. Median age at administration was 29 years (IQR 18–43). Median FVIII:C baseline level was 0.17 IU/mL (IQR 0.09–0.27), median FVIII:C peak level 0.64 IU/mL (IQR 0.46–1.03) and after 6 h 0.41 IU/mL (IQR 0.27–0.61). Median AUC0-6 was 2.99 IU/mL/h (IQR 1.99–4.52). FVIII:C baseline level was significantly associated with AUC0-6 (β = 2.68 IU/mL/h [95%CI 1.49; 3.87], $P > 0.001$) as was FVIII:C peak level (β = 3.20 IU/mL/h [95%CI 2.87; 3.54], $P > 0.001$). Age (continuous and > 18 >), body weight, blood type and VWF:Ag were not associated with AUC0-6. The most frequently occurring *F8* gene mutations were studied separately; Amino acid change Asn637Ser ($n = 9$) was positively associated with AUC0-6 (β = 0.45 IU/mL/h [95%CI 0.10; 0.85], $P = 0.014$). Arg2169His ($n = 19$), Pro149Arg ($n = 5$), Arg612Cys ($n = 13$) were not associated with AUC0-6. No association was found with familial response, possibly due to small sample size. Patients with > 0.50 IU/mL FVIII:C at peak level and after 6 h, had an AUC0-6 ≥ 4 IU/mL/h. Patients with > 0.50 IU/mL FVIII:C at peak level and > 0.30 IU/mL after 6 h, had an AUC0-6 ≥ 3 IU/mL/h.

Summary/Conclusion: AUC is mainly determined by FVIII:C baseline and peak level. The AUC also distinguishes between patients with and patients without a clinically relevant maximum and duration of desmopressin response. Currently, we are constructing a model to predict the AUC based on these factors to apply in daily clinical practice.

OC 48.2

FVIII-targeting specific regulatory T-cell therapy: a novel translational approach for tolerance in Hemophilia A patients

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Background: Foxp3$^+$ regulatory T cells (Tregs) have been recognized as an important T subset, responsible for self-tolerance and homeostasis of our immune system. Clinical applications using Treg's are now considered as the next-generation cellular therapy in a variety of undesirable immune disorders such as type 1 diabetes, GVHD, and transplantation, and potentially as a treatment for hemophilia inhibitors. However, expanded polyclonal Tregs are generally non-specific immunosuppressive therapies. Specific Tregs are clearly desirable.

Aim and Hypothesis: To produce specific human Tregs, we hypothesized that we could render expanded Tregs specific using T-cell receptors (TCR) cloned from hemophilia A patient's effector CD4 clones; these T cells should recognize defined HLA-restricted factor VIII (FVIII) epitopes.

Methods: FVIII-reactive T cell clones were generated from patient's PBMCs by tetramer isolation, and FVIII-reactive mouse T hybridomas also have been produced by standard fusion with immunized mouse spleens. From the patient's clones, nucleotide and amino acid sequences were identified to design FVIII-specific TCR construction and retroviral constructs developed for transduction into expanded polyclonal human T effector (Teff) and Treg populations.

Results: Upon retroviral transduction, we show tetramer binding and reactivity to the FVIII epitope by transduced human T cells; functional assays demonstrate peptide-specific expansion of transduced human Teff cells. Importantly, polyclonal human T cells expanded under Treg conditions and then transduced with patient's TCR showed up-regulation of FoxP3 and GARP upon recognition of FVIII peptide and MHC class II.

Summary: We demonstrate that specific Tregs can be generated by expression of TCR from a hemophilia patient's T cells. Potential application of 'FVIII- specific' Tregs in HLA-transgenic humanized mice will be an important step toward future clinical application. (Supported by NIH grant RO1 HL061883-15 to DWS and unrestricted research funding from Bayer, Pfizer and CSL Behring to KPP).

OC 48.3

Diagnosis and management challenges in patients with mild hemophilia A and discrepant FVIII measurements

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Background: Patients with mild hemophilia A (MHA) have FVIII:C levels of 5–40 IU/dL and usually exhibit fewer bleeding episodes than those with the severe or moderate forms of the disease. 30% of patients with MHA present markedly different FVIII:C level when assayed by one-stage clotting and two-stage chromogenic assays. It is therefore a real clinical challenge to predict the individual bleeding risk of these patients.

Aim: The aim of the present study was to correlate bleeding tendency of these patients with the results of a panel of phenotypic and genotypic tools.

Methods: Fifty five patients with MHA were included in this multicenter prospective clinical study. The severity of bleeding symptoms was evaluated using the ISTH/SSC score developed by Rodeghiero et al. FVIII:C levels were measured using an aPTT based one-stage FVIII assay (FVIII:C1) and three commercial chromogenic kits (FVIII:C2). FVIII antigen levels and FVIII gene mutation analysis were also performed in all patients. Thrombin generating capacity was determined using a low tissue factor concentration of 1pM in 28 patients.

Among 55 patients, nine had a history of spontaneous hemarthroses, which is a very unusual symptom and discrepant clinical presentation of MHA. Twenty three patients were denoted discrepant because the difference between FVIII:C1 and C2 modified the diagnosis of MHA i.e. results above 40 IU/dL or below 5 IU/dL. Finally, 24 patients were also denoted discrepant and studied because there was a twofold lower or greater difference between FVIII:C1 and C2 i.e. ratio of ≥ 2.0 and ≤ 0.5.

Results: Our results showed that a one-stage FVIII: C assay cannot rule out the diagnosis of MHA, a combined use of FVIII:C1 with a FVIII:C2 is suitable for detecting MHA. We observed a better correlation between clinical bleeding tendency and FVIII:C2 results compared to FVIII:C1. Thus, in patients with a history of spontaneous hemarthroses, FVIII:C2 results were three times lower than FVIII:C1. Patients with a bleeding score > 2, considered as non-bleeders had a thrombin generating capacity > 50% of normal and significantly higher (ETP = 723 ± 147 nM.min; peak IIa 65 ± 23 nM) than those with a score > 4, severe bleeders (ETP = 439 ± 142 nM.min; peak IIa35 ± 16 nM) ($P = 0.0002$ and 0.004 respectively). Twelve patients had at least one FVIII:C > 40 IU/dL; in most of them FVIII:C2 was higher than FVIII:C1 and 10 of them had a low bleeding tendency with a score > 2. Ten patients had at least one FVIII:C > 5 IU/d; nine of them had a FVIII:C2 lower than FVIII:C1 with a bleeding

score > 4. In these patients thrombin generation was very low (ETP = 392 ± 126 nM.min; peak IIa28 ± 14 nM). FVIII gene mutation analysis showed mutations previously reported in MHA patients with discrepant FVIII:C measurements, but with no predictive value of the individual bleeding phenotype of patients.
Conclusion: Overall, we observed a correlation between chromogenic FVIII:C results, thrombin generation assay and bleeding tendency of patients with discrepant FVIII:C measurements, whilst FVIII:C1 was not well correlated with clinical bleeding phenotype in this particular population.

OC 48.4

Improvement of fibrin clot structure after FVIII injection in hemophilia A patients treated on demand

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Patients with hemophilia A have seriously impaired thrombin generation due to the inherited deficiency of FVIII and thereby form loose and instable fibrin clots which are unable to maintain the hemostasis. However, there are still limited data considering fibrin clot structure and function in hemophilia patients.
Methods: Permeability of the fibrin network, assessed by the flow measurement technique, was investigated in plasma samples from 20 patients with severe hemophilia A treated on demand. Blood samples from the patients were taken before and 30 min after FVIII injection. The results were correlated with fibrinogen concentration, FVIII concentrations, thrombin activatable fibrinolysis inhibitor (TAFI) and the results of global hemostatic assays: endogenous thrombin potential (ETP) and overall hemostatic potential (OHP). The fibrin structure was visualized using scanning electron microscopy (SEM).
Results: Permeability coefficient Ks decreased significantly after FVIII treatment (20.2 ± 5.1 to 9.7 ± 3.4 respectively, $P > 0.0001$). Ks correlated significantly with the levels of investigated parameters (Pearson′s test was used -R values and only results with P values > 0.05 are presented): fibrinogen (−0.43), FVIII dose (−0.54), OHP (−0.81), OCP (−0.83), OFP (0.63), ETP (−0.73), TAFI/TAFIi (−0.35) and FVIII level (−0.72). SEM images revealed irregular porous fibrin clots composed of thicker and shorter fibers in plasma from patients before treatment with FVIII. The clots were recovered after FVIII replacement almost to the level of fibrin structure in a control sample, reveling compact fibrin with smaller intrinsic pores, composed of thinner fibers with increased branch-points which create resistance to fibrinolysis.
Conclusion: To the best of our knowledge, this is the first description of fibrin clot porosity and structure before and after the treatment of well selected hemophilia patients treated on demand. Fibrin clot structure was seriously impaired in patients with hemophilia while on demand treatment with FVIII normalized fibrin clot permeability and improved the fibrin clot structure. It seems that thrombin generation is the main determinant of fibrin structure in hemophilic plasma.

OC 49 – Prothrombin

OC 49.1

Crystal structure of the prothrombinase complex from the venom of *Pseudonaja textilis*

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Background: Formation of thrombin from prothrombin is catalysed by the prothrombinase complex, which comprises factor Xa (fXa), its cofactor, factor Va (fVa), calcium ions and negatively-charged phospholipid surfaces, such as those found on activated platelets. This reaction is the central process of blood coagulation and is of great medical relevance; insufficient thrombin generation results in bleeding, including haemophilia, whereas excessive thrombin generation causes thrombosis. A detailed structural and mechanistic understanding of the prothrombinase complex is of great interest within the field.
Interestingly, the Australian eastern brown snake (*Pseudonaja textilis*) has evolved a 'weaponised' prothrombinase for use in self-defense and hunting prey. In addition to the regulated plasma prothrombinase responsible for maintaining normal hemostasis within the blood of the snake, gene duplication and a few mutations have resulted in a venom form of prothrombinase (traditionally known as Pseutarin C). This complex has evolved away four of the main regulatory mechanisms known to control the activity of human prothrombinase, including a drastic reduction in the size of the inhibitory B domain of fV (approx. 100 residues compared to > 800 in human), the ability to form a high-affinity complex in the absence of membranes, and resistance to inhibition by APC and antithrombin. Envenomation thus leads to unregulated thrombin formation and ultimately death by disseminated intravascular coagulation.
Aims: In this work we sought to determine the crystal structure of the prothrombinase complex from the venom of *Pseudonaja textilis*, in order to understand the molecular basis of thrombin generation.
Methods: We produced recombinant *P. textilis* fV (full-length, with the R742Q and R788Q mutations) in BHK cells, and recombinant *P. textilis* fX (truncated, comprising the EGF2-catalytic domains) in *E. coli*. The proteins formed a high-affinity complex, which was purified by size exclusion chromatography and crystallised. X-ray diffraction data were collected at a synchrotron, and the crystal structure was solved by molecular replacement.
Results: Our structure reveals the specific binding interface of fV and fX, and allow us to understand how the snake has evolved both fX and fV in order to weaponise them for use in the venom. It has also allowed us to construct a high-quality model of full-length prothrombinase, which reveals the likely mode of prothrombin binding and consequently explains why the presence of fVa mediates specific, sequential cleavage of prothrombin.
Summary/Conclusion: We present here the first structure of a vertebrate prothrombinase complex, which is likely to represent how mammalian complexes assemble and function. Knowledge of this has far-reaching implications for diseases relating to incorrect spatiotemporal regulation of thrombin generation, such as haemophilia and thrombophilia.

OC 49.2

NMR and crystallographic studies demonstrate that GpIba interacts exclusively with thrombin's exosite II

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Activation of platelets by the serine protease thrombin is one of the critical events in haemostasis. This process is initiated by cleavage of the protease-activated receptor (PAR) 1 and is accelerated in the pres-

ence of the cofactor glycoprotein Ibα (GpIbα). The *N*-terminal extracellular domain of GpIbα contains an acidic stretch that has been identified as the main thrombin binding site. Thrombin's two anion binding exosites have been implicated in GpIbα binding, but it remains unclear which of the two, or even if both participate. Determining which thrombin exosite mediates the interaction with GpIba is critical for the function of thrombin in platelet activation. Depending on the binding mode, thrombin could act as a platelet adhesion molecule or receptor dimerization trigger, or GpIbα could act as a cofactor for PAR-1 activation by thrombin. To determine how thrombin binds to GpIbα, we employed the methods of X-ray crystallography, solution NMR spectroscopy and biophysical binding studies. Our results conclusively demonstrate that GpIbα binds exclusively to thrombin's exosite II, thereby recruiting thrombin activity to the platelet surface and leaving exosite I available for PAR-1 recognition and enhanced platelet activation.

OC 49.3

Elevated prothrombin promotes venous, but not arterial, thrombosis in mice: implications for investigating vascular bed-specific mechanisms in humans

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Background: Individuals with elevated prothrombin levels, including those with the G20210A mutation in the prothrombin 3'-untranslated region, have increased venous thrombosis risk. However, the risk of arterial thrombosis is less clear with some clinical studies showing a modest correlation and others not. Although these hyperprothrombinemic individuals do not have increased circulating prothrombotic biomarkers at baseline, their plasma demonstrates increased tissue factor-dependent thrombin generation *in vitro*.
Aims: We investigated the pathologic role of elevated prothrombin in *in vivo* models of venous and arterial thrombosis and distinguished mechanisms differentiating thrombogenesis in these vessels.
Methods: Human prothrombin was infused into mice to raise circulating levels to that associated with increased thrombosis risk in humans. Venous thrombosis was induced by inferior vena cava ligation or electrolytic stimulus to the femoral vein. Arterial thrombosis was induced by ferric chloride application or electrolytic stimulus to the carotid artery. Procoagulant activity was assessed by circulating thrombin-antithrombin complexes and calibrated automated thrombography. The kinetics of platelet and fibrin accumulation during thrombus formation was followed in real-time by intravital fluorescence microscopy and time to vessel occlusion. Thrombus composition was assessed by thrombus weight and erythrocyte and fibrin content.
Results: Elevated prothrombin did not cause spontaneous thrombosis or increase baseline prothrombotic markers in unchallenged mice, but significantly increased thrombin generation during tissue factor-triggered coagulation *ex vivo*. Elevated prothrombin also significantly increased thrombin generation following venous injury *in vivo* (2.3-fold, $P > 0.009$). Arterial injury models showed faster platelet accumulation than venous models (3.2-fold, $P > 0.002$). Elevated prothrombin did not accelerate platelet accumulation following either arterial or venous injury. Elevated prothrombin also did not increase the rate of fibrin accumulation or shorten the time to occlusion following arterial injury. However, compared to control mice, elevated prothrombin increased the rate of fibrin accumulation (2.3-fold, $P > 0.02$) following venous injury, producing extended thrombi with significantly increased mass (31.3 ± 6.6 vs. 20.7 ± 6.4 mg, mean ± SD, $P = 0.01$).
Conclusions: These findings reconcile previously discordant studies regarding thrombin generation in hyperprothrombinemic individuals measured *ex vivo* and *in vitro*, and are the first to show that elevated prothrombin promotes venous thrombosis *in vivo*.

OC 49.4

Prothrombin activation intermediates bind thrombomodulin demonstrating sequential capacitation of exosite 1

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Background and Aims: The interaction of thrombin with thrombomodulin (TM) triggers anticoagulant and antifibrinolytic pathways. Thrombin binds TM via exosite 1, highlighting the critical role of this exosite in the regulation of hemostasis. Hirugen (hirudin 54–65) binds exosite 1 on thrombin but not on prothrombin, suggesting that exosite 1 undergoes maturation upon prothrombin activation. Consistent with this concept, prothrombin activation intermediates demonstrate progressive capacity to bind hirugen. However, we have shown that HD1, an exosite 1-binding DNA aptamer, not only inhibits thrombin activity, but also attenuates prothrombin activation because it binds prothrombin and thrombin with comparable affinity. To explore the functional consequences of this differential maturation, we compared the capacity of prothrombin intermediates to bind thrombomodulin with those of prothrombin and thrombin.
Methods: After activating protein C (PC) with thrombin in the presence of a TM peptide consisting of epidermal growth factor domains 4–6 (TM456) and calcium, activated PC (APC) was quantified by chromogenic assay in the presence of hirudin to inactivate thrombin. APC generation was determined in the presence of increasing concentrations of prothrombin, prothrombin intermediates (prethrombin 1 and 2, and active site-blocked (FPR) meizothrombin and meizothrombin desF1), FPR-thrombin, hirugen, and aptamer HD1.
Results: PC was activated by thrombin in a TM456-dependent fashion with a Kd(app) of 16.5 ± 9.1 nM. Activation was inhibited by hirugen and HD1, thereby verifying the exosite 1-dependence of the reaction. The affinities of prothrombin and prothrombin intermediates for TM456 were determined based on their capacity to attenuate PC activation. FPR-thrombin inhibited TM456-dependent, but not TM456-independent, PC activation with a Ki(app) of 45.9 ± 24.9 nM. Prothrombin at concentrations up to 2 μM failed to attenuate PC activation. In contrast, FPR-meizothrombin and FPR-meizothrombin desF1 attenuated PC activation with Ki(app) values comparable to that of FPR-thrombin (64.2 ± 5.7 and 52.4 ± 12.8 nM, respectively, $P > 0.05$). Prethrombin 1 and 2 also attenuated PC activation with Ki (app) values of 497.9 ± 188.5 ($P = 0.051$) and 346.1 ± 90.2 nM ($P > 0.025$), respectively, demonstrating weaker affinity for TM456.
Summary: Whereas prothrombin does not bind TM456, activation cleavage at Arg 320 of prothrombin is sufficient to capacitate exosite 1 for binding. The progressive maturation of exosite 1 for binding TM456 upon prothrombin activation is similar to that for hirugen. Progressive maturation of exosite 1 provides another regulatory switch that modulates the dynamic balance between thrombin's coagulant and anticoagulant roles.

OC 50 – Rare Bleeding Disorders – I

OC 50.1

Antisense-based RNA therapy of severe coagulation factor V deficiency: *in vitro* and ex vivo rescue of a F5 deep-intronic splicing mutation

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Background: Antisense molecules are emerging as a promising tool to correct splicing defects. This form of molecular therapy may be a

valid alternative to fresh frozen plasma in the management of coagulation factor V (FV) deficiency, for which no specific factor concentrate or recombinant preparation is available. Recently, we investigated a patient with undetectable FV and multiple life-threatening bleeding episodes. This patient was homozygous for a deep-intronic splicing mutation (*F5* IVS8+268A>G) which activates a cryptic donor splice site and causes the inclusion of a pseudo-exon with an in-frame stop codon in the mature *F5* mRNA.

Aim: To design antisense molecules targeting the aberrant splice site and to test their efficacy and safety in an *in vitro* minigene model and *ex vivo* on patient-derived megakaryocytes.

Methods: COS-1 (kidney) and HepG2 (liver) cells transfected with a *F5* minigene construct containing the IVS8+268A>G mutation were treated with an antisense morpholino oligonucleotide (MO, 1–5 μM) or with a construct (0.25–2 μg/mL) expressing antisense U7 small nuclear RNA (U7snRNA). After 48 h, mRNA was analysed by real-time qPCR and gel electrophoresis. *Ex vivo* experiments were approved by the Ethics Committee of Padua Academic Hospital and conducted with the patient's informed consent. Patient's megakaryocytes were obtained by *ex vivo* differentiation of circulating haematopoietic progenitors and FV expression was visualised by immunofluorescence analysis. Cytotoxicity of the antisense molecules and/or transfection agents was evaluated *in vitro* using xCELLigence technology.

Results: Expression of the mutant *F5* minigene in COS-1 and HepG2 cells produced aberrant mRNA and normal mRNA in the proportion of ~10:1. Treatment with mutation-specific antisense MO and U7snRNA dose-dependently increased the relative amount of correctly spliced mRNA up to ~30-fold and ~100-fold, respectively, whereas control MO and U7snRNA with irrelevant sequences had no effect. Patient's megakaryocytes did not express FV, but became positive for FV after treatment with mutation-specific antisense MO or U7snRNA. *In vitro* cytotoxicity assays showed that treatment with antisense molecules adversely affects viability/proliferation of COS-1 cells but not of HepG2 cells. Cytotoxic effects were largely attributable to the transfection agents used to deliver the antisense molecules to the cells.

Conclusions: Our findings provide *in vitro* and *ex vivo* proof-of-principle for the efficacy of antisense-based RNA therapy in severe FV deficiency. However, additional studies on the potential cytotoxic effects of antisense molecules and/or transfection agents are needed to ensure the safety of this form of molecular therapy.

OC 50.2

Delivery of a modified U1 small nuclear RNA alleviates splicing-defective coagulation Factor VII expression in mouse models

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Background: The small nuclear RNA U1 (U1snRNA), the component of the spliceosomal U1snRNP,is an attractive therapeutic molecule because of its ability to rescue mRNA splicing impaired by mutations. However, the correction efficacy has been proven only in cellular models. Coagulation factor deficiencies represents ideal models to test the U1snRNA-based strategy since even modest increase of functional protein levels would ameliorate the patients' clinical phenotype. We previously demonstrated in cellular models that the modified U1snRNA U1+5a restores mRNA splicing and coagulant function of coagulation factor VII (FVII) impaired by the *F7* c.840+5G>A splicing mutation, which causes severe FVII deficiency.

Aims: To demonstrate the U1+5a-mediated correction of the F7 c.840+5G>A mutation in mouse models.

Methods: Mouse models of FVII deficiency caused by splicing mutations are not available. We created a novel mouse model of human FVII (hFVII) deficiency by liver-restricted expression, either transient (by hydrodynamic injection of plasmids) or prolonged (by adeno-associated viral [AAV] vectors), of the hFVII splicing-competent cassette harbouring the FVII c.840+5G>A mutation (FVII+5A) in C57BL/6 mice. The rescue was assessed by co-expression of the U1+5a, under the control of its own promoter. To avoid competition for AAV receptor binding, two AAV serotypes with liver tropism were used (AAV2-FVII+5A and AAV8-U1+5a). hFVII expression was evaluated by human-specific assays.

Results: While delivery of plasmid pFVII+5A alone was ineffective, co-delivery of pFVII+5A with a molar excess (1.5X) of pU1+5a resulted in a significant increase of circulating hFVII levels (178 ± 126 ng/mL), with a peak of 367 ng/mL corresponding to 17% of pFVII-wt. This finding was corroborated by the appearance of hFVII-positive staining cells and of correctly spliced hFVII transcripts (26 ± 10% of total transcripts) in mouse liver, thus indicating the ability of the U1+5a to efficiently re-direct usage of the mutated hFVII splicing site *in vivo*.

To assess prolonged correction we exploited AAV. Mice were injected with 1.2×10^{12} vector genomes (vg)/mouse of AAV2-FVII+5A alone or with the AAV8-U1+5a (1.2×10^{11} or 6×10^{11} vg/mouse). Noticeably, circulating hFVII antigen levels were appreciable only in mice treated with the AAV8-U1+5a. The U1+5a-mediated effect was dose-dependent, as measured by hFVII levels of 3.9 ± 0.8 ng/mL (1.2×10^{11} vg/mouse) or 23.3 ± 5.1 ng/mL (6×10^{11} vg/mouse) at 2 weeks post-injection. These findings were corroborated by the appearance of correctly spliced hFVII transcripts (4 ± 0.5% or 16 ± 3% of the total transcripts, respectively) and of hFVII-positive cells in mouse hepatocytes.

Worth noting that in our experimental model hFVII expression results from hepatocytes simultaneously transduced by both AAV2-FVII+5A and AAV8-U1+5a. When mice were injected with an increased dose of template AAV2-FVII+5A (6×10^{12} vg/mouse) and the lowest AAV8-U1+5a dose, the correction effect (6.9 ± 1.7 ng/mL) was more pronounced. This suggests that in FVII deficient patients, expressing the target *F7* pre-mRNA in all hepatocytes, the rescue would be likely more robust.

Conclusions: Altogether, we propose a novel methodology to model human FVII deficiency caused by splicing defects and to evaluate correction approaches *in vivo*. Our data provide the first *in vivo* proof-of-principle of the U1snRNA-mediated rescue of gene expression, and highlight its therapeutic potential in coagulation factor disorders.

OC 50.3

A very rare simultaneous presence of a ring chromosome 13 and a splicing site mutation on Factor X gene

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Background: Factor X (FX) coagulation disorder is are rare autosomal recessive disease with an incidence of 1 in10^6 in the general population. Patients affected with the severe form of this defect tend to be the most seriously affected among those with rare bleeding disorders. The bleeding tendency may appear at any age, although the more severely affected patients present early in life with umbilical-stump or central nervous system (CNS) bleeding. *F10* gene is located on chromosome 13q34 and spans 27 kb; to date, more than 100 mutations were reported to cause FX deficiency and most of them (80%) are missense mutation located at the catalytic domain.

Aims: Molecular characterization of a 2 years-old boy with severe FX deficiency (FX:C > 2%) associated to severe clinical symptoms such as spontaneous CNS subdural haemorrhages, and his family parents.
Methods: Direct sequencing analysis of FX gene by ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Milan, Italy); analysis of seven highly polymorphic short tandem repeats (STRs) selected from the Genethon human linkage map (ABI PRISM Linkage Mapping Set MD10) to verify the presence of parental alleles; investigation of aberrant splicing mechanisms by expression of FX minigenes in mammalian cells; FISH on metaphase chromosomes using Vysiss LSI probe for 13q34 and Vysis TelVysion probe for 13q (Abbott Molecular, Hoofddorp, the Netherlands).
Results: The proband resulted to be homozygous for the c.370+2T>C mutation occurring at the conserved GT dinucleotide of the donor splice site (5′ss) of intron 4. This genetic change significantly reduced the efficiency of the splicing from a score of 39 to undetectable. Expression studies with minigenes indicated that the mutation c.370+2T>C abolishes the canonical 5′ss and activates a cryptic 5′ss in intron 4 (position +55), leading to the insertion of an intronic sequence with a premature stop codon therefore preventing synthesis of a functional protein.
However, while the proband's father confirmed to be heterozygous for the same mutation, the mother was wild type. The presence of a gross deletion on the second allele of the proband or an uniparental disomy (UPD) were then hypothesized. Six out of seven analyzed STRs indicated that the proband had both paternal and maternal alleles; the remaining STR (D13S1265) indicated the possibility of a reduction to homozygosity in the proband due to a partial UPD or of a partial deletion of chromosome 13q34. A further cytogenetic analysis on 80 cells showed 46 chromosomes including a small ring chromosome 13 with breakpoints at p13 and q34. The karyotype was 46, XY, r(13)(p11q34). The karyotypes of both parents were normal and confirm the absence of subtelomere 13q and 13q34 region.
Conclusions: The molecular characterization in this family identified a very rare simultaneous presence of a ring chromosome 13 causing the loss of the entire FX gene associated with a splicing site mutation resulting in an aberrant splicing mechanism. These abnormalities lead to a severe case of FX deficiency who required regular prophylaxis.

OC 50.4

Characterisation of an apparently synonymous F5 mutation causing aberrant splicing and factor V deficiency

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Background: Coagulation factor V (FV) deficiency is a rare bleeding disorder inherited as an autosomal recessive trait. In this study we investigated a 20 year old man with severe FV deficiency (FV:Ag > 3%, FV:C > 3%). The patient's initial diagnosis was made at the age of 15, when he presented with prolonged epistaxis. Subsequently, he was treated with FFP for several muscle bleeds induced by mild trauma.
Aim: To elucidate the molecular basis of FV deficiency in this patient.
Methods: Ethical approval was obtained from the Human Subjects Committee of the University of North Carolina. Mutation screening was performed by amplifying and sequencing each exon of the *F5* gene (including splicing junctions) from the patient's genomic DNA. *F5* mRNA was obtained from blood cells, reverse-transcribed to cDNA, amplified in 20 overlapping fragments and analysed by agarose gel electrophoresis and direct sequencing. The impact of the identified variant on *F5* pre-mRNA splicing was investigated *in silico* using NNsplice and ESE Finder, and verified experimentally in a minigene model. Moreover, the aberrantly spliced *F5* cDNA was expressed in COS-1 cells to evaluate the effects of the mutation on protein secretion and function.
Results: *F5* mutation screening identified two novel heterozygous transversions in the patient's DNA: 668 G>C in exon 4, predicting the substitution of Cys165 by a Ser and consequent destruction of a disulfide bridge; and 1371 C>G in exon 8, which was initially regarded as a neutral variant because it does not predict an amino acid change (Val399Val) and it is located far from splicing junctions. However, cDNA analysis showed that half of the patient's *F5* mRNA was spliced incorrectly between exons 8 and 9 and lacked the last 18 nucleotides of exon 8. Subsequent re-evaluation of the 1371 C>G mutation by *in silico* sequence analysis indicated that this mutation activates a cryptic donor splice site in exon 8 and abolishes an exonic splicing enhancer (ESE) possibly contributing to exon 8 definition. These findings suggested that the 1371 C>G mutation may be responsible for the splicing defect observed in the patient's *F5* mRNA, which was confirmed by expressing a *F5* minigene containing the mutation in COS-1 and HepG2 cells. The aberrantly spliced *F5* mRNA, whose stability was similar to that of the normal mRNA, encodes a putative mutant form of FV lacking amino acids 399–404. Expression experiments in COS-1 cells are currently ongoing to determine whether and to what extent the mutant protein is secreted and functional.
Conclusions: The patient is doubly heterozygous for the *F5* 668 G>C (Cys165Ser) and 1371 C>G (Val399Val) mutations. The latter mutation, though translationally silent, causes FV deficiency by disrupting pre-mRNA splicing. Our findings illustrate a frequently overlooked molecular mechanism of disease and underscore the importance of cDNA analysis for the correct assessment of exonic mutations.

OC 51 – Treatment for Haemophilia

OC 51.1

A novel prediction model for inhibitor development in severe hemophilia A

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Background: Treatment of previously untreated patients (PUPs) with severe hemophilia A is complicated by the formation of inhibitory antibodies against FVIII in. Adequate identification of PUPs with high risk for inhibitor development is a valuable clinical tool for possible alternative treatment strategies to prevent inhibitor development.
Aims: To develop a novel prediction model for inhibitor development at first exposure in PUPs with severe hemophilia A in a large dataset.
Methods: Validation of the original prediction model was performed in a dataset of PUPs born in 2000–2007 (n = 503) with severe hemophilia A (FVIII > 0.01 IU/mL). The novel model was developed in the pooled dataset of the derivation and validation datasets: PUPs born in 1990–2007 (n = 825). All five variables of the original prediction model were used. The variables family history of inhibitors and F8 gene mutation were reclassified to include a reference category with the lowest independent probability for inhibitor development and an additional 'unknown' category to better correspond with clinical practice. The variable intensive treatment at first exposure was redefined as a continuous variable. The variables age at and reason for first exposure were not redefined. Model performance was assessed by calculating positive and negative predictive values (PPV/NPV; calibration) and the AUC of the ROC (discrimination).
Result: Validation of the original prediction model showed an AUC of the ROC of 0.65 (95% CI 0.600.70) and PPVs ranging from 0.12 (low risk) to 0.48 (high risk). In the multivariable logistic regression model the variables family history of inhibitors, F8 gene mutation and number of exposure days at first exposure were significantly associated with inhibitor development. Age at and reason for first exposure were not significantly associated with inhibitor development. AUC of the ROC

was 0.71 (95% CI 0.670.75) and the PPVs of the model were 0.12 (low risk), 0.28 (average risk), 0.35 (increased risk) and 0.66 (high risk).
Summary/conclusions: The novel prediction model for inhibitor development showed improved validity and clinical applicability. The model represents the maximum performance with the current existing clinical parameters at first exposure. However, to increase further the discriminative power of the model other variables like immune regulatory genes need to be added.

OC 51.2
An *in vitro* model studying the effects of rivaroxaban and dabigatran on clot formation in factor VIII-depleted plasma mimicking the plasma of hemophilia A patients

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Background: Hemophilia A patients have impaired hemostasis due to deficiency or dysfunction of clotting factor VIII (FVIII). Despite the bleeding tendencies, thrombosis is a common complication in these patients undergoing factor replacement therapy. Consequently, this necessitates the use of anticoagulants. Rivaroxaban and dabigatran are new, direct, factor-specific anticoagulants. Dosing of FVIII to a target level of > 50% is often required to achieve adequate hemostasis so that anticoagulants can be administered safely. However, there are conflicting recommendations from the American College of Chest Physicians and the American Academy of Orthopaedic Surgeons regarding the optimal FVIII replacement levels in hemophilia A patients requiring anticoagulant therapy. This further complicates the decisions regarding antithrombotic treatment in these patients.
Aim: To investigate the effects of rivaroxaban and dabigatran on clot formation using FVIII-depleted plasma reconstituted with varying levels of FVIII to mimic haemophilia A conditions.
Methods: A turbidometric assay was used to assess clotting time (CT), which was defined as time to reach an absorbance of 0.55. Corn trypsin inhibitor (CTI) was used to inhibit clinically irrelevant FXIIa. Briefly, a 10 μL mixture containing 5 μg/mL phosphatidylcholine and phosphatidylserine (75%/25%) vesicles, 25 nM FIXa, 30 μg/mL CTI, and varying levels of FVIII in the presence or absence of anticoagulant (150 ng/mL rivaroxaban or 180 ng/mL dabigatran), was incubated with 85 μL of vWF-supplemented FVIII-deficient plasma at 37°C for 60 min. The mixture was then added to 5 μL of 15 mM $CaCl_2$ in a 96-well plate, and absorbance at 350 nm was continuously monitored for 2 h. A Williams' *t*-test was used to statistically compare multiple data points to the corresponding baseline control. A $P > 0.01$ was considered statistically significant.
Results: Without anticoagulant, the mean CT at 100% FVIII was 1.9 ± 0.05 min and there were no significant differences in CT until FVIII was > 40%. When FVIII decreased to 5%, corresponding to the upper threshold of moderate hemophilia, the CT increased fourfold to approximately 8.58 ± 0.56 min. At 1% FVIII, which represents the critical level at which hemophilic patients may exhibit spontaneous bleeding, CT further prolonged to 28.1 ± 0.84 min, a 15-fold prolongation as compared to controls with 100% FVIII. Both rivaroxaban and dabigatran increased the CT throughout all the different FVIII levels. In order to achieve a CT equivalent to the values with 1% FVIII in the control, ~10% FVIII was required for samples containing rivaroxaban and ~25% FVIII for dabigatran.
Conclusion: In patients with severe hemophilia A (> 1% FVIII levels) requiring anticoagulant therapy, it is a common practice to supplement these patients with FVIII until the plasma concentration reaches at least 50% that of normal. Our *in vitro* data suggest that, relative to the control with 1% FVIII, we achieved a similar prolongation of CT when FVIII levels were ~10% and ~25% for rivaroxaban and dabigatran, respectively. This suggests that less FVIII replacement may be required to achieve adequate hemostasis in patients receiving new factor-specific anticoagulants, thereby avoiding over-utilization of costly and potentially prothrombotic FVIII in hemophilia A patients requiring anticoagulants therapy.

OC 51.3
Binding and inhibition of cell surface tissue factor pathway inhibitor by an inhibitory fusion peptide

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Background: Tissue factor pathway inhibitor (TFPI) is a key regulator of factor X (FX) activation in the extrinsic pathway of blood coagulation. It inhibits FXa generation by formation of a quaternary complex consisting of factor VIIa (FVIIa), tissue factor (TF), FXa and TFPI. The main portion (~80%) of TFPI in humans is reportedly associated with endothelial cells.
Aim: We used human umbilical vein endothelial cells (HUVECs) as a model to demonstrate improved cell surface TFPI inhibition by a fusion peptide consisting of two peptides targeting two different binding epitopes on TFPI.
Methods: Binding studies of an inhibitory fusion peptide to TFPI on living cells were performed using fluorescence activated cell sorting (FACS) and fluorescence microscopy. HUVECs were incubated directly on the plates with different concentrations of a biotinylated TFPI inhibitory fusion peptide, and subsequently stained with streptavidin FITC. Competition experiments were performed using simultaneous incubation of biotinylated fusion peptide with a molar excess of non-biotinylated fusion peptide. To demonstrate binding of the fusion peptide to TFPI alpha and TFPI beta, cells were treated, before peptide incubation, with phosphatidylinositol phospholipase C (PI-PLC) to remove glycosylphosphatidylinositol (GPI) anchored TFPI beta from the cell surface. Inhibition of cell surface TFPI was analyzed in an FX activation assay performed on living cells. Incubation with an FVIIa-peptide mix and subsequent addition of FX and a FXa-specific fluorogenic substrate monitored the extrinsic tenase activity *in situ* in a fluorescence reader.
Results: Our findings clearly demonstrate that the fusion peptide binds TFPI in a concentration dependent manner; the specificity of its binding to cell surface TFPI was confirmed by competition experiments. We also showed that the fusion peptide binds TFPI alpha as well as TFPI beta on the surface of living HUVECs. This is consistent with its binding epitopes located on Kunitz domain (KD) 1, KD2 and their linker, which result in inhibition of cell surface TFPI in the cell based FX activation assay. FX activation assays demonstrated maximal inhibition of cell surface TFPI at fusion peptide concentrations as low as 10 nM. In contrast, the two peptides comprising the fusion peptide, alone or in combination, did not completely inhibit TFPI, even at very high concentrations.
Summary/conclusions: We provide evidence that a fusion peptide efficiently binds and inhibits endothelial cell surface TFPI as well as plasma TFPI, strongly suggesting that it may inhibit all vascular TFPI forms. Importantly, the molecular fusion of two peptide entities translates to a far more potent inhibition of cell surface TFPI than its two single peptide constituents; thus, the introduction of bivalency resulted in a synergistic effect. Highly potent inhibition of all intravascular TFPI forms is likely to ameliorate hemostasis in hemophilia.

OC 51.4

Plasma-derived factor IX concentrates, but not rFIX, support direct platelet activation and microparticle formation and, as a result of this, increase platelet mediated endogenous thrombin potential

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Bachground: In pharmacokinetic studies the *in vivo* mean incremental recovery of BeneFIX was shown to be about 30% lower compared to plasma derived products although the mean half-life was similar. This indicates towards a different platelet activation and/or binding pattern of recombinant FIX in comparison to pdFIX. Surface-associated PDI is an important regulator of coagulation factor ligation to thrombin-stimulated platelets and of subsequent feedback activation of platelet thrombin generation.
Aims: The binding behaviour of rFIX Benefix and different plasma-derived factor IX (pdFIX) concentrates to platelets were studied and the ability to support thrombin formation in the presence of platelets was compared.
Methods: The influence of rFIX and pdFIX on platelet activation, microparticle formation and endogenous thrombin potential of gelfiltered human platelets resuspended in FIX depleted plasma was studied.
Results: rFIX and the different pdFIX concentrates did not differ in the promotion of endogenous thrombin potential in FIX deficient plasma that was depleted of cells, cell fragments and microparticles by ultracentrifugation. In contrast, the endogenous thrombin potential induced by a minute amount of thrombin was markedly increased by pdFIX in comparison to the rFIX Benefix. The observed increase in thrombin formation by pdFIX in comparison to the rFIX Benefix did not occur when external tissue factor was added. Several of the pdFIX products bound, in contrast to BeneFIX, already to non-activated platelets. In contrast, to induce rFIX binding to platelets, these needed to get activated by high concentrations of thrombin or collagen plus thrombin as described in the literature for FIX inside human plasma. PdFIX supported platelet microparticle formation induced by low concentrations of agonists, while rFIX did not.
Conclusion: We assume that support of platelet activation, microparticle formation and platelet mediated endogenous thrombin potential by pdFIX leads to the pharmacokinetic differences observed for rFIX and pdFIX.

OC 52.1

Intensive care outcomes of patients with thrombotic thrombocytopenic purpura: a single centre study

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Background: Thrombotic thrombocytopenic purpura (TTP) is a rare haematological disorder, associated with significant morbidity and mortality. Specifically, severe neurological or cardiac complications may require a high level of supportive care in the intensive care unit (ICU) setting.
Aims: To describe the TTP population admitted to ICU in a tertiary referral hospital.
Materials and Methods: Single-centre retrospective cohort study of consecutive patients admitted with TTP to ICU between September 2006 and October 2012. Patients were followed-up for a minimum of 3 months after hospital discharge.
Patient demographics, clinical, physiological and laboratory parameters were collected from admission and follow-up data. Acute Physiology And Chronic Health Evaluation II (APACHE II; ICU admission severity score) and Sequential Organ Failure Assessment (SOFA; daily organ dysfunction score) were determined, with normal scores being zero in healthy individuals.
ADAMTS13 activity using Frets and IgG auto-antibody to ADAMTS 13 (ELISA), were measured at presentation and throughout patients course and follow-up.
Results: There were 151 admissions with confirmed TTP, of which 58 acute episodes (57 patients) were admitted to ICU, representing 38% of TTP hospital admissions. 69% ($n = 39$) were female. Median age was 45 (IQR 33–59) years. 51% ($n = 29$) were Caucasian.
APACHE II was 16 (IQR 11.3–20.0), SOFA*tmax* (the cumulative organ insult during ICU) was 9.0 (IQR 5.0–14.8). Mean ICU and hospital length of stay (LOS) was 4.9 $\pm$ 9.8 and 16.7 $\pm$ 14.6 days respectively.
Mortality was 23% ($n = 13$). All deaths occurred in ICU, within 10 days of admission and 31% ($n = 4$) within 24 h. The most common reason for admission to ICU was neurological dysfunction (66%, $n = 38$), with decreased GCS accounting for 45%($n = 27$). ECG changes, ischemic cardiac pain, and cardiac monitoring represented 17% ($n = 10$) of admissions.
Mechanical ventilation was required for 41.7% ($n = 25$) (initiated in the referring centre in 12 cases), cardiovascular support for 23.3% ($n = 14$) and renal replacement therapy for 3.3% ($n = 2$). ADAMTS13 activity at presentation was > 5% in 86.7% ($n = 52$). 98% ($n = 57$) were treated by plasma exchange, 90% ($n = 54$) received steroids and 80% ($n = 48$) rituximab.
Death was the primary outcome. Patients who died had greater median APACHE II score (22.5 [IQR 16.3–31.5] vs. 13.0 [IQR 11.0–18.8]), SOFA*tmax* (19.0 [IQR 13.5–20.5] vs. 7.0 [IQR 5.0–10.5]), IgG anti-ADAMTS 13 antibody titre (70.0 [IQR 38.5–86.5] vs. 38.5 [IQR 12.3–73.0]) and troponin T (0.16 [IQR 0.08–0.43] vs. 0.05 [IQR 0.01–0.17]).
Decreased GCS ($P > 0.01$), mechanical ventilation ($P > 0.001$), cardiovascular support ($P > 0.001$) and troponin T > 0.05 μg/L ($P = 0.011$) were strongly associated with death.
Conclusion: From this large patient cohort, mortality is higher in patients admitted to ICU, a reflection of their greater physiological derangement and associated organ dysfunction. Neurological and cardiac involvements were the most common precipitants to ICU admission. Increasing severity of organ dysfunction, and hence corresponding organ support, as well as biochemical markers of disease severity were associated with death in ICU. Severity scores may be of use in triaging ICU admissions, with APACHE II predicted mortality (22.3%) closely matching actual mortality. We advocate close collaboration between haematologists and intensivists to develop the expertise required to manage this challenging groups of patients.

OC 52.2

ADAMTS13 and Von Willebrand factor antigen levels in patients with severe leptospirosis

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Background: Leptospirosis is an acute febrile illness caused by infection with pathogenic *Leptospira* sp. The clinical spectrum of the disease varies from sub-clinical infection or flu-like episodes to severe disease characterized by jaundice, acute kidney injury and serious hemorrhages. Hematological manifestation is the hallmark of leptospirosis with thrombocytopenia and bleeding of multi-organs are common in the severe cases. Previous studies have shown the elevated soluble E-selectin and von Willebrand Factor (vWF) antigen in severe leptospirosis, reflecting endothelial cell dysfunction. The pathogenesis of thrombocytopenia in leptopirosis is not well understood. We do not

know whether ADAMTS13 has important role in the pathogenesis of thrombocytopenia in patients with leptospirosis.
Aims: To know the ADAMTS13 and vWF antigen levels in severe leptospirosis patients with and without bleeding manifestations.
Methods: A clinical cohort study was conducted in Dr Kariadi hospital, from 2011 to 2012. Sixty severe leptospirosis patients with bleeding, 10 severe leptospirosis patients without apparent bleeding manifestations, and 10 mild anicteric leptospirosis patients were included in the study. The levels of ADAMTS13 and vWF antigens were measured by ELISA.
Results: Fifty six (70%) patients were male, mean age: 48 years (range: 16–81). The levels of ADAMTS13:Ag were significantly different ($P = 0.024$) between the groups of severe leptospirosis with bleeding median: 352 ng/mL (29–885), without apparent bleeding 318 ng/mL (120–572) and mild leptospirosis 562 ng/mL (283–851). No statistical difference between the ADAMTS13:Ag levels in severe patients with bleeding compared to that of severe patients without apparent bleeding ($P = 0.163$). The levels of vWF: Ag were significantly different among the three groups, with median values of 1.1 (0.14–3.4) vs. 0.93 (0.46–1.84) vs. 0.97 (0.56–1.38) ng/mL. The low platelet count was correlated positively with the low levels of ADAMTS13:Ag (r = 0.389, $P = 0.000$), but there was a negative correlation between the platelet count and the level of VWF: Ag. (r = −0.349, $P = 0.002$).
Conclusion: In severe leptospirosis patients with bleeding, the levels of ADAMTS13:Ag decreased while the level of von Willebrand Factor: Ag elevated. The levels of ADAMTS13:Ag and vWF:Ag were associated with the thrombocytopenia.

OC 52.3
Very early onset of autoimmune thrombotic thrombocytopenic purpura in five children with polynesian origin in four combined with immunodeficiency

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Background: Autoimmune thrombotic thrombocytopenic purpura (aTTP) is a rare disease characterized by thrombocytopenia, microangiopathic hemolytic anemia with schistocytes on the peripheral blood smear and a variable degree of end organ ischemia. The seriousness of this disease is underlined by the high mortality if left untreated (80–90%) and relapsing courses in 44% of survivors which demands an optimal treatment regimen. The incidence is about 1.7 cases/million/year. Underlying is a severe ADAMTS13 deficiency (> 10% of the normal activity) due to inhibitory autoantibodies preventing normal processing of strong procoagulative unusually large Von Willebrand factor multimers. The first onset of aTTP typically occur in women between 20 and 40 years of age and are rarely seen in children.
Observation: Strikingly, over the last 7 years we observed frequently relapsing aTTP with severe acquired ADAMTS13 deficiency and inhibitory autoantibodies in five children 1–11 years of age of Polynesian origin. At least four of the five cases have additional signs and symptoms indicative of a common variable immune deficiency (CVID).
Results: Case 1, 2 and 3 have hypogammaglobulinaemia requiring regular intravenous immunoglobulin infusions (IvIg) for at least 5 years now. While in case 1 hypogammaglobulinaemia and other symptoms indicative of immunological dysregulation (eosinophilic colitis, asthma, eczema…) had been documented prior to the first aTTP episode, in cases 2 and 3 (siblings) this was noted after Rituximab treatment for relapsing aTTP episodes. A poor response to single vaccinations (Diphtheria and Tetanus, Pneumococcus) was documented in case 1 and 2. Case 4 has a negative pneumococcal serology. Case 1 and 3 suffer from recurrent invasive infections including pneumococcal pneumonia, meningitis and otitis media. Flowcytometry was done in two children revealing low CD4 (Case 1) and decreased switched- and memory B-cell levels (case 3), respectively.
Conclusions: Although cytopenias such as idiopathic thrombocytopenia or Evans' syndrome, which are both differential diagnoses of aTTP, and various other autoimmune diseases have been reported in CVID, the association with aTTP is novel (or underreported). The common Polynesian ethnicity and the shared clinical phenotype with the unusual early onset of aTTP hints at a new genetic underlying mechanism. Further detailed diagnostics to confirm CVID or similar according to standard criteria (www.esid.com), family studies and the search for common underlying genetic aberrations are underway.

OC 52.4
Genetic predisposing factors in five portuguese patients with atypical hemolytic uremic syndromeidentification of a new mutation in the CFH gene

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Introduction: Atypical Hemolytic uremic syndrome (aHUS) is a rare disease characterized by thrombotic microangiopathic anemia (TMA) predominant renal impairment and absence of Shiga toxin infection as a trigger. Complement alternative pathway dysregulation plays an important role in the pathogenesis of aHUS; approximately 60% of aHUS patients have loss-of-function mutations in the genes encoding complement regulatory proteins (*CFH, CFI, MCP and THBD*) and gain-of-function mutations in complement activators, like factor B (*CFB*) and complement component 3 (*C3*). However, in nearly 40% of the patients no mutations have been identified, in these genes raising the possibility of other genetic causes. Polymorphisms in *CFH* gene have been associated with aHUS, age-related macular degeneration (AMD) and membrane-proliferative glomerulonephritis (MPGN). ADAMTS13 (disintegrin and metalloprotease with thrombospondin motifs) mutations have been associated to thrombotic thrombocytopenic purpura (TTP), characterized by TMA with neurologic symptoms, fever and kidney failure. TTP and aHUS overlap some of the phenotypic features and sometimes their clinical distinction is not clear.
Aim: In order to characterize the clinical diagnosis and molecular background in five patients with aHUS, we performed the screening of *ADAMTS13, CFH, CFI and MCP* genes.
Patients and Methods: Five unrelated Portuguese patients with aHUS were investigated: three children (age: 2, 7 and 10 years) and two adults (age: 35 and 39 years). The children and one adult male had more than two episodes. One adult patient died at presentation. Diagnosis of aHUS was considered in patients with microangiopathic hemolytic anemia, thrombocytopenia, acute renal failure, not associated with Shiga toxin, who presented ADAMTS13 antigen, activity and antibody within the normal range. *ADAMTS13*, *CFH*, *CFI* and *MCP* mutations were screened by PCR/direct sequencing of the promoter regions, exons and exon-intron boundaries. Hemolytic assay was performed using liposome immunoassay (LIA) and C3 and C4 serum levels were determined.
Results: (i) *Clinical and laboratory data:* In 4/5 patients the first episode of aHUS occurred at childhood (≤ 11 years). Plasma complement levels and plasma ADAMTS13 activity (45–107%; RV: 40–130) were normal in all patients. (ii) G*ene analysis: ADAMTS13*- 3 patients presented polymorphisms: Q448E; Q448E/A900V and R7W. Complement regulatory genes – three patients (two children and one adult) have mutations in the heterozygous state: *CFH*- p.N1050Y and p.R1215L; *MCP*c.287-2A>G. CFH p.R1215L, in the short consensus repeat-20, has not been previously reported and is considered 'proba-

bly damaging' by *in silico* analysis. All patients present 3–5 combined polymorphisms in *CFH* (V62I, A307A, H402Y, A473A, Q672Q, E936D) and *MCP* (IVS8+23 G>T). Two of the patients only presented polymorphisms in *ADAMTS13, CFH* and *MCP* genes.
Conclusion: All the five patients' with aHUS have polymorphisms in the complement regulatory genes and three of them also have mutations in *CFH* or *MCP* genes. Three of the patients have polymorphisms in *ADAMTS13*. The adult patient with *MCP* mutation also has two *ADAMTS13* polymorphisms in the heterozygous state. The study of the other complement genes is underway. Genetic screening of patients with aHUS is important to elucidate the diagnosis and to lead their management and specific treatment, eventually with complement inhibitors.

OC 53 – Contact activation

OC 53.1
Polyphosphate stimulates FXIIa-mediated fibrinolysis

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Background: Factor XII (FXII) is classified as a coagulation factor, but is structurally homologous to the tissue-type plasminogen activator and urokinase. The activated form, FXIIa, is reported to function in fibrinolysis, but its role is not well characterised. FXII is activated upon binding to an anionic surface generating αFXIIa, a two-chain enzyme that retains the surface binding site within the heavy chain. Platelet polyphosphate (polyP) is known to bind FXII and accelerate autoactivation and activation by kallikrein.
Aim: This study investigates whether polyP regulates the function of FXIIa in fibrinolysis.
Methods: Fibrin clots were formed with fibrinogen (3.8 μM), glu- or lys-plasminogen (240 nM), αFXIIa (100 nM) ± polyP with an average chain length of 65 phosphates ($polyP_{65}$; 1 μM). In some experiments zymogen FXII (100 nM) was preincubated for 30 min with $polyP_{65}$ (2 μM) before addition to the fibrinogen-plasminogen mixture. Clotting was initiated with thrombin (0.25 U/mL) and $CaCl_2$ (10 mM) and fibrinolysis monitored at 405 nm. 50% clot lysis times were calculated and expressed as mean ± SEM. Plasminogen (200 nM) activation by αFXIIa (100 nM) ± polyP (1 μM) or FXII (100 nM) preincubated ± polyP (2 μM) was monitored using a chromogenic substrate (S2251).
Results: $PolyP_{65}$ significantly enhanced αFXIIa-mediated clot lysis (137 ± 3 min vs. > 360 min; $P > 0.0001$). Clots prepared with lys-plasminogen were more susceptible to αFXIIa-mediated lysis than clots formed with glu-plasminogen (154 ± 2.6 min vs. > 360 min; $P > 0.0001$); consistent with superior binding of the truncated form, lys-plasminogen, to fibrin. $PolyP_{65}$ enhanced αFXIIa-mediated lysis of clots formed with glu-plasminogen (137 ± 3 min) and lys-plasminogen (60 ± 4.1 min) to a similar degree (~2.6-fold). Therefore the ability of polyP to stimulate αFXIIa-mediated fibrinolysis does not arise from increased transition of the closed (glu) to open (lys) form of plasminogen. Chromogenic studies revealed that $polyP_{65}$ accelerates rates of activation of both glu-plasminogen and lys-plasminogen. $PolyP_{65}$ alone was unable to stimulate plasminogen activation and does not directly modify plasmin activity, indicating that $polyP_{65}$ is acting as a cofactor in αFXIIa-mediated cleavage of plasminogen to plasmin. We have previously shown that platelet polyP stimulates autoactivation of FXII. We therefore monitored the ability of $polyP_{65}$ to activate zymogen FXII and subsequently function as a cofactor in fibrinolysis. Addition of FXII, that had been pre-incubated with $polyP_{65}$, to clots containing fibrinogen and glu-plasminogen yielded clot lysis times of 203 ± 13 min. In the absence of $polyP_{65}$ FXII was unable to induce lysis within the 480 min time frame analysed. Consistent with the effects observed on clot lysis, accelerated rates of plasminogen activation were apparent in chromogenic assays when FXII was preincubated with $polyP_{65}$.
Conclusions: PolyP provides an appropriate surface for FXII activation and remains associated with the heavy chain of the enzyme. PolyP promotes the plasminogen activator function of FXIIa, thereby accelerating plasmin generation and downstream fibrinolysis. FXII circulates at high concentrations in plasma and, in the presence of platelet polyP, may be a potent regulator of fibrinolysis.

OC 53.2
A nanobody-based method for tracking factor XII activation in plasma

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Introduction: Factor XII (FXII) initiates blood coagulation, but can also independently trigger the inflammatory kallikrein-kinin system when plasma contacts anionic materials. While all contact activators induce inflammation, only few induce coagulation. Interestingly, active FXII (FXIIa) can occur in two forms: full-size α-FXIIa (80 kDa) triggers both coagulation and inflammation simultaneously, whereas truncated β-FXIIa (28 kDa) only displays proinflammatory activity. This suggests that the formation of trigger-specific FXIIa isoforms underlies each of the two contact activation responses. At present no method is available to detect free FXIIa in patient plasma.
Aim: To develop a bioassay for tracking FXII activation by various triggers in plasma.
Methods: FXIIa specific nanobodies (17 kDa monovalent camelid antibody fragments) were selected using a phage display system and implemented in an ELISA-based bioassay.
Results: Two nanobodies were selected for their ability to recognize FXIIa, but not FXII zymogen. Nanobody A10 recognizes the catalytic domain of purified α-FXIIa, but not that of purified β-FXIIa, whereas nanobody B7 recognizes both. This suggests minute differences in the catalytic domain between these two isoforms of FXIIa.
Capturing of FXIIa from human plasma is strongly enhanced by the small-molecular protease inhibitor PPACK. This molecule occupies the FXIIa active site and prevents the association of macromolecular plasma protease inhibitors, which prevents nanobody recognition.
Upon *in vitro* activation of the plasma contact system, our nanobody-based assays distinguish activation products of FXII that vary with the type of activator present: whereas procoagulant activators solely trigger the formation of a species that is captured by B7, proinflammatory activators first generate a species that is recognized by B7, which is later converted into a second species that is recognized by A10.
Conclusions: Our studies shows that the development of nanobodies offers new opportunities to investigate the activated enzymes in health and disease. Findings from our new bioassay indicate that a progressive proteolysis of FXIIa converts it from a coagulation factor into an inflammatory enzyme. The sensitivity achieved in this new assay is far greater than that of purified chromogenic substrate assays, enabling us to further examine the role of FXII in biocompatibility issues, cardiovascular disease or inflammatory conditions such as angioedema.

OC 53.3
Aberrant contact system activity causes hereditary angioedema type III

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Background and Aim: Hereditary angioedema (HAE) is an inherited rare disorder that is clinically characterized by life-threatening swelling attacks. In contrast to the classical HAE types I and II that are caused by deficiency in functional C1-esterase inhibitor, HAE type III patients have normal C1-esterase inhibitor. A single point mutation at position

Thr328 in coagulation factor XII (FXII) has been associated with HAE type III, however, mechanism and therapy of the disease have remained enigmatic.
Methods and Results: Here, we analyze mechanism of HAE type III in patient plasma and in edema models in genetically altered humanized mice. Chromogenic assays and substrate conversions indicated largely increased enzymatic activity of mutated vs. normal FXII. Recombinant and patient-derived FXII variants showed that the Thr328 substitution in FXII causes excessive activation of the plasma contact system resulting in overshooting production of the inflammatory mediator bradykinin. Addition of C1-esterase inhibitor dose-dependently blocked bradykinin production in HAE types I and II, but was inactive in HAE type III. Biological and small molecule FXII inhibitors interfered with aberrant contact system-triggered bradykinin formation in HAE type III plasma. To analyze the mechanism of HAE type III *in vivo*, we reconstituted FXII null mice with recombinant human FXII mutants and established a transgenic mouse that tetracycline-regulatable expresses Thr328-mutated human FXII in the liver. Intravital confocal scanning microscopy and tracer extravasation-based methods showed excessive bradykinin-mediated vascular leakage in HAE type III mice challenged by infused FXII-contact activators and in IgE/allergen-stimulated cutaneous anaphylaxis models, respectively.
Conclusion: This study elucidates the disease mechanism of HAE type III *in vivo* and *in vitro*, and suggests that FXII inhibition might be a rational therapeutic strategy for prevention and treatment of edema.

OC 53.4
Identification of leukocyte factor XII: a critical regulator of WBC function

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Background: Leukocytes from Factor XII (FXII) deficient patients migrate less into skin windows. Zymogen FXII binds to uPAR and stimulates cell signaling through β1 integrins, leading to endothelial cell proliferation and angiogenesis. *In vivo* FXII KO mice have reduced wound angiogenesis (Blood 115:4979, 2010).
Aims: Since FXII modulates wound angiogenesis, we examined FXII in inflammation and wound repair *in vivo*.
Methods: Skin wound (5 mm punch biopsies) and thioglycolate (TG)-induced chemical peritonitis were performed on *F12*−/−, uPAR-deleted (*Plaur*−/−), and Bradykinin B2 Receptor-deleted (*Bdkrb2*−/−)mice. Wounds were harvested from days 1 to 7 (D1-7) and frozen sections were stained with anti-CD11b, Gr-1, and F4-80. Analysis determined the relative number of neutrophils and macrophages in the wounds. In TG-induced peritonitis, the number of peritoneal exudate cells (PECs) was measured by cell counting and flow cytometry. Bone marrow (BM) transplantation experiments determined if the defect in murine *F12*−/− leukocytes was host- or bone marrow-derived. Determination of a leukocyte pool of FXII was performed by isolating bone marrow cells' mRNA followed by PCR on its cDNA. Leukocyte FXII antigen was determined by immunofluorescence. The contribution of plasma/liver- vs. leukocyte/bone marrow-derived FXII was determined by intravenous FXII siRNA injection creating plasma FXII deficiency in WT mice. The *in vivo* functions of leukocyte FXII were examined in wound healing experiments.
Results: On Day 1 after punch biopsy wounds, *F12*−/− mice exhibit decreased recruitment ($P = 0.0136$) of CD11b-labeled inflammatory cells to injury sites. *F12*−/− mice have reduced wound Gr-1(+) neutrophils and F4-80(+) macrophages. Similar findings were observed with *Plaur*−/− but not with *Bdkrb2*−/− mice. In the TG-induced peritoneal inflammation assay, *F12*−/− mice exhibited significantly decreased number of PECs on D1 and D7. *Plaur*−/− and *Bdkrb2*−/− mice had no defects in this assay. In *F12*−/− mice, there was a disproportionate decrease in neutrophil recruitment in peritoneal fluid. *F12*−/− macrophages have reduced adherence to plastic. On adoptive BM transplantation experiments, WT BM transplanted to *F12*−/− mice corrected the TG-induced PEC migration defect for D1 and D7 from instillation. Alternatively, *F12*−/− BM transplanted into WT, recapitulated the leukocyte defect observed in *F12*−/− mice. WT mice treated with FXII siRNA reduced plasma FXII activity, > 10% at 24 h ($T_{1/2} = 6.7$ h). Plasma FXII deficient mice did not have a PEC recruitment defect. Additionally, *in vivo* infusion of purified FXII did not correct the PEC recruitment defect in *F12*−/− mice. BM cell-derived mRNA from WT mice, reverse-transcribed to cDNA, demonstrates XII cDNA that shares sequence homology to hepatic XII from exons 2 through 14. Immunofluorescence staining of BM-derived leukocytes demonstrates XII in cells with nuclear morphology resembling monocytes and neutrophils. *In vivo*, murine FXII deficiency is associated with an improved rate of punch biopsy wound healing.
Conclusions: These data show that *in vivo*, there is a unique pool of leukocyte FXII (BM-derived) distinct from plasma (hepatic) FXII. Leukocyte FXII is responsible for leukocyte function in wounds and inflammation sites. *F12*−/−mice have attenuated wound injury and, paradoxically, improved wound healing rates. Modulation of leukocyte FXII promotes wound healing.

OC 53.5
Targeting the polyphosphate-coagulation factor XII pathway prevents cardiac ischemia-reperfusion injury without influencing bleeding risk

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Background and Aims: Ischemia and reperfusion-elicited cardiac injury contributes to mortality in myocardial infarction. We have identified a critical role for the plasma protease coagulation factor XII (FXII) and its platelet-derived inorganic activator polyphosphate in cardiac ischemia-reperfusion injury.
Methods and Results: FXII deficiency and neutralization of polyphosphate with phosphatase largely reduced fibrin deposition and infarct size in ischemic myocardium. *Vice versa*, reconstituting FXII null mice with human FXII restored fibrin formation and myocardial injury in ischemic heart tissues. Factor XII deficient mice, animals lacking the FXII substrate factor XI, and mice with combined deficiency in factors XII and XI, are protected from myocardial infarction to similar degrees, indicating that FXII contributes to cardiac ischemia-reperfusion injury via the intrinsic pathway of coagulation. Deficiency in FXII and degradation of polyphosphate attenuated procoagulant platelet activity, impaired clot stability, and blocked thrombus formation under flow, resulting in significant protection from myocardial infarction. Despite its critical role in thrombus formation and tissue ischemia, genetic and pharmacologic targeting of the polyphosphate-FXII pathway did not increase bleeding risk.
Conclusion: The data reveal a fundamental role for polyphosphate-initiated, FXII-dependent coagulation in cardiac ischemia and implicate this pathway as a target for safe interference with ischemia-reperfusion injury.

OC 53.6

A pivotal role of regulatory pathways of autophagy during the formation of neutrophil extracellular traps (NETs) in human neutrophils

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Background: Activated neutrophils can form neutrophil extracellular traps (NETs) to sequester microbes in the extracellular environment through the mechanism called NETosis. The steps of NETosis are characterized by massive vacuolization, chromatin decondensation, membrane disruption and extracellular release of chromatin fibers armed with antimicrobial granular cargos. These dynamic cellular events require both activities of oxidative burst and autophagy; in fact, the lack of either axis leads to neutrophil apoptosis. However, the mechanistic detail of the regulation of NETosis is still poorly understood.

Aims: We aimed to determine the molecular mechanisms that coordinate neutrophil NETs formation in response to inflammatory stimuli. In particular, we investigated roles of the inhibitory pathways of autophagy, mTOR and calpain during NETosis.

Methods: Purified human neutrophils were stimulated with phorbol 12-myristate 13-acetate (PMA; an activator of protein kinase C), or f-Met-Leu-Phe (fMLP; a bacterial-derived peptide). In selected experiments, neutrophils were pretreated with pharmacological inhibitors. NETs formation was monitored in the presence of the cell-permeable DNA-binding dye, Hoechst 33342 and/or the cell-impermeable DNA dye, Sytox Green, using fluorescence microscopy.

Results: We found that the kinetics of PMA-driven NETosis was accelerated in the presence of inhibitors for the mTOR pathway (rapamycin) or calpain (ALLN), and that the combination of these two inhibitors resulted in an additive increase of NET formation as compared to vehicle-treated cells. Stimulation of neutrophils with fMLP alone failed to produce NETs; however the presence of the calpain inhibitor ALLN, but not the mTOR inhibitor rapamycin, resulted in vacuolization and efficient induction of NETosis in response to fMLP. Our data show that inhibitors for cytoskeletal machinery, such as myosin II (blebbistatin), microtubule (nocodazole) and actin polymerization (cytochalasin D), blocked PMA-induced NETs formation, as well as the effect of ALLN on NETosis downstream of fMLP.

Conclusions: Our findings suggest that mTOR and calpain pathways, which serve as inhibitory pathways of autophagy, can negatively regulate the initial stage of PMA-induced NETosis. This calpain activity may prevent neutrophils from undergoing NETosis downstream of the fMLP-receptor signaling pathways, thus playing a pivotal role in driving neutrophils toward either apoptosis or NETosis. Moreover, we show that NETs formation requires the activity of cytoskeletal machinery, highlighting multiple layers of regulatory mechanisms underlying NETosis.

OC 54 – Endothelial cells

OC 54.1

Fuelling the knowledge of blood coagulation signaling with time resolved quantitative phosphoproteomics of thrombin-stimulated endothelial cells

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Thrombin is the central enzyme involved in blood coagulation. It serves an additional important function beyond coagulation by cleaving protease-activated receptors (PARs) on the cell surface of endothelial cells, thereby initiating signal transduction cascades. Through these signal transduction cascades, thrombin regulates the barrier function of the endothelium and the release of storage organelles, called Weibel-Palade bodies that contain various hemostatic and vasoactive substances. The identification of downstream effectors of PARs is critical for understanding the interactions between signaling cascades and for developing new pharmacological approaches for controlling thrombin- and PAR-mediated responses. Due to the complexity of signal transduction networks, the molecular mechanisms underlying endothelial signaling in response to thrombin have remained incompletely understood. Here we have combined Stable Istotope Labeling with Amino acids in Cell culture (SILAC), affinity-based phospho-peptide enrichment and high resolution mass spectrometry to perform a quantitative and time-resolved analysis of the thrombin induced signaling events in endothelial cells. Blood outgrowth endothelial cells (BOECs) were SILAC labeled and stimulated with 1 U/mL thrombin for 2, 5, 10 or 30 min in a three-way reverse labeling experiment. Digested peptides were separated using Strong Cation Exchange chromatography, enriched for phospho-peptides by titanium dioxide precipitation, and analyzed at the LTQ-Orbitrap Elite mass spectrometer. We identified and accurately quantified 7791 class I phosphorylation sites localized on a total of 2593 gene products. Of those, 2224 phosphorylation sites were found significantly regulated over the 30-min time course. Amongst others, we found the established PAR1 (thrombin) receptor, Rho-associated kinase 2, protein kinase C and phospholipase C, which provide a positive control for our quantitative approach. Further bio-informatic analysis highlighted enrichment of KEGG pathways, such as leukocyte transendothelial migration, focal adhesion and regulation of actin cytoskeleton, as well as various gene ontology categories, including GTPase regulator activity, regulation of Ras and Rho protein signal transduction and adherens junction. In addition to the identification of these well-established events, a wealth of previously unidentified phosphorylation sites and processes were found to be regulated. Our study clearly shows the enormous potential of using a global phosphoproteomic approach to unravel endothelial cell signaling, and we anticipate that the present study will be a powerful resource for cell biologists working in the fields of endothelial barrier function, Weibel-Palade body release and PAR-mediated signaling. Moreover, our study demonstrates that the key enzyme of the coagulation cascade thrombin initiates a powerful signal transduction cascade that results in the phosphorylation of a large array of downstream effectors and therefore emphasizes the intimate relation between coagulation and vascular biology.

OC 54.2

Aldosterone decreases thrombin generation via enhancement of thrombomodulin-mediated protein C activation

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Background: Aldosterone (aldo) and its receptor, the mineralocorticoid receptor (MR) are implicated in several physiopathological processes such as heart failure. Aldo has been proposed to be prothrombotic through impairment of fibrinolysis in animal models. On the other hand, aldo, via MR activation, upregulates the endothelial protein C receptor (EPCR) in bone marrow endothelial cells and prolong clotting times in presence of activated protein C (APC). These findings suggest an imbalance of the hemostatic system, modulated by the extent of MR activation on endothelial cells. However, the role of MR, a ligand-activated transcriptional factor, in endothelial anticoagulant function remains to be elucidated.

Aims: Our aim was to investigate whether MR activation decreases *in vitro* thrombin generation at the surface of endothelial cells and modifies the thrombotic risk *in vivo*.

Methods: *In vitro*, we used human aortic endothelial cells (HAECs) to explore the mechanisms of MR activation on the dynamic anticoagulant APC pathway. HAECs were treated for 24 h with 10^{-8} M aldo in the presence or not of 10^{-6} M MR or glucocorticoid receptor antagonists. *In vivo* we used our mouse model with conditional overexpression of the MR in endothelial cells (MR-EC) (Nguyen Dinh Cat et al., FASEB J. 2010; 24:2454–63). Thrombin generation in mouse plasma or at the surface of adherent HAEC was measured using calibrated automated thrombography (CAT). Thrombus formation was evaluated in MR-EC mice after $FeCl_3$ injury.
Results: At the surface of cultured HAECs, thrombin generation was attenuated (area under the curve values (ETP): 785 ± 16 vs. 661 ± 16 nM.min, $P > 0.05$) and EPCR synthesis and secretion were increased by 20 and 30% upon stimulation with aldo. Blocking the MR, but not the glucocorticoid receptor, reversed these effects. Inhibition of protein C activation by an anti-thrombomodulin antibody resulted in an enhancement of thrombin generation, which was not impaired by aldo. Similar results were obtained with protein C-deficient plasma. When washed HAECs were incubated with purified protein C and increasing concentrations of thrombin, aldo enhanced APC generation on these cells in a thrombin dose-dependent manner. Vessel occlusion times after exposure of the carotid artery surface to ferric chloride was delayed in MR-EC compared with control mice (7.4 ± 0.5 vs. 4.4 ± 0.3 min, $n = 6$ in each group). In the absence of the APC system, thrombin generation parameters did not differ between MR-EC and CT mice. There was a greater ability of added APC to diminish thrombin generation in plasma of MR-EC mice compared to plasma of controls (ETP: 57 ± 10 vs. 145 ± 30 nM.min).
Summary/Conclusions: These findings reveal that MR activation in the vascular endothelium of healthy vessels can protect against thrombosis and demonstrate that MR-mediated EPCR overexpression drives this anti-thrombotic property through enhancement of thrombomodulin-mediated protein C activation. This anti-thrombotic property of MR activation may have clinical implications in drospirenone-containing oral contraceptives since drospirenone is a potent MR antagonist.

OC 54.3
Endothelium but not platelet derived thiol isomerase ERp57 is required for thrombosis *in vivo*

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Background: The thiol-disulfide oxidoreductase ERp57 is a member of the endoplasmic reticulum resident protein disulfide isomerase (PDI) family. ERp57 is also found on the activated platelet surface. Like PDI, ERp57 mediates platelet activation and thrombus formation.
Aims: Here we determine the contributions of endothelial- and platelet-derived ERp57 to thrombus formation and fibrin generation in the mouse laser injury model.
Methods: To study contributions from different cellular sources ERp57 was depleted in mouse platelets using targeted gene deletion by crossing $ERp57^{flox/flox}$ mice with PF4 $Cre^{+/0}$ mice. Knockdown of ERp57 in all adult mouse tissues was achieved by blocking translation of ERp57 protein using vivo-morpholinos. Finally thrombus formation was examined in the presence of a function blocking antibody to ERp57.
Results: *In vitro* studies using cultured endothelial cells show that ERp57 is secreted rapidly into the culture medium on thrombin stimulation. ERp57 was also detected on the surface of endothelial cells by flow cytometry. Morpholino mediated knockdown of ERp57 did not affect the translation of other thiol isomerases: PDI, ERp5 and ERp72. Knockdown of ERp57 resulted in diminished platelet aggregation *in vitro*. Aggregation of platelets by 50 μM AYPGKF, a PAR4 agonist was also inhibited in platelet specific conditional knockouts for ERp57. This defect in aggregation was similar to inhibition observed in the presence of a function-blocking antibody to ERp57. There was also a defect in hemostasis observed by increased tail bleeding times in mice with platelet conditional knockout or morpholino knockdown of ERp57. Using intravital microscopy, we observed that ERp57 is detectable on activated endothelium of the vessel wall after laser injury and in the developing thrombus. The median integrated fluorescence intensity for ERp57 in wild-type mice was compared to wild-type mice injected with eptifibatide, an inhibitor of platelet activation, and thus an inhibitor of the contribution of ERp57 derived from platelets. ERp57 expression was similar in both treated and untreated animals up to 20 sec after injury. After 20 sec, the expression of ERp57 in eptifibatide-treated mice decreased significantly compared to that in untreated mice, indicating that initial ERp57 was derived from the endothelium and later additional ERp57 was derived from platelets following their accumulation in the thrombus. Infusion of a function-blocking antibody to ERp57 prior to vessel wall injury led to dose-dependent inhibition of platelet thrombus formation and fibrin generation in the mouse laser injury model of thrombosis. Deletion of ERp57 by gene targeting of PF4 in platelets as well as vivo-morpholino mediated translational block demonstrated a reduction in platelet thrombus formation as well. However, the rate and amount of fibrin generation were diminished only in mice with global ERp57 knockdown either by antibody mediated functional inhibition or morpholino mediated translational block.
Summary/Conclusion: These results provide evidence for a role of platelet and endothelium derived thiol isomerase, ERp57, in thrombus formation. Early in the time course of laser-induced thrombus formation the primary source of ERp57 is the endothelium. As we have observed previously in our laser-induced injury model the activated endothelium at the injury site can support maximal fibrin formation.

OC 54.4
Parmodulins act at PAR1 to stimulate cytoprotective genetic program in endothelial cells

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Background: Activation of PAR1 in endothelial cells by thrombin elicits its loss of barrier function and apoptosis. In contrast, activation of PAR1 by APC stimulates cytoprotective signaling in endothelium. We have identified and characterized a novel class of small molecules, termed parmodulins, which modulate PAR1 function by acting at the intracellular face of the receptor. In platelets, parmodulin-1 (aka JF5, PNAS, 108:2951) and parmodulin-2 (aka ML161, ACS Med Chem Lett, 3:232) selectively block signaling downstream of PAR1 and interfere with thrombosis, but not hemostasis in murine models.
Aims: Our goal was to determine whether parmodulins act on PAR1 to modify the function of endothelial cells.
Results: Initial studies demonstrated that parmodulins blocked thrombin-induced loss of barrier function as assessed by immunofluorescence microscopy of phalloidin-stained human umbilical vein endothelial cell (HUVEC) monolayers. Unexpectedly, parmodulins also protected against apoptosis induced by TNF-α or staurosporine as assessed by staining with YO-PRO-1 dye. To confirm that parmodulins acted at PAR1 to achieve their cytoprotective activity, we tested their ability to prevent TNF-α-induced apoptosis in HUVECs transfected with either PAR1 siRNA or a vector control. Parmodulins and APC both protected mock-transfected HUVECs from TNF-α-induced apoptosis. In contrast, neither parmodulins nor APC protected against apoptosis in PAR1 knockout HUVECs. A 4 h exposure to parmodulins was required to achieve maximum protection against TNF-α-induced apoptosis, raising the possibility that parmodulins stimulate a cytoprotective genetic program in endothelial cells. Transcript profiling of > 30,000 genes was performed in HUVECs exposed to vehicle alone, parmodulin-2, TNF-α, or parmodulin 2 then TNF-α. TNF-α elicited the upregulation of 748 genes. Parmodulin-2 inhibited upregulation of 107 of these genes by > 1.5-fold and elicited upregulation of 26 genes by > 1.5-fold. RT-PCR confirmed parmodulin-2-mediated inhibition of MMP10, END1, TL2, and RSPO3 transcripts as well as parmodulin-2-mediated upregulation ofSTC-1 transcripts. Several of

the genes downregulated by parmodulin-2 are under the control of NF-kB. Both parmodulins and APC blocked TNF-α-induced expression of a GFP reporter construct under the control of a NF-kB sensitive promoter, confirming the transcript profiling results indicating that parmodulins act in part by inhibiting signaling to NF-kB. Stanniocalcin-1, which was upregulated in response to parmodulin-2, has cytoprotective effects in endothelial cells. We therefore evaluated stanniocalcin-1 protein expression in response to parmodulins. Exposure to parmodulins 1 and 2 resulted in a 3.7 + 1.1-fold and 4.4 + 0.4-fold increase in stanniocalcin-1, respectively. Stimulation with APC, low dose thrombin and SFLLRN stimulated stanniocalcin-1 levels 5.0 + 0.8-fold, 3.0 + 0.3-fold, and 2.8 + 0.8-fold, respectively. Since the cytoprotective effects of parmodulins resemble those of APC and APC blocks inflammation and sepsis *in vivo*, we evaluated parmodulin-2 in a mouse model of inflammation. In these studies, parmodulin-2 completely blocked the sixfold increase in leukocyte rolling observed over the 70 mins following surgical exposure of cremaster venules.

Conclusions: Parmodulins are the first small molecules identified to activate a cytoprotective genetic program in endothelial cells through PAR1. They interfere with NF-kB-mediated transcription and stimulate expression of stanniocalcin-1. These biased PAR1 endothelial cell agonists represent a new strategy in the treatment of inflammatory disorders and sepsis.

OC 54.5

Endothelial Protease Nexin-1 potentiates the cytoprotective effects of activated Protein C by preventing endothelial Protein C receptor shedding

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Background: Human protein C is a plasma serine protease that plays a key role in haemostasis, and activated protein C (aPC) is known to elicit protective responses in vascular endothelial cells. This cytoprotective activity requires the interaction of the protease with its cell membrane receptor, endothelial protein C receptor (EPCR), followed by the activation of the protease-activated receptor-1 (PAR-1). However, the mechanisms regulating the beneficial cellular effects of aPC are not well-known.

Aims: The scientific question we address is to determine whether a serine protease inhibitor called protease nexin-1 (PN-1) or serpinE2, expressed by vascular cells, can be considered as a new player in the cytoprotective aPC pathway.

Methods: We studied the cytoprotective activities of aPC *in vitro*, by analyzing its effect both in the model of staurosporine-induced apoptosis and in thrombin-induced permeability in a dual-chamber system, using EAhy926 endothelial cells whose PN-1 expression was silenced by siRNA transfection or blocked with a specific neutralizing anti-PN-1 antibody. Dermal vascular hyperpermeability was also explored *in vivo* by using a modified Miles assay. In this assay we compared the leakage of Evan's Blue in response to local intradermal injection of VEGF in both wild-type and PN-1-deficient mice.

Results: We observed that vascular barrier protective and anti-apoptotic activities of aPC were reduced both in endothelial cells underexpressing PN-1 and in endothelial cells whose PN-1 function was blocked by a neutralizing antibody. Our *in vitro* data were further confirmed *in vivo*, since local intradermal injection of aPC could abolish VEGF-mediated hypermeability in the skin of wild-type mice whereas it had no protective effect in the skin of PN-1-deficient mice. These data can be explained by a previously unknown protective role of endothelial PN-1 on EPCR shedding. We indeed provided evidence that PN-1 inhibits furin activity, a serine protease known to activate a disintegrin and metalloproteinase 17 (ADAM17) involved in the shedding of EPCR. We evidenced *in vitro* by using recombinant proteins that PN-1 forms a SDS-stable complex with furin which leads to the inhibition of furin activity, and thereby reduces the furin-dependent maturation of proADAM17. Moreover double immunofluorescence revealed that furin colocalized with PN-1 both at the intracellular level and at the surface of the cells, indicating a direct interaction between PN-1 and furin in endothelial cells.

Conclusions: Our results demonstrate an original role of endothelial PN-1 as a furin convertase inhibitor, providing new insights for understanding the regulation of EPCR-dependent aPC endothelial protective effects. Both PN-1 and furin are expressed in numerous cells, and since furin represents a processing enzyme of various cell surface receptors and growth factors, its inhibition by PN-1 open new fields of investigation in many different disciplines.

OC 54.6

Platelet endothelial aggregation receptor-1 is a critical determinant of endothelial cell function

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Background: Platelet endothelial aggregation receptor-1 (PEAR1), a type I transmembrane receptor, is expressed on platelets and endothelial cells (ECs) but its contribution to EC function is unknown. We aimed at unraveling its physiological and pathophysiological role in ECs.

Methods and Results: PEAR1 expression was analyzed by qPCR in cultured human endothelial progenitors (blood outgrowth endothelial cells or BOECs), in ECs from human umbilical cord and in ECs freshly isolated from multiple vascular beds. PEAR1 mRNA expression was heterogeneous, with the lowest expression in BOECs and the highest in macrovascular and microvascular ECs from heart and liver tissue. ECs from umbilical cord showed intermediate expression levels. Stainings of fast growing hemangiomas (human pyogenic granuloma) revealed low expression of PEAR1 in the young, rapidly proliferating ECs of the granuloma compared to normal subcutaneous ECs.

The role of PEAR1 in EC proliferation was evaluated in the hybrid cell line EA.hy926. Knockdown of PEAR1 by lentiviral transduction with a short-hairpin anti-PEAR1 construct (shPEAR1) doubled the EA.hy926 EC proliferation rate, as compared to control lentiviral-transduced cells. This was associated with significantly increased baseline Akt-P levels (PI3K-activity) in shPEAR1 cells and significantly decreased levels of the Akt-P phosphatase PTEN. Expression of the nuclear proliferation suppressor p21/CIP1 in shPEAR1 ECs was reduced by 60 ± 2% (mean ± SEM, qPCR and Western blot), whereas the band intensity on Western blots for the cyclin-dependent kinase CDC2 significantly increased, consistent with increased mitosis. During *in vitro* matrigel tube formation assays, using human umbilical artery endothelial cells (HUAECs), PEAR1 expression rose fivefold. This was associated with a threefold increase of PTEN. In constrast, during tube formation of PEAR1-knockdown HUAECs, PTEN-levels dropped 1.5-fold. PEAR1-knockdown significantly ($P > 0.005$) increased total tube length, the number of tube branching points and the number of tubes compared to control HUAECs. These findings are in accordance with the increased baseline phosphorylation of Akt in shPEAR1 ECs.

Anti-PEAR1 antibodies behaved as a pseudo-ligand in EA.hy926 ECs and HUAECs and triggered PI3-kinase activation, resulting in PEAR1 phosphorylation and a short-term elevation of Akt-P levels. This elevation enhanced the phosphorylation of eNOS (activating site P1177 on Western blot), an enzyme responsible for rapid production of NO in ECs. Correspondingly, anti-PEAR1 antibodies mildly stimulated tube formation of control HUAECs.

Conclusions: Our study provides the first evidence that PEAR1 is expressed in ECs from multiple vascular beds and that PEAR1 sup-

presses proliferation, modulates eNOS-activation and controls tube formation processes via the PI3K/Akt/PTEN- pathway in ECs. The upregulation of PEAR1 during EC maturation is compatible with a role for PEAR1 in endothelial cell differentiation.

OC 55 – Immune Thrombocytopenia Purpura

OC 55.1

Modified HPA-1a monoclonal antibody to prevent fetal-alloimmune thrombocytopenia

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Background: Fetal/neonatal alloimmune thrombocytopenia (FNAIT) is often caused by maternal alloantibodies against the human platelet antigen (HPA)-1a. Subsequent platelet destruction is mediated via the Fc-part of the alloantibodies. The monoclonal antibody SZ21 binds to the HPA-1a-epitope and inhibits binding of maternal alloantibodies. However, it also promotes complement activation and phagocytosis. Deglycosylation of antibodies abrogates the Fc-related effector functions but may still allow transplacental transport.
Aims: To investigate the use of *N*-glycan modified HPA-1a antibody in preventing platelet destruction in FNAIT.
Methods: To modify the *N*-glycan of SZ21, deglycosylation was performed under native conditions using Endo F, and the modification was evaluated by Coomassie Blue and lectin blotting. To investigate transplacental transport, pregnant BALBc mice were injected with SZ21, *N*-glycan modified (NGM)-SZ21 or isotype-matched control IgG antibody at gestation day 17. The impact of *N*-glycan removal on the binding affinity of SZ21 for the HPA-1a-epitope on GPIIb-IIIa was analyzed using Surface Plasmon Resonance (SPR). The ability of NGM-SZ21 to prevent anti-HPA-1a-mediated platelet clearance *in vivo* was investigated using the NOD/SCID mouse model of alloimmune thrombocytopenia.
Results: Endo F treatment only removed the *N*-glycan attached to Asn 297 of the antibody heavy chain without altering the protein structures. When injected into pregnant mice, both native SZ21 and NGM-SZ21 were transported equally into fetal circulation (8.9% vs. 8.7%, $p > 0.05$). The binding properties of NGM-SZ21 to HPA-1a were not affected after *N*-glycan modification (fitted equilibrium dissociation constant 1.17×10^9 vs. 4.36×10^9 M, $p > 0.05$). NGM-SZ21 prevented platelet-destruction induced by maternal anti-HPA-1a antibodies *in vivo* in the NOD/SCID mouse model (Elimination of HPA-1a-platelets: 62% vs. 18%, $P = 0.013$).
Summary/Conclusion: Deglycosylation of SZ21 abrogates Fc-moiety related effector functions, while neither its transplacental transport nor its capacity to block binding of maternal HPA-1a antibodies to platelets was impaired. Humanized, deglycosylated anti-HPA-1a monoclonal antibodies may represent a novel treatment strategy to prevent anti-HPA-1a mediated platelet destruction in FNAIT.

OC 55.2

Anti-GPIb antibody induces platelet desialylation: a novel mechanism of Fc-independent immune thrombocytopenia, and a potential new diagnosis and therapy against refractory ITP

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Background: Autoimmune thrombocytopenia (ITP) is characterized by patients' antibodies targeting their own platelet antigens, primarily glycoprotein (GP)IIbIIIa-integrin (70–80%) and GPIb-complex (20–40%). Current paradigm suggests that clearance of opsonized platelets through the reticuloendothelial system via Fcγ-receptors results in thrombocytopenia and bleeding disorders. However, evidence from our group and others have demonstrated that anti-GPIbα, but not anti-GPIIbIIIa, can induce thrombocytopenia via an Fc-independent pathway, which is resistant to intravenous IgG (IVIG) therapy in murine ITP-models (Blood 2006). These observations are consistent with subsequent IVIG studies in human ITP patients. Interestingly, human anti-GPIb-mediated ITP patients seem also resistant to steroid therapy in our recent retrospective study (American Journal of Hematology 2012). This suggests that anti-GPIbα antibodies may induce platelet clearance through a different mechanism which is currently poorly understood.
Methods: We developed unique mouse anti-mouse monoclonal antibodies (mAbs) in GPIIIa or GPIbα deficient mice, which also cross-react with human GPIIbIIIa and GPIbα. Flow cytometry was used to evaluate whether these mAbs were able to induce platelet activation, apoptosis and desialylation. Fc-independent phagocytosis of anti-GPIbα opsonized platelets was examined using FcγR blockers. The role of desialylation on GPIbα in platelet clearance was assessed using neuraminidase (NA) and it's inhibitor *N*-acetyl-2,3-dehydro-2-deoxy neuraminic acid (DANA). We also repeated these experiments with human platelets and plasma from human ITP-patients.
We also investigated the effects of anti-GPIbα antibodies on platelet activation, apoptosis and clearance *in vivo*. Briefly, BALB/c mice were injected with anti-GPIbα or anti-GPIIIa mAbs and 24 h later, platelet desialylation, activation and apoptosis were measured by flow cytometry. The effect of desialylation on platelet clearance was assessed with DANA. The significance of Ashwell-Morell Receptors (AMR) in anti-GPIbα-mediated platelet clearance in the liver was examined using asialofetuin, a blocker of AMR.
Results and Discussion: We found that anti-GPIbα, but not anti-GPIIbIIIa mAbs, induced significant platelet desialylation, P-selectin expression, phosphatidylserine (PS)-exposure, and increased inner membrane mitochondrial depolarization (ΔY_m). Moreover, we found that desialylation of GPIbα is functionally coupled with platelet activation, as prior treatment with activation inhibitors abrogated desialylation and treatment with DANA diminished PS-exposure, and P-selectin expression. Most importantly, incubations of human platelets with ITP-patient plasma showed similar effects. *In vivo*, we found significant increases in PS-exposure and ΔY_m induced by anti-GPIbα, but not by anti-GPIIIa mAbs. Interestingly, prior injection with DANA rescued platelets numbers in anti-GPIbα, but not in anti-GPIIIa injected mice. A significant role for AMR and MAC-1 receptors in the clearance of deglycosylated platelets was observed; blocking of AMR by asialofetuin, decreased platelet clearance in anti-GPIbα, but not anti-GPIIbIIIa antibody injected mice and Fcγ-independent phagocytosis occurred with anti-GPIbα opsonized platelets but not anti-GPIIbIIIa. Thus, we demonstrate for the first time that anti-GPIbα antibodies induce GPIbα desialyation, platelet activation and apoptosis. Therefore, we identified novel

Fc-independent platelet clearance pathways, more specifically, via AMR and MAC-1 receptors in the liver. These findings may lead to novel therapeutic regimens including the potential use of sialidase inhibitors as a solution for anti-GPIb-mediated ITP patients previously refractory to both steroid and IVIG therapies.

OC 55.3

Dendritic cells differently phagocytose activated or apoptotic blood platelets

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Background: In several auto-immune diseases, platelets are destroyed by auto-antibodies, resulting in hemorrhagic syndromes. Among the cells involved in the immune response, dendritic cells (DCs) are crucial antigen presenting cells, as they may initiate autoimmunity through the stimulation of naïve T lymphocytes (TL). The ability of DCs to phagocytose platelets and further to present platelet antigens to T cells has still to be studied in depth.
Objectives: The aim of our project was to define whether resting, activated or apoptotic platelets could be internalized by DCs, which would be critical for the promotion of platelet antigen presentation to TL.
Methodology: Human immature DCs were derived from blood monocytes and co-cultured with resting, 1U/mL thrombin-activated or 1 μM ABT737-treated apoptotic platelets for 18 h at 37 °C. The cells were then fixed and analysed by confocal microscopy to establish a phagocytosis index. Immunolabeling and electron microscopy were performed to analyse the localization of the internalized platelets.
Results: After 18 h of co-incubation, the percentage of DCs that had phagocytosed resting platelets was $3 \pm 1\%$. This percentage reached $12 \pm 1\%$ and $18 \pm 2\%$ when activated or apoptotic platelets were incubated with DCs respectively, the difference between activated and apoptotic platelets being statistically significant ($n = 5$, $P > 0.01$). This process depends on the exposure of phosphatidylserine at the platelet surface, as it was inhibited ($7 \pm 1\%$ vs. 13 ± 2 for activated platelets, $8 \pm 2\%$ vs. 21% for apoptotic platelets), in the presence of recombinant annexin V ($n = 3$, $P > 0.05$). Interestingly, electron microscopy observation revealed that, after 18 h, activated platelets were found in Major Histocompatibility Class II (MHC-II)-positive compartments, while apoptotic platelets were found in MHC II-negative phagosomes, suggesting different routes of phagocytosis.
Conclusion: Our study provides evidence that DCs differentially internalize activated or apoptotic platelets which may result in different mechanisms of antigen handling, processing and presentation.

OC 55.4

Autoantibody binding to glycoprotein Iba induces Fc?RIIa-mediated platelet activation in a patient with immune thrombocytopenia

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Background: The primary targets of autoantibodies in immune thrombocytopenia (ITP) are the platelet receptors αIIbβ3 (70–80%) and glycoprotein (GP) Ibα (20–40%). Anti-αIIbβ3 antibodies are thought to induce thrombocytopenia through increased platelet clearance by Fcγ receptor-bearing macrophages, which is prevented with intravenous immune globulins. The molecular mechanism of thrombocytopenia observed in patients with autoantibodies against GPIbα, who are often refractory to this treatment, is less clear. Anti-GPIbα antibodies are known to induce platelet activation, which may be a cause for accelerated platelet destruction in ITP patients with autoantibodies against this receptor.
Methods: We investigated the effects of anti-GPIbα autoantibodies on platelet activation with fluorescent activated cell sorting. GPIbα translocation, clustering and colocalization with FcγRIIa after autoantibody binding were determined by Förster Resonance Energy Transfer (FRET) using time-gated Fluorescence Lifetime Imaging Microscopy (FLIM).
Results: Platelets from an ITP patient with anti-GPIbα antibodies showed elevated surface expression of P-selectin (12-fold), fibrinogen (twofold) and phosphatidylserine (fivefold). Phagocytosis of patient platelets by matured monocytic THP-1 cells was 21-fold increased compared with controls. Incubation of normal platelets with patient plasma caused platelet activation, which was prevented by removal of the GPIbα ectodomain or IgG depletion. FRET/FLIM analysis revealed that patient antibodies induced translocation of GPIbα to lipid rafts, where it colocalized with the low affinity Fc-receptor FcγRIIa. A blocking antibody against FcγRIIa, or inhibition of GPIbα translocation to lipid rafts with ganglioside GM3 prevented antibody-induced platelet activation.
Conclusions: The results demonstrate that binding of autoantibodies induces GPIbα translocation to lipid rafts where it clusters. This facilitates autoantibody ligation to FcγRIIa, resulting in platelet activation and destruction by macrophages. Blockage of autoantibody-FcγRIIa interaction in lipid rafts may be a therapeutic target for the treatment of ITP.

OC 55.5

Therapeutic efficacy of rapamycin in patients with chronic immune thrombocytopenia

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Introduction: Although immune thrombocytopenia (ITP) is an autoantibody-mediated disease characterized by destruction and impaired platelet production, dysfunctional cellular immunity has also great importance in ITP pathogenesis. Rapamycin, as an immunosuppressant that inhibits T-cell proliferation but selectively spares regulatory T (Treg) cells, has broad prospects in the field of transplantation immunology and autoimmune diseases. In this study, we investigated the effects of rapamycin on the chronic ITP (cITP) patients and to further explore its possible mechanisms.
Design and Methods: Peripheral blood samples were collected from cITP patients. The rate of $CD4^+$ $CD25^+$ Treg cells was analyzed using flow cytometry (FCM), and the Foxp3 mRNA expression was assessed by quantitative reverse-transcription polymerase chain reaction (RT-PCR). In addition, the levels of relevant cytokines were measured by enzyme-linked immune sorbent assay (ELISA) before and after the rapamycin treatment. Furthermore, the suppressive ability of Treg cells isolated from patients and healthy controls was evaluated via coculturing Treg cells and their autologous $CD4^+$ T or $CD8^+$ T cells in the culture medium.
Results: Among thirty-five treated patients who did not respond to previous steroid and immunosuppressant agents, thirteen patients achieved complete response and eight patients achieved partial response. Following treatment with rapamycin, the levels of Treg cells increased ($P > 0.05$); the Foxp3 mRNA expression greatly enhanced and the concentrations of IL-10 and TGF-β(beta) also increased in the responsive group ($P > 0.05$); the suppressive ability of Treg cells isolated from cITP patients was significantly lower than that of the con-

trol group ($P > 0.05$), meanwhile, the Treg cells displayed enhanced function for $CD8^+$ T cells after treatment ($P > 0.05$).
Conclusions: For cITP patients who displayed impaired activity and frequencies of Treg cells, rapamycin could suppress proliferation of both $CD4^+$ T and $CD8^+$ T cells, promote expansion of functional Treg cells and increase their suppressive capacity. Our results suggest that rapamycin might be a potentially therapeutic tool for treating cITP patients.

OC 55.6

A novel monoclonal antibody against GPIbalpha (Gi10) inhibits human platelet clearance induced by autoantibodies against GPIb/IX from ITP patients in a NOD/SCID mice model

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Background: Idiopathic thrombocytopenic purpura (ITP) is an autoimmune disease caused by autoantibodies (aabs) against platelet glycoproteins (GPs) IIb/IIIa and Ib/IX. Recent evidence indicates that aabs against GPIb/IX mediate platelet destruction by a different mechanism in comparison to aabs against GPIIb/IIIa (Fc-independent vs. Fc-dependent pathway) (Webster et al., 2006). Accordingly, patients with ITP mediated by anti-GPIb/IX aabs seem to be less responsive to IVIG treatment (Li et al., 2012). Thus, alternative therapy option should be considered for these refractory patients.
Aims: In this study, we aimed to analyse the capability of a novel mouse monoclonal antibody (mab) specific for GPIb (mab Gi10) to interfere with the binding of aabs against GPIb/IX *in vitro*. In addition, the capability of this mab to inhibit platelet clearance *in vivo* was investigated in a previously established NOD/SCID mouse model (Newman et al., 2007).
Methods: Only sera from well-characterized ITP patients containing GPIb/IX aabs ($n = 10$) were included. All sera were tested by the glycoprotein specific immunoassay, MAIPA, using a panel of mabs against different GPIb/IX subunits (Ibalpha, Ibbeta and IX). The inhibitory capacity of mab Gi10 toward anti-GPIb/IX aabs was investigated by flow cytometry. Analysis of human platelet clearance by anti-GPIb/IX aabs and mab Gi10 was performed in the NOD/SCID mouse model.
Results: Mab Gi10 was characterized in our laboratory and showed specific reaction against GPIbalpha remnant on platelets. Mab Gi10 did not affect platelet function *in vitro* (Kiefel et al., 1991). Analysis of aabs against GPIb/IX in the MAIPA assay using mab Gi10 as capture antibody showed significant reduction of aabs reactivity when compared to other capture mabs against GPIbalpha (clone AP1, SZ2), GPIX (clone FMC25) and GPIbbeta (clone Gi22), indicating binding competition between mab Gi10 and GPIb/IX aabs. These results could be confirmed by flow cytometric analysis; pre-incubation of platelets with mab Gi10 blocked the binding of GPIb/IX aabs. When purified IgG fractions of GPIb/IX aabs or mab Gi10 were injected into the NOD/SCID mouse, clearance of human platelets from mice blood circulation were observed. However, a significant reduction of platelet clearance was detected when mice were pre-treated with deglycosylated mab Gi10 (NGM-Gi10) prior to injection of aabs against GPIb/IX.
Summary and Conclusions: These data demonstrate the capability of mab Gi10 to hinder the binding of GPIb/IX aabs and thereby to inhibit platelet clearance *in vivo*. The use of modified mab Gi10 (such as NGM-Gi10) may represent an alternative therapy option for selected ITP patients.

OC 56 – Inhibitor development in haemophilia A

OC 56.1

Induction of antigen-specific tolerance upon infusion of Fc-fusion proteins via the Materno-fetal interface

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Background: Under physiological conditions, the organism is tolerant to self-constituents. The thymus provides the basis for so called '*central tolerance*' mechanisms, which both delete autoreactive T effectors (Teff) cells and rescue natural T regulatory (Treg) cells. Regulatory T cells (Tregs) that developed via central tolerance mechanisms in the thymus play a major role in the maintenance of peripheral tolerance. Several pathological situations result from a break of tolerance towards endogenous molecules, as seen in autoimmune diseases, or from the inability to mount tolerance towards exogenously administered innocuous molecules, as observed in alloimmune responses to protein therapeutics like in Hemophilia A. Self tolerance is probably established as early as during fetal life, at the time of lymphocyte development. Maternal IgG are transported across the placenta to fetal circulation via transcytosis through the neonatal Fc receptor (FcRn). This process shapes the immune repertoire of the newborns and imparts on the nature of the immune responses in adult life.
Aim: We exploited this physiological process of *materno-fetal* interface to shape the immune repertoire during the ontogeny of the immune system, by introducing the Ag of interest during fetal life, in order to induce tolerance to an immunogenic protein therapeutic: coagulation factor VIII (FVIII).
Methods: To this end, mouse Ig-Fc-fusion chimeric molecules were developed using immunodominant domains of FVIII (A2-Fc and C2-Fc) and using the first domain of hemagglutinin (HA1-Fc). The chimeric proteins were produced by stably transfected HKB11 cells, purified by affinity chromatography and characterized by ELISA and western blots. *In vivo* imaging and ELISA validated the placental transfer of the chimeric proteins into the fetal circulation during gestation, after intravenous infusion in pregnant mice. The functional avidity, thymic presentation, time window and therapeutic dose were determined in HA-specific T-cell receptor (TcR) transgenic (Tg) mice, where T cells specific for the $HA_{111\text{-}119}$ peptide can be identified using the 6.5 anti-clonotypic antibody.
Results: Intravenous injection of 100-μg chimeric proteins in pregnant mice during gestational day 16–18 was sufficient to significantly induce HA1-specific Tregs and to delete HA1-specific autoreactive T cells in the progeny at 2 weeks of age. These validated parameters were then applied to a mouse model of hemophilia A (FVIII−/−) utilizing A2-Fc, C2-Fc and a monoclonal mouse IgG1 as control. The progeny of treated mothers was then challenged with therapeutic doses of the FVIII neo-antigen at 6 weeks of age. The progeny of A2-Fc and C2-Fc treated mothers showed a drastic reduction in the levels of total anti-FVIII IgG with abrogation of the proliferation of FVIII-specific T cells.
Conclusion: Our study provides the first proof of concept towards exploiting the materno-fetal interface to shape the immune repertoire and to induce tolerance in allo-immune pathological conditions.

OC 56.2

Role of mannose-ending glycans in the endocytosis and presentation of FVIII to T cell by human and mouse antigen-presenting cells

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Background: Development of FVIII inhibitors is the major complication in hemophilia A patients. The anti-FVIII immune response involves uptake and processing of therapeutic FVIII by antigen presenting cells (APCs), which leads to FVIII peptide presentation to naïve CD4+ T cells. Previously, using two models of APCs, human monocyte-derived dendritic cells (Mo-DCs) and murine bone marrow-derived dendritic cells (BM-DCs), we had shown that both APCs internalize FVIII. While FVIII endocytosis by Mo-DCs was mediated in part through the macrophage mannose receptor (CD206), FVIII endocytosis by BM-DCs did not involve mannose-sensitive pathways.

Aim: The aim of this study was to compare the importance of FVIII mannose-ending sugars for endocytosis and presentation of FVIII to T cells by mouse splenocytes and human Mo-DCs.

Methods: We produced human B domain-deleted FVIII lacking oligomannose carbohydrates at Asn239 and Asn2118 (DM-FVIII), and wild-type B domain-deleted FVIII (WT-FVIII, Δ2FVIII, LFB, Les Ulis, France) by transducing CHO-S cells with lentiviral vectors. We also generated a FVIII-specific mouse T-cell hybridoma restricted to the HLA DRB1*0101, following immunization with B domain-deleted FVIII (BDD-FVIII) of HLA-A2.1-/HLA-DRB1-transgenic H-2 class I/class II KO mice (SureL1). Lymph node cells from immunized mice were stimulated 3 days *ex vivo* with BDD-FVIII and fused with BWZ36 (TCR−/−) cells by polyethylene glycol fusion. Screening of hybridomas was performed using BDD-FVIII and splenocytes from SureL1 mice. Hybridoma activation was assessed by measuring secreted Il-2. To compare FVIII presentation by mouse and human APCs, T-cell stimulation assays were performed using either splenocytes from SureL1 mice or human Mo-DCs generated from a healthy donor with the DRB1*0101/0301HLA class II locus.

Results: We generated 13 FVIII-specific hybridomas. One FVIII-specific hybridoma was sub-cloned and used for all *in vitro* antigen presentation assays. This sub-clone was activated in a dose-dependent manner by WT-FVIII when presented by HLA DRB1 0101/0301 human Mo-DCs or splenocytes from SureL1 mice. Mouse splenocytes activated equally the T-cell hybridoma whether WT-FVIII or DM-FVIII was used in the assay (117 ± 13 vs. 97 ± 23 pg/mL of Il-2, respectively). In contrast, T-cell activation by Mo-DCs was drastically reduced when DM-FVIII was used instead of WT-FVIII (4 ± 2 and 21 ± 7 pg/mL of Il-2, respectively).

Conclusion: This study highlights the differences in FVIII endocytosis pathways by mice and human APCs. This work also confirms the relevance of mannose-sensitive pathways in FVIII uptake and presentation to CD4+ T cells by Mo-DCs.

OC 56.3

Inhibitor eradication therapy in non-severe hemophilia A

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Background: In patients with non-severe hemophilia A (HA) presence of an inhibitor may exacerbate the bleeding phenotype dramatically. There are very limited data on the optimal therapeutic approach to eradicate inhibitors in non-severe HA patients.

Methods: All non-severe HA patients (FVIII:C 2–40%), who developed a clinically relevant inhibitor between 1980 and 2011 in the participating centers of the INSIGHT study, were included. Data on clinical and inhibitor characteristics and eradication therapy were collected. High titer (HT) inhibitor was defined as a Bethesda assay of > 5 BU/mL and low titer (LT) as ≤ 5 BU/mL. 'Re-challenge' was defined as exposure to FVIII concentrates after the inhibitor became undetectable (with or without inhibitor eradication treatment) and 'sustained success' was defined as a negative inhibitor assay after re-challenge. Proportions were analyzed using chi-square or Fischer's exact tests, as appropriate.

Results: In total, 108 non-severe HA patients from 30 centers in Europe and Australia were included. Inhibitors occurred at a median age of 37 years (inter quartile range (IQR) 1560) after a median cumulative exposure days (ED) to FVIII concentrates of 29 days (IQR 1470). There were 56 HT inhibitors, 45 LT inhibitors and in seven patients the peak titer was unknown. The median historical peak titer was 7.0 BU/mL (IQR 230).

Data on eradication treatment were available in 101 patients. Eradication treatment was administered in 27 patients: 16 patients were treated with Immune Tolerance Induction (ITI) exclusively, four were treated with immunosuppressives exclusively and seven were treated with both ITI and immunosuppressives. Eradication treatment resulted in undetectable inhibitor levels in 21/27 patients (78%). Sustained success was obtained in 15/17 patients (88%) that were re-challenged. A majority of 74 inhibitor patients did not receive any inhibitor eradication treatment. The inhibitor became undetectable in 52/74 patients (70%) and was still present in the other 22 patients at end of follow-up. In 21/35 patients (60%) with undetectable inhibitors that were not treated with eradication treatment and re-challenged with factor VIII, sustained success was attained. Use of eradication treatment was associated with sustained success in comparison to no eradication treatment: relative risk (RR) 1.47, 95% confidence interval (CI) 1.072.03.

Next, we calculated the effect of eradication treatment separately for the HT and LT group. In the HT group, sustained success was attained in all eight patients that were re-challenged after eradication treatment, compared to 6/14 patients without eradication that were re-challenged (RR 2.33, CI 1.274.27). In the LT group 7/9 patients that were re-challenged attained sustained success after eradication, compared to 15/21 patients that were re-challenged without eradication treatment (RR 1.09, CI 0.701.69).

Conclusion: In this unique cohort of 108 non-severe HA patients with inhibiting antibodies inhibitor eradication treatment was associated with sustained success after re-challenge with factor VIII in HT inhibitor patients, but not in LT inhibitor patients.

OC 56.4

Mechanisms of immune tolerance induction by rFVIIIFc in hemophilia A mice

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Background and Aims: Alloantibodies to factor VIII (FVIII) are a significant impediment in treating hemophilia A (HemA). Previously, we reported that repeated administration of therapeutic doses of rFVIIIFc in HemA mice resulted in significantly lower subsequent antibody responses to FVIII, compared with B-domain-deleted and full length FVIII. Reduced antibody responses to rFVIIIFc are consistent with the presence of higher percentage of Tregs and tolerogenic cytokines in the spleens of these mice. In this study we further elucidate the mechanisms by which rFVIIIFc induces these tolerogenic effects, including identifying the genes regulated by rFVIIIFc and related to either immune or tolerance responses and the effects of interaction of rFVIIIFc with Fc receptors (FcγR and FcRn).

Methods: HemA mice were injected with rFVIIIFc at therapeutic (50 IU/kg) or supraphysiological doses (250 IU/kg) or vehicle once weekly for 4 weeks followed by two injections every 2 weeks. cDNAs from splenocytes were used for immune tolerance gene profiling. In addition, two rFVIIIFc mutants were constructed containing either FcγR non-binding mutation (N297A) or FcRn non-binding mutation (IHH). Splenic T-cells were isolated from HemA mice treated with these variants and used for T-cell proliferation and IFNγ secretion assays.

Results: Microarray analysis revealed that genes related to tolerance (Foxp3, CTLA4, and IL-10) and anergy (Egr2, Dgka, and Cblb) were upregulated and pro-inflammatory genes such as CCL3 and STAT3 were down-regulated in splenocytes of mice receiving 50 IU/kg rFVIIIFc in comparison to vehicle and 250 IU/kg treatments. The results were validated using real time PCR. CD4+ T-cells from the 250 IU/kg group elicited higher proliferation and IFNϒ secretion compared to those from the 50 IU/kg and vehicle-treated groups, whereas the IFNγ secretion from 250 IU/kg of rFVIIIFc N297A group was significantly reduced. IFNγ production from the 50 IU/kg groups of rFVIIIFc or rFVIIIFc N297A was comparable to vehicle. Nevertheless, T-cells from mice receiving 50 IU/kg rFVIIIFc maintained robust proliferation and IFNϒ secretion in response to anti-CD3/CD28 antibody suggesting the absence of non-specific immunosuppression.

Summary/Conclusions: Therapeutic doses of rFVIIIFc induced expression of tolerance-specific genes in comparison to supraphysiological doses of rFVIIIFc. Gene candidates identified here that are regulated by rFVIIIFc provide insight into the mechanism of tolerance induction to FVIII. This is supported by findings that T-cells from the 250 IU/kg displayed robust proliferation and IFNγ production, in contrast to the 50 IU/kg and vehicle treated groups, in response to rFVIII. Failure to interact with FcgR family of receptors (comprising of both activating FcϒR and inhibitory FcϒR2B receptors) by rFVIIIFc N297A dampened the T-cell response in mice immunized with supraphysiological doses of rFVIIIFc. Moreover, rFVIIIFc treated mice maintained robust T-cell responses to anti-CD3/CD28 antibody suggesting the lack of non-specific immunosuppression. The evaluation of rFVIIIFc IHH variant will further elucidate the role of FcRn in immune tolerance. In summary, these findings provide insight on the possible mechanisms of rFVIIIFc in inducing immune tolerance to rFVIII in hemA mice.

OC 56.5

Novel strategies to target long-lived plasma cells for treating Hemophilia A inhibitors

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Background: Formation of inhibitory antibodies is a critical and challenging problem for therapeutic treatment in hemophilia A patients. It is hypothesized that long-lived plasma cells (LLPCs) play an important role in the persistent production of anti-FVIII antibodies in hemophilia A (HemA) inhibitor patients. The migration of plasma cells to the BM, where they become the LLPCs, is largely controlled by an interaction between the C-X-C type chemokine ligand 12 (CXCL12) produced by bone marrow (BM) stromal cells and its receptor CXC receptor4 (CXCR4; CD184) on plasma cells. Our previous data showed that administration of anti-murine CD20 (IgG2a) can deplete B cells significantly and reduce anti-FVIII inhibitor titers in FVIII plasmid-treated HemA mice with pre-existing inhibitors, however, complete tolerance to FVIII was not achieved probably due to the persistence of LLPCs.

Aims: We sought novel therapeutic strategies that target CXCL12/CXCR4 pathway to reduce/eliminate LLPCs and achieve the goal for long-term tolerance to FVIII in the HemA inhibitor mice.

Methods: AMD3100, the CXCR4 antagonist, plus G-CSF inhibit the interaction of CXCL12 and CXCR4, thus facilitating the mobilization of CD34+ cells and blocking the homing and retention of LLPCs. We also combined these reagents with the specific IL-2/IL-2mAb (JES6-1) complexes to target both B and T cell-dependent anti-FVIII immune responses. Two groups of FVIII-primed inhibitor mice were treated with different combined immunomodulation regimens: (i) IL-2 complexes+AMD3100+G-CSF+anti-CD20, (ii) AMD3100+G-CSF+anti-CD20. Control mouse groups were treated with each of the single regimens and FVIII only, or untreated as the naïve control. All the treatments were administered one cycle per 2 weeks for 4 weeks and the therapeutic effects (FVIII activities) as well as immune responses (anti-FVIII inhibitors) were evaluated at different time points after treatment.

Results: Significant expansion of Treg cells reaching a 5~7-fold increase on the peak days (day 3–7 after treatment) was observed in the IL-2/IL-2mAb complexes treated groups, whereas ~98% of B cell populations were depleted in the anti-CD20 treated groups. In addition, administration of AMD3100 plus G-CSF significantly reduced circulating and bone marrow CXCR4+ plasma cells. Except for the control groups, the two mouse groups treated with combined immunosuppressive regimens showed a significant reduction of inhibitory titers following the treatment. Long-term responses are being followed and second challenge with FVIII plasmid will be used to evaluate the induction of long term tolerance to FVIII.

Summary/Conclusions: These combination regimens are highly promising in modulating/eliminating pre-existing anti-FVIII antibodies and inducing long-term tolerance in FVIII primed subjects.

OC 56.6

Co-administration of factor VIII and dexamethasone prevents anti-factor VIII antibody development in a mouse model of hemophilia A

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Background: Antibodies that inhibit the coagulant function of factor VIII (FVIII) are a major complication of treatment for patients with hemophilia A. Inhibitor development depends on components of adaptive immunity, such as B cells and $CD4^+$ T cells, and on components of innate immunity, which activate antigen-presenting cells. The immunologic 'decision' for immunity or tolerance may occur early during exposure to FVIII. Corticosteroids, which are used extensively in humans, have suppressive effects on adaptive and innate immunity, but the effect of immunosuppression at the time of initial FVIII exposure on the risk of inhibitor development has not been investigated.

Aims: To determine if dexamethasone administered during initial exposure to FVIII will prevent the occurrence of anti-FVIII antibodies in a mouse model of hemophilia A, and to assess if dexamethasone-induced immunologic tolerance will withstand a subsequent exposure to FVIII.
Methods: Hemophilia A mice with knockout of exon 17 of the *F8* gene and with a chimeric human/murine major histocompatibility complex type II transgene carrying the binding sites of the human HLA-DRB1*1501 allele were used. Recombinant human FVIII (Advate, approximately 0.1 mcg/IU) was given by tail vein injection at 6 IU per dose, dexamethasone was given by intraperitoneal injection at 75 mcg per dose, and lipopolysaccharide (LPS) was given IV at 2 mcg per dose. The experiment consisted of two phases: in Phase 1, mice were given either FVIII and Dexamethasone (Dex group) or FVIII alone (Control group) for 5 days; in Phase 2, which began 7 weeks after the start of Phase 1, Control mice without evidence of anti-FVIII antibodies were given FVIII for 3 days, and Dex mice were given either FVIII (FVIII group) or FVIII and LPS (FVIII-LPS group) for 3 days. Plasma samples were collected 6 weeks after the start of Phase 1 by retro-orbital venous plexus, and 3 weeks after the start of Phase 2 by cardiac puncture. Plasma was examined for evidence of anti-FVIII antibodies and FVIII inhibitory activity with ELISA for anti-FVIII IgG and Bethesda assay, respectively. Proportions of mice with positive titres were analyzed using Fisher's exact test.
Results: After Phase 1, 0/17 Dex mice had detectable anti-FVIII IgG, as compared to 5/17 Control mice ($P = 0.04$). A subset of these mice had completed Phase 2: 3/6 Control mice had detectable anti-FVIII IgG, compared to 2/5 FVIII-LPS mice and 0/4 FVIII mice ($P = 0.31$). Bethesda assays were performed after Phase 2: 1/6 Control mice had detectable FVIII inhibitory activity, compared to 2/5 FVIII-LPS mice and 0/4 FVIII mice ($P = 0.57$).
Conclusions: Dexamethasone administration at the time of initial exposure to FVIII prevented the development of anti-FVIII IgG in all treated mice. It may be the case that this tolerance withstands a subsequent exposure to FVIII, and may even blunt the immunostimulatory effect of LPS. The mechanisms leading to this effect warrant further study, as there is an obvious possible translational application for these findings.

OC 57 – Modifications in Factors VIII, IX and XI

OC 57.1

Selective mutagenesis of the heparin and antithrombin exosites on human factor IX(a) enhances thrombin generation in human plasma

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Background: Hemophilia B is an X-linked genetic disorder characterized by defective factor IX activity. Recombinant factor IX (rFIX) is employed as protein replacement for the treatment and prophylaxis of bleeding episodes. Antithrombin is the primary plasma inhibitor of factor IXa, and inhibition is enhanced by endothelial heparan sulfate. We hypothesize that selective disruption of protease interactions with heparin and antithrombin may enhance rFIX efficacy by prolonging the protease half-life *in vivo*.
Aims: The aim of these studies was to assess the effect of mutations in the factor IX(a) heparin and antithrombin-binding exosites on activity in human plasma.
Methods: Human rFIX cDNA constructs with alanine substitutions (chymotrypsinogen numbering) in the heparin binding exosite (K126A, K132A, K126A/K132A), antithrombin-binding exosite (R150A), or both (K126A/K132A/R150A) were expressed in HEK293 cell lines. Recombinant zymogens were purified from conditioned media, and a portion activated to protease with human factor XIa. Both zymogen and protease forms were characterized in APTT-based clotting assays, and in tissue factor (TF) and factor IXa-initiated thrombin generation (TG) assays in pooled human factor IX-deficient plasma. Comparisons were made with human plasma-derived factor IX(a)(pFIX) and recombinant factor IX(a) wild type (WT).
Results: APTT based clotting activities for zymogens expressed as a% $\pm$ SEM relative to rFIX WT were: pFIX 105.2 $\pm$ 2.8, WT 100.8 $\pm$ 7.1, K126A 63.3 $\pm$ 2.3, R150A 62.4 $\pm$ 4.0, K132A 30.9 $\pm$ 1.1, K126A/K132A 20.6 $\pm$ 9.2, and K126A/K132A/R150A 7.3 $\pm$ 3.8. Likewise, APTT clotting activities for the proteases were: WT 100 $\pm$ 6.1, pFIXa 98.4 $\pm$ 11.4, K132A 91.4 $\pm$ 1.6, R150A 77.1 $\pm$ 5.8, K126A 39.5 $\pm$ 2.4, K126A/K132A/R150A 10.9 $\pm$ 0.6, and K126A/K132A 9.3 $\pm$ 0.6. In contrast to their clotting activities, both FIX R150A and K126A demonstrated markedly enhanced TF-initiated TG relative to WT protein ($\geq$ 2-fold). Likewise, the magnitude of TG by FIX K132A was similar to FIX WT. FIX K126A/K132A had reduced thrombin generation proportionate to clotting activity (~20%), and addition of R150A (triple mutant), increased TG to about half that of FIX WT. In the FIXa-initiated assay, FIXa K132A and R150A demonstrated TG roughly proportional to their clotting activities, FIXa K126A had moderately enhanced TG (60–70%) relative to clotting activity, and FIXa K126A/K132A had reduced TG proportionate to clotting activity, with a modest increase with addition of R150A (triple mutant).
Conclusion: Selected disruption of the heparin and antithrombin exosites on human factor IX by alanine substitution reduces traditional clotting activity to a variable extent. Despite moderate reductions in clotting activity, the mutations K126A and R150A enhanced TF-initiated TG in human plasma relative to WT protein. Combined mutations in the heparin-binding site (K126A/K132A) significantly reduced both clotting activity and plasma TG, likely due to disruption of the protease-cofactor interaction. Mutation of the antithrombin-binding site (R150A) tended to increase plasma TG relative to clotting activity, either alone or in the context of combined mutations in the heparin-binding exosite (double vs. triple mutant). These results suggest that optimization of these exosite mutations will enhance TG by rFIX in human plasma.

OC 57.2

Optimizing factor IX-Triple clotting activity *in vitro* and *in vivo*

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Background: Using gain-of-function factor IX (FIX) for replacement therapy for hemophilia B (HB) has been an attractive strategy. We previously reported a high activity FIX, FIX-Triple (FIX-V86A/E277A/R338A) as a good substitute for FIX-WT (wild-type) in protein replacement therapy and gene therapy as well as cell therapy.
Aims: To test whether FIX Padua mutation improves the clotting activities of FIX-Triple, a novel recombinant FIX named FIX-TripleL (FIX-V86A/E277A/R338L) was generated in this study by modifying FIX-Triple at residue 338 from alanine to leucine as identified in FIX-Padua (FIX-R338L).
Methods: Both *in vitro* and *in vivo* models were used to demonstrate the clotting function of FIX-TripleL.
Results: Purified FIX-TripleL exhibited a 22-fold higher specific activity and a 14-fold increased binding affinity to activated FVIII compared to FIX-WT. FIX-TripleL improved the therapeutic potential of FIX-Triple increased by 100% as demonstrated by calibrated automated thrombogram and thromboelastography. With normal clearance rate in HB mice, the clotting activities of FIX-TripleL were consistently 2–3-fold higher than those of FIX-Triple or FIX-R338L. Tail-vein administration of adeno-associated virus (AAV) expressing FIX in HB mice showed that FIX-TripleL had a 14-fold higher specific activity than FIX-WT, which was significantly better than FIX-Triple

(ninefold) or FIX-R338L (sixfold). Moreover, there were no signs of adverse thrombotic events in long-term AAV-FIX-treated C57Bl/6 mice. Abnormality in AAV-FIX-treated livers was observed exclusively in some of the mice injected with high but not medium or low dose AAV-FIX, indicating the advantages of using hyperfunctional FIX variants to reduce viral doses and meanwhile maintain therapeutic clotting activities.

Conclusions: These studies suggest that incorporation of FIX Padua mutation into FIX-Triple significantly improves the clotting function of FIX-Triple so as to optimize protein replacement therapy and gene therapy.

OC 57.3

Increasing the binding affinity between FVIIIa subunits results in higher stability and greater thrombin generation in plasma

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Factor (F)VIIIa is a heterotrimer of A1, A2 and A3C1C2 subunits. FVIIIa activity is labile due to the tendency of A2 subunit to dissociate from the A1/A3C1C2 dimer. This dissociation of A2 subunit inactivates FVIIIa activity, thereby down-regulating FXase. Thus retention and dissociation of A2 subunit reflect FVIIIa stability and FXase activity. Earlier results from our laboratory showed that replacing the acidic residues D519, E665, and E1984, that localize in hydrophobic patches at the interfaces between the A2 and either the A1 or A3C1C2 subunits, with either Ala or Val reduced rates of FVIIIa decay and yielded increased thrombin generation (Wakabayashi et al., 2008, 2009). We now show that the observed increase in FVIIIa stability and thrombin generation with these FVIIIa variants result from an increased binding affinity between A2 subunit and A1/A3C1C2 dimer in FVIIIa, as well as a higher stability of FVIIIa in plasma. For these studies, the isolated A2 domains with Ala or Val substitution for D519 and/or E665 were expressed in a baculovirus system. Ala and Val substitution for E1984 (A3 domain) was achieved by mutating this residue in FVIII, expressing the intact protein in BHK cells, activating the FVIII to FVIIIa, purifying the A3C1C2 subunit, and combining it with A1 subunit to form the A1/A3C1C2 dimer. To assess inter-subunit affinity, FVIIIa was reconstituted from variable concentrations of either the isolated A2 subunit or A1/A3C1C2 dimer, using either the WT or variant subunit. Resultant FVIIIa activity was determined in a FXa generation assay. The FXa generation curve were fitted to a single-site binding equation, and the dissociation constant (Kd) for A2 was determined for WT and each variant. The Kd value for the interaction of WT A2 combined with WT A1/A3C1C2 (44 $\pm$ 3 nM) was 3–7-fold higher than Kd values observed for the A2 variants at D519 (16 $\pm$ 1 nM, Ala; 11 $\pm$ 1 nM, Val) and E665 (13 $\pm$ 1 nM, Ala; 6 $\pm$ 1 nM, Val) and for the A3C1C2 variant at E1984 (16 $\pm$ 3 nM, Ala; 6 $\pm$ 1 nM, Val). Overall, Val containing variants showed higher affinity interactions than the Ala variants. Furthermore, the double mutant (D519V/E665V) showed an approximately 40-fold lower Kd (0.9 $\pm$ 0.7 nM) than WT. In another series of experiments, the reconstituted WT and A2 variant FVIIIa forms at various dilutions were incubated in hemophilic plasma for 1 min, and the remaining activity was determined by clotting assays. All variants showed greater activity levels than WT FVIIIa with 9- and 22- fold increases for the D519A and D519V, respectively and 15- and 21-fold increases for the E665A and E665V, respectively. Again, the double mutant D519V/E665V showed the greatest increase (42-fold) relative to WT. Therefore, these hydrophobic mutations at the A2-A1 and A2-A3C1C2 subunit interfaces result in a high binding affinity for the A2 subunit. This high binding affinity is responsible for the high plasma stability and correlates well with the previously observed reduced rates in FVIIIa decay and increased thrombin generation parameters.

OC 57.4

Transcriptional and post-transcriptional targeting of FVIII expression to overcome immunological responses to gene therapy for Hemophilia A

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Background: Hemophilia A (HA) is an X-linked bleeding disorder due to mutations in clotting factor VIII (FVIII) gene. To date HA patients are treated with recombinant or plasma-derived FVIII with a high probability of developing inhibitors (20–40%). Several efforts have been focused on the improvement of lentiviral vectors (LV) to obtain selective targeted expression by transcriptional and post-transcriptional regulation. However, immune responses to FVIII remain the major obstacle. The liver is known to induce tolerance rather than immunity towards antigens presented locally to T cells by specialized resident cells, such as liver sinusoidal endothelial cells (LSEC) and Kupffer cells (KC).

Aims: To investigate the possible role of LSEC and KC in gene therapy for HA using LV expressing human FVIII under the control of cell-specific promoters $\pm$ specific microRNA target sequence (miRTs).

Methods: We implemented two different strategies of targeting LV to LSEC or KC in hemophilia A mice, preparing LVs containing GFP or FVIII under the control of the ubiquitous PGK promoter, or the CD11b (surface integrin, monocyte/macrophage-specific) or VEC (vascular endothelial cadherin, endothelial-specific) promoters in combination with miRTs: miR142 (silenced in hematopoietic cells), miR126 (silenced in endothelial cells), miRT122 (silenced in hepatocytes). These sequences were inserted in the LV alone or in pairs.

Results: Co-staining with F4/80 or CD31 and GFP antibodies on liver sections taken at different time points after LV-GFP injection in mice confirmed a widespread pattern of GFP expression for up to 3 m, the longest time tested. Immunofluorescence showed GFP expression restricted to specific cell types within the liver by addition of one or two miRTs. After injection of LV.VEC.GFP and LV.CD11b.GFP, transgene expression was restricted to LSEC and KC respectively, although some off-target expression was detected. The addition of the miRT122-142 combination to LV.VEC.GFP restricted GFP-expression to LSEC and miRT126 to LV.CD11b.GFP further increased specific expression in KC, with no off-target expression and sustained GFP expression at all-time points analyzed up to 6 months. We then injected HA mice with LV.PGK.FVIII $\pm$ miRT142 and LV.VEC.FVIII $\pm$ miRT122-142. In the first group anti-FVIII antibodies were detected starting at 2 weeks after vector delivery, however the presence of miRT142 alone halved the titer of neutralizing antibodies in LV-injected mice. In HA mice injected with LV.VEC.FVIII $\pm$ miRT122-miRT142 long term phenotypic correction was shown by functional assay in both groups of the latter injected mice for up to 6 months with reduced clotting time and an average of 5% FVIII activity in injected mice and virtually no inhibitors were detected. Finally, we recently injected LV.CD11b.hFVIII $\pm$ miRT126 in HA mice to verify if overexpression of FVIII by KC is associated with tolerance induction as reported after FVIII-expression by LV-transduced LSEC.

Conclusion: In our study, endothelial or monocyte/macrophage specific-promoters in combination of selected miRT sequences in LV were able to overcome FVIII off-target expression limiting immune responses and providing phenotypic correction in treated HA mice.

OC 57.5

Factor VIII C1-domain spikes 2092–2093 and 2158–2159 comprise regions that modulate cofactor function and cellular uptake

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The C1-domain of factor VIII (FVIII) has recently been implicated in phospholipid binding of activated FVIII. It has further been found to contribute to Low density lipoprotein Receptor-related Protein (LRP)-dependent FVIII endocytosis, and uptake of FVIII by antigen-presenting dendritic cells. We have previously established that anti-C1 domain antibody KM33 blocks all these biological processes. In the present study, we employed hydrogen-deuterium exchange (HDX) mass spectrometry to identify the molecular hot-spot on FVIII to which this antibody binds. The results showed that the FVIII regions 2091–2103 and 2157–2162 are protected by KM33 from HDX. These peptides comprise the two C1-domain spikes 2092–2093 and 2158–2159, which have previously been suggested to contribute to phospholipid binding. We have recently demonstrated that the spike 2092–2093 contributes to assembly with lipid membranes with low PS content. This raises the question as to whether spike 2158–2159 would serve a similar role. To investigate this, we took advantage of the fact that replacement of R2159 for an asparagine would introduce an N-linked glycosylation motif in the KM33 binding region. Upon expression of this variant in human 293 cells, the purified mutant indeed proved to be glycosylated in this position. Binding studies revealed that the glycosylated R2159N variant did not display any interaction with antibody KM33. We further found that the R2159N variant exhibits reduced binding to LRP. In accordance with this finding, FVIII R2159N also displayed reduced uptake by LRP expressing cells, including human monocyte-derived dendritic cells. While cellular uptake was reduced, FVIII cofactor function of FVIII R2159N was maintained on phospholipid membranes containing 15% phosphatidylserine (PS). On membranes with low, i.e. 5% PS content, however, FVIII function was reduced, in accordance with the apparently reduced affinity of the R2159N variant for membranes of cells that are capable of FVIII uptake. These findings imply that the two C1 domain spikes 2092–2093 and 2158–2159 both modulate the assembly of FVIII with PS-containing membranes. These data further demonstrate that the two spikes in the FVIII C1-domain regulate FVIII function by a subtle, PS-dependent mechanism. The reduced uptake of FVIII R2159N by dendritic cells suggests that this mutant may display reduced immunogenicity. Collectively, these data suggest that the two spikes of the FVIII C1-domain are structure elements that contribute to both membrane-driven FVIII function and immunogenicity. While previous studies have proposed that it is predominantly the FVIII C2-domain that drives membrane assembly, the present data contribute evidence for an increasingly important role of the C1-domain role in FVIII biology.

OC 57.6

The role of lysine residues in the interaction of blood coagulation factor VIII with its clearance receptor low-density lipoprotein receptor-related protein

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Members of the LDL receptor family contribute to the rapid clearance of blood coagulation factor VIII from the circulation. It has been implicated that positively charged lysine residues, but not arginine residues, drive the interaction between the LDL receptor family members and their ligands. However, it has remained unclear which residues on factor VIII contribute to the interaction and how the specificity of the interaction is regulated. Using two independent approaches, we now study the interaction of factor VIII with the largest member of the LDL receptor family, low-density lipoprotein receptor-related protein (LRP1) in detail, with particular emphasis on lysine residues. Hydrogen deuterium exchange-mass spectrometry (HDX-MS) experiments identified eight regions in the FVIII light chain that display significant protection from exchange with solvent in the presence of LRP1 cluster II. These regions include residues 1671–1689, 1737–1746, 1804–1835, 2057–2069, 2078–2099, 2213–2235, 2266–2271, and 2293–2296 and are likely to constitute the core of the interface between factor VIII and LRP1. Intriguingly, these regions are scattered throughout the *a3*, A3, C1 and C2 domains of the factor VIII light chain. To further systematically address the contribution of individual lysine residues within the *a3*, A3, C1 and C2 domains of the factor VIII light chain, we constructed a library of FVIII variants carrying lysine to arginine (KR), alanine (KA) or glutamic acid (KE) replacements using site-directed mutagenesis. The interaction of the variants with LRP cluster II was evaluated using Surface Plasmon Resonance (SPR) analysis. Factor VIII light chain variants were expressed in 293 Freestyle cells, captured on the SPR chip (17 fmol/mm^2) using an anti-C2 antibody and LRP cluster II (0.2–200 nM) was passed over in solution. These experiments revealed that multiple (11) lysine residues within the factor VIII light chain including 1673/1674, 1693, 1813/1818, 1827, 1967, 1972, 2065, 2092 and 2136 contribute to the interaction with LRP1 cluster II. However, none of these residues accounts completely for LRP1 cluster II binding. Replacements of contributing lysine residues by positively charged arginine, uncharged alanine or negatively charged glutamic acid residues indicated that reversing the positive charge had the strongest effect on the interaction. In addition, an additive effect of combining multiple lysine replacements was observed. The majority of the lysine residues that were identified in the mutagenesis study are located within those regions that were also identified using the HDX-MS study and are therefore considered 'hot spots'. Taken together, our combined experimental approach using HDX-MS and site-directed mutagenesis suggests that the interaction of factor VIII with LRP1 occurs over an extended surface containing multiple lysine residues in the factor VIII light chain. Crystal structure analysis and docking studies proved to be compatible with a model in which the acidic binding pockets of the ligand binding regions of the receptor spatially align with the putative 'hot spot' lysine residues in such a way that the receptor engages the bottom of the C1 domain and curls around the factor VIII molecule.

OC 58 – New Developments in Thrombus Formation

OC 58.1

Polo-like kinase 3 regulates *in vivo* thrombosis through the regulation of thromboxane A2 generation, granular secretion, and integrin aIIbβ3 outside-in signaling

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Background: Polo-like kinase (Plk) family members are serine/threonine kinases involved in cell cycle regulation. Their expression and function in platelets are not known. We identified for the first time the presence of Plk3 in human and mouse platelets.
Aim: To evaluate the functional role of Plk3 in platelet activation and thrombosis.
Methods: We used *Plk3−/−* mice in C57Bl/6 background. *In vivo* thrombosis was assessed by tail bleeding assay, 10% $FeCl_3$-induced

carotid artery injury, and pulmonary thromboembolism induced by collagen/epinephrine. Wild-type (WT) mice were used as controls.
Results: We found that Plk3 is localized to the filopodia of activated platelets. Furthermore, Plk3 co-immunoprecipitated with integrin $a_{IIb}b_3$ in an aggregation-dependent manner, suggesting that it may play a role in integrin outside-in signaling. Since Plk3 is a mitotic kinase, we evaluated the effect of loss of Plk3 on platelet counts in *Plk3−/−* mice. We found that ablation of Plk3 significantly elevated the platelet number, indicating a thrombocytotic phenotype. We next evaluated the degree of megakaryocyte (MK) ploidy and found no difference between WT and *Plk3−/−* mice. When analyzed for the total MK number, we found that lack of Plk3 resulted in a significantly increased number of MKs in the bone marrow, which may account for the increased platelet number. We next examined the effect of lack of Plk3 on platelet functions induced by physiological agonists. We found that platelet aggregation induced by low dose of collagen, thrombin, or PAR4 peptide was significantly augmented ($P > 0.02$) in Plk3 null platelets compared to WT. This was further supported by the significantly increased ($P > 0.05$) fibrinogen receptor exposure on platelets. To determine the molecular mechanism of the observed hyperaggregation, we analyzed signaling events such as ERK1/2 and Akt, upstream regulators of integrin $a_{IIb}b_3$ activation. Interestingly, we found that agonist-induced activation of ERK2 and Akt was significantly enhanced in the absence of Plk3, suggesting that lack of Plk3 may cause a prothrombotic phenotype. When we tested the *in vivo* effect of Plk3 ablation, to our surprise, we found that *Plk3−/−* mice showed an anti-thrombotic phenotype as assessed by a significantly ($P > 0.001$) delayed average tail bleeding time, extended carotid vessel occlusion time ($P > 0.001$), as well as a marked protection ($P > 0.0004$) from pulmonary thromboembolism compared to WT mice. Thrombin-induced generation of TxA2 ($P > 0.03$) as well as a- and d- granules secretion was significantly attenuated ($P > 0.007$) in *Plk3−/−* mice compared to WT. Furthermore, phosphorylation of cPLA2, a key enzyme involved in TxA2 generation, was significantly attenuated ($P > 0.05$) in Plk3 null platelets. When analyzed for fibrin clot retraction, a function of outside-in signaling, we found that Plk3 null platelets failed to retract the fibrin clot, supporting the observed anti-thrombotic phenotype *in vivo*. The severity of the anti-thrombotic phenotype in *Plk3−/−* mice may have been dampened due to the opposing role of Plk3.
Summary: The results presented here suggest that Plk3, a mitotic kinase, plays a significant role in the regulation of platelet function such as TxA2 generation, granular secretion, and clot retraction, thus affecting the process of thrombosis.

OC 58.2
Disruption of ICAM-4 mediated direct erythrocyte-platelet interaction leads to reduced thrombus formation

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Background: Direct platelet-erythrocyte interaction has been published but no information is available on the mechanism of the interaction and its physiological function. Intercellular adhesion molecule 4 (ICAM-4) is an erythroid-specific membrane component that belongs to the family of immunoglobulin superfamily of proteins and it has been shown to be a ligand for platelet activated $\alpha_{IIb}\beta_3$.
Aims: Our aim is to investigate the involvement of ICAM-4 and $\alpha_{IIb}\beta_3$ in the direct erythrocyte-platelets interaction under near physiological conditions *in-vitro*. Secondly we aim to explore the physiological function of the ICAM-4 mediated erythrocyte-platelet interaction *in-vivo*.
Methods: An *in-vitro* perfusion system connected to a light microscope and a digital camera was used to study erythrocyte binding to platelets adhered to surfaces coated with different adhesive proteins at different flow-rates. Flow cytometry analysis was performed to further study the binding of an ICAM-4 mimetic peptide (resembling an extracellular domain of ICAM-4) to $\alpha_{IIb}\beta_3$ on platelets. A $FeCl_3$-based experimental thrombosis model in mice was applied to study the effect of an anti-ICAM-4 antibody and an ICAM-4 blocking peptide on thrombus formation. Platelet deposition and fibrin formation was recorded under a fluorescence microscope with calcein labelled donor platelets and FITC labelled anti-fibrin antibody.
Results: Erythrocytes bind to platelets both in buffer and in whole blood under low shear flow. Erythrocytes attached to platelets with a sort of 'focal adhesion point', resulting in a tear-drop shape. This adhesion was inversely correlated with flow shear rate and predominately occurred at shear rates lower than 300/s. The addition of platelet agonists during the flow experiments increased erythrocyte binding to platelets 3–6-folds indicating that platelet activation is involved in capturing erythrocytes from the circulation. An Arg-Gly-Asp (RGD) containing peptide (d-RGDW), known to inhibit $\alpha_{IIb}\beta_3$ mediated platelet aggregation inhibited erythrocyte-platelet adhesion up to 72%, depending on the agonist used ($P > 0.05$, $n = 4$). Erythrocyte-platelet adhesion under flow was inhibited with both an anti-ICAM-4 antibody (40%, $P > 0.01$, $n = 8$) and an anti- integrin β_3 (CD61) antibody (46%, $P > 0.001$, $n = 8$). In addition, the ICAM-4 mimetic peptide demonstrated a significant inhibitory effect on erythrocyte-platelet adhesion. Flow cytometry experiments showed that this ICAM-4 peptide also reduced fibrinogen binding to platelets (43%, at 125 μM ADP, $P > 0.05$, $n = 5$), suggesting that the ICAM-4 mimetic peptide competes with fibrinogen for binding to activated $\alpha_{IIb}\beta_3$. The *in-vivo* thrombosis model in mice demonstrated that the anti-ICAM-4 antibody profoundly inhibited platelets deposition and fibrin formation at the site of injury. Comparing to control mice, which shows a vessel occlusion time of approximately 10 min, the anti-ICAM-4 antibody treated mice shows no vessel occlusion in the first 20 min. The ICAM-4 mimetic peptide demonstrated similar inhibitory effect *in-vivo*.
Conclusions: We observed a direct erythrocyte-platelet interaction under conditions of low shear. This interaction is partly mediated via erythrocyte-receptor ICAM-4 and $\alpha_{IIb}\beta_3$ on platelets. In addition, we found that disruption of the ICAM-4 mediated erythrocyte-platelet interaction *in vivo* by an anti-ICAM-4 antibody or a ICAM-4 mimetic peptide led to reduced platelet deposition and fibrin formation at the injury sites in mouse vessels.

OC 58.3
PDK1 regulates platelet activation and arterial thrombosis

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The effects of PDK1, a master kinase in PI3K/Akt pathway, on platelet activation are unknown. Accordingly, platelet-specific PDK1 deficient mice were characterized to elucidate the platelet-related function (s) of PDK1. We found that PDK1 deficiency caused a mild thrombocytopenia. Also, the aggregation of $PDK1^{-/-}$ platelets was diminished in response to low levels of α-thrombin, U46619 and ADP, respectively. Further results demonstrated that PDK1 regulates thrombin-induced platelet activation by affecting αIIbβ3-mediated outside-in signaling. This result provided an explanation for the diminished spreading of $PDK1^{-/-}$ platelets on immobilized fibrinogen (Fg) and the decreased rate of clot retraction in platelet rich plasma containing $PDK1^{-/-}$ platelets. PDK1 deficiency diminished agonist-induced Akt Ser473 phosphorylation, and thoroughly abolished Akt Thr308 and Gsk3β Ser9 phosphorylation in response to agonist treatment, and

platelet spreading, respectively. A Gsk3β inhibitor fully restored the aggregation of PDK1$^{-/-}$ platelets in response to low levels of thrombin, the normal spreading of PDK1$^{-/-}$ platelets on Fg, and normal clot retraction in PRP containing PDK1$^{-/-}$ platelets. Those results indicated that Gsk3β is a major downstream effector of PDK1 in αIIbβ3-mediated outside-in signaling and thrombin-induced platelet activation. Finally, the *in vivo* data demonstrated that PDK1 is an important regulator in arterial thrombosis formation.

OC 58.4

Thrombus formation *in vivo* can occur independently of Syk kinase function

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Background: Spleen tyrosine kinase (Syk) and ζ-chain associated protein kinase of 70 kDa (Zap-70) are both spleen tyrosine kinase family members, which phosphorylate key adapter proteins and phospholipase C γ isoforms. Both kinases display partly overlapping expression patterns, but the intrinsic kinase activity of Zap-70 is lower. In contrast to Syk, which is highly expressed by haematopoietic cells, Zap-70 expression is largely confined to T cells and natural killer cells. In platelets, Zap-70 is not expressed, but Syk is present in the immunoreceptor tyrosine-based activation motif (ITAM) and hemITAM signalling pathways mediating signal transduction by glycoprotein (GP) VI and the C-type lectin-like receptor 2 (CLEC-2). Furthermore, Syk is involved in platelet integrin outside-in signalling. To assess if the two kinases exhibit interchangeable functions *in vivo*, we generated mice, which express Zap-70 under the control of intrinsic Syk promoter elements (*SykZap70/Zap70* mice).
Aims: In this study, we aimed to determine the consequence of the kinase replacement for platelet activation, integrin outside-in signalling and thrombus formation *ex vivo* and *in vivo*.
Methods: Platelet activation *in vitro* was analysed by flow cytometry and aggregometry. Platelet outside-in signalling was assessed using a platelet spreading assay with and without second wave inhibitors. Thrombus formation *ex vivo* was determined by a flow adhesion assay. To investigate the impact of the kinase exchange on haemostasis, tail bleeding times were measured. In addition, *SykZap70/Zap70* mice were subjected to two different models of arterial thrombosis.
Results: *SykZap70/Zap70* platelets spread normally on a fibrinogen-coated surface, indicating that Zap-70 fully compensated for the loss of Syk in the integrin outside-in signalling pathway. Consistent with our data on the abrogated GPVI and CLEC-2 signalling *in vitro* in *SykZap70/Zap70* platelets, thrombus formation under flow on a collagen-coated surface was abolished. The tail bleeding times in the *SykZap70/Zap70* mice were significantly increased, which is in agreement with the recently reported severe haemostatic defect in GPVI/CLEC-2 double-deficient mice. However, in stark contrast to GPVI-, CLEC-2- or double-deficient mice, occlusive arterial thrombus formation *in vivo* was unaltered in *SykZap70/Zap70* mice.
Summary/Conclusion: Syk signalling is essential for maintaining haemostasis. Remarkably, however, Syk appears to be dispensable for arterial thrombus formation *in vivo*. Further studies are required to elucidate whether Zap-70 compensates for the loss of Syk or whether the two platelet receptors GPVI and CLEC-2 have signalling-independent roles in arterial thrombus formation.

OC 58.5

Hemostasis and thrombosis in JAK2V617F-KI mice

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Background: Hemostasis disorders represent a leading cause of mortality and morbidity in patients suffering from myeloproliferative neoplasms (MPN) with a high incidence of thrombosis and a lower incidence of hemorrhagic disorders.
The pathophysiology of these anomalies is not known but some risk factors have been identified including the V617F mutation of JAK2 found in 95% and 60% of PV and TE patients, respectively. The specific effect of JAK2^{V617F} on hemostasis deregulation is difficult to characterize in patients because of their heterogeneity in JAK2^{V617F} allele burden and prophylactic treatments. Recently, animal models of MPN were developed as JAK2^{V617F} transgenic and knock-in mice.
Aims: Our objective was to determine, the effect of the JAK2^{V617F} mutation on the *in vitro* and *in vivo* hemostatic responses using JAK2^{V617F}mice.
Methods: We used two KI models, the Vav-Cre and Scl-CreERt/JAK2^{V617F} KI that constitutively and upon tamoxifen induction express the mutation in hematopoietic cells, respectively. Both models mimic a PV-like disease with its evolution into secondary myelofibrosis. The effect of the mutation on platelets was analyzed *in vitro* by flow cytometry, and by measuring adhesion and aggregation in flow conditions on collagen-coated area. Hemostatic responses were assessed *in vivo* by measuring the tail bleeding time and FeCl3-induced thrombosis on mesenteric vessels.
Results: Platelets from Vav-Cre/JAK2^{V617F} mice did not exhibit elevated CD62P level and had normal surface expressions of the major glycoproteins to the exception of GPVI that was significantly decreased ($P > 0.01$). Platelets adhesion and aggregation, in arterial flow conditions (1200/s) on immobilized collagen was profoundly impaired with a platelet surface decreased by up to 80% ($P > 0.01$). *In vivo*, Vav-Cre/JAK2^{V617F} mice exhibited an enlargement of mesenteric vessels and have a prolonged tail bleeding time ($P > 0.01$). After FeCl3-induced injury, platelet aggregates formed rapidly but were highly unstable.
Interestingly, using Scl-CreERt/JAK2^{V617F} mice, platelet GPVI deficiency, morphological vessel abnormalities, the increased tail bleeding time, and the *in vivo* accelerated rate of thrombosis with low thrombus stability, only appeared 2 months after induction of the mutation by the tamoxifen suggesting no direct link with it.
Secondary polycythemia induced by EPO-treatment of WT mice occurred without hemostasis disturbance; Thrombocytemia induced by retroviral expression of TPO in WT mice reproduced the GPVI deficiency, the prolonged bleeding time and the absence of *in vivo* occluding thrombi.
Conclusions: Our results in JAK2^{V617F} mice are consistent with the complex hemostasis disorders observed in MPN patients i.e. thrombosis, embolism and hemorrhages. They seemed not directly associated with the mutation but rather with the development of MPN. Further work is needed to evaluate the cause and consequence of the GPVI and collagen adhesion deficit.

OC 58.6

The JAK2V617F mutation causes an increase in platelet reactivity in a knock-in mouse model of essential thrombocythaemia

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Background: The major clinical challenge in treating patients with myeloproliferative diseases (MPDs) such as polycythaemia vera (PV)

and essential thrombocythaemia (ET) is the prevention of thrombotic events. The JAK2V617F mutation is found in > 90% and ~50% of patients with PV and ET respectively. Initial studies to identify whether platelet reactivity was modified in MPD patients were inconclusive as they were performed before identification of the V617F mutation, and hampered by the co-existance of normal and clonal haematopoiesis. We have generated a knock-in Cre-inducible JAK2V617F mutant mouse in which the human JAK2V617F (huJAK2V617F) has been knocked into one of the endogenous mouse alleles of the JAK2 gene. After induction, comparable transcript levels of the huJAK2V617F and the normal mouse allele have been shown, phenocopying the heterozygous nature of ET patients. This is in contrast to previous studies in transgenic animal models in which the mutation was highly overexpressed.

Methods and Results: Using this model, we have shown increased platelet aggregation to collagen-related peptide (CRP), and mid-range concentrations of collagen in platelet rich plasma (PRP). Fibrinogen binding is increased in whole blood from JAK2V617F mice in response to CRP and thrombin, but decreased in response to ADP compared to WT, with P-selectin expression also increased in response to CRP in JAK2V617F mice. Thrombus formation on a collagen-coated surface increases in whole blood laminar flow assay. Platelet spreading under static conditions on a fibrinogen-coated surface also increased. *In vivo* studies showed a marked decrease in tail bleeding time in mutant animals.

Conclusion: This mouse model offers a unique opportunity to dissect the differences in platelet biology in the context of the JAK2V617F mutation. Our data strongly suggests that JAK2V617F platelets are hyper-reactive, and therefore we postulate that solely reducing the platelet count in ET patients to normal levels is not sufficient to reduce the hyperthrombotic effect of the disease.

OC 59 – Non Inherited Risk Factors for Venous Thrombosis

OC 59.1

A randomized study on 1 vs. 4 weeks prophylaxis for venous thromboembolism after laparoscopic surgery for colorectal cancer

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Background: Extending antithrombotic prophylaxis beyond 1 week reduces the incidence of venous thromboembolism (VTE) in patients undergoing open abdominal surgery for cancer. Whether extended prophylaxis is required after laparoscopic surgery for cancer is unknown.

Aims: The aim of the study was to evaluate the efficacy and safety of 4 weeks compared to 1 week antithrombotic prophylaxis in patients who underwent laparoscopic surgery for colon-rectal cancer.

Methods: Complete compression ultrasonography (cCUS) of the lower limbs was performed in consecutive patients after 8 ± 2 days of antithrombotic prophylaxis. Patients with no evidence of VTE were randomized to two prophylaxis regimens: short (heparin discontinuation) or extended (heparin for three additional weeks). cCUS was repeated at day 28 ± 2 after surgery by a study physician blinded to treatment allocation. The study primary outcome was the incidence of VTE at day 28 ± 2 from surgery.

Results: Overall, 301 patients were evaluated for inclusion in the study and 225 were randomized. VTE occurred at day 28 ± 2 in 11 out of 113 patients randomized to short (9.7%) and in none of the 112 patients randomized to extended heparin prophylaxis (RRR 53%, 95% CI 46–59%, $P = 0.001$). The incidence of VTE at 3 months was 0.9% and 9.7% in patients randomized to extended or to short heparin prophylaxis, respectively (RRR 91%, 95% CI 30–99%, $P = 0.003$). The rate of bleedings was similar (0.9% in the two groups).

Conclusions: After laparoscopic surgery for colon-rectal cancer, extended antithrombotic prophylaxis is safe and reduces the risk for VTE, as assessed by cCUS, compared to 1 week prophylaxis (NCT01589146).

OC 59.2

Increased risk of venous thrombosis after arterial thrombosis: causal or explained by common risk factors?

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Background: Venous and arterial thrombosis are traditionally considered as two different diseases. However, recently associations between the two entities have been described.

Aim: We aimed to investigate explanations for the increased risk of venous thrombosis (VT) after an arterial event (AT), such as presence of common risk factors or immobilisation during the acute phase of a myocardial infarction or stroke.

Methods: The study was performed in two large cohorts from Denmark and Norway, i.e. the Diet, Cancer and Health cohort (DCH) and the Tromsø study. The DCH cohort included individuals recruited between 1993 and 1997 aged 50–64 years and without prior cancer at the time of recruitment. The Tromsø study is a population-based cohort conducted between 1994 and 1995. Both cohorts were regularly linked to their national registries by means of the civil registration number. Incidence rates of VT were calculated for the overall cohorts and for subjects with arterial thrombosis, starting from the date of the arterial event, to study the overall risk and the effect of time on the incidence. The ratios between the two events were calculated using a time-dependent Cox regression model. To investigate the mechanism we adjusted for the following common risk factors: sex, age, Body Mass Index, smoking, alcohol intake, hypertension, diabetes, and education level. All participants gave informed consent, and both studies were approved by their local ethical committee.

Results: Out of 6200 patients with an arterial thrombosis, 108 developed a venous thrombosis, leading to an overall incidence of VT of 4.0 (CI95 3.3–4.8) per 1000 person years in this group, compared to an incidence of 1.2 (CI95 1.1–1.3) per 1000 person years in subjects without AT. This led to an overall 2.7-fold (CI95 2.2–3.3) higher risk of VT in patients with AT. When we studied the time-trend between the two events we found an incidence rate for VT of 39.8 per 1000 person years (CI95 24.7–64.0) in the first 2 months after arterial thrombosis compared with an incidence of 1.1 per 1000 person years (CI95 1.0–1.2) in those without AT. This incidence rate decreased over time to 1.9 per 1000 person years in the period of more than 6 years after the arterial event. When we adjusted the overall hazard ratio for common risk factors, it attenuated from 2.7 to 1.5 (CI95 1.2–1.9), indicating that common risk factors partly explained the increased risk. Patients in whom the VT occurred within 2 months after AT were less likely to

have common risk factors for arterial and venous thrombosis than those who had a VT more than 2 months after an AT.
Conclusion: In the first 2 months after an arterial thrombosis, the risk of a venous event was strongly increased. This risk decreased over time but remained higher also the long term. The long-term association was partly explained by common risk factors. However, in the high-risk period shortly after AT, the role of common risk factors seemed to be limited.

OC 59.3

Arthroscopy of the knee and risk of venous thrombosis: results from the MEGA study

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Background: Knee arthroscopy is the most common orthopaedic procedure performed worldwide. In contrast to most other orthopaedic surgeries, guidelines generally recommend against the use of thromboprophylaxis, because the reported estimates vary widely and the exact risk of venous thrombosis (VT) after knee arthroscopy remains unclear. Furthermore, the influence of genetic and acquired risk factors, as well as the indication for knee arthroscopy on this risk is not known, even though identification of such high-risk groups can individualise prophylactic treatment.
Aims: To estimate the risk of venous thrombosis after knee arthroscopy and to identify high risk groups.
Methods: We used data from a large population based case-control study (MEGA-study) into the aetiology of venous thrombosis (4418 cases, 6150 controls, consent and ethical approval obtained). Odds ratios (OR) with 95% confidence intervals (CI95) were calculated and adjusted for age, sex, body mass index, regular exercise and rheumatic disease. We used varying exposure time windows for reasons of statistical precision. Absolute risks were estimated from the ORs, assuming an incidence of VT of 1–2 per 1000 person years in the general population. Analyses of joint effect all have those with none of the risk factors in the analysis as reference group.
Results: Knee arthroscopy (105 cases, 24 controls) was associated with an almost sevenfold increased risk of VT in the following year (OR 6.7 [CI95; 4.3–10.5]) and an 18-fold increased risk over a 3 months period (OR 18.3 [CI95; 8.8–37.8]). This corresponds to an incidence of VT of 0.5–0.9% in the 3 months after the procedure.
Considering events during 1 year after knee arthroscopy showed that chondroplasty, meniscal surgery and diagnostic knee arthroscopy resulted in a sixfold increased risk (OR 6.1 [CI95; 3.8–9.7]) whereas ligament reconstructions led to a 20-fold increased risk (OR 20.3 [CI95; 2.6–157]). An even more pronounced risk was found in those with genetic or other acquired risk factors. The combination of knee arthroscopy and oral contraception use led to a 64-fold elevated risk over the following year (CI95; 8.5–481); with carriership of the factor V Leiden mutation, prothrombin G20210A mutation or a non-O blood type the OR was 16.6 (CI95; 8.9–30.7). There was no excess risk conferred by obesity (BMI > 30 kg/m^2 and arthroscopy: OR 7.4 [CI95; 2.5–21.5]). No information was available on thromboprophylaxis, while a proportion of the patients most likely did receive this. Therefore our results are most likely an underestimation of the true risk.
Conclusion: Knee arthroscopy strongly increases the risk of venous thrombosis, especially in the first months after the procedure. Patients with genetic predispositions or who have other acquired risk factors have an additionally increased risk. Prophylactic treatment can be individualised and optimised when the indication for knee arthroscopy and the presence of genetic and acquired risk factors are taken into account. This will substantially reduce thrombosis morbidity, especially considering the high frequency of the procedure.

OC 59.4

Optimal risk estimation for DVT requires measurement of coagulation protein concentrations

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Background: he risk of developing deep venous thrombosis has been linked to a number of separate risk factors, ranging from clinical risk factors like leg injury or immobility to genetic factors like SNPs and plasma coagulation protein concentrations such as the concentration of Factor (F)VIII. In a recent publication, de Haan et al. (Blood, 2012) presented a risk score based on clinical and genetic risk factors, which showed good discrimination results between the high (such as hospitalized patients) and low risk individuals in the MEGA (Blom et al., JAMA, 2005) and LETS (van der Meer et al., Thromb Haemost, 1997) studies. The inclusion of coagulation protein concentrations may further improve the discrimination power of these risk models however.
Aims and Methods: To this end, we applied neural network machine learning methods to the data that has been collected in the MEGA study and constructed an algorithm to translate clinical risk factors (recent surgery, leg injury, immobility, recent travel, pregnancy, use of oral contraceptives, obesity, cancer and family history of thrombosis), genetic risk factors (FV Leiden, blood group non-O, prothrombin G20210A and mutations in FXI and fibrinogen) and coagulation protein concentrations (antithrombin, FII, FVII, FVIII, FIX, FX, FXI, fibrinogen, protein C, protein S) into one risk score. In order to test the robustness of our method, we validated the risk score algorithm that was optimized on the MEGA data on the independent LETS data set.
Results: The machine learning methods based on the complete set of inputs (including coagulation protein concentrations) outperformed the published method based on clinical risk factors and SNPs alone (area under the ROC curve of 0.86, 95% confidence interval 0.84–0.87 vs. 0.81 (0.79–0.82) in a cross-validation study on MEGA). The validation on LETS required the exclusion of cancer patients from the MEGA cohort, since these patients were excluded from the original LETS study. Without malignancies as a discriminative factor, the AUC on MEGA dropped to 0.82 (0.80–0.84) with and 0.78 (0.76–0.80) without coagulation protein concentrations. Without further adaptation of the algorithm, the discriminative power when applied to the LETS cohort remained high with an AUC of 0.81 (0.77–0.85) and 0.78 (0.74–0.82) for the same method with clinical and genetic risk factors only. All increases in AUC upon inclusion of protein concentrations were statistically significant (p-values > 0.05).
Conclusion: These results indicate that including concentration measurements of coagulation proteins holds a strong, additional value over current methods. We showed that earlier published results can be improved when concentrations of coagulation proteins into the thrombosis risk estimation are included.

OC 59.5

Validation study of the IMPACT-ILL venous thromboembolism risk assessment model in the acutely ill medical patient

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Background: For Venous Thromboembolism (VTE) prevention, American College of Chest Physicians (ACCP) and International Union of Angiology (IUA) guideline recommendations are primarily based off of risk factors utilized for entry into randomized controlled trials (RCT) or post-hoc analysis of these RCTs. These guidelines rec-

ommend a group-based, as opposed to an individualized risk assessment, approach. It is currently unknown how these risk factors interact in a quantitative manner.

A recent retrospective VTE risk assessment model (RAM) (IMPACT-ILL) was derived from the multinational IMPROVE registry in hospitalized medical patients. The '*VTE-VALOURR*' is a retrospective, multi-center, case-control, validation study of this RAM and is also assessing other VTE and bleeding risk factors. Many RAMs are available with none being appropriately externally validated in the acutely ill medical patient.

Methods: Reports from ICD-10 coding and the McMaster Transfusion Registry for Utilization Surveillance and Tracking (TRUST) database, which contains demographics, transfusion data, and approximately 50 clinical variables including thrombotic outcomes of inpatients, were used as the data source at three hospitals. Inclusion criteria were hospitalized medical patients ≥ 18 years with ≥ 3 days length of stay (LOS) while exclusion criteria were patients with pregnancy, mental health disorders, atrial fibrillation/flutter, trauma, spinal cord injury, surgery within 90 days, VTE within 24 h of admission, treatment dose anticoagulants (including warfarin) within 48 h of admission, or transferred from a non-McMaster acute care facility. Pulmonary embolism and lower extremity deep vein thrombosis (DVT) within 3 months of admission were the outcomes of interest and verified by chart review. Upper extremity DVT was excluded. Descriptive statistics, including proportions and frequencies, were used to summarize binary variables. A VTE cohort and a VTE free control cohorts' receiver operator curves will be compared assessing the validity of the IMPACT-ILL RAM.

Results: From January 1st, 2005 to February 28th, 2011, 247,241 hospitalizations occurred at three McMaster hospitals. After exclusionary criteria were applied, 779 VTE events were identified. Of these, 419 were excluded because they were VTE events not related to a previous hospitalization (i.e. community-acquired), leaving a VTE cohort of 139 patients. A VTE-free control cohort at a 2:1 ratio was also evaluated for VTE and bleeding risk factors. Approximately 80% of the current VTE cohort appears to have a score of 2 or above and be at moderate to high risk of VTE. Final results of the validity of the IMPACT-ILL VTE RAM will be presented.

Conclusions: Testing for validity of the IMPACT-ILL VTE RAM will be useful and may help identify medical patients at risk of VTE that do not readily fit into group-specific VTE risk categories. Further, validation may identify subsets of medical patients at especially high risk of VTE and focus future randomized controlled trials. Other VTE risk factors may be identified with the study.

OC 59.6

Impact of incident atrial fibrillation on future risk of venous thromboembolismthe Tromsø study

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Background: Epidemiological and clinical studies have provided strong evidence that atrial fibrillation is associated with thrombus formation in the left atrium with subsequent increased risk of systemic embolism, particularly ischemic stroke. Previous autopsy and retrospective studies have shown that pulmonary embolism may originate from right arterial thrombi. Similarly, a retrospective cohort reported higher prevalence of pulmonary embolism in subjects with atrial fibrillation than without atrial fibrillation. Although these observations support the concept that atrial fibrillation is associated with risk of pulmonary embolism, no prospective cohort study has investigated the association between incident atrial fibrillation and future risk of venous thromboembolism (VTE).

Aims: We wanted to investigate whether incident atrial fibrillation is associated with future risk of incident VTE in a prospective cohort study with subjects recruited from a general population.

Methods: A total of 29 774 men and women participated in at least one survey of the Tromsø study (1994–95, 2001–02 or 2007–08), where information was obtained by questionnaires, blood samples and a physical examination. The study participants were followed from date of enrolment until December 31, 2010, and all events of atrial fibrillation and VTE were recorded and validated. Cox proportional hazard regression models, using age as time scale and atrial fibrillation as a time-dependent variable, were used to obtain crude and multivariable hazard ratios (HR) for VTE with 95% confidence intervals (CI). The analyses were adjusted for sex, body mass index, smoking, physical activity, cholesterol, self-reported arterial cardiovascular disease and diabetes. The study was approved by the regional committee for research ethics, and all participants gave their informed written consent to participate.

Results: Of the study participants, 1604 (5.4%) were diagnosed with atrial fibrillation and 621 (2.1%) had an incident VTE event during a median of 15.6 years follow-up. Among those with atrial fibrillation, 65 (4.1%) developed a subsequent VTE yielding an age-adjusted incidence rate (IR) per 1000 person-years of 9.17 (95% CI: 7.19–11.17), whereas the corresponding IR in subjects without atrial fibrillation was 6.39 (95% CI: 5.69–7.18). Subjects diagnosed with atrial fibrillation had increased risk of VTE compared to subjects without atrial fibrillation in crude (HR: 2.09, 95% CI: 1.60–2.73) and multivariable adjusted analyses (HR: 1.95, 95% CI: 1.48–2.56). Atrial fibrillation was associated with risk of both pulmonary embolism and deep vein thrombosis, but as expected, the risk estimates for pulmonary embolism were higher (multivariable HR: 2.54, 95% CI: 1.74–3.72) than for deep vein thrombosis (multivariable HR: 1.50, 95% CI: 1.01–2.23). The risk of VTE was highest during the first 6 months after the atrial fibrillation diagnosis (age-adjusted IR: 32.06, 95% CI: 21.27–48.32).

Conclusion: We found that atrial fibrillation was associated with increased risk of VTE, and pulmonary embolism in particular. Our findings support the concept that pulmonary embolism may originate from right atrial thrombi formed in an environment of atrial fibrillation.

OC 60 – Novel Platelet Receptors

OC 60.1

Syk is essential for anti-CLEC-2 antibody-induced thrombocytopenia, but not receptor depletion

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Background: The C-type lectin-like receptor 2 (CLEC-2) plays an important role in haemostasis and thrombosis. Ligation of CLEC-2 induces phosphorylation of the hemITAM motif of the receptor and the recruitment of Syk, thereby initiating a signalling cascade that involves the adapter LAT and culminates in the activation of phospholipase (PLC) gamma 2. We have previously shown that treatment of mice with the CLEC-2 specific INU1 antibody leads to a transient thrombocytopenia and depletion of CLEC-2 in circulating platelets. Such CLEC-2 depleted mice are protected from occlusive thrombus formation but do not show a major bleeding defect. The mechanisms underlying these anti-CLEC-2 antibody induced effects have not been identified.

Aims: By the use of mouse lines deficient in central molecules in the CLEC-2 signalling pathway the mechanisms underlying anti-CLEC-2 antibody-induced thrombocytopenia and receptor depletion were assessed *in vivo*.

Methods: Mice were treated with the monoclonal antibody INU1 (anti-CLEC-2, 200 μg i.v.) to deplete CLEC-2 in circulating platelets. Platelets were analysed up to 7 days after injection using flow cytometry, biochemical methods and functional assays.

Results: INU1-treatment induced severe thrombocytopenia in wild-type and *Lat*−/− mice to the same extent and this was not altered by

pre-treatment with integrin αIIbβ3-blocking antibodies. In sharp contrast, no thrombocytopenia was observed in INU1-treated mice lacking either CLEC-2 or Syk in platelets demonstrating that binding of INU1 to platelet CLEC-2 directly induces platelet depletion *in vivo* through a Syk-dependent mechanism that does, however, not require αIIbβ3-dependent aggregation. Strikingly, INU1 induced the irreversible loss of CLEC-2 in wild-type, *Lat*−/− and Syk-deficient mice to the same extent and irrespective of αIIbβ3 blockade. In Syk-deficient platelets, the INU1-induced CLEC-2 loss occurred through internalisation and intracellular clearing demonstrating for the first time an active mechanism of CLEC-2 down-regulation in platelets.

Summary/Conclusions: Our results reveal that anti-CLEC-2 antibody induced thrombocytopenia and receptor depletion can be mechanistically uncoupled and that the latter occurs independently of Syk. These findings may have important implications for the possible development of therapeutic agents that modulate CLEC-2 function.

OC 60.2

Expression of exogenous proteins in platelets: all you need is Yop

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Background: While primarily known for their role in hemostasis, platelets are also essential components of the innate immune system. Their ability to detect and capture blood-born bacterial pathogens, in particular, is well established. These interactions are varied in nature and depend on the specie and strain a given bacterium belongs to. Yersinia enterocolitica is one of three pathogenic Yersinia species capable of injecting large amounts of plasmid-encoded cytotoxins known as Yops into immune cells, a defense mechanism that is known to disrupt the phagocytic and lytic responses of leukocytes. The injection of Yops into mammalian cells is dependent on the type III secretion system (T3SS) of Yersinia and requires both a *N*-terminal secretion domain and the co-expression of a chaperone protein.

Aims. Because blood platelets are known to bind virulent strains of Yersinia enterocolitica, we hypothesized that these bacteria could be used as a vector to inject proteins into platelets.

Methods: Attenuated, Yop-deficient Yersinia enterocolitica strains were engineered to produce massive amounts of fusion proteins bearing the N-terminal secretion domain of YopE along with the chaperone protein SycE. Expressed proteins included, amongst others, fluorescent proteins and intracytoplasmic epitopes-targeted peptides. Ex vivo infection of blood platelets with fusion protein-expressing Yersinia enterocolitica strains was performed on both activated and resting platelets. After bacteria removal, the amount of injected material present in platelets was quantified by western blotting, immunofluorescence and flow cytometry. Simultaneously, platelets were tested in various assays to ascertain any altered functional response.

Results: Yersinia enterocolitica was able to bind to both resting and activated platelets. Protein injection was very successful in both cases with success rates ranging from 98% to 100%. Within 1 h of infection, fluorescent proteins such as eGFP could be readily detected by immunofluorescence and flow cytometry, demonstrating the efficiency of protein transfer. The infection procedure had no detectable effect on platelet responsiveness. As for inhibitory peptides: platelets that were injected with a peptide spanning part of the talin-1 FERM domain presented with impaired integrin aIIbb3 responses; platelets injected with a peptide targeting calcium channels had normal intracytoplasmic calcium oscillations in their resting state but impaired calcium mobilization when treated with thrombin.

Conclusion: In nucleated cells, expression of exogenous proteins, fluorescent reporters or bioactive peptides is just a transfection step away, a sadly unattainable commodity to all platelet biologists. Bearing this mind, we have sought a methodology that would allow us to overcome this limitation. Our studies show that Yersinia enterocolitica can be engineered to rapidly deliver proteins and peptides into blood platelets without affecting their responsiveness. This new tool should prove especially useful to anyone interested in studying platelet intracytoplasmic signaling.

OC 60.3

A novel double heterozygous substitution p.(Val207Ala; Thr223Arg) in the P2Y12 receptor is associated with reduced receptor expression and platelet dysfunction

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Background: The Genotyping and Phenotyping of Platelets consortium (GAPP) has developed an approach for rapidly identifying and characterizing rare function disrupting variations in genes encoding platelet proteins, including GPCRs which are critical regulators of platelet function and are anti-thrombotic drug targets. This has yielded two novel thromboxane receptor variants (Mumford et al., 2010; Mumford et al., 2013) and three novel $P2Y_{12}$ variants (Nisar et al., 2011; Daly et al., 2009; Patel et al., in preparation) which result in reduced platelet responsiveness. We now report a novel double heterozygous $P2Y_{12}$ receptor variant associated with reduced platelet responsiveness to ADP.

Aims: To characterise the functional effect of the double heterozygous p.[(Val207Ala; Thr223Arg)] $P2Y_{12}$ variant in platelets and cell lines.

Methods: Platelet function was assessed by measuring platelet aggregation responses in platelet rich plasma (PRP). The candidate $P2Y_{12}$ variations were identified by capture of the *P2Y12R* coding exons from genomic DNA using an Agilent-SureSelect targeted array of 216 platelet genes and sequencing on an Illumina GA-II. For the cell expression studies, HA-tagged wild type (WT), V207A, T223R and V207A/T223R constructs were generated and transiently expressed in HEK293 cells and stably expressed in human 1321N1 astrocytoma cells.

Results: The 41 year old female subject with no history of pathological bleeding displayed diminished aggregation responses to 2 and 5 μM ADP compared to healthy controls, but similar aggregation responses to high concentrations of other agonists. Analysis of *P2Y12R* showed heterozygous non-synonymous variations predictive of V207A and T223R substitutions in the 5th transmembrane domain and the 3rd intra-cellular loop respectively. A family study showed that both variations p.([Val207Ala; Thr223Arg]) were present on the same *P2Y12R* allele. Immunoblotting and imaging experiments in both HEK293 and 1321N1 cells showed that total expression of the double mutant V207A/T223R was significantly reduced and accompanied with a loss of receptor function. Surface and total expression of the single mutants, however, was comparable to WT.

Conclusions: We have identified a novel double heterozygous mutation within the $P2Y_{12}$ receptor, associated with reduced ADP-induced platelet aggregation. This is the first description of a naturally occurring double variant in the same $P2Y_{12}$ allele. Our data suggest that it is the combination of the V207A and T223R substitutions that causes reduced total receptor expression, resulting in reduced platelet responsiveness to ADP.

This work was supported by the British Heart Foundation (RG/09/007).

OC 60.4

Endothelial protein C receptor is expressed on activated platelets and contributes to FVIIa binding

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Background: At pharmacologic concentrations recombinant Factor VIIa (rFVIIa, NovoSeven®) binds to activated platelets and mediates activation of FX directly on the platelet surface. We originally proposed that this binding was mediated by surface-expressed phosphatidyl serine (PS). More recently, Lismann et al. demonstrated that rFVIIa can also bind to the platelet surface glycoprotein (GP) Ib/IX/V complex. However, binding to GPIb/IX/V on other membrane surfaces did not recapitulate the pattern of rFVIIa binding to activated platelets. Therefore, we sought other possible contributors to platelet rFVIIa binding.

Aims: Our unpublished data show that Protein C can partially compete with rFVIIa and rFVIIa variants for binding to activated platelets. The group of Pendurthi and Rao have shown that rFVIIa binds to endothelial protein C receptor (EPCR) and that binding to EPCR modulates the proteolytic activity of rFVIIa against cell-surface targets. The goal of our current work is to determine whether EPCR is expressed on platelets and whether it contributes to rFVIIa binding (and activity) on platelets.

Methods: Human platelets were isolated from blood samples collected from normal donors after obtaining informed consent. The binding of rat monoclonal (Santa Cruz) and goat polyclonal (Affinity Biologicals) antibodies raised against human EPCR was assessed by flow cytometry. EPCR was also immunoprecipitated from platelet lysates using the monoclonal antibody and detected on PAGE followed by Western blotting with the polyclonal antibody.

Results: We found that EPCR could not be detected on the surface of unactivated platelets, and activation with thrombin alone resulted in a low level of EPCR expression. By contrast, activation with the combination of thrombin and convulxin (a collagen receptor agonist) resulted in the appearance of a discrete population of platelets with high expression of EPCR and a residual population of platelets that are negative for EPCR. The population of EPCR-positive platelets is the same as the platelets that bind high levels of rFVIIa, and comprise from 30% to 60% of the platelets, depending on the blood donor. Kjalke et al. have previously reported that COllagen And Thrombin activated (COAT) platelets preferentially bind rFVIIa. An anti-EPCR antibody was able to partially block FVIIa binding to activated platelets. A protein with the correct molecular weight for full length EPCR could be immunoprecipitated from thrombin + convulxin-activated platelets.

Conclusion: EPCR is expressed on the population of highly procoagulant COAT platelets induced by dual stimulation with thrombin and a collagen agonist. EPCR plays a role in mediating binding of rFVIIa to activated platelet surfaces.

OC 60.5

Regulation of the hemostatic and inflammatory responses triggered by ligands of Toll like receptor 2 and 4 is another non-genomic role of nuclear factor-?B in platelets

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Background: Platelets express toll like receptors (TLRs), a family of proteins that recognize molecular components of pathogens. Although several studies reported platelet activation mediated by activation of TLR2 and 4, the data are controversial and the molecular mechanisms are not fully elucidated. In nucleated cells, one of the downstream molecules of the TLRs signaling pathway is the transcription factor nuclear factor-κB (NF-κB), a major regulator of inflammatory genes transcription. We have previously shown that activation of NF-κB mediates platelet activation triggered by classical platelet agonists, indicating that this transcription factor exerts non-genomic functions in platelets.Whether NF-κB is involved in the signaling pathway of TLR2 and 4 have not yet been investigated.

Aim: To further examine platelet activation mediated by TLR2 and TLR4 stimulation and determine if activation of NF-κB is a downstream signal.

Methods: Washed human platelets were stimulated with Pam(3)CSK (4) or lipopolysaccharide (LPS) (agonists for TLR2 or 4 respectively) in the absence or presence of BAY11-7082 and Ro106-9920, two non-structurally related inhibitors of NF-κB. Aggregation and ATP release were measured using a Lumi-aggregometer and platelet-leukocyte aggregates were enumerated by cytometry. Activation of NF-κB was examined by the degradation of its inhibitor, I-kB, and the phosphorylation of the p65 subunit, by Western Blot. The release of IL-1b was determined by ELISA. Results are expressed as X ± SEM (Student T test $*P > 0.05$ vs. C and ANOVA $\#P > 0.05$ vs. Pam(3)CSK(4), LPS or Thrombin (Thr) $n = 4–5$).

Results: Stimulation of platelets with Pam(3)CSK(4) (0.1–15 μg/mL) triggered platelet aggregation, ATP release, as well as proinflammatory responses such as IL-1b secretion (C: 51 ± 13, Pam(3)CSK(4) (1 μg/mL): 288 ± 96* pg/mL) and the formation of platelet-leukocyte aggregates (C: 17 ± 2, Pam(3)CSK(4): 38 ± 4*% of PMN associated with platelets). All Pam(3)CSK(4)-mediated responses were completely inhibited by preincubation of the platelets with BAY11-7082 or Ro106-9920. LPS (10 μg/mL) did not have a direct effect but potentiated aggregation and ATP release induced by threshold Thr concentrations (0.01 U/mL). This synergism was partially blocked by the NF-κB inhibitors (BAY11-7082: 39 ± $3^{\#}$, Ro106-9920: 37 ± $5^{\#}$% of inhibition). While platelet-leukocyte aggregates were no triggered by LPS, it efficiently induced IL-1b release that was prevented in platelets pretreated with BAY11-7082 or Ro106-9920 (C: 51 ± 13, LPS: 460 ± 110*, LPS+BAY11-7082: 24 ± $16^{\#}$, LPS+Ro106-9920: 32 ± $17^{\#}$ pg/mL). Stimulation of platelets with 1 μg/mL of Pam(3) CSK(4) or LPS resulted in the degradation of I-kB and the phosphorylation of the p65 subunit of NF-κB (Pam(3)CSK(4): 5 ± 1*, LPS: 6 ± 1*, fold increase). Both responses elicited by Pam(3)CSK(4) or LPS were completely inhibited by an antibody against TLR2 or 4 respectively, and synergized by threshold concentrations of Thr (phosphorylation of p65: Thr: 4 ± 1, Thr+Pam(3)CSK(4): 9 ± $1^{\#}$, Thr + LPS: 12 ± $4^{\#}$).

Conclusion: Activation of TLR2 and 4 trigger platelet hemostatic and inflammatory responses that are mediated by the transcription factor NF-κB. These data reinforce the notion of the relationship between the immune response and platelets, reveal another non-genomic function of NF-κB in platelets and highlight this molecule as a potential target to prevent platelet activation in inflammatory and infectious diseases.

OC 60.6

The newly identified platelet receptor DCBLD2 is involved in platelet activation and thrombus formation

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Background: Recent genome wide association and -omics studies revealed new platelet proteins potentially related to platelet disorders. One of these is the discoidin, CUB and LCCL domain containing 2 protein (DCBLD2), a transmembrane receptor which we previously found to be involved in thrombus formation in a zebrafish model [1].

Aim: Our aim is to further characterize the role of DCBLD2 in thrombosis and haemostasis and to uncover its platelet-specific function.
Methods: To study the *in vivo* role of DCBLD2 in thrombosis and haemostasis, DCBLD2 knock-out (KO) mice were subjected to well established models of $FeCl_3$-induced thrombosis, tail clipping bleeding and ischemic stroke (transient middle cerebral artery occlusion, tMCAO). *In vitro* agonist-induced platelet aggregation experiments and flow cytometric analysis of murine and human platelets provided further clues about the mechanisms responsible for the effects observed *in vivo*.
Results: Although DCBLD2 KO mice have normal platelet counts and no distinct phenotype, the absence of DCBLD2 clearly resulted in a significant decrease in the time to occlusion upon arterial vessel damage in the $FeCl_3$-induced thrombosis model. These data confirm our earlier findings in zebra fish and point towards an attenuating effect of DCBLD2 on thrombus formation. Interestingly, absence of DCBLD2 did not affect tail-clipping times. It is known that certain platelet receptor-ligand interactions contribute to stroke progression after reperfusion of previously occluded major arteries. This is however not the case for DCBLD2 as the infarct volume and functional outcomes after ischemic stroke in DCBLD2 KO mice were not significantly different from those in wild-type animals. Involvement of DCBLD2 in platelet activation was further validated by *in vitro* agonist-induced platelet aggregation experiments. Aggregation of platelet rich plasma (PRP) from DCBLD2 KO mice showed a significant increase of $13 \pm 0.5\%$ ($n = 5$; $P > 0.01$) and $10 \pm 1\%$ ($n = 3$; $P > 0.01$) compared to controls when stimulated with ADP or collagen respectively. When stimulated with threshold concentrations of thrombin, the maximum aggregation level of DCBLD2 KO platelets was up to $40 \pm 4\%$ ($n = 4$; $P > 0.01$) higher. In addition, flow cytometric analysis of washed platelets from the DCBLD2 KO mice, activated with threshold concentrations of thrombin, showed increased expression of both P-selectin and activated $\alpha_{IIb}\beta_3$, further demonstrating the dampening effect of DCBLD2 on platelet activation. Aggregation of platelets in human PRP was found to be stronger in response to several agonists in the presence of recombinant human DCBLD2-extracellular domain (ECD) indicating not only a similar role for DCBLD2 in man as in mice, but also suggesting that the recombinant domain is competing with the receptor for an extracellular ligand.
Conclusions: Our data demonstrate a down-regulating function of DCBLD2 in platelet activation and platelet-dependent thrombus formation. Our experiments also suggest the presence of an extracellular ligand for DCBLD2, which remains to be identified.
1. O'Connor MN, Salles II, Cvejic A, Watkins NA, Walker A, Garner SF, et al. Functional genomics in zebrafish permits rapid characterization of novel platelet membrane proteins. Blood 2009; 113:4754–62.

OC 61 – Pregnancy and Coagulation

OC 61.1
Qualitative changes in VWF are acquired during pregnancy

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Background: Levels of the critical clotting protein von Willebrand Factor (VWF) increase markedly during pregnancy. The VWF changes observed during pregnancy have long been thought to be the result of direct hormonal stimulation of VWF production. We hypothesized that pregnancy also induces qualitative changes in VWF.
Aims: To test this hypothesis, we investigated molecular properties of VWF in healthy pregnancies.
Methods: Plasma samples were obtained from a healthy pregnancy repository; all pregnancies exhibit increased VWF:Ag levels, consistent with the literature, and all have stable ADAMTS13 activities. For this study, subjects who had samples available from multiple trimesters and a > 6 week postpartum sample were selected. Samples were normalized for VWF:Ag (ELISA, GTI Diagnostics) in VWF-deficient plasma (Affinity Biologicals). VWF multimers were examined by 1% agarose gel electrophoresis, blotted to PVDF, and detected by immunoblotting (HRP-conjugated rabbit anti-human VWF, Dako). Isoelectric focusing (IEF) of native plasma was performed in precast 5% acrylamide gels pH range 3–7 (Invitrogen), blotted to PVDF, and detected by immunoblotting (HRP-conjugated rabbit anti-human VWF, Dako). VWF blood group A glycan burden was determined by ELISA (mouse anti-A primary antibody, Immucor; HRP-conjugated donkey anti-mouse secondary antibody, Jackson ImmunoResearch).
Results: VWF multimer patterns shifted over the course of pregnancy towards higher order multimers by the third trimester. These higher order multimers were no longer detectable in postpartum samples. IEF analysis of native VWF demonstrated two distinct bands which focused at pH ~5.6 and 5.7 in normal plasma and early pregnancy samples. The VWF upper band (pH 5.7) became less distinct, shifted focus towards the lower band (pH 5.6), and the ratio of top band density to lower band density decreased in the third trimester. The VWF focusing pattern returned to two distinct bands at pH 5.6 and 5.7 in the postpartum sample. Blood group A glycan burden on VWF shifted to a progressively to higher detectable A glycan over the course of two pregnancies and returned to a lower A glycan burden postpartum. However, this pattern of increased A glycan burden was not consistent among several other blood group A pregnancies tested.
Conclusions: VWF multimer structure and isoelectric point changed over the course of these pregnancies. These shifts were most evident in the third trimester and resolved postpartum. Interestingly, VWF blood group A carbohydrate burden also demonstrated a capacity to shift over the course of pregnancy, but this finding was not consistent in other pregnant blood group A subjects. We conclude that VWF can acquire complex molecular qualitative changes during gestation. These data support a new model in which VWF shifts both quantitatively and qualitatively as a result of pregnancy. We speculate that pregnancy-induced qualitative changes correspond with differences in VWF function. The shift towards higher order VWF multimers later in pregnancy suggests greater VWF hemostatic activity. This new model may have significant clinical implications for pregnant women, particularly in the assessment of patients at risk for bleeding or thrombotic complications.

OC 61.2
Aspirin or anticoagulants for treating recurrent miscarriage in women without antiphospholipid syndrome: an updated systematic review

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Background: Approximately 5% of women attempting to conceive experience two or more miscarriages (recurrent miscarriage, RM). RM is associated with thrombophilia and anticoagulant therapy appears to increase the chance of live birth in subsequent pregnancies in women with antiphospholipid syndrome. Whether this is also true in women with unexplained RM or women with RM and inherited thrombophilia is uncertain.
Aims: To evaluate the efficacy and safety of aspirin and anticoagulant agents in women with a history of at least two miscarriages without apparent causes, other than inherited thrombophilia.
Methods: We searched the Cochrane Pregnancy and Childbirth Group's Trials Register (30 April 2012) and scanned bibliographies of all located articles for any unidentified articles.

Randomised and quasi-randomised controlled trials that assessed the effect of anticoagulant treatment on live birth in women with a history of at least two miscarriages without apparent causes other than inherited thrombophilia were eligible. Interventions included aspirin, unfractionated heparin, and low-molecular-weight heparin (LMWH) for the prevention of miscarriage. One treatment could be compared with another or with placebo. Two authors assessed the studies for inclusion in the review and extracted the data. If necessary they contacted study authors for more information.

Result: Nine studies, including data of 1238 women, were included in this review evaluating the effect of either LMWH or aspirin or a combination of both, on the chance of live birth in women with recurrent miscarriage, with or without inherited thrombophilia. Studies were heterogeneous with regard to study design and treatment regimen and three studies were considered to be at high risk of bias. Two of these three studies showed a benefit of one treatment over the other, but in sensitivity analyses (in which studies at high risk of bias were excluded) anticoagulants did not show a beneficial effect on live birth. Obstetric complications such as preterm delivery, preeclampsia, intrauterine growth restriction and congenital malformations were not significantly affected by any treatment regimen. Treatment with LWMH combined with aspirin increased the risk of bleeding significantly in one study. Local skin reactions to LMWH were reported in almost 40% of patients in the same study. Subgroup analyses in women with inherited thrombophilia were underpowered for firm conclusions.

Conclusion: There is a limited number of studies on the efficacy and safety of aspirin and heparin in women with a history of at least two miscarriages without apparent causes other than inherited thrombophilia. Of the nine reviewed studies quality varied, different treatments were studied and of the studies at low risk of bias only one was placebo-controlled. No beneficial effect of anticoagulants in studies at low risk of bias was found. Therefore, the use of anticoagulants in women with unexplained recurrent miscarriage is not recommended. The effect of anticoagulants in women with unexplained recurrent miscarriage and inherited thrombophilia needs to be assessed in randomised placebo controlled trials; at present there is no evidence of a beneficial effect.

OC 61.3

Calibrated automated thrombography does not reveal hypercoagulability in women with unexplained recurrent pregnancy loss

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Background: Unexplained recurrent pregnancy loss (RPL) is often attributed to an underlying maternal prothrombotic state. Consequently it is common practice for such patients to receive antenatal thromboprophylaxis despite randomized controlled trials (RCTs) demonstrating no benefit of aspirin ± heparin over placebo for live birth rate.

Aims: To ascertain whether a global assay of coagulation, calibrated automated thrombography (CAT) could discriminate between women with a history of RPL when compared with parous controls who have never experienced pregnancy loss.

Methods: Between March 2011 and September 2012 we prospectively recruited 50 consecutive women from the recurrent miscarriage clinic at King's College Hospital. Those with ≥ 3 losses prior to 14 weeks' gestation or 1 loss after 14 weeks that remained unexplained were eligible, and were compared to 41 controls. Exclusion criteria comprised prior venous thromboembolism, antiphospholipid syndrome, inherited thrombophilia, previous placenta mediated pregnancy complication, use of hormonal contraception, anticoagulants and immunosuppressants. Venous blood was collected (> 6 weeks after a miscarriage) with minimal stasis and the second draw divided into BD vacutainers containing 0.109 M trisodium citrate. Each participant also provided a sample to which corn trypsin inhibitor (CTI) at a final concentration of 18.3 μg/mL was added. Platelet poor plasma (PPP) prepared following double centrifugation at 4750 *g* for 10 min was stored at −40°C. Hemker's thrombin generation method was carried out utilising manufacturer reagents (Thrombinoscope BV, the Netherlands). PPP was tested alone (5 pM) and with thrombomodulin (5 pMTM) at a final concentration of 6 nM (5 pM TF and 4 μM PL) and with 1 pM TF for samples containing CTI at a mean ± SD time period of 5.3 ± 1.3 weeks following freezing. Peak thrombin and estimated thrombin potential (ETP) are expressed as normalised ratios against NHCP (Technoclone, Austria) which was run in tandem with each assay as a reference plasma. Velocity index (peak thrombin/[time to peak thrombin-lag time]) and percentage suppression of both peak thrombin and ETP in the presence of TM were also determined ([variable in the absence of TM-variable in the presence of TM]/variable in the absence of TM).

Results: Normalised ratios for peak thrombin (5 pM: 0.88 vs. 0.83, $P = 0.42$; 5 pMTM: 0.79 vs. 0.69, $P = 0.19$; 1 pM: 0.43 vs. 0.36, $P = 0.11$) and ETP (5 pM: 0.81 vs. 0.75, $P = 0.27$; 5 pMTM: 0.67 vs. 0.57, $P = 0.19$; 1 pM: 0.68 vs. 0.61, $P = 0.17$) were comparable between subjects and controls in all assays respectively. There was no statistical difference in velocity index (5 pM: 104.7 nM/min vs. 100.1 nM/min, $P = 0.93$; 5 pMTM: 85.2 nM/min vs. 73.9 nM/min, $P = 0.37$; 1 pM: 28.1 nM/min vs. 24.8 nM/min, $P = 0.65$) or TM-related suppression of peak thrombin (34.6 vs. 38.5, $P = 0.63$) and ETP (46.7 vs. 49.8, $P = 0.86$) between subjects and controls respectively. *P* values adjusted for potential confounding factors of age, ethnicity, smoking status, phase of menstrual cycle, BMI, factor VIII:C and antithrombin levels.

Summary: Calibrated automated thrombography was not able to identify an underlying prothrombotic state in women with unexplained RPL. These results do not provide a rationale for use of prophylactic anticoagulation to safeguard pregnancy in such women and support the findings of recently published RCTs which failed to show the benefit of such an approach.

OC 61.4

Risk of recurrence during pregnancy after a first VTE: a french cohort

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Background: Pregnancy is a high risk period for venous thromboembolism (VTE) especially for women with previous VTE. However, risk stratification is difficult and recommendations for prophylaxis are of low grade of evidence because randomized studies are lacking during pregnancy.

Aims: We aimed to study the risk of recurrent VTE during pregnancy after a first VTE.

Methods: We studied the risk of recurrent VTE during pregnancy in a cohort of young women under 50 years with first proved VTE, admitted from 1992 to 2010 in BrestUniversityHospital. Follow-up information was collected annually about medical events and thrombosis risk factors. In case of pregnancy, we collected information about prophylaxis with low-molecular-weight heparin (LMWH).

Results: Of the 338 women under 50 years with a first VTE, 62 had a total of 96 pregnancies starting before January 2011. At beginning of pregnancy, median age was 29 years and 29% of women were obese. Initial VTE was associated with hormones (pregnancy, postpartum or contraception) in 92%. We observed recurrent VTE in 7.3% of

pregnancies. Women received prophylaxis with LMWH in 79% of pregnancies. Dose and duration of prophylaxis were consistent with international recommendations in 65% of cases. Recurrent VTE occurred in early pregnancy, before starting prophylaxis, and postpartum sometime despite LMWH. Risk factors for recurrence were obesity, varicose veins and hormone-related initial VTE.
Conclusion: Risk of recurrent VTE during pregnancy is high, which would justify early prophylaxis in pregnancy, especially for women with initial hormone-related VTE. Discontinuation of anticoagulants for delivery and LMWH dosage may explain failures of prophylaxis during postpartum. Risk stratification and randomized trials would solve this issue.

OC 61.5

Risk factors for acute VTE in pregnancy and the postpartum period: a retrospective case control study of 14 years at National Women's Health, Auckland, New Zealand

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Background: This was a retrospective study of acute venous thromboemboli (VTE) occurring during pregnancy or within 8 weeks postpartum, from 1998 to 2011, at National Women's Health, New Zealand.
Aim: The assessment of the risk factors for acute pregnancy associated VTE (PA-VTE).
Methods: Medical ethics approval was obtained. The study group included women who received enoxaparin for the treatment of acute PA-VTE. The control group was women who delivered before and after study group patients. Data was collected from patients' records.
Results: Seventy-eight events occurred in 76 women and 123 controls were identified. The incidences of age > 35 years, parity ≥ 3, multiple pregnancies, smoking, BM > 30, comorbidities or preeclampsia were not statistically increased in this study group compared to the control group but the incidence of antiphospholipid syndrome was statistically higher in the study group. There was a history of previous VTE in 12.8% of the study group, of which 80% were hormone related. A family history of VTE was present in 21% of the women and half of the VTEs in mothers or sisters were pregnancy related. Of those tested, Protein S deficiency was confirmed in two patients (3%), four patients (6.3%) were FVL heterozygous and 9 (15.3%) were PGM heterozygous. The incidence of emergency caesarian deliveries was significantly greater in those with postpartum VTEs than the control group. Of the 21 postpartum VTEs, three patients had received thromboprophylaxis. Postpartum prophylaxis may have prevented six of eighteen VTE. There was associated systemic infection in 11 cases, surgery in one case and severe varicose veins in two cases. Twelve women were immobilized for ≥ 3 days. Of the 29 women with first trimester VTEs, six (20.7%) experienced hyperemesis. Regional anaesthesia was not significantly different between the postpartum VTE and control groups. There was a higher incidence of VTE in those of European ethnicity and a lower incidence in those of Asian ethnicity. The ACCP[3] and RANZCOG[2] guidelines were assessed for thromboprophylaxis recommendations in women with a personal history of VTE, those with inherited thrombophilias and in the postpartum setting. Eight patients had previous pregnancy or COC related VTE and should have received thromboprophylaxis according to both guidelines. Postpartum thromboprophylaxis may have prevented 6 (33%) of the postpartum VTEs. Of the three patients with a known thrombophilia and a family history of VTE, the VTE may have been prevented by thromboprophylaxis in accordance with the ACCP guidelines in one case and with the RANZCOG guidelines in two cases. None of the ten women, with known thrombophilias but no personal or family history of VTE, would have received thromboprophylaxis according to either guideline.
Conclusion: A family history of VTE is highlighted as a risk factor for PA-VTE. Testing for heritable thrombophilias should be considered if there is a history an unprovoked or hormone-related VTE in a first degree relative. A third of the postpartum VTE occurred within 7 days of delivery and may have been prevented by thromboproprophylaxis. There was a significant association between emergency caesarean deliveries and postpartum VTE.

OC 61.6

Tranexamic acid inhibits fibrinolysis-induced coagulopathy associated with post-partum hemorrhage

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Background: Beneficial effects of the preventive use of tranexamic acid (TA) have been established in major surgery and trauma. In ongoing post-partum haemorrhage (PPH), a high dose of TA reduced blood loss, duration of bleeding and transfusion needs in the French randomized controlled EXADELI trial. During this study (Controlled Trials ISRCTN09968140), markers of fibrinolysis were investigated.
Aims: To investigate the biological effects of tranexamic acid on hemostasis in post partum haemorrhage.
Methods: women with PPH > 800 mL following vaginal delivery were assigned to receive either TA (loading dose4 g over 1 h, then infusion of 1 g/h over 6 h) (TA [n = 72]), or no prohaemostatic treatment (untreated controls [n = 72]). At four time-points (T1 inclusion, T2 30 min, T3 2 h, T4 6 h), blood loss and biological coagulation parameters (D Dimers, factor II (FII), factor V (FV) and plasmin antiplasmin complexes [PAP]) were recorded. Results are expressed as median and quartiles.
Results: In the control group, D Dimers were increased at T1 (3730 ng/mL [2468–8493]) as soon as the PPH began and became maximal at T3 (7495 [4400–15,772]; P = 0·001 T1 vs. T3). Fibrinogen, FII and FV levels decreased from T1 to T4 with the lowest values at T3 (fibrinogen 3.34 g/L [2.6–4], FII 78 UI/mL [68–88], FV 77 UI/mL [66–90]; P > 0.0001 when compared to T1 values). In the TA group, the increase in D Dimers was blunted and T3 levels were kept as low as T1 (3888 ng/mL [2688–6172] vs. 3645 ng/mL [2222–6223]; T3 and T1 values respectively) and were significantly reduced when compared to controls (P > 0.0001). PAP complexes were significantly reduced in the TA group compared to controls (902 μg/mL [516–1289] vs. 2254 μg/mL [1113–3394] respectively; P = 0.03 at T2, trend for T3). The time course of fibrinogen, FII and FV was not significantly modified by the use of TA.
Summary/Conclusions: This study provides a biological evidence of hyperfibrinolysis-induced coagulopathy associated with PPH and its improvement by the use of Tranexamic acid.
[AEK1]A quel temps T1 ou T3

OC 62 – Rare Bleeding Disorders – II

OC 62.1

First report of a CalDAG-GEFI gene (RASGRP2) mutation in humans that affects platelet function and causes severe bleeding

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Background: Inherited platelet disorders are rare diseases that give rise to bleeding when platelets fail to fulfill their hemostatic function upon vessel injury. Study of these pathologies provides an unique opportunity to delineate mechanisms of platelet signal transduction by highlighting defects in specific pathways.
Aims: To investigate the molecular basis of an inherited platelet disorder in three siblings affected by severe bleeding and a platelet aggregation defect in the absence of mutations in *ITGA2B* and *ITGB3* genes coding $\alpha II_b\beta 3$
Methods: Exome sequencing; Genotyping; Protein sequence modeling; Flow cytometry; Platelet function testing (aggregation, adhesion and spreading); GTPases activation assays; Thrombin generation; Leukocyte phenotyping.
Results: Exome sequencing of two affected siblings and three non-affected relatives (focusing on single nucleotide or ins/del variants affecting coding regions) revealed 114 candidate variants following the recessive hypothesis. Only four candidates were absent or at low frequency (> 1‰) in public databases. Among these one stood out, a non-synonymous cG742T mutation (exon 8) in the *RASGRP2* (RAS guanyl releasing protein 2) gene leading to p.G248W in the encoded protein, Calcium and DAG-regulated Guanine Exchange Factor I (CalDAG-GEFI). Modeling a closely related structure, SOS-2, reveals that the mutation causes the formation of a protrusion within the cavity of the catalytic domain of the GEF. The mutation affects platelet's ability to effect proper $\alpha II_b\beta 3$ integrin inside-out signaling. In homozygous carriers, maximal aggregation in response to all tested doses of ADP or low doses of any other agonists was reduced except for ristocetin and PMA (phorbol 12-myristate 13-acetate). Dense and alpha granule numbers and content were normal and procoagulant properties were moderately affected. The mutation strongly dampened the activation of the CalDAG-GEFI prototypical substrate in platelets, Rap1, induced by low and intermediate doses of ADP and TRAP-6. PMA induces the same Rap1 activation in patients and controls highlighting, the existence of CalDAG-GEFI bypassing pathways dependent on protein kinase C (PKC). An ADP-mediated pathway able to overcome CalDAG-GEFI dysfunction was also shown.
Under arterial flow and over fibrillar collagen, the surface covered by homozygous platelets was strongly reduced. Although there was no obvious defect in aggregation, the adhesion kinetics were also strongly altered for obligate heterozygotes whose have normal tethering but defective ability to form thrombi.
On immobilized fibrinogen, homozygous and heterozygous platelets exhibit a reduced number of filopodia and failed to form lamellipodia. Accordingly, Rac1 activation was decreased. Stimulation with high doses agonists only partially reversed the spreading defect indicating that CalDAG-GEFI activity is required for full spreading. The functional deficiency induced by the *RASGRP2* mutation is confined to platelets and has only little impact on leukocyte functions.
Conclusion: We report the first case of a *RASGRP2* mutation in humans causing severe bleeding without affecting leukocytes. Remarkably, the functional deficiency was found to be confined to platelets and can be bypassed under some conditions. We observed that the presence of only one normal allele is sufficient to support normal aggregation but not adhesion under flow and spreading. This makes CalDAG-GEFI an interesting therapeutic target to prevent thrombosis without causing bleeding.

OC 62.2

A comprehensive approach for the study of a rare bleeding disorder: factor VII deficiency, the IRF7 and STER experiences.

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Background: Understanding clinical features and assessing optimal treatment for the Rare Bleeding Disorders (RBD) is hampered by their rarity and, in general, by the lack of specific treatment tools. The latter issue is not true for FVII deficiency, a bleeding disorder for which several replacement options are available: Recombinant, activated FVIIa (rFVIIa), Plasma Derived FVII concentrates, Fresh Frozen Plasma and the four factor Prothrombin Complex Concentrates.
Methods: We have evaluated a large series of patients with FVII deficiency within the International Registry for FVII (n = 514) and the Seven Treatment Evaluation Registry (STER) (n = 225) initiatives, using novel approaches to evaluate homogeneous clinical phenotypes and to assess the optimal management. Genotype assessment was available for 332 individuals of whom 104 (31.3%) were homozygous, 86 (25.9%) compound heterozygous and 142 (42.8%) heterozygous for mutations. The *Age at disease presentation* and the *type of first symptom* were the elements used to assess the disease severity; FVII coagulant activity levels (FVIIc) were employed to further refine patients' categorization.
Results: Three main phenotypes were identified: asymptomatic (≈30%), mild (55–60%) characterized by muco-cutaneous bleeds and the severe, life and limb threatening bleeding phenotype (10–15% characterized by CNS, GI and joint bleeds), with a good clinical consistency over time. Treatments were followed up prospectively through an online protocol.
Surgical interventions were 136 in total, 69 'major', 67 'minor' (including eight adeno-tonsillectomies). For the major interventions three doses of at least 13 μg/Kg of rFVIIa on the day of operation were suggested; because of the lack of events, no data concerning the optimal overall post-surgical RT duration is available. As for the minor surgeries (dental extractions and invasive & diagnostic procedures) a single dose of 20 μg/Kg was found sufficient to avoid bleeding.
For most of the spontaneous bleeding episodes (n = 101), apart from the life-threatening bleeds, a single, intermediate dose of rFVIIa (60 μg/Kg) was proven efficacious. Prophylaxis courses were reported in 37 cases, mostly subsequent to life- and limb-threatening bleeds, and rFVIIa was shown to be very effective when 'frequent' schedules (at least three times weekly) and doses of 90 μg/Kg were used. This appears to be in contrast with the pharmacokinetic (PK) data obtained for rFVIIa, characterized by very low *in vivo* recoveries (n = 116 evaluations), half-life (n = 10, median 1.97 h) and AUClast (214.3 Uh/dL); however, the high volume of distribution on the terminal phase, much higher than the plasma volume, indicate that a large extravascular diffusion of FVIIa and binding to extravascular receptors takes place, a fact that may well explain a prolonged pharmacodynamic effect. The latter finding is particularly evident when PK is evaluated using the Prothrombin Time ratio instead of the FVIIc assay. Treatment adverse events, as reported in the 225 STER patients, were the occurrence of inhibitors to FVII 3/225 (1.3%) and of thrombotic episodes 4 (1.7%), the latter clearly related to surgery and replacement therapy.
Conclusions: We have prospectively evaluated the clinical and management features of FVII deficiency providing settings and treatment schedules focused on disease severity.

OC 62.3

The ThromboGenomics Next Generation Sequencing Platform for the DNA-based diagnosis of known inherited rare bleeding and platelet disorders

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Background: There are over 140 Bleeding and Platelet Disorders (BPDs) registered in OMIM. For many BPDs, diagnostic laboratory algorithms are cumbersome or inconclusive. Molecular diagnosis is often missing in many cases, preventing targeted treatments and family counselling. Next Generation Sequencing Technologies (NGSTs) have resulted in the development of fast, accurate and cost effective DNA tests to diagnose several inherited disorders. For BPDs, NGSTs enable the exciting opportunity to use DNA testing early on in the diagnostic algorithm, rather than as a final confirmatory step, thereby reducing time to diagnosis and healthcare costs. To achieve this goal, ISTH experts under the umbrella of the ThromboGenomics working group (http://www.isth.org/group/genomics) are collaborating on a set of 97 genes harbouring mutations underlying most known BPDs.

Aims: Bringing NGST-based diagnostics to healthcare delivery requires: (i) gene annotation to clinical standards providing a widely accessible, stable and sustainable frame of reference for cataloguing clinically significant mutations; (ii) proof of principle that the BPD gene panel can substantially improve the diagnosis of rare inherited BPDs.

Methods: LRG (www.lrg-sequence.org) records entailing the DNA sequence, transcripts and clinically relevant variants of all 97 genes are agreed through curation by ISTH-ThromboGenomics gene-specific experts and given an immutable ID. The guiding principle for the creation of an LRG record is that the ISTH-ThromboGenomics community will have the final say in defining the sequences and their annotation. In parallel, a Roche/Nimblegen custom panel capturing nearly one million bp has been designed to contain all transcribed sequences, the 2000 bp centred around transcription start sites, and all BPD variants present in the Human Gene Mutation Database (www.hgmd.cf.ac.uk). To validate the platform and assess its diagnostic performance, 500 idiopathic BPDs and 500 BPDs with known causative variants are being sequenced with it.

Results: So far 12 ISTH experts have agreed to join the curation effort. The curation for four disorders is complete and LRGs have been created for the following genes: ITGA2B, ITGB3, GP1BA, GP1BB, GP9, WAS, and MYH9. BPD-causative variants have been agreed for these genes.A first generation platform has been tested with 12 DNA samples, achieving 940X average coverage across BPD gene regions. This level of coverage ensures that multiplexing 96 samples in a single HiSeq2000 lane results in 92% of the target been covered at > 30X, and 98% of the 'capturable' regions. Less than 2% of the 8740 HGMD mutations in the panel wouldbe covered at > 30X. To improve the design even further, baits have been rebalanced toimprove capture uniformity.

Summary/Conclusions: Early sequencing results are extremely promising in terms of panel coverage, capturehomogeneity, and potential level of multiplexing. This means that a low-cost, comprehensivegenetic test for BPDs seems achievable. At the meeting we will share the analysis of thesequencing of at least 500 samples in the validation experiment. The gene curation processalready underway will be accelerated and LRG records for 2/3 of the genes will have beendeposited prior to the ISTH conference.

OC 62.4

Constitutive activation of integrin alphaIIb-beta3 due to an inherited mutation of integrin beta3 leads to defective receptor function and impaired thrombopoiesis

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Background: Mutations of integrin alphaIIbbeta3 lead to Glanzmann Thrombasthenia (GT), an autosomal recessive bleeding disorder characterized by absent platelet aggregation associated with a normal platelet count and volume. We have recently described a novel autosomal dominant hereditary macrothrombocytopenia with platelet dysfunction and mucocutaneous bleeding associated with a heterozygous mutation (2134+1 G>C) of the ITGB3 gene, leading to the deletion of the entire exon13 and consequently to del647-686 of integrin beta3 (Gresele P et al. Haematologica. 2009; 94:663–9): a variant form of GT.

Aims: Aim of the present study was to unravel the mechanism by which a heterozygous mutation of integrin beta3 induces the phenotype of a typical autosomal recessive GT and to clarify the role of this mutation in the generation of macrothrombocytopenia.

Methods: CHO cells expressing the wild type alphaIIbbeta3, the mutant form or both were generated. Integrin expression and receptor internalization were studied by Western blotting and flow cytometry. AlphaIIbbeta3 activation was studied by aggregation in the presence of fibrinogen, PAC-1- and fibrinogen-binding. Outside-in signaling was investigated by spreading assay, protein phosphorylation and clot retraction. Additional studies on patient's platelets were also carried out. The wild type and the mutant alphaIIb-beta3 receptor were also transduced by retrovirus in fetal liver-derived murine megakaryocytes on which spreading and proplatelet formation were studied.

Results: Studies in CHO cells reveal that the mutation exerts a dominant negative effect and induces constitutive activation of alphaIIb-beta3. The co-expression of the mutant beta3 subunit together with the normal protein decreases the number of alphaIIb-beta3 receptors expressed on the cell surface. Mutant integrin-bearing CHO cells bind fibrinogen and PAC-1 without the need of stimulation and aggregate spontaneously in the presence of fibrinogen, thus showing constitutive activation of the receptor. The mutant, activated receptor is internalized without the need of agonist activation, and this explains its reduced surface expression. Beta3 cytoplasmic tail and Focal Adhesion Kinase are constitutively phosphorylated in CHO cells expressing the mutant beta3 subunit, spreading on fibrinogen is faster while clot retraction is defective, all results compatible with defective alphaIIb-beta3 mediated outside-in signalling. Patients' platelets show constitutive activation of outside-in signalling with increased actin polymerization in resting platelets and spontaneous spreading on Von Willebrand factor.

Fetal liver-derived murine megakaryocytes expressing the mutant beta3 show abnormal proplatelet formation, with tips decreased in number and larger in size than those of controls, and abnormal spreading on fibrinogen. In addition, platelet maturation from preplatelets is impaired, with the generation of asymmetric barbell-proplatelets and consequently the formation of platelets of heterogeneous dimensions.

Summary/Conclusions: Taken together these data show that a defective outside-in signaling generated by a constitutively activated alphaIIb-beta3 receptor is the cause of both macrothrombocytopenia and defective platelet function in our GT variant.

OC 62.5

A new type of hereditary bleeding disorder resulting from a premature stop codon in thrombomodulin (p.Cys537*)

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Background: Thrombomodulin is a single pass type-1 transmembrane protein with key roles in the regulation of haemostasis and inflammation. We describe a novel thrombomodulin variant within a family with an undiagnosed autosomal dominant phenotype of delayed post traumatic bleeding.
Aims: Investigations were performed to identify and characterise the previously observed clinical haemostasis defect within an individual prior to scheduled abdominal surgery such that an appropriate treatment plan could be devised.
Methods: Coagulation screening tests (prothrombin time, APTT, thrombin time), coagulation factor assays (fibrinogen, factor II, V, VII, VIII, IX, X, XI, XII, XIII, von Willebrand factor antigen/activity), platelet function tests, endogenous thrombin potential (ETP), antithrombin, protein S and protein C assays were performed. Plasma thrombomodulin levels were quantified by ELISA and genomic DNA was screened for thrombomodulin mutations by polymerase chain reaction amplification followed by direct DNA sequencing.
Results: All coagulation screening tests, coagulation factor assays and platelet function test results were within normal limits. The ETP using 5 pM tissue factor (TF) was normal (1300 nmol/min, normal range 1114–2272 nmol/min), but markedly reduced using 1 pM TF (354 nmol/min, normal range 851–1759 nmol/min). Addition of normal plasma failed to correct the ETP, indicating the presence of a coagulation inhibitor. Addition of 10-fold excess of protein C abolished thrombin generation in the patient plasma but left normal plasma unaffected. The plasma thrombomodulin level was elevated (130 ng/mL, normal range 2–8 ng/mL). A heterozygous c.1611 C>A mutation was detected within the thrombomodulin gene sequence (THBD) which coded for a change from cysteine 537 to a premature stop codon (p.Cys537*). The mutations lies within the transmembrane region of thrombomodulin (p.Cys537*) and results in a truncated thrombomodulin molecule lacking the *C*-terminal cytoplasmic domain. The same thrombomodulin mutation and laboratory findings have been identified in two close family members who share the same bleeding predisposition.
Summary/Conclusions: We have identified a new thrombomodulin mutation (c.1611 C>A) associated with a decrease in thrombin generation at low tissue factor concentration, elevated plasma thrombomodulin levels and an accompanying increase in protein C activation. We predict that one of the consequences of the p.Cys537* truncated thrombomodulin molecule would be a reduction of its binding to the endothelial membrane and its shedding into the plasma. This would facilitate protein C activation, factor Va/VIIIa cleavage and early cessation of thrombin generation within a developing haemostatic clot. The likely consequence of this scenario would be the formation of smaller and weaker haemostatic clots, thus explaining the phenotype of delayed post traumatic bleeding observed within this family. To our knowledge this is the first case of a thrombomodulin mutation causing a bleeding disorder.

OC 62.6

Characterization of four novel β3 integrin defects associated with Glanzmann's thrombasthenia

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Background: Glanzmann's thrombasthenia (GT) is a recessively inherited bleeding disorder characterized by quantitative and, or, qualitative abnormalities of αIIbβ3, an integrin receptor for adhesive proteins that mediates platelet aggregation. The α- and β-subunits of αIIbβ3 are encoded by *ITGA2B* and *ITGB3* respectively, and many genetic defects resulting in reduced expression or dysfunction of αIIbβ3 have been described. As part of a study to investigate the molecular basis of GT in a cohort of UK patients, we identified four novel *ITGB3* mutations predicting amino acid substitutions in the ligand binding domain (E200K, W264L, S317F) and signal peptide (W11R) of β3. We have now used *in silico* and *in vitro* methods to characterise these defects further.
Aims: To confirm the pathogenicity of four *ITGB3* mutations using *in silico* methods to predict their effects on αIIbβ3, and by analysing αIIbβ3 receptor levels after expression of the corresponding β3 variants in heterologous cells.
Methods: b3 integrin sequences from 12 vertebrate species were aligned with the human sequence using the ClustalW2 tool available at http://www.ebi.ac.uk. The effects of candidate mutations were predicted using the Polymorphism Phenotyping v2 (http://genetics.bwh.harvard.edu/pph2/), Sorting Intolerant from Tolerant (http://sift.jcvi.org/), Align Grantham Variation Grantham Deviation (http://agvgd. iarc.fr/agvgd_input.php) and SNPs&GO (http://snps-and-go.biocomp.unibo.it/snps-and-go/) tools. The SignalP 4.0 tool (http://www.cbs.dtu.dk/services/SignalP) was used to examine the effect of the W11R substitution on signal peptide cleavage. Site-directed mutagenesis was used to introduce *ITGB3* mutations into pcDNA3.1(-)neo-ITGB3 expression construct. Chinese Hamster Ovary (CHO) cells were then co-transfected with recombinant *ITGB3* and WT-*ITGA2B* expression vectors and expression of the recombinant αIIbβ3 receptors assessed by flow cytometry.
Results: Alignment of β3 integrin sequences revealed conservation of tryptophan (W) 264 and serine (S) 317, while tryptophan (W) 11 was conserved in seven of the 10 species where sequence was available, suggesting an important role for these amino acids in β3 integrin. Supporting this, the W264L and S317F substitutions were predicted to significantly affect protein function. The W11R substitution was not expected to disrupt signal peptide cleavage but was predicted to affect protein function. In contrast, glutamate (E) 200 is not conserved and is replaced by lysine (K) in eight of 12 species examined and the E200K substitution was predicted to be benign by three of the tools used. There was an almost complete loss of cell surface expression of αIIbβ3 levels in CHO cells expressing the W11R, W264L and S317F variants ($P > 0.0006$), and 37% and 23% reductions in intracellular expression of the W264L ($P > 0.005$) and S317F ($P > 0.05$) variants, while the intracellular expression of the W11R variant was reduced to 99% of that of WT ($P > 0.0005$). In contrast, cells expressing the E200K variant showed similar cell surface and intracellular expression to those cells expressing the WT receptor ($P = 0.39$).
Conclusion: Our findings confirm the pathogenicity of the W264L, S317F and W11R mutations in GT. Assessment of the functional consequences of the E200K substitution is required to confirm whether it is responsible for the diagnosis of GT in the patient with this defect.

OC 63 – Recurrent Venous Thrombosis – II

OC 63.1

Current statin use and its association with recurrent venous thrombosis

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Background: Evidence from randomized clinical trials suggest that the use of statin therapy is associated with a decreased risk of incident

venous thrombosis (VT), particularly in the setting of use for primary prevention of cardiovascular disease (CVD). The association of statins with risk of recurrent VT is not known.

Aims: The aim of this study is to assess the relationship between current statin use and the risk of recurrent VT. We hypothesize that current statin use is associated with a decreased risk of VT recurrence, especially among those without CVD.

Methods: In a population-based study, we identified an inception cohort of adults 30–89 years of age who were members of a large health maintenance organization (HMO) and who suffered an incident VT (deep vein thrombosis or pulmonary embolism) from 2002 through 2010. Participants were followed for VT recurrence by reviewing the full medical record. A recurrent VT was defined as a physician-diagnosed recurrence more than 2 weeks after the incident event and with clinical and/or imaging evidence of a new or expanded clot. Statin use was identified from prescription fills within the HMO. Using failure time models with baseline (age, BMI, sex, race, idiopathic incident event, estrogen use in women) and time-varying follow-up covariates (cancer, smoking, prevalent CVD, vitamin K antagonist [VKA] and aspirin use), we estimated the relative hazard of a recurrent VT comparing current statin users and recent statin stoppers (≤ 2 years) with never or past (> 2 years) statin users. Time scale was time since incident event and censoring occurred at last follow-up or death. The study is expected to include 2600 participants with incident VT. Preliminary, cleaned data on 1854 participants (71%) with incident VT were available at the time of abstract submission.

Results: The average age was 65.6 years and 45% were male. Among the incident events, 57% were idiopathic. At cohort entry, 23% of participants were using statin therapy; during follow-up, 22% initiated treatment while 57% of users permanently stopped therapy. Among users, simvastatin was used by 54% and lovastatin by 43%. We identified 275 recurrent events during an average of 3.2 years of follow-up. A recurrent VT occurred in the first 2 years in 10% of the cohort. Male sex (hazard ratio [HR] = 1.3), idiopathic status of incident event (HR = 1.3), and cancer (HR = 3.9) were associated with higher risk of a recurrent event, and VKA use (HR = 0.2) was associated with a lower risk. Compared with never or past (> 2 years) use, current statin use was associated with a 20% lower risk of recurrent VT (HR = 0.8; 95% CI: 0.6–1.1); recent discontinuation of statins was not associated with risk (HR = 1.0; 95% CI: 0.6–1.6). When participants with CVD (myocardial infarction, stroke, or angina) at baseline were excluded (n = 408), the HR was 0.6 (95% CI: 0.4–0.9).

Summary/Conclusion: In interim analyses of observational data, we found a lower risk of recurrent VT associated with current statin use but confidence intervals were wide. Additional data collection is underway and subgroup analyses by drug type and dose will be performed.

OC 63.2

The risk of recurrent venous thromboembolism among women in relation to estrogen intake: results from a prospective cohort study

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Background: Women have a lower risk of recurrent venous thromboembolism (VTE) than men. The risk of recurrence among women with VTE in association with female hormone intake is not well studied.

Aim: to investigate the risk of recurrent VTE among women who had their incident VTE associated with estrogen use.

Methods: The Austrian Study on Recurrent Venous Thromboembolism (AUREC), is a large prospective cohort study. Patients with a first objectively confirmed deep vein thrombosis (DVT) of the leg and/or pulmonary embolism (PE) who had received anticoagulants for 3–18 months were included. Exclusion criteria were: age > 18 years; VTE associated with surgery, trauma, cancer or pregnancy; long-term anticoagulation; natural inhibitor deficiency; lupus anticoagulant; homozygousity or double heterozygousity for factor V Leiden and/or the prothrombin mutation. Women were advised to refrain from further estrogen use and were excluded in case of non-adherence. The study end point was recurrent symptomatic DVT and/or PE verified by imaging. The local ethics committee approved the study and all patients gave written informed consent.

Results: 1179 patients (54% females) were followed for a mean of 63 months. Recurrent VTE was recorded in 72 of the 631 females (11%) and in 186 of the 548 males (33%). Compared to men, women had a relative risk of recurrence of 0.3 (95%CI 0.2–0.4) after adjustment for age, location of VTE and factor V Leiden mutation.

Two hundred and seventy-four women used combined contraceptives and 72 used estrogen containing hormone replacement therapy (HRT) at time of initial thrombosis. Recurrent VTE was seen in 15 contraceptive users. Women using contraceptives were at lower risk of recurrence than non-users (adjusted RR 0.3 (95% CI 0.2–0.7, P = 0.01). After 5 years the probability of recurrence was 4% (95% CI 1–7) among contraceptive users and 16% (95% CI 8–23) among non-users (P = 0.002).

Ten of the 72 women with HRT had recurrent VTE. Women using HRT were at lower risk of recurrence than those in the same age group with idiopathic VTE (adjusted RR 0.6 [95% CI 0.3–1.4] P = 0.2). After 5 years the probability of recurrence was 13% (95% CI 3–23) among HRT users and 18% (95% CI 12–24) among non-users (P = 0.1).

Conclusion: The risk of recurrent VTE is lower in women than in men. The risk of recurrence is particularly low among women with VTE during combined contraceptive use. In these women extended anticoagulation is not justified provided that they refrain from further estrogen use.

OC 63.3

Safety of dabigatran vs. warfarin for acute venous thromboembolism: pooled analyses of RE-COVER and RE-COVER II

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Background: In the RE-COVER and RE-COVER II trials, patients with acute venous thromboembolism (VTE) were started on parenteral therapy and were then randomized to receive overlapping warfarin (W) or warfarin-placebo (single-dummy). After discontinuing parenteral therapy, they either continued warfarin or received dabigatran etexilate (DE), respectively (double-dummy). Safety may be assessed from the start of randomization on any study drug (scenario 1); thus, bleeding events related to parenteral therapy alone (before administration of DE) will be included in the dabigatran arm. Alternatively, to assess safety of DE itself, scenario 2 is to count bleeding from the first intake of active study drug (W in single-dummy, DE in double-dummy period), thus excluding events associated with parenteral therapy. Scenario 3 is to count events from the start of the double-dummy period to compare DE with W at its full pharmacological potential (but omitting the W/parenteral overlap). DE is not currently approved for this indication.

Aims: Using the pooled trial data we investigated the impact of counting bleeding events by the three different approaches on the safety comparison between DE and W.

Methods: Patients with acute VTE received parenteral anticoagulation and were randomized to the addition of W or placebo for ≥ 5 days until INR or sham INR was ≥ 2.0 on two consecutive days followed by continued W (INR range 2.0–3.0) or DE 150 mg twice daily, respectively, with corresponding placebos for 6 months. Major bleeding events (MBE) and the composite of MBE or clinically relevant non-major bleeding events (CRBE) were counted from three starting points until 6 days after the last intake of any study drug.
Results: Scenario 1: MBE for DE in 37 patients (1.4%) vs. 51 (2.0%) for W; hazard ratio (HR) 0.73 (95% CI 0.48, 1.11). There were 13 patients who had not yet taken DE and 11 taking W in the single-dummy period with MBE; 5 and 3 of these, respectively, continued into the double-dummy phase. Scenario 2: MBE (DE vs. W) in 24 patients (1.0%) vs. 51 (2.0%); HR 0.48 (95% CI 0.29, 0.78). Scenario 3: MBE in 24 patients (1.0%) vs. 40 (1.6%), respectively; HR 0.60 (95% CI 0.36, 0.99). All three analyses favored DE and were statistically significant in scenarios 2 and 3. The HR for MBE/CRBE favored DE and for all comparisons the upper bounds of the CIs were below 1.0. There was no difference in symptomatic VTE or VTE-related mortality (counted from the start of randomization on any study drug) between DE and W: 2.7% vs. 2.4%, HR 1.09 (95% CI 0.77, 1.54).
Conclusions: Three safety comparisons were made: from the start of the two treatment regimens, from the start of each active study drug (excluding bleeds caused by heparin in the absence of DE), and from the start of the double-dummy period (after W had reached therapeutic levels). Regardless of the calculation, pooled data from RE-COVER and RE-COVER II consistently showed a profile of numerically or even statistically significant lower major bleeding rates with DE than with W.

OC 63.4
Risk factors for recurrent venous thromboembolism: VTE Epidemiology Group (VEG) Study

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Background: Recurrence of venous thromboembolism (VTE) has important implications for VTE management and outcomes. Only a few risk factors for recurrence have been recognized consistently. To simplify decision making, these factors must be assessed allowing for the presence or absence, and quality, of anticoagulant therapy.
Aims: To explore potential risk factors for recurrent VTE.
Methods: Patient data were retrieved from the subset of general practices in England contributing to the Clinical Practice Research Datalink (CPRD) linked to data from the Hospital Episodes Statistics (HES) and to the Office for National Statistics (ONS). From January 2001 to October 2011, all VTE cases in the CPRD were verified with an algorithm based on review of medical records and notes, hospital diagnoses, cause of death and anticoagulation therapy. We conducted a nested case-control study defining all patients with recurrent VTE as cases and those without recurrence as controls; the date of VTE recurrence was recorded as the index day. For each case we selected five patients without recurrence on the index day with the same duration of observation as the respective case. Patients with a record indicating vitamin K antagonist (VKA) use before the first VTE were excluded. The independent association between the prevalence of potential risk factors at the first VTE and at the index day, and the recurrent VTE were derived from conditional multivariate logistic regression models and presented as adjusted odds ratios (ORs) with 95% confidence intervals (CIs). Adjustment included the type of antithrombotic therapy: VKA, low molecular weight heparin or antiplatelet.
Results: A total of 24,795 patients with first VTE were at risk of recurrent VTE. The mean age of patients was 63.4 years and 46.0% were male. Of these, 3678 cases of recurrent VTE occurred during the entire observational period. The risk of VTE recurrence was greater in the following groups: age > 30 years (OR = 1.43; 95% CI 1.20–1.71), males (OR = 1.26; 95% CI 1.17–1.36), body mass index ≥ 30 kg/m^2 (OR = 1.14; 95% CI 1.05–1.25) and current smokers (OR = 1.12; 95% CI 1.01–1.24). Deep vein thrombosis with pulmonary embolism compared with deep vein thrombosis alone was associated with an increased risk (OR = 1.51; 95% CI 1.30–1.75). When compared with provoked VTE, patients with active cancer or with unprovoked VTE at the time of the first VTE had an increased risk of recurrence (OR = 1.59; 95% CI 1.38–1.83, and OR = 1.52; 95% CI 1.39–1.65, respectively). The new occurrence of a provoking event after the initial VTE augmented the risk of recurrence, e.g. for a new active cancer (OR = 5.60; 95% CI 4.51–6.96), new major trauma (OR = 2.89; 95% CI 1.83–4.54) or new pregnancy (OR = 2.09; 95% CI 1.27–3.44) compared with those without a new provoking event. Other predictors for recurrent VTE were renal disease (OR = 1.17; 95% CI 1.04–1.32) and superficial thrombophlebitis (OR = 3.50; 95% CI 1.97–6.21).
Summary/Conclusions: The recognition of risk factors for recurrent VTE should guide decisions on the duration of anticoagulation therapy.

OC 63.5
Mortality following Venous thromboembolism. Risk factors from a large cohort. VTE Epidemiology Group (VEG) Study

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Background: Mortality is the commonest outcome at 5 and 10 years following both pulmonary embolism (PE) and deep vein thrombosis (DVT). Mortality remains higher following index PE but is significant following DVT also. The recognition of risk factors and the use of a risk score may help to evaluate high risk groups and guide therapy type and duration. To simplify decision making these factors for mortality must be assessed allowing for the presence, quality and absence of anticoagulant therapy.
Aim: To explore potential risk factors for mortality following VTE.
Methods: Patient data were retrieved from the subset of general practices in England contributing to the Clinical Practice Research Database (CPRD) that has been linked to data from the Hospital Episodes Statistics (HES) and to the Office for National Statistics (ONS).From January 2001 to October 2011, all VTE cases in the CPRD were verified with an algorithm based on review of medical records and notes, hospital diagnoses, cause of death and anticoagulation therapy. We conducted a nested case-control study in patients with a first VTE. Deaths during the entire observational period from any cause during the entire observational period were defined as cases and the date of death as the index day. For each case we selected five patients alive on the index day and with the same duration of observation as the respective case. The independent association between the prevalence of potential risk factors associated with first VTE for all-cause mortality was derived from conditional multivariate logistic regression models and presented as adjusted odds ratios (ORs) with 95% confidence intervals (CIs). Adjustment included age, gender, socioeconomic risk factors, components of the Charlson index, and the type of antithrombotic therapy (VKA, low molecular weight heparin or antiplatelet therapy).
Results: Of 35,373 patients with first VTE 29,550 patients survived more than 7 days and had 81,906 person-years of observation were at risk of mortality following the acute presentation. Over the observational period and after at least 90 days following first VTE 6714 deaths were identified.
The adjusted odds ratio for all-cause mortality was increased for the following VTE-related risk factors: having both PE and DVT, OR 1.21 (1.031.43) compared to a primary DVT; recurrent DVT, OR 1.51 (1.291.76) and recurrent PE, 1.52 (1.281.82) compared to no recurrence, severe post-phlebitic syndrome, OR 1.73 (1.272.37) and new

onset of chronic pulmonary hypertension 2.44 (1.733.43). The odds of mortality were lower in the following conditions: TTR > 60%, OR 0.47 (0.370.60); TTR 40 ≤ 60%, OR 0.80 (0.641.01) both compared with TTR > 40%.

Summary/Conclusions: The recognition of risk factors for mortality should facilitate decisions for managing therapy in VTE patients.

OC 63.6

Red cell distribution width and blood monocytes are associated with an increased risk of venous thrombosis

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Background: Recent studies have suggested that leukocytes and erythrocytes play a role in the process of coagulation and their presence is clearly observed in the anatomy of a venous clot. Furthermore, leucocytosis is a predictor of venous thrombosis during follow-up of patients with polycythemia vera and essential thrombocytemia.

Aim: The aim of this study was to investigate whether peripheral leukocytes, erythrocytes and other hematological variables (hematocrit, hemoglobin and red cell indices) are associated with the risk of venous thrombosis.

Methods: To study this, we used data from 2473 patients with venous thrombosis and 2935 controls from the MEGA study. The MEGA (Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis) study is a population-based case-control study on risk factors for venous thrombosis. The variables assessed were: total leukocytes, granulocytes, lymphocytes, monocytes, hematocrit, hemoglobin, erythrocytes and red cell indices (mean corpuscular volume [MCV], mean hemoglobin volume [MCH], mean corpuscular hemoglobin volume [MCHC] and red cell distribution width [RDW]). Odds ratios and their 95% confidence intervals (95% CI) were calculated for erythrocytes and leukocytes counts, hematocrit, hemoglobin and red cell indices and adjusted for age, sex, malignancy, co-morbidities and CRP using logistic regression. Cut-off points of variables were established at the 1st, 5th, 95th, 97.5th and 99th percentilesin the control subjects.

Results: We found a strong dose-response relation for higher RDW and monocytes with risk of venous thrombosis, with odds ratios of 3.1 (95% confidence interval [CI], 2.0–4.8) and 2.8 (95%CI, 1.3–5.8), respectively, after adjustment for age, sex, C-reactive protein, malignancy and co-morbidities. A low monocyte count (> 0.12 × 109/L) was associated with a lower venous thrombosis risk after full adjustment (odds ratios 0.6; 95%CI, 0.4–0.8). In our study, RDW was associated with an increased risk for venous thrombosis, even when RDW was still within the usual reference range (above 14.1%). This association was held after full adjustment (OR 3.1; 95% CI 2.0–4.8 for percentile above 99) and for all subgroup analyses. Further adjustments for anemia did not reduce the odds ratio. Recently, an elevated RDW was reported to be associated with an increased risk for all-cause mortality and cardiovascular disease. To our knowledge, this is the first study to report an association between venous thrombosis and higher RDW.

Conclusion: High RDW and blood monocytes, two unexpensive and easily obtainable tests, are associated with increased risk of venous thrombosis. Future studies should evaluate the underlying mechanism and the use of these variables in prediction models for first and recurrent thrombosis.

OC 64 – Thrombin Generation Tests

OC 64.1

Quantitative imaging of thrombus formation under shear

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Background: When vessel walls are damaged, exposed extracellular matrix proteins and tissue factor (TF) trigger a series of events that lead to the formation of a hemostatic plug through platelet recruitment and concurrent blood coagulation. The extrinsic pathway of blood coagulation initiates thrombin generation that clots fibrinogen and activates platelets, coagulation cofactors, and factor XI (FXI) of the intrinsic pathway, which can further enhance thrombin formation and assist in the development of a stable hemostatic plug. Under pathological conditions, platelet and coagulation activation pathways can extend into the lumen of the blood vessel, resulting in the formation of a thrombus that can occlude the vessel. How these pathways relate to the mass, volume, and density of the growing thrombus inside blood vessels remains ill-defined. This is in part due to a lack of a quantitative technique to measure the physical parameters of thrombus formation.

Aims: To create a measurement technique for determining the relative roles of the intrinsic and extrinsic coagulation pathways in regulating the basic physical parameters of mass, volume, and density of platelet aggregates and thrombi formed under shear, using whole blood in an *ex vivo* model.

Methods: Citrate-anticoagulated whole blood with or without added calcium was perfused through a flow chamber at a shear rate of 200/s over immobilized fibrinogen, collagen, and/or TF (0.1 or 1 nM). In selected experiments, whole blood was pretreated with inhibitors of FXIIa (CTI, 40 μg/mL), FXIIa activation of FXI (14E11, 20 μg/mL), or FXIa activation of FIX (1A6, 20 μg/mL). Non-interferometric quantitative phase microscopy (NI-QPM) and Hilbert transform differential interference contrast (HT-DIC) microscopy were used to determine mass and volume, respectively, of formed aggregate or thrombi.

Results: Platelet aggregates formed on surfaces of fibrinogen and collagen, but not on TF alone, under shear in the absence of coagulation. The extent of platelet adhesion and aggregation remained constant when fibrinogen or collagen was coated in combination with TF. Recalcification of the sodium-citrate anticoagulated blood resulted in fibrin formation on both fibrinogen and collagen surfaces. Mean volume of platelet aggregates and thrombi did not significantly differ on the surfaces. However, the mean mass and density dramatically increased for thrombi formed on surfaces of fibrinogen or collagen that had been coated in combination with TF. For instance, mean mass of thrombi increased from 122.8 ± 14.1 pg to 325.0 ± 10.7 pg and density increased from 30 ± 0.002 to 70 ± 0.003 fg/μm^3 (p-value ≤ 0.01) when TF was immobilized in combination with collagen. The presence of FXIIa and FXIa inhibitors abrogated fibrin formation on fibrinogen and collagen surfaces only in the absence of TF, resulting in a corresponding reduction in mass and density.

Conclusion: We have developed a quantitative imaging platform to measure the mass, volume and density of platelet aggregate and thrombus formation under shear. This platform may provide a novel screening method for rapid quantitative efficacy/safety assessments of various antithrombotic or pro-hemostatic agents.

OC 64.2

Delayed and decreased thrombin generation is associated with stroke in the elderly: results from the prosper study associate

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Background/Aim: Experimental as well as clinical data suggest a contribution of hypercoagulability to the pathophysiology of atherosclerosis and -thrombosis. As thrombin fulfills a central role in the coagulation process and links to several mechanisms involved in arterial vascular disease and (models) of ischemic brain damage, we investigated the association between thrombin generation (TG) and ischemic stroke in the elderly.
Method: We studied the relationship between TG and stroke in the PROspective Study of Pravastatin in the Elderly at Risk (PROSPER), a multi-center prospective cohort study including 5804 individuals aged 70–82 years with known cardiovascular risk factors or previous cardiovascular disease. TG was measured in platelet poor plasma with addition of 1 and 5 pM tissue factor, using the calibrated automated thrombogram. The associations between TG and incident stroke were investigated using Cox proportional hazard models adjusted for country, treatment, age, sex, conventional cardiovascular risk factors, interleukin-6, and CRP levels. Subjects on warfarin ($n = 105$) were excluded from analysis.
Results: Plasma samples for TG were available from 4932 (85%) subjects of whom 227 subjects (4.6%) had incident stroke within the 3.2 years of follow-up. In 156 strokes (69%) ischemic etiology was confirmed. TG was lower in patients who developed stroke than in control patients; normalized peak height (nPH): 71 ± 41% vs. 82 ± 45% and normalized endogenous thrombin potential (nETP): 79 ± 23% vs. 87 ± 25%; means and SDs. Moreover TG showed an independent and inverse relationship with stroke risk. Hazard ratios (95% confidence intervals) for the highest vs. the lowest tertile for nPH, nETP, log lag time and log time to tail were 0.71 (0.60–0.85), 0.68 (0.58–0.79), 1.27 (1.09–1.47) and 1.32 (1.13–1.54), respectively. Hazard ratios did not change when analysis were restricted to confirmed ischemic strokes.
Conclusion: In elderly people at increased risk of cardiovascular disease, a delayed and decreased potential to generate thrombin is an independent risk factor for stroke. Although the role of thrombin formation in the pathogenesis of stroke remains unknown, these data support an effect of plasma coagulation activity on progression of arterial vascular disease, related to stroke.

OC 64.3

Evaluation of the thrombin generation potential of a recombinant factor VIII Fc fusion protein (rFVIIIFc) in a phase III multi-national clinical trial

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Background: The recombinant coagulation factor VIII Fc (rFVIIIFc) consists of a single molecule of human factor VIII covalently linked to the dimeric Fc domain of human immunoglobulin G1 (IgG1). The rFVIIIFc fusion protein utilizes the FcRn receptor-mediated immunoglobulin cycling pathway to extend plasma half-life. In a global phase 3 clinical study (A-LONG), rFVIIIFc demonstrated effective prevention and control of bleeding episodes with a longer prophylactic dosing interval than current short acting FVIII products. In addition to the one-stage and chromogenic clotting assays, pharmacokinetics (PK) samples were analyzed by a thrombin generation assay (TGA) in order to gain additional insights into the coagulation potential of rFVIIIFc in a potentially more relevant physiological setting.
Aims: To compare the *ex vivo* thrombin generation potential of rFVIIIFc to a currently marketed rFVIII product (Advate®) in post-infusion patient plasma samples by a standardized TGA in a Phase 3 multi-center clinical trial.
Methods: A total of 1207 samples from 137 subjects were collected from 48 clinical study sites in 15 countries for TGA analysis. Blood samples were collected into special tubes containing 3.2% citrate and 50 μg/mL corn trypsin inhibitor (CTI), followed by centrifugations to produce platelet-poor plasma. The TGA was performed at a central laboratory using a modified calibrated automated thrombogram (CAT®) method. Before testing, the thawed samples were centrifuged to remove any remaining cell debris or microparticles. Each assay contained 80 μL sample, 20 μL 1:6000 dilution of Innovin®+4 μM synthetic phospholipids and 20 μL fluorogenic substrate with calcium. This study was approved by local site medical ethics committees and informed consent was obtained from all participating subjects.
Results: Results of 643 samples (29 subjects) from sequential PK profiling for both rFVIIIFc and rFVIII were included for this report. The responses of major TGA parameters (peak thrombin and endogenous thrombin potential) were proportional to the one-stage and chromogenic FVIII activity throughout the measurable range. At early time points post-dosing, rFVIIIFc demonstrated comparable thrombin generation responses to rFVIII, and the decay of thrombin generation activity post-dosing was markedly slower for rFVIIIFc than that of rFVIII. The average thrombin generation profile for rFVIIIFc compared to rFVIII correlated well with the half-life extension observed for plasma rFVIIIFc activity by the one-stage and chromogenic assays. Regression analysis of the thrombin generation profiles vs. FVIII activity indicated comparable thrombin generation responses for rFVIIIFc and rFVIII at equivalent FVIII activities.
Summary/Conclusions: Our large scale, global clinical evaluation of rFVIIIFc by TGA using a standardized sample collection procedure and an optimized and validated assay performed at a central laboratory demonstrated that, despite inherent patient-to-patient differences in thrombin generation potential, (i) The TGA results confirmed the longer duration of activity of rFVIIIFc relative to rFVIII. (ii) The pooled patient data showed comparable TGA responses for rFVIIIFc and rFVIII at equivalent FVIII activities. (iii) The comparable *ex vivo* thrombin generation potential of rFVIIIFc and rFVIII observed in this study supports our method for rFVIIIFc potency assignment against the WHO FVIII concentrate standard.

OC 64.4

Whole blood thrombin generation in aging mice

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Background: Thrombin generation (TG) is increasingly being recognized as a versatile diagnostic tool in the field of thrombosis and haemostasis. TG in plasma is most accurately measured with the Calibrated Automated Thrombogram assay (CAT) and is sensitive in detecting conditions of hyper- and hypocoagulabilty. By modification of the classic plasma CAT assay, we were able of measuring TG in whole blood (WB-CAT assay). The advantage of using this assay in mice is that only small amounts of blood are needed (> 100 μL) to perform triplicate measurements including calibration. As a result there is no need to sacrifice the mice and TG can be measured in one mouse repeatedly in time. As a proof of principle, we studied the TG in Bmal1-knock out (KO) and wild type (WT) mice. Bmal1-KO mice were known to have a prothrombotic phenotype due to accelerated aging compared to WT mice. However, standard coagulation tests reveal opposite results (e.g. decreased prothrombin time and prolonged activated partial thromboplastin time).

Aims: To technically validate the WB-CAT assay in mice and to investigate TG in prothrombotic Bmal1-KO mice compared to WT mice.
Methods: Mice blood samples were taken from the orbital sinus using a capillary coated with a dimethyldichlorosilane solution. Blood was anticoagulated with citrate (3.8%) and reactivated during the experiment with a solution containing calcium, tissue factor (TF) and the substrate. Hirudin was also added to the calibrator wells to prevent any TG that could disturb the calibrator signal.
Results: When activating the blood using different TF concentrations, we found that 0.5 pM TF was sufficient to start coagulation. Addition of corn trypsin inhibitor (CTI) to prevent contact activation did not have a profound effect indicating that contact activation does not play a significant role in the initiation phase. The mean intra-assay variation ($N = 24$) of the peak height, lagtime, time-to-peak and velocity index revealed to be fairly precise as the coefficient of variation (CV) was 10% or less. The CV of the ETP was higher (12%). Studying TG in the Bmal1-KO mice revealed that the mean ETP of Bmal1-KO mice (ETP [SD] = 437 [78]) was significantly higher ($P > 0.001$) than measured in WT mice (ETP [SD] = 220 [45]). We found comparable differences for the peak height ($P = 0.027$). No difference was observed in lagtime, TTP and velocity index (p-values were respectively 0.45, 0.15 and 0.74).
Conclusion: We have developed a reproducible assay to monitor TG in whole blood of mice using only minimal amounts of blood. In addition, with this method we have established an increased TG in Bmal1-KO mice compared.

OC 64.5

A systematic review to evaluate thromboelastography for characterization of bleeding patients with advanced liver diseases

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Background: The liver produces clotting proteins, but thrombocytopenia commonly occur in patients with advanced liver disease. Traditional coagulation tests, such as PT or aPPT, only measure the interactions of clotting proteins without evaluating the roles of cellular components during clot formation. In contrast, thrombelastography (TEG) monitors real-time clot formation of whole blood, thereby allowing global evaluation of the hemostatic system and providing more information regarding the integrity of hemostasis in patients with liver diseases and concomitant thrombocytopenia.
Aims: To evaluate whether patients with bleeding complications due to advanced liver diseases have distinctive TEG abnormalities as compared with those without bleeding.
Method: The OVID MEDLINE and EMBASE databases from 1946 to 2012 were searched with keywords 'thromboelastography' and 'liver disease'. 113 studies were obtained from OVID MEDLINE and 216 studies from EMBASE. Duplicates, non-English, non-primary articles such as reviews, meta-analysis and animal studies were excluded. We only included studies that evaluated the association between TEG parameters and objective bleeding outcomes. Data regarding study design, number of subjects, TEG settings, TEG values, clinical outcomes, and results of other conventional coagulation test were extracted. The data from both bleeders and non-bleeders were standardized to the normal values given in each study to adjust for the variations in study design and TEG methodology. To account for different sample sizes, normalized values were weighted according to each study's sample size. Weighted means and weighted standard deviations were calculated for each of the 4 TEG parameters (R, K, α, MA) and Student's *t*-test was conducted to compare the bleeders with non-bleeders. A *P*-value of ≤ 0.05 was considered statistically significant.
Results: Three prospective and one retrospective studies involving a total of 172 patients with 21 bleeders and 151 non-bleeders were identified. Three studies assessed bleeding following an invasive procedure, while one assessed recurrence of bleeding. All bleeding outcomes were evaluated independently of TEG measurements. Of the four studies, one study used kaolin-activated TEG, one used native TEG, while the other two were unstated.
The R in the patients with bleeding was $132.3 \pm 37.2\%$, significantly longer than $93.2 \pm 4.0\%$ in those without bleeding. The K of the bleeders was $228.4 \pm 168.7\%$; again, significantly higher than the value of $132.23 \pm 29.16\%$ among the non-bleeders. Those with bleeding also had a significant lower α of $68.5 \pm 36.1\%$, as compared with the value of $92.1 \pm 8.5\%$ in those without bleeding. In contrast, there was no significant difference in the MA between the patients with or without bleeding; the values were $83.9 \pm 11.0\%$ and $85.69 \pm 11.96\%$ respectively.
Summary/Conclusion: The results suggested that patients with clinical bleeding took longer to initiate clotting and the clot propagated slower, but the clot achieved similar maximum strength once it formed. Patients suffering from severe liver diseases with bleeding complications have a distinctively different TEG profile as compared with those without bleeding complications. These abnormal TEG parameters are compatible with coagulopathy mainly due to insufficient procoagulant proteins for hemostasis. Yet, the advantages of using TEG, instead of traditional plasma-based coagulation tests, in predicting bleeding in patients with advanced liver diseases warrant further investigation.

OC 64.6

Increased thrombin generation in patients with premature myocardial infarction is linked to tissue factor microparticles in the circulation

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Background: The extent of the haemostatic response may determine the risk of forming an occlusive thrombus, particularly in patients who suffer MI at an early age, when the atherosclerotic burden is relatively low. While levels of individual haemostatic factors have been linked to increased risk of MI, measurement of the endogenous thrombotic potential (ETP) may better indicate the overall haemostatic response of an individual. Previously we have shown, using a thrombin generation assay sensitive for plasma coagulation factor levels (triggered with 5 pM tissue factor [TF] and 4 μM phospholipid [PL]) that the ETP in plasma from individuals with an MI > 50 years was significantly higher than that in age/sex-matched controls with no history of CHD. Moreover there was a significantly higher level of TF in the cases compared to the controls and levels of TF were strongly correlated between the ETP in the MI cases and the level of TF in their plasma which was not seen in the control subjects.
Aims: Here we sought to determine whether the higher level of TF seen in the MI cases was predominantly in the form of microparticles (MPs).
Methods: Plasma from 198 individuals with an MI under the age of 50 years and 194 age/sex-matched controls with no history of CHD was analysed for thrombin generation by the Calibrated Automated Thrombogram (CAT) MP assay triggered with 4 μM PL that is designed to measures the activity of endogenous TF+ve MPs. The plasma samples were also passed through a 0.22 μm filter (Ceveron) to remove MPs and reanalysed in the same assay.
Results: All four parameters from the CAT MP assay were significantly different in the cases compared to the controls. The lag time (LT) and time to peak (ttP) were faster in the patients (for LT 32.31 ± 1.45 vs. 38.5 ± 1.62 min; $P = 0.005$ and for ttP 34.78 ± 1.42 vs. 40.95 ± 1.51 min; $P = 0.0032$), indicative of higher numbers of TF+ve MPs in the patients' samples. As previously the ETP levels were higher in the patients (980 ± 45 vs. 697 ± 42 nM.min; $P > 0.0001$) as was the level of peak thrombin (PT; 160.2 ± 8.3 vs. $105.0 \pm$

7.0 nM.min; $P > 0.0001$). Following filtration to remove MPs the LT and ttP (which are the most sensitive parameters to detect MP-derived TF in the assay) increased significantly (p£0.03) and after filtration there was no difference in these parameters between the patients and the controls (for LT 37.56 ± 1.76 vs. 41.54 ± 1.81 min; $P = 0.01159$ and for ttP 39.50 ± 1.66 vs. 43.36 ± 1.70 min; $P = 0.1058$). Filtration also caused the mean ETP and PT to fall slightly for both patients and controls but for both measures the patients still generated significantly more thrombin that the controls (for ETP 824 ± 49 vs. 643 ± 45 nM.min^{-}; $P = 0.0070$ and for PT 145.2 ± 9.4 vs. 109.1 ± 8.5 nM.min; $P = 0.0047$).

Conclusions: These data are consistent with a higher level of TF+ve MPs in the plasma of patients who have suffered an MI at an early age compared to matched controls. Although the cellular source of the TF +ve MPs was not determined these prothrombotic MPs may be a contributing factor the increased thrombotic risk in the patients.

OC 65 – Von Willebrand Factor – I

OC 65.1

Accelerated uptake of VWF/platelet complexes in macrophages contribute to VWD-type 2B associated thrombocytopenia

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Background: Von Willebrand Disease (VWD)-type 2B is caused by so-called *gain-of-function* mutations, clustered in exon 28 of von Willebrand factor (*VWF*) gene and results in spontaneous binding to glycoprotein (GP)Ib. Bleeding tendency is associated with heterogeneous clinical manifestations such as the decrease of high molecular weight-VWF multimers, presence of giant platelets and/or platelet aggregates, and moderate to severe thrombocytopenia.

Aims: One of the major predictor of the bleeding risk in VWD-type 2B patients is the severity of the thrombocytopenia, the causes of which have not yet been identified. An *in vivo* mouse model of VWD-type 2B obtained via hydrodynamic gene transfer of plasmids to express murine VWF carrying type 2B mutations was applied. This model was used to investigate the molecular mechanism underlying the thrombocytopenia.

Methods and Results: Mutation-specific conformational changes in the VWF molecule increase its affinity for the platelet receptor GPIb, allowing spontaneous VWF-platelets interactions. Indeed, VWF-type 2B mutants but not wild type (wt)-VWF were adsorbed to platelets, as assessed via immunofluorescence analysis. Moreover, the presence of VWF-type 2B/platelet complexes and aggregates was associated with the occurrence of thrombocytopenia.

Given that liver and spleen macrophages have already been identified as important actors in the clearance of VWF and platelets, we studied their possible involvement in the elimination of VWF-type 2B/platelet complexes. *In vivo* chemical depletion of macrophages was successfully obtained through liposome-clodronate injection and converted thrombocytopenia in VWD-type 2B mice to near normal platelet counts. Furthermore, CMTMR-*in vivo* cell tracker was used to localize exogenous type-2B platelets infused in VWD-type 2B mice. Microscopical qualitative evaluation of liver and spleen cryo-sections revealed that the vast majority of type 2B-platelets co-localized with Kupffer cells (CD68+) in the liver and with marginal metallophilic macrophages (CD169+) in the spleen. Quantitative analysis confirmed that significant more platelets are directed to liver and spleen in VWD-type 2B mice than in wt-mVWF expressing mice.

Finally, platelet half-life was determined in our mouse model of VWD-type 2B and proved significantly shorter (34 ± 4 h) compared to the half-life of platelets in wt-mVWF-expressing mice (53 ± 7 h, $P > 0.0001$), possibly contributing to the lower platelet counts in VWD-type 2B mice.

Conclusions: Our data indicate that VWF-type 2B/platelet complexes are rapidly targeted to macrophages, which could contribute to the thrombocytopenia in patients with VWD-type 2B.

OC 65.2

The vicinal disulphide bond and calcium coordination site of the von Willebrand factor A2 domain have distinct and additive roles in domain stabilisation that govern proteolysis by ADAMTS13

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Background: Rheological shear forces in the blood trigger von Willebrand factor (VWF) unfolding which exposes the Y1605-M1606 scissile bond within the A2 domain for cleavage by ADAMTS13. The VWF A2 domain contains two main structural features that influence its ability to unfold: a calcium coordination (Ca^{coord}) site and a vicinal disulphide bond (VicCC) between C1669-C1670.

Aim: To investigate the functional dependency of Ca^{coord} and VicCC in the unfolding of the VWF A2 domain and subsequent proteolysis by ADAMTS13.

Methods: VWF A2 domain variants with point mutations in the Ca^{coord} site (D1498A, D1596A, N1602A) were combined with deletion (ΔCC) and substitution mutations (C1669G/C1670G) of the VicCC and expressed in HEK293 cells both as VWF A2 domain fragments and full-length VWF protein. Differential scanning fluorimetry assays were used to determine T_m of the VWF A2 domain variants during thermodynamic unfolding to assess protein stability. ADAMTS13 cleavage assays in the absence/presence of denaturing agent were used to assess susceptibility to proteolysis.

Results: The expression and secretion of the isolated VWF A2 domain containing the VicCC (A2VicCC) was unaffected by mutation of Ca^{coord} residues. The presence of calcium ions stabilised the WT A2VicCC domain, causing a shift in T_m of 19.2 °C from 1 mM EDTA (46.3 °C) to 5 mM $CaCl_2$ (65.5 °C). The T_m of the Ca^{coord} mutants was not affected by addition of calcium/EDTA and these fell into two distinct groups: 'stabilising mutations' that had a similar stability to the WTA2VicCC in the presence of calcium (T_m in the range of 60–65 °CD1596A, D1596A/N1602A), and 'destabilising mutations' that had a similar stability to WTA2VicCC in the absence of calcium (T_m in the range of 46–51 °CD1498A, N1602A, D1498A/D1596A, D1498A/N1602A, D1498A/D1596A/N1602A). When these Ca^{coord} mutants were introduced into the A2ΔCC, all the 'destabilising mutations' prevented secretion and resulted in intracellular retention of the A2ΔCC protein. In contrast, the WTA2ΔCC and A2ΔCC containing 'stabilising mutations' were successfully secreted. Introduction of the Ca^{coord} mutants into the full length VWF protein in both the presence (WT) and absence (VicGG) of the VicCC did not prevent secretion. In cleavage assays with ADAMTS13 in the absence of denaturant, the presence of either the VicCC or 'stabilised' Ca^{coord} prevented cleavage. Substitution of VicCC and 'destabilisation' of the Ca^{coord} site (VWF VicGG D1498A or VicGG N1602A) resulted in cleavage of full length VWF in the absence of denaturant or shear.

Conclusions: Mutation of both the VicCC and specific residues of the Ca^{coord} site destabilises the isolated A2 domain to such extent that it is no longer secreted. Secretion of A2 domain is recovered in the full length protein. VWF with a substituted VicCC and 'destabilised' Ca^{coord} site is proteolysed by ADAMTS13 without a need for induced unfolding, whereas VWF containing either the VicCC or the Ca^{coord} site is resistant to proteolysis in the absence of induced unfolding. The distinct and additive effects of both the VicCC and Ca^{coord} provide the VWF A2 domain with stability, whilst allowing it to unravel and expose its cleavage site for ADAMTS13 under high shear.

OC 65.3

Comprehensive characterization of loss and gain-of-function von Willebrand factor collagen binding variants and the role of GPVI using a mouse model system

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Background: von Willebrand factor (VWF) is a multifunctional multimeric protein that plays an important role in primary hemostasis where binding of VWF to exposed subendothelial collagens is the first step towards thrombus formation at the site of injury. To date, four point mutations within the VWF A3 domain affecting collagen-binding have been described in 11 patients: S1731T, W1745C, S1783A, and H1786D. However, given the rarity of collagen-binding mutants and the heterogeneous genetic backgrounds of the affected subjects, the analysis of these aberrant VWF-collagen interactions has been relatively limited.
Aims: To characterize VWD collagen binding defects and analyze the role of collagen, we developed a comprehensive inbred mouse model system. In addition to the analysis of collagen binding via the VWF A3 domain, we further analyzed the impact of the direct collagen binding of platelets by knocking down GPVI.
Methods: Full-length murine VWF cDNA was cloned into the pCIneo plasmid to produce recombinant mouse VWF (r-mVWF) and into the pSC11 plasmid with the liver-specific Enhanced murine Transthyretin (ET) promoter for hydrodynamic delivery. Mutagenesis was performed to introduce the gain-of- function mutation (L1757A) and loss-of-function mutations (S1731T, W1745C, S1783A, H1786D, A3 deletion). All these amino acids are conserved between human and mouse. r-mVWF was obtained by calcium phosphate transient transfection. The function of r-mVWF was analyzed by the following methods a static collagen binding assay, flow chamber assay and *in vivo* intravital thrombosis analysis. The collagen binding assay is a conventional ELISA based assay. In the flow chamber assay, whole blood from VWF knockout mice was perfused into a collagen/r-mVWF coated flow chamber and real-time thrombus formation was analyzed by Quorum WaveFX- X1 spinning disk confocal system. In the intravital experiments, VWF cDNA was delivered to VWF knockout mice by hydrodynamic injection. GPVI knockdown was performed by intraperitoneal injection of anti-GPVI antibody. After rhodamine 6G infusion, injury was induced on arterioles in the cremaster muscle by 10% ferric chloride for 3 min. Following injury, a single arteriole in the injured area was observed for 40 min.
Results: The gain-of-function (L1757A) variant showed consistent enhanced collagen binding. Collagen binding assay demonstrated 2.7- and 2.0-fold-binding to collagen type I and III and the flow chamber experiment showed 3.0- and 1.5-fold-increase in thrombus size. The loss-of-function mutants showed variable degrees of functional deficit (10–88% reduction compared to wild type VWF). In the intravital experiment, the wild type and gain-of-function (L1757A) variant showed 100% occlusion at 30 and 25 min respectively, while a loss of function mutant (H1786D) showed 66% occlusion at 40 min and did not fully occlude. VWF, GPVI and combined VWF/GPVI knockout mice showed significant loss of thrombus formation (28.8%, 34.1%, 33.8% occlusion at 40 min, respectively).
Summary/Conclusion: We have performed a comprehensive evaluation of the collagen binding variants described in patients. Our results are consistent with an important role for collagen binding in thrombus formation. Nevertheless, VWF/GPVI double knockout mice still demonstrated significant amounts of thrombus formation, suggesting the role of alternative pathway(s) for thrombogenesis, independent of collagen binding.

OC 65.4

The role of platelet von Willebrand factor in mice

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Background: von Willebrand factor (VWF) is a large adhesive multimeric glycoprotein that mediates binding of platelets to sites of vascular injury and protects clotting factor VIII from degradation in circulation. VWF is constitutively secreted from endothelial cells in plasma and subendothelium but is also stored as ultra-large VWF multimers in endothelial Weibel-Palade bodies and platelet a-granules. The exact role of ultra-large platelet VWF is still not completely understood.
Aim: Given the fact that ultra-large VWF has been reported to contribute to both thrombosis and inflammation, we aimed to study the role of platelet VWF in hemostasis, thrombosis and thrombo-inflammatory disorders such as stroke.
Methods: To determine the role of platelet VWF, we transplanted bone marrow mononuclear cells from wild-type (WT) mice in VWF knockout (KO) mice and *vice versa*, resulting in chimeric mice with only platelet VWF and chimeric mice that lack platelet VWF. To study the role of platelet VWF we used our chimeric mice to perform tail clipping bleeding assays, $FeCl_3$-induced carotid artery thrombosis models and a stroke model of transient focal cerebral ischemia.
Results: The bleeding time in chimeric mice with only platelet VWF was prolonged and was not different from the one in VWF KO mice (> 600s). Chimeric mice that lack platelet VWF on the other hand had bleeding times similar to those of WT mice (148 $\pm$ 60 vs. 155 $\pm$ 48 s, respectively). These studies suggest that (i) platelet VWF alone is not able to support normal hemostasis and (ii) lack of platelet VWF does not induce a bleeding phenotype in mice. After injury of the carotid artery, arterial occlusion times in chimeric mice with only platelet VWF were prolonged, similar to VWF KO mice (> 60 min), thus indicating that platelet VWF alone cannot support normal thrombus formation, corroborating the bleeding assay results. Thrombosis experiments with chimeric mice lacking platelet VWF are still ongoing. Interestingly, using a mouse model of transient middle cerebral artery occlusion, we observed that cerebral infarct sizes in chimeric mice with only platelet VWF (79.6 $\pm$ 32.3 mm^3) were similar as in WT mice (71.3 $\pm$ 38.3 mm^3). These results are surprising and point towards the contribution of platelet VWF in cerebral ischemia/reperfusion injury. Stroke studies using mice lacking platelet VWF are in progress.
Conclusion: Our data suggest that whereas platelet-derived VWF does not play a crucial role in thrombosis and hemostasis, it might aggravate thrombo-inflammatory diseases such as stroke. Further studies will assess how platelet VWF mediates stroke progression.

OC 65.5

Common and rare VWF coding variants are associated with von Willebrand Factor and Factor VIII phenotypes in African Americans: the NHLBI Exome Sequencing Project

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Background: von Willebrand Factor (VWF) is a critical clotting protein which stabilizes Factor VIII (FVIII), adheres at sites of vascular

injury, and binds platelets. VWF and FVIII levels vary widely among individuals and are generally higher in individuals of African ancestry (AA) than European ancestry (EA). Differences between AAs and EAs may be partly due to genetic factors, but large studies of the effects of *VWF* gene variants have not been reported in AA.

Aims: To study the association of *VWF* coding variants with VWF and FVIII levels in AA.

Methods: Using *VWF* sequence data from 6515 individuals (4298 EA, 2217AA) in the NHLBI Exome Sequencing Project (ESP), we employed genotype imputation to study the association of *VWF* coding variants with VWF and FVIII levels in 4468 AA from four population-based cohorts: the Atherosclerosis Risk in Communities (ARIC), Coronary Artery Risk Development in Young Adults (CARDIA), Multi-Ethnic Study of Atherosclerosis (MESA), and Women's Health Initiative (WHI). In ESP, 452 EA and 409 AA *VWF* variants were identified. In AA, 147 *VWF* variants had a minor allele frequency (MAF) > 0.1%. Of these, 114 were successfully imputed (30 missense, 28 synonymous, 56 intronic). Genetic association analysis was performed using the 30 missense variants and data in the four cohorts for VWF:Ag (n = 2989, mean 132 ± 58 IU/dL) and FVIII:C (n = 4450, mean 155 ± 64 IU/dL).

Results: Eight of 30 *VWF* missense variants, including a novel variant (Ser1486Leu), were significantly associated with levels of VWF, FVIII, or both, in AA after Bonferroni correction ($P > 0.0017$). Five>/i> VWF variants were common (MAF > 10%), three were rare (MAF > 1%), and in ESP all were more common in AA than EA. The variant Thr789Ala (rs1063856, MAF = 43.2%) was associated with higher VWF and FVIII levels ($P = 10^{-9}$ and 10^{-6}, respectively), with each additional copy of the G allele (Ala789) associated with ~8 IU/dL higher VWF and FVIII levels. The variants Ile1380Val (rs11063988, MAF = 10.6%) and Asn1435Ser (rs11063987, MAF = 10.3%) were in strong linkage disequilibrium ($r^2 = 0.89$) and associated with ~6–9 IU/dL higher FVIII and VWF levels. Conversely, Arg2185Gln (rs2229446, MAF = 19.9%), was associated with lower VWF and FVIII levels ($P = 10^{-13}$ and 10^{-17}, respectively); each additional copy of the A allele (Gln2185) was associated with ~13 IU/dL lower VWF and FVIII levels. His817Gln (rs57950734, MAF = 11.7%) was associated with lower FVIII levels ($P = 10^{-21}$) but no difference in VWF; each A allele (Gln817) was associated with ~18 IU/dL lower FVIII. The three rare missense variants (Met1439Val [rs150077670], Ser1486Leu [rs149424724], Arg2287Trp [rs61750625]) were each significantly associated with lower VWF ($P = 10^{-4}$, 10^{-7}, 10^{-6}, respectively) but not FVIII level. The presence of a single copy of each of these rare variants were associated with an average per allele difference of 35–40 IU/dL lower VWF.

Conclusions: Using an exome sequencing and genotype imputation approach in a large AA population sample, we identified several common and rare *VWF* coding sequence variants which were associated with effects on VWF and FVIII levels in AAs. These *VWF* variants may contribute to the genetic complexity underlying ethnic differences in VWF and FVIII.

OC 65.6
High density lipoprotein and apolipoprotein A1 modulate VWF secretion and self-association

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Background: Several studies have shown that VWF multimers in solution can homotypically associate with VWF multimers immobilized onto collagen, and endothelial and platelet surfaces. Association of newly secreted ultra-large VWF multimers into strands and cables on the endothelial surface, and adsorption of VWF to artificial surfaces represent additional forms of VWF self-association. While working on the properties of purified VWF, we observed that VWF disappeared from the solution rapidly. Through steps of fractionation and inactivation, we identified apolipoprotein A1 (ApoA1), the primary component of high density lipoprotein (HDL), as one possible VWF stabilizer in plasma.

Aim: To characterize the effect of ApoA1 and HDL on VWF self-association.

Methods: VWF self-association was studied by two Methods (i) adsorption to and selective elution from artificial surfaces, and (ii) formation of ULVWF strings on the surface of endothelial cells. Purified VWF was exposed to surfaces with or without stabilizing proteins. At specified times, the following fractions were analyzed by Western blotting or ELISA: VWF remaining in solution, VWF eluted from surfaces by 2% sodium dodecyl sulfate, and VWF eluted by 4% CHAPS-5 mM DTT-8M urea. Confluent endothelial cells in flow chambers were stimulated with phorbol myristate acetate (50 ng/mL), with or without HDL (2 mg/mL). ULVWF was visualized by binding to platelets that were perfused over the stimulated cells with or without HDL. The cells were then fixed and imaged. Platelet-decorated VWF strings on the endothelial surface exceeding 25 mm were counted.

Results: Substantial amounts of purified VWF adsorbed to plastic and glass surfaces through VWF-surface interactions (~15%), followed by VWF self-association (~70%). Human ApoA1 and HDL stabilized VWF in solution (~90%) and prevented its adsorption to surfaces. While pre-treating surfaces with ApoA1 or HDL moderately reduced adsorption, maximal stabilization required the continued presence of ApoA1 or HDL during incubation. These results suggest that ApoA1 or HDL not only prevented surface adsorption, but also prevented self-association of solution VWF with surface-bound VWF. In cultured endothelial cells, the presence of HDL during stimulation by phorbol myristate acetate significantly reduced the amount of secreted and cell surface-bound ultra-large VWF (ULVWF), suggesting that HDL modulates VWF secretion and self-association of the secreted VWF into ULVWF. The binding of platelets to ULVWF was slightly affected by the presence of HDL. Fluorescence depolarization studies showed that ApoA1 and HDL bound to a specific sequence in VWF, suggesting that exposure and interaction of this region with ApoA1 and HDL regulate VWF self-association. Further, we observed that the levels of ApoA1 were below normal in patients with thrombotic complications in the microvasculature, including thrombotic thrombocytopenic purpura, sickle cell disease, and sepsis.

Summary: These results show that ApoA1 or HDL may modulate platelet adhesion by modulating both VWF secretion from endothelial cells and self-association into ULVWF strands on the endothelial surface. This may be another mechanism by which HDL protects against cardiovascular disease and extends its protective effects from large arteries to the microvasculature.

OC 66 – Alternative Treatments of Haemophilia A

OC 66.1
Fusion of two TFPI inhibitory peptides yields in total inhibition of human TFPI even at highly elevated TFPI levels

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Background: TFPI is a Kunitz (K)-type protease inhibitor that inhibits both, FXa and TF-FVIIa and is an important physiological inhibitor of the extrinsic coagulation pathway. Direct inhibition of TFPI in hemophilia models alleviates bleeding challenges and may become an

important approach in hemophilia treatment. Recently we presented *de novo* peptide inhibitors of TFPI that enhance coagulation in hemophilia models. Two optimized peptides JBT-A7 and JBT-B5 efficiently blocked inhibitory activity of TFPI and bind to K1 and K1-K2, respectively, which was shown by crystallography. The X-ray structures offered starting points for peptide optimization and provided atomic details for linking the two single peptides to generate a fusion peptide that maximally blocks TFPI activities.

Aims: We aimed to generate a TFPI 'super-inhibitory' fusion peptide, which totally blocks TFPI activity even at high TFPI plasma levels.

Methods: The interaction of TFPI inhibitory peptides to TFPI fragments was assessed by Biacore. Recently we showed the crystal structure of JBT-A7 bound to K1 of TFPI. Moreover, JBT-B5 was co-crystallized with K1-K2 of recombinant human (h)TFPI. The structure of K1 in the K1-K2/JBT-B5 and the NTermK1/JBT-A7 complex were overlayed. The TFPI inhibitory potential of the peptides was tested in model systems (FXa inhibition and TF-FVIIa catalyzed FX activation) and in plasma (TF-triggered thrombin generation). The assays were carried out at TFPI concentrations up to 10 nM, which is 50-fold higher than the physiological flTFPI plasma concentration.

Results: Overlay of the structure of K1 in the K1-K2/JBT-B5 and the NTermK1/JBT-A7 complex revealed no significant differences between the two crystal structures of the Kunitz domain. This implies that the peptides do not induce conformational changes on the domains of TFPI responsible for the antagonistic effect. The model confirms non-overlapping epitopes and close proximity of the termini of both peptides. The distance could be bridged by an approximately ten amino acid linker. A fusion peptide with a 10-serine-linker was synthesized. The fusion peptide showed highly improved dissociation in Biacore experiments and fully inhibited TFPI activity in the model assays. In contrast, single peptides are partial inhibitors of high TFPI concentrations. In thrombin generation assays, the fusion peptide showed a substantially higher ability to increase the thrombin peak even at elevated TFPI compared with the single peptides. Although a mixture of the single peptides showed an improved response over each monomeric peptide, it did not reach the effect of the fusion peptide. Thus, we provide clear evidence that a molecular fusion is required for most efficient inhibition of TFPI activity.

Summary/Conclusions: The fusion of two single peptides binding to different epitopes on TFPI can inhibit the interaction of TFPI with both, FXa and FVIIa resulting in a full inhibitor of TFPI activity even at highly elevated TFPI. The fusion peptide has the potential to block local high TFPI concentrations as it occurs during platelet activation. Thus, the fusion peptide can be useful to prevent bleeding in hemophilia patients. Moreover, this FVIII and FIX independent approach would allow treatment of inhibitor patients via a non-i.v. route of application.

OC 66.2

Influence of TFPI levels on the different parameters of thrombin generation and impact of a TFPI neutralization on the correction of thrombin generation in haemophilic patients

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Background: TFPI is a potent direct inhibitor of factor Xa, inhibiting the factor VIIa/TF complex in a factor Xa-dependent manner. TFPI feedback inhibition of factor VIIa/TF is consistent with a deficient factor Xa production in haemophilic plasma. It has already been shown that TFPI is involved in haemorrhagic manifestations in haemophilia and that blocking TFPI can restore haemostasis.

Aim of the study: To evaluate prospectively the influence of TFPI on the different parameters of TG and to evaluate the impact of TFPI neutralization on the correction of thrombin generation (TG).

Patients and Methods: Twenty-four haemophilic patients (14 HA and 10 HB). TG assay (TGA) was evaluated using the CAT system described by C Hemker on platelet poor plasma (PPP) and on platelet rich plasma (PRP) at low TF concentration (1 pM) before and after adding anti human TFPI antibody (R &D System) at different concentrations. Blood was taken on citrated tubes containing CTI. Free TFPT (fTFPI) levels were measured by ELISA (Asserachrom free TFPI, Asnières France).

Results: When all haemophilic patients are taken together, the correlation (evaluated with R^2 Spearman correlation; * if $P > 0.05$) between Lag Time (LT) and fTFPI is much better in PRP ($R^2 = 0.247$*) than in PPP ($R^2 = 0.07$). In PRP, the correlation between LT and fTFPI is much better in haemophilia A ($R^2 = 0.440$*) and in severe haemophilia ($R^2 = 0.503$*). This correlation does not exist in PPP. When all haemophilic patients are taken together, the correlation between endogenous thrombin potential (ETP) and fTFPI is much better in PPP ($R^2 = 0.239$*) rather than in PRP ($R^2 = 0.022$). However, in haemophilia A patients the correlation between ETP and fTFPI in PRP is high ($R^2 = 0.486$*) but still better in PPP ($R^2 = 0.655$*). The same correlation is obtained in PPP between free TFPI and the Thrombin Peak.

We demonstrated that blocking TFPI by an anti-TFPI Antibody (Ab) allows a complete correction of the TG profile in PRP and in PPP; the correction depends on the anti TFPI Ab concentration that was used. Anti TFPI Ab (1 μg/mL) induced a 70% reduction of the LT when TG is performed in PRP.

With 1 μg/mL of anti TFPI Ab, the correction of ETP expressed as ETP + Ab/initial ETP is better in PPP (3.7) than in PRP (2.5) ($P = 0.02$). The correction of the Peak expressed as Peak + Ab/initial Peak is better in PPP (6.6) than in PRP (3.9) ($P = 0.05$).

Conclusion: A major influence of fTFPI is observed on LT in PRP and on ETP in PPP. There is a good correlation between fTFPI and ETP and Peak especially in haemophilia A. Anti TFPI are able to restore thrombin generation in haemophilic patients; their efficacy should be evaluated by TGA *ex vivo* rather in PRP to take into account platelet TFPI fraction.

OC 66.3

Effect of bypassing agent therapy with and without tranexamic acid in haemophilia A patients with inhibitors, a prospective crossover study using thromboelastography and thrombin generation assay

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Background: Activated prothrombin complex concentrate (aPCC) and recombinant activated factor VII (rFVIIa) are the only two drugs that are available to treat bleeds in haemophilia A patients with high titer inhibitors. The haemostatic response to treatment is widely variable and often difficult to predict. The antifibrinolytic agent tranexamic acid (TXA) may improve the haemostatic outcome but has been little studied due to a lack of standardized methods to monitor the effect.

Aims: The objective of the present study is to evaluate the effect of aPCC and rFVIIa with and without TXA, clot stability and thrombin generation capacity, using thromboelastography (TEG) and thrombin generation assay (TGA), respectively, and to assess the risk of thrombosis and disseminated intravascular coagulation (DIC) following these treatment regimens.

Methods: We conducted a prospective *in-vivo* crossover study including haemophilia A patients with high titer inhibiors ($n = 6$), age between 22 and 61 that had signed informed consent. The study was approved by the Regional Committee for Medical and Health

Research Ethics. On the first study day the participants received aPCC (75 IU/kg intravenously) while on the second study day aPCC was given concomitantly with TXA (20 mg/kg orally). Blood sampling was performed at baseline, 15, 30, 60, 120, 180 and 240 min post-treatment on both days for TEG, TGA analysis and DIC monitoring. After a washout period of 14 days the subjects were crossed over to rFVIIa (90 μg/kg intravenously) without and with TXA. Blood sampling and experimental set up was repeated as for the aPCC study arm.

Endogenous thrombin potential (ETP) was the main parameter used for evaluating the coagulable state whereas maximum clot firmness (MCF) and area under the curve (AUC) assessed clot stability. Comparisons between different treatment regimens were analysed with linear mixed models due to the crossover study design with repeated measurements. Post hoc comparisons were done with Bonferroni correction. A value of $P > 0.05$ was considered to indicate statistical significance.

Results: We found that both MCF and AUC increased by threefold ($P > 0.05$) following the combined treatment of aPCC or rFVIIa with TXA compared to aPCC or rFVIIa alone. There was no significant difference in ETP for the treatment with and without TXA ($p > 0.05$). In addition, DIC parameters were within normal range following all treatment regimens.

Conclusion: The results from our study suggest that the combined treatment strategy of aPCC/rFVIIa and TXA provides a beneficial haemostatic effect by increasing clot stability without increasing the thrombin generation potential and thereby the risk of thrombosis. There was no laboratory or clinical signs implying the occurrence of DIC. Moreover, the study also demonstrated that TEG and TGA seemed to be valuable methods in monitoring the therapeutic effects of bypassing agents. These findings may have important implications for the management of haemophilia A patients with inhibitors as they add evidence regarding the efficacy and safety of this novel treatment regimen.

OC 66.4

RISE- response to DDAVP in moderate/mild haemophilia A patients: in search for determinants

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Background: Large inter-individual variation in DDAVP response is observed in moderate/mild haemophilia A (MHA) patients. Identification of determinants of the DDAVP response may support optimal use of DDAVP and increase our understanding of the biological mechanisms associated with the release of VWF and rise in FVIII:C.

Aim: We aim to identify clinical and genetic determinants of the DDAVP response in MHA patients.

Methods: RISE is an international multicenter cohort study which aims to include 800 MHA patients (baseline (b)FVIII:C 0.02–0.40 IU/mL) who have received DDAVP between 1980 and 2013. Data on potential determinants were collected: ethnicity, family history of DDAVP response, age at receiving DDAVP, blood group, *F8* mutation, bVWF:Ag and bFVIII:C. Main outcome is the response 1 h after DDAVP administration, classified as complete (CR; FVIII:C > 0.50 IU/mL), partial (PR; FVIII:C 0.30–0.50 IU/mL) or no response (NR; FVIII:C > 0.30 IU/mL). Data were analyzed by univariate/multivariate analyses (adjusted for age, CR in family members, bFVIII:C, Body Mass Index) or Chi-Square test where appropriate.

Results: Data on the first 311 patients are presented. Median age was 31 years (IQR 1746) and median bFVIII:C and bVWF:Ag levels were 0.16 IU/mL (IQR 0.11–0.24) and 0.97 IU/mL (IQR 78122) respectively. The majority ($n = 260$; 84%) of patients received intravenous administration of DDAVP (0.3 μg/kg). *F8* genotype, known in 233 patients (75%), displayed 67 different missense mutations. Most prevalent were Arg593Cys ($n = 74$), Asn618Ser ($n = 20$) and Arg2150His ($n = 11$).

CR was observed in 200 patients (64%) and associated with bFVIII:C level (adjusted relative risk for 0.01 IU/mL rise in bFVIII:C (aRR) 1.2; 95% CI 1.081.43) and CR in family members (aRR 16.05; CI 3.867.1). CR was achieved in 33% ($n = 1$) with bFVIII:C level > 0.05 IU/mL, in 26% ($n = 13$) with bFVIII:C level 0.05–0.09 IU/mL, in 43% ($n = 33$) with bFVIII:C level 0.1–0.14 IU/mL and in 85% ($n = 153$) with bFVIII:C level ≥ 0.15 IU/mL. CR in family members was present in 75 of the 122 patients (62%).

Differences in the proportion of patients achieving CR in the three most prevalent mutations were observed. The best response was observed for Asn618Ser (median bFVIII:C = 0.24 IU/mL; IQR 0.170.32): 95% achieved CR. CR was present in 66% of the Arg593Cys patients (median bFVIII:C = 0.16 IU/mL; IQR 0.12–0.21). Patients with Arg2150His (median bFVIII:C = 0.08 IU/mL; IQR 0.06–0.09) had a significantly lower response than the rest of the population ($P > 0.043$): only 36% achieved CR, possibly explained by the FVIII secretion and VWF binding defect of this mutation.

None of the patients with the following four of the 14 most prevalent mutations achieved CR: Pro130Arg, Gly1960Val, Trp2229Cys and Pro2300Leu (median bFVIII:C = 0.07 IU/mL; IQR 0.06–0.10). Structural defects of FVIII are likely present in Gly1960Val and Trp2229Cys. Pro2300Leu causes a FVIII secretion and VWF binding defect. The defect caused by Pro130Arg is still unclear.

Conclusion: The response to DDAVP is associated with FVIII mutation, baseline FVIII:C levels and the response in family members.

OC 67 – Coagulation Factor XIII

OC 67.1

Major roles for FXIII-A and transglutaminase 2 in maintaining cardiovascular tissue integrity

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Introduction: In addition to its key role in cross-linking fibrin to stabilize blood clots, factor FXIII-A may be involved in tissue repair. This function is also undertaken by the closely homologous enzyme, transglutaminase 2 (TG2). TG2 is widely expressed while FXIII-A is expressed in cells of the megakaryocyte, monocyte and chondrocyte lineages. Both enzymes are expressed in cardiac tissue and arterial wall. Besides tissue repair, FXIII-A and TG2 have been suggested to play overlapping roles in pathways that are essential for normal development, including bone deposition and macrophage function.

Aim: To determine the roles of these transglutaminases in cardiovascular repair by breeding mice deficient in one or both enzymes upon an apoE negative background.

Methods: Basal expression of mRNA levels in tissues from saline-perfused mice ($n = 10$ per genotype) was assessed in animals at 8–10 weeks. Starting at 6–8 weeks, male mice ($n = 30$ per genotype) were fed a rodent diet containing 21% lard and 0.15% cholesterol for 12 weeks. Female mice at 6–8 weeks ($n = 12$ per genotype) were subjected to left carotid artery ligation or to sham ligation for a period of 28 days. At termination, fat-fed and ligated mice were perfusion fixed with buffered formalin.

Results: Mice of all genotypes, including apoE/FXIII-A/TG2 triple knockout mice, displayed normal skeletal development as assessed by dual energy X-ray absorption measurements. No compensatory induction of mRNA encoding other transglutaminases was observed in the hearts, spleens, bones or livers of transglutaminase knockout mice. Increased expression of mRNAs encoding haeme oxygenase and CD163 was observed in the hearts of apoE/FXIII-A/TG2 triple

knockout mice, suggesting an influx of macrophages. Upon fat feeding, there was no difference between genotypes in plaque area in the aortic sinus or in buried fibrous caps per unit plaque area in the brachiocephalic artery, the latter being an index of plaque rupture. However extensive cardiac fibrosis (averaging 17% of ventricular volume) was observed in the hearts of 20/28 surviving apoE/FXIII-A/TG2 triple knockout mice, while milder fibrosis (4% of ventricular volume) was detected in 3/30 apoE/FXIII-A double knockout mice, but not in mice of other genotypes, ($P > 0.0001$). Fibrosis appeared to be secondary to extravasation of blood. Carotid ligation led to frequent arterial rupture (6/12 by 10 days) in apoE/FXIII-A/TG2 triple knockout mice, but not in mice of other genotypes, ($P = 0.004$). Although frequent elastic lamellar breakages and vessel dilation occurred in apoE/TG2 double knockout mice, these lesions did not progress to rupture.

Conclusions: Neither FXIII-A nor transglutaminase 2 protects against atherosclerotic plaque expansion and rupture in a mouse atherosclerosis model. However both transglutaminases maintain the integrity of the cardiac microvasculature against leakage and protect the arterial wall against mechanical damage.

OC 67.2

Functional characteristics of coated platelets

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Introduction: Collagen and thrombin stimulate platelets into a population known as 'coated' platelets, which have been characterized in three distinct ways: (i) irreversible binding of factor V, fibrinogen and other proteins via transglutaminase-mediated cross-linking; (ii) high $\alpha_{IIb}\beta_3$-independent binding of fibrinogen; (iii) surface exposure of phosphatidylserine (PS). Functional studies suggest roles for coated platelets in the local assembly of α-granular proteins and in the stimulation of thrombin generation. However, it is unclear how 'coated' platelets differentiate from 'procoagulant', PS-exposing platelets formed under different conditions.

Aim: Investigate the functional characteristics of 'coated' and 'procoagulant' platelets.

Methods: Washed human platelets in $CaCl_2$-containing buffer were stimulated with combinations of thrombin, PAR1/PAR4 peptides, convulxin (collagen receptor agonist) or ionomycin (Ca^{2+} ionophore). Platelets in partially fibrinogen-depleted plasma were triggered with tissue factor plus $CaCl_2$. Subpopulations of activated platelets were identified by dual-color flow cytometry, using labeled fibrinogen, coagulation factors and a fluorescently labeled transglutaminase-dependent probe, A14 α2-antiplasmin (A14-αAP). Thrombin generation was measured using the calibrated automated thrombogram (CAT) assay.

Results: Stimulation with convulxin/thrombin resulted in a platelet population binding annexin A5, factor Va and factor Xa. These platelets persistently showed high fibrinogen binding (the level of which increased with concentration of thrombin), but gradually decreased PAC1 mAb binding, indicating inactivation of $\alpha_{IIb}\beta_3$. The same platelets stained positively for fibrin with an anti-fibrin mAb, and readily incorporated A14-αAP via transglutaminase activity, as indicated by specific inhibition of either factor XIIIa or tissue-type transglutaminase. Platelet stimulation in plasma with tissue factor/$CaCl_2$ resulted in a large platelet population with the same characteristics. Formation of these platelets correlated with the extent of thrombin generation. Addition of factor XIII increased fibrinogen binding to these platelets, but was without effect on thrombin generation.

Platelet stimulation with the Ca^{2+}-elevating agonists, convulxin/PAR1 peptide, convulxin/PAR4 peptide or ionomycin provoked high binding of annexin A5 and coagulation factors. These agonists again enhanced thrombin generation, but did not produce high fibrinogen binding, fibrin staining or A14-αAP incorporation. Finally, platelet stimulation with convulxin or thrombin alone resulted in minimal binding of annexin A5, low binding of fibrinogen and PAC1 mAb, and no binding of coagulation factors nor A14-αAP.

Summary/Conclusion: These findings indicate that A14-αAP, incorporated via factor XIIIa and tissue-type transglutaminase, specifically detects platelets with high fibrinogen binding and fibrin staining, which are generated by collagen/thrombin co-stimulation. By implication, 'coated' platelets should be considered as platelets with a fibrin coat, collecting multiple functional proteins. These platelets are highly active in supporting thrombin generation.

OC 67.3

Pharmacokinetics of recombinant FXIII at steady-state in patients with congenital FXIII A-subunit deficiency

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Background: A comprehensive understanding of the steady state pharmacokinetics (PK) of recombinant FXIII (rFXIII) has been obtained in patients with congenital FXIII A-subunit deficiency, and the haemostatic coverage during long-term prophylaxis is described in detail.

Aims: The objective of this investigation was to obtain and thoroughly describe the PK of rFXIII at steady-state in patients with congenital FXIII A-subunit deficiency, as part of a once monthly prophylaxis dosing schedule.

Methods: Steady-state PK has been investigated in this on-going, multi-centre, multi-national, open-label, single-arm, repeat dose phase 3b, safety and efficacy trial in patients with congenital FXIII A-subunit deficiency. Each patient is participating for at least 52 weeks during which they are receiving monthly (28 ± 2 days) intravenous (i.v.) injections of rFXIII (35 IU/kg).

All patients were offered to participate in the steady-state PK sampling and in total 23 out of 55 patients consented. Blood samples for PK assessments were collected at the following time points: Pre-dose (immediately prior to dosing), 1, 2 h, 3, 7, 14, 21 and 28 days post-dose. The Berichrom® FXIII activity assay was used for measurement of FXIII activity and thus the method used for the primary PK evaluation. The PK parameters were calculated using non-compartmental statistical methods, without prior baseline adjustment. At each PK sampling time point information regarding adverse events and bleeding episodes were collected and additional blood samples for FXIII enzyme linked immunosorbent assay were drawn. Furthermore, antibody assessments were performed pre-dose and and at day 28 post-dose (prior to the subsequent rFXIII administration).

Results: The PK results show a mono-exponential decline in FXIII levels, following first-order elimination with a mean half-life of 13.9 days. The mean FXIII activity levels for the cohort were above 0.1 IU/mL throughout the 28-day PK period, including pre-dose. The mean clearance was 0.15 mL/h/kg, with a C_{max} level of 0.89 IU/mL and mean C_{trough} value of 0.17 IU/mL. From the total of 139 individual activity measurements obtained during the PK session, all but two were above 0.1 IU/mL throughout the entire dosing interval. One of the two low activity levels occurred at day 28, in a patient with a few previous occasions of low activity levels, and the other occurred at day 14 in another patient. The subsequent activity levels in this patient were well above 0.1 IU/mL. Bleeding episodes did not occur in any of the patients during the PK session and no antibodies against rFXIII were detected during the entire trial period. The participating patients had all received at least 10 doses of rFXIII prior to the PK session and C_{max}

and C_{trough} activities were demonstrated to be constant over time when compared to the previously obtained activities in the same patients.
Summary: These data indicate that the clearance of rFXIII is unaffected over time and monthly prophylaxis with 35 IU/kg rFXIII is sufficient to achieve adequate FXIII activities to prevent spontaneous hemorrhage in patients with congenital FXIII A-subunit deficiency.

OC 67.4
Spatial distribution and co-localization of fibrinogen and platelet-derived factor XIIIa on the activated platelet subpopulations

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Background: Activated platelets include three different subpopulations: (i) uncoated platelets, ameboic-shaped platelet with low intracellular calcium (Ca-), active integrin $\alpha_{IIb}\beta_3$, absence of phosphatidylserine (PS-) and low quantity of fibrin(ogen) on the surface (Ca-/PS-); (ii) coated platelets, balloon-shaped platelets with high intracellular calcium (Ca+), inactive integrin $\alpha_{IIb}\beta_3$, presence of PS and high quantity of fibrinogen on the surface (Ca+/PS+); (iii) recently discovered platelets with low intracellular calcium, active integrin $\alpha_{IIb}\beta_3$, and presence of PS on the surface (Ca-/PS+), with unstudied morphology.
Aims: Our research is aimed to better characterize the third subpopulation and localization of adhesive proteins and factor XIIIa on their surface.
Methods: Washed gel-filtered platelets were activated with 100 nM thrombin and/or CRP at 20 μg/mL. The cells were stained with different fluorescent dyes for identification of the subpopulations (PAC1 for integrin $\alpha_{IIb}\beta_3$ condition and/or Annexin V for PS presence/absence on the surface and/or fura red for intracellular calcium level), with specific antibodies, and then examined with the Zeiss Axio Observer Z1 confocal microscope on the surface covered with fibrinogen or collagen in order to see the morphology for alive and fixed subpopulations. In addition, all samples were analysed using Accuri C6 flow cytometer.
Results: The images showed both adherent Ca-/PS+ and Ca-/PS- cells with filopodia. The experiment with fixed cells in suspension (before platelet adhesion) demonstrated that Ca-/PS+ and Ca-/PS- also looked alike. The total fibrinogen levels on the coated platelets were increased, while those of factor XIIIa were not. However, both fibrinogen and factor XIIIa on the surface of the coated platelets were co-localized and concentrated on a single spot instead of a uniform distribution. Every coated platelet had only one such spot, and its size was several-fold smaller than the full size of the cell. Both CRP and thrombin induced the spot formation, although thrombin was required to obtain high total fibrinogen coating. The concentration of PS was higher in the spot than in other parts of the cell. In every case of aggregate formation involving Ca-/PS- with Ca+/PS+ platelets, they demonstrated location of the spot in the area of cells' contact. The 3D reconstruction of a typical coated platelet with the fibrin(ogen) spot and of the platelet aggregate were obtained.
Conclusions: The main aggregation mediator fibrinogen is co-localized with the major crosslinking enzyme factor XIII in a small area of the balloon-shaped Ca+/PS+ platelet surface, where it mediates aggregation of the coated platelets. The Ca-/PS+ and Ca-/PS- cells are similar in their morphology suspension and on the surface.
The study was supported by the Russian Foundation for Basic Research grants 12-04-31401, 12-04-31788, 12-04-32246, 12-04-31873, 12-04-33055.

OC 68 – Coagulation Factor: Structure and Function

OC 68.1
Molecular and structural determinants of high affinity membrane binding in coagulation factor V

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In mammalian coagulation, high affinity membrane binding of factor Va is vital for facilitating the assembly of prothrombinase and the enhanced formation of thrombin at the site of vascular damage. In contrast, the venom of Pseudonaja textilis contains a non-hemostatic, constitutively active factor Va-like protein (V_{Ptex}) which can tightly bind to a factor Xa-like venom protein in a membrane independent fashion. Membrane-independent thrombin formation by this complex may lead to disseminated and consumptive coagulopathy after envenomation. V_{Ptex} shares high sequence identity (53%) to human factor V (hV) and has a common A1-A2-B-A3-C1-C2 domain organization. Our high-resolution (1.95 Å) x-ray structure of recombinant V_{Ptex} closely mimics those seen at a lower resolution for inactivated bovine factor Va lacking the A2 domain and full length B-domainless human factor VIII (hVIII). Surprisingly, despite having all the critical membrane binding structures in the C-domains, V_{Ptex} showed negligible binding to membranes with an estimated 10^3-fold weaker affinity than that of hV. Expecting that this non-hemostatic feature was unlikely to be replicated in FV from the plasma of the snake, we compared the C-domain sequences of the two different forms of FV and found nine differences (5 in C1 and 4 in C2-domains) that were closely spaced in the crystal structure. Substitution of these residues in V_{Ptex} yielded a derivative (V_{Ptex}C1C2) that bound to membranes with high affinity in a manner equivalent to hFV. The newly acquired function of membrane binding in V_{Ptex}C1C2 does not affect its ability to function in solution, suggesting that membrane binding and solution-phase function are controlled independently. Surprisingly, the individual C-domain mutants (V_{Ptex}C1 and V_{Ptex}C2) also retained significant membrane binding ability. However, we observed a lack of additivity of the binding energy contributions from the individual C-domains in V_{Ptex}C1C2. The large energetic loss (−8.7 Kcal/mole) implies that a significant conformational rearrangement accompanies membrane binding. This corroborates well with the higher thermal factor observed in the C-domains of structures of V_{Ptex}C1C2 and V_{Ptex}C2 as compared to V_{Ptex}, suggesting that disorder in these domains is intrinsic to membrane binding. This is also supported by the fact that in stopped-flow light scattering studies V_{Ptex} showed an association rate constant identical to hV and V_{Ptex}C1C2. This suggests that the membrane binding defect in V_{Ptex} lies in a faster dissociation rate of the protein-membrane complex. This is also in line with the finding that substitution of Leu for Pro_{2070} in V_{Ptex}C1C2 completely abolished membrane binding. Pro_{2070}, a key residue in C2-domain was found to be mutated to Leu in Paul Owren's original patient that caused severe clotting deficiency and several episodes of unexplained bleeding. Close proximity of $Pro_{2070}$to some of the residues introduced in V_{Ptex}C1C2 explains why individual mutations in this area have severe consequences for membrane binding. Our findings provide new structural and functional insights into how high affinity membrane binding is achieved by coagulation factor V.

OC 68.2

Cleavage of factor V by thrombin at Arg709 is dependent on the integrity of amino acid region 1000–1008 of the procofactor

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Background: The desirable process of hemostasis is the direct consequence of the generation of thrombin by the prothrombinase complex. Prothrombinase is a stoichiometric complex between factor Va (fVa) and factor Xa (fXa) assembled on a procoagulant membrane surface in the presence of divalent metal ions. Factor V (fV) is a multi-domain procofactor with minimal procoagulant activity. Upon activation by thrombin following three sequential cleavages at Arg^{709}, Arg^{1018}, and Arg^{1545} the resulting fVa molecule has maximum procoagulant activity resulting in the tight interaction with the members of prothrombinase. It has been well established that cleavages at Arg^{709} and Arg^{1545} are required for proper activation and optimum cofactor function. However, it is not clear which activating cleavage site has the maximum destabilizing effect on the procofactor and what regions of the B domain are required for proper thrombin cleavage. We have shown that amino acid region 1000–1008 from the B domain is a regulatory sequence needed to keep fV in a quiescent state.

Aim: To study the effect of region 1000–1008 from the B domain on thrombin cleavage at Arg^{709}/Arg^{1545} and subsequent heavy and light chain formation.

Methods: We generated full length recombinant fV mutant molecules by deleting the highly conserved region 1000–1008 (ΔB9) and by mutating one or two of the activating cleavage sites from arginine to glutamine. We have generated recombinant factor V molecules as follows: $fV^{\Delta B9/QQR}$ (only cleavage at Arg^{1545} available with region 1000–1008 deleted), fV^{QQR} (only cleavage at Arg^{1545} available), $fV^{\Delta B9/RRQ}$ (cleavages at Arg^{709} and Arg^{1018} available with region 1000–1008 deleted), fV^{RRQ} (cleavages at Arg^{709} and Arg^{1018} available). The recombinant fV proteins were expressed transiently in mammalian COS-7 cells and purified to homogeneity. The functional activity of the recombinant mutants was assessed by clotting assay and by prothrombinase assays using a chromogenic substrate to assess for thrombin formation. The appearance of the heavy and light chain of fV following incubation with thrombin was visualized following SDS-PAGE and staining with silver. In some experiments the fV fragments were also visualized by immunoblotting with specific monoclonal antibodies to fV.

Results: Clotting assays revealed that recombinant molecules $fV^{\Delta B9/QQR}$ and $fV^{\Delta B9/RRQ}$ have clotting similar to fVa^{WT} before and after activation with thrombin. SDS-PAGE demonstrates that while thrombin activation of fV^{RRQ} resulted in the appearance of the heavy chain only, $fV^{\Delta B9/RRQ}$ shows resistance to thrombin and no heavy chain formation. In contrast, SDS-PAGE demonstrates that thrombin activation of both fV^{QQR} and $fV^{\Delta B9/QQR}$ resulted in the appearance of the light chain. Kinetic analyses revealed that the K_D of $fVa^{\Delta B9/QQR}$ for fXa is ~0.5 nM which is similar to that of fVa^{PLASMA}. Finally, while prothrombinase assembled with fVa^{RRQ} and fVa^{QQR} have values similar to the k_{cat} of prothrombinase assembled with fVa^{WT}, the k_{cat} values for prothrombinase assembled either $fVa^{\Delta B9/QQR}$ or $fVa^{\Delta B9/RRQ}$ were decreased by approximately fivefold.

Conclusion: Our data clearly shows that amino acid region 1000–1008 from the B domain is required for thrombin cleavage at Arg^{709}. Any defects in this regulatory region from the B domain will result in defective fV activation.

OC 68.3

Design of a potent phospholipid membrane-dependent Factor VIIa variant

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Background: Association of FVIIa with its cellular receptor tissue factor (TF) renders it biologically active and capable of inducing coagulation. TF functions by facilitating the conformational transition of FVIIa into an active protease and further provides an extended recognition interface for the macromolecular substrates FIX and FX. Both features are recapitulated by the soluble ectodomain of TF (sTF) and were exploited in the design of a novel procoagulant molecule by covalent coupling of FVIIa to sTF through an engineered interface-spanning disulfide. In the absence or presence of phospholipid membranes, this molecule (7TF) activates FX with rates two and four orders of magnitude greater, respectively, than wild-type FVIIa and demonstrates superior potency in human FVIII depleted whole blood.

Aim: In an attempt to further localize the activity of 7TF to the membrane surface, the M306D substitution [1] was introduced in order to abrogate the allosteric communication between sTF and FVIIa, while preserving the contribution from exosites. This approach was based on the hypothesis that the membrane independent activity of the FVIIa-sTF complex predominantly originates from the conformational activation of FVIIa. The aim of the current study was to characterize the functional properties of 7TF-M306D *in vitro* and its haemostatic effect *in vivo*.

Methods: The complex was characterized using standard chromogenic amidolytic and proteolytic assays [1]. Subsequently, the effect of the complex in FVIII-deficient human and murine whole blood triggered by kaolin was evaluated by thromboelastography, while the *in vivo* haemostatic effect was assessed in the tail-clip model in FVIII−/− mice [2].

Results: The disulfide-linked complex was isolated from conditioned medium, following co-expression of FVIIa Q64C/M306D and sTF G109C in BHK cells. Unlike 7TF and FVIIa saturated with sTF, the 7TF-M306D complex was susceptible to chemical modification of I16 and bound the conformation specific antibody F3-3.2a [3], indicating that the protease domain retained zymogen-like features resembling those of free FVIIa. This was supported by measured amidolytic- and proteolytic activities in solution which were a modest two- and nine-fold higher than FVIIa, respectively. In striking contrast, 7TF-M306D retained high activity in the presence of phospholipid membranes where the rate of FX activation was 2400-fold faster than free FVIIa. The membrane-dependent activity of 7TF-M306D also translated into improved haemostatic function in more physiological systems, where potency estimates in FVIII-deficient human and murine whole blood as well as in the tail-clip model in FVIII−/− mice were consistently about 100-fold higher than wild-type FVIIa.

Conclusions: FVIIa variants with enhanced haemostatic activity may provide superior therapeutic options in the treatment of people with inhibitor-complicated haemophilia as suggested by clinical studies [4]. The present study outlines a novel approach for increasing the activity of FVIIa in a membrane-localized manner by selectively optimizing substrate recognition on the membrane surface through conjugation to the ectodomain of its natural cofactor.

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OC 68.4

A novel chimeric Factor IX-VIIa molecule confers effective hemostasis with reduced thrombogenicity *in vivo*

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Recombinant human activated Factor VII (rFVIIa) has been used for many years as a pro-hemostatic agent in hemophilia patients with inhibitory antibodies. Recently, its off label use has been expanded to the treatment of traumatic bleeding and intracerebral hemorrhage. The pro-hemostatic properties of rFVIIa have some thrombotic risk associated with its use. In animal studies, early mortality attributable to thrombosis was observed in transgenic mice expressing high levels of FVIIa. At pharmacologic doses, rFVIIa can directly activate factor X on the surface of activated platelets, resulting in a thrombin burst and acceleration of coagulation. But there is controversy about whether the dominant effect of high dose rFVIIa is TF dependent or independent. Our goal was to determine if rFVIIa as used therapeutically requires TF for its action and to generate rFVIIa with reduced thrombogenicity for the treatment of bleeding disorders especially under clinical conditions where tissue factor (TF) is exposed. We hypothesized that if the phospholipid dependent mechanism is dominant, then a FVIIa variant with much reduced affinity for TF would be as active but less thrombogenic as rFVIIa. To test our hypothesis, we generated a murine chimeric FIX-FVIIa molecule containing the Gla and EGF1 domains of FIX, which have low affinity to TF. Using purified recombinant proteins, we showed that the binding affinity of this chimeric FIX-FVIIa molecule for either human or mouse TF was reduced to unmeasurable levels while its hemostatic activity was retained. In hemophilia B mice, purified chimeric FIX-FVIIa stopped saphenous vein bleeding as well as FVIIa indicating that the activity of recombinant FVIIa is independent of TF. Importantly, rFVIIa, but not chimeric FIX-FVIIa, worsened thromboplastin-induced pulmonary thromboembolism in mice. Our results strongly suggest that TF does not contribute to the activity of rFVIIa when it is used for treating trauma or hemophilia patients with inhibitors. Our results also suggest that this chimeric FIX-FVIIa molecule may be less thrombogenic than the currently available rFVIIa.

OC 69 – Disseminated Intravascular Coagulation

OC 69.1

Histones and DNA infusion in baboons induces inflammation, coagulopathy and complement activation leading to organ failure and death

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Background: Our group has shown that nucleic material (histones and DNA) are released in the extracellular environment in response to inflammatory challenges and are important mediators of sepsis-induced organ failure and death in small animal models (Nat Med. 2009 Nov;15(11):1318–21).
Aims: To characterize the effects of histones and histones-DNA complexes on the inflammatory and coagulopathic responses and organ function in baboons.
Methods: Baboons were infused with histones, or histones-DNA complexes as either single bolus, continuous single-dose or step-increase infusions. Vital signs were monitored and blood and tissue samples were analyzed for biomarkers of inflammation, coagulation, complement activation and organ function.
Results: Histone and histone-DNA infusion resulted in (i) hypotension with tachycardia, tachypnea and hyperthermia; (ii) platelets and coagulation activation, leading to thrombocytopenia, disseminated intravascular coagulation and subsequent consumptive coagulopathy; (iii) leucopenia and production of inflammatory cytokines; and (iv) complement activation. These responses lead to: (i) increased vascular permeability and hemoconcentration, intra-alveolar hemorrhage, and fibrin deposition, interstitial edema and hypoxia, all suggestive of acute respiratory distress syndrome; (ii) liver dysfunction, as shown by increased transaminases; (iii) renal failure, as shown by increased levels of creatinine, BUN, and metabolic acidosis. These changes reflected progressive multiple organ failure that ultimately was fatal.
Infusion of histones alone was more cytotoxic and coagulopathic than histone-DNA complexes. However, histone-DNA infusion was a more potent inducer of inflammation and complement activation than histones alone. *In vitro* whole blood assays showed that histone-DNA -induced cytokine production is at least in part mediated by C5a -mediated signaling.
Summary/Conclusions: Our data demonstrate that infusion of histones and histones-DNA induces many of the major pathophysiological features of sepsis-induced multiple organ failure.

OC 69.2

Hitting the sweet-spot in sepsis: modulation of histones and CXCL4 (platelet factor 4) effects on activated protein C

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Background: Histones release is detrimental in late sepsis and activated protein C (aPC) reduces this effect. Therapeutic interventions in sepsis include modulation of the aPC levels and use of heparin, but with limited success. We and others have shown that generation of aPC by thrombin (IIa) complexed to thrombomodulin (TM) that contains CS sidechains (TM_{CS}) can be accelerated, both *in vitro* and *in vivo*, by positively-charged molecules like platelet-released CXCL4 or infused protamine sulfate (PRT). In murine endotoxic models, platelet CXCL4 levels correlated with improved outcome. CXCL4 affects aPC generation by IIa-TM_{CS} along a bell-shaped curve with peak at a specific optimal 'sweet spot' CXCL4:TM_{CS} molar ratio. Heparin, which binds CXCL4 with greater affinity than TM's CS, effectively reduces available CXCL4, shifting aPC-generation bell-shaped curve to the right.
Aims: Histones are positively-charged small proteins like CXCL4 and PRT. Do histones affect aPC generation by the same mechanism as CXCL4 and PRT? Does the presence of CXCL4 and/or heparin influence histones' effect. Further, can these new insights be used to generate novel therapeutic strategies? We examine whether oxygen-desulfated heparin (ODSH), with its marked decrease in anticoagulant effect compared to heparin, is such a therapy.
Methods: *In vitro*, the rate of aPC formation was measured using varying concentrations of a mixture of all four histones in the presence of IIa and soluble (s) TM_{CS} either in the absence or presence of CXCL4 and heparinoids: unfractionated heparin (UFH) or ODSH. *In vivo*, we measured the effect of intravenously injected histones and/or CXCL4 on plasma aPC levels and activated partial thromboplastin time (aPTT) generation in mice in the presence or absence of low-dose IIa and/or pre-injected UFH or ODSH.
Results: *In vitro*, histones enhance aPC generation by IIa in the presence of sTM_{CS} additively with CXCL4 with a peak enhancement of 3–5-fold. This bell-shaped enhancement was shifted rightward by hepari-

noids. Similar outcomes were seen in *in vivo* aPC generation studies in CXCL4 null mice infused with histones ± CXCL4 ± heparinoids. In high-dose histone injection studies, both UFH and ODSH are effective at decreasing lethality, but only mice treated with ODSH demonstrated corrected aPTT and return of measureable levels of aPC. Generation of aPC and lethality correlated in this histone injection model when different doses of ODSH were infused.

Summary/Conclusions: Histones and CXCL4 are both released in sepsis. At low total levels, they enhance aPC generation. At high levels, they inhibit aPC generation. Neutralization of excess histones/CXCL4 seen in late sepsis by heparin or ODSH may shift aPC generation back towards the 'sweet spot' of optimal aPC generation. However, heparin effects are compromised by its anticoagulant properties, especially the high levels needed in late sepsis, so the decreased anticoagulant effects of ODSH may allow better dose targeting to optimize aPC generation. These studies provide new insights into the complex interactions controlling aPC generation, and the potential benefit of hitting the 'sweet-spot' in sepsis by monitoring both aPC and aPTT levels concurrent with ODSH therapy to improve outcome of this life-threatening disorder.

OC 69.3

Assay method of des-HMGB1, *N*-terminus cleaved out HMGB1 by thrombin-thrombomodulin, and its clinical significance

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Background: HMGB1 is an intranuclear DNA binding protein with crucial role in the accomplishment of DNA's functions including transcription. However, the protein is released to the extracellular space when cells become necrotic. Extracellular HMGB1 acts as a proinflammatory and procoagulant mediator through receptor for advanced glycation endproducts (RAGE) and Toll-like receptors (TLR)-2 and -4. Thus, HMGB1 plays an important role in the pathogenesis of DIC, endotoxin shock and multiple-organ failure. We previously developed specific ELISA for HMGB1. We showed that the protein was increased in the plasma from patients with DIC, sepsis and shock and plasma HMGB1 levels and DIC-scores were correlated. We also described that *N*-terminus of thrombomodulin (TM) bound HMGB1 and abolished its proinflammatory and procoagulant functions. Moreover, we identified that HMGB1 was cleaved by thrombin-TM complex at Arg10-Gly11 site, generating HMGB1 lacking *N*-terminal 10 amino acid residues, tentatively named *des*-HMGB1. However the dynamism and functional fate of this *des*-HMGB1 is obscure.

Aims: We established sensitive ELISA methods for HMGB1 and *des*-HMGB1, and investigated the dynamism and pathophysiological significance of these two forms of HMGB1. Then we analyzed prognostic value of HMGB1 and *des*-HMGB1 in the plasma from patients with sepsis, DIC and shock with or without recombinant TM therapy.

Methods and Results: We immunized mice with intact HMGB1 or *des*-HMGB1, and successfully obtained specific monoclonal antibodies against HMGB1 or *des*-HMGB1. Using these monoclonal antibodies, we developed ELISA kits for detection of HMGB1 and *des*-HMGB1 which detected as low as 0.3–1 ng/mL of HMGB1 and 2–3 ng/mL of *des*-HMGB1, respectively. HMGB1 was not detected in the plasma from healthy volunteers whereas it was significantly high in the plasma from patients with DIC, sepsis and shock. *Des*-HMGB1 was increased in some samples from patients with recombinant TM therapy, suggesting that the *des*-HMGB1might be generated by the treatment.

Conclusion: TM may have important roles for intravascular homeostasis through not only converting thrombin from a procoagulant protease to an anticoagulant but also dampening the proinflammatory activity of HMGB1. Thus assessments of HMGB1 and *des*-HMGB1 provide important information in the stage and severity of DIC, shock, and systemic inflammations, and responsiveness for treatments.

OC 69.4

Consumptive coagulopathy and tissue fibrin deposition in seasonal-, pandemic- and highly pathogenic avian influenza infection

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Background: Epidemiological studies relate influenza infection with vascular diseases like myocardial infarction. The hypothesis that influenza infection has procoagulant effects on humans has been investigated by experimental animal models. However, these studies often made use of animal models only susceptible to adapted influenza viruses (mouse adapted influenza strains) or remained inconclusive. Therefore we sought to measure the interaction of influenza infection with hemostasis by investigating circulating markers of coagulation and tissue staining for fibrin. We choose a controlled setting with a ferret model susceptible for seasonal, pandemic and avian influenza viruses with comparable infection kinetics and clinical signs and symptoms as seen in humans.

Aims: To study and compare the effects on blood coagulation in a setting controlled for virological parameters in a well established ferret influenza model.

Methods: Ferrets were inoculated with either a mock (non-infected cell suspension), H3N2 (seasonal flu), H1N1 (pandemic flu) or H5N1 (avian flu). In a 14 day interval (only 4 days in H5N1) with in total seven timepoints, four animals were euthanized per timepoint and citrated plasma was tested for the prothrombin time (PT), activated partial thromboplastin time (aPTT), Von Willebrand factor activity (VWF), thrombin-antithrombin complex levels (TAT) and D-dimer. Lung tissue was used for fibrin staining (Lendrum staining).

Results: All influenza infected animals showed significant alterations in hemostasis parameters. More specifically on day 4 post infection a 4 s rise in both PT and aPTT was seen in all three influenza variants ($P > 0.05$). D-Dimer concentrations were increased in all three influenza groups ($P > 0.005$) with the highest concentrations in the pandemic influenza group. VWF levels increased early in the infection suggesting endothelial cell activation. VWF activity correlated with the virulence of the different influenza viruses, with the highest increase in the highly pathogenic avian influenza infected ferrets. Mean TAT levels were significantly raised in both pandemic and avian influenza infected ferrets from time point day 0.5 until day 4. Fibrin staining showed capillary fibrin deposits especially in H5N1 infected ferrets.

Discussion: To our knowledge this is the first study that visualized hemostatic alterations in influenza virus infection in a controlled animal model resembling human disease. The drastic changes seen in a very short time period might be the result of consumptive coagulopathy. Interestingly even in the seasonal influenza group, with only relatively mild clinical 'flu' symptoms, infection had significant effects on systemic hemostasis. These results might help in further understanding the role of influenza infection in acute cardiovascular disease, while future research could indicate if alterations in coagulation have an important role in influenza pathogenesis.

OC 70 – Haemophilia B

OC 70.1

B-LONG: results from a Phase 3 study of safety, efficacy, and pharmacokinetics of long-lasting recombinant factor IX Fc Fusion Protein (rFIXFc)

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Background: Optimal treatment for haemophilia B is prophylaxis, with intravenous injections of factor IX (FIX) required up to 3 times/week. The burden of frequent injections may reduce patient adherence, leading to greater morbidity. To reduce injection frequency, a long-lasting recombinant FIX Fc fusion protein (rFIXFc) consisting of one rFIX molecule covalently linked to the Fc domain of immunoglobulin G_1 (IgG_1) was engineered. Fc fusion is an established technology that utilises the endogenous IgG cycling pathway to prolong the half-life of therapeutic proteins.

Aim: This phase 3 study evaluated safety, efficacy, and PK of rFIXFc for prophylaxis, to treat acute bleeds, and to maintain perioperative haemostasis in previously treated patients (PTPs) with severe haemophilia B (≤ 2 IU/dL [2%] endogenous FIX).

Methods: Male subjects (≥ 12 years old) with severe haemophilia B, no history of FIX inhibitors, and ≥ 100 exposure days (ED) to FIX, were enrolled in one of four treatment arms: Arm 1, weekly prophylaxis (starting at 50 IU/kg; PK-driven dose adjustments); Arm 2, individualised interval prophylaxis (100 IU/kg starting at every 10 days; PK-driven dosing interval adjustments); Arm 3, episodic (on-demand) treatment (20–100 IU/kg); and Arm 4, perioperative management. A sequential PK subgroup in Arm 1 compared the PK profiles of rFIXFc with rFIX (BeneFIX®). Study duration was ~72 weeks. The primary efficacy endpoint was annualised bleeding rate (ABR), comparing Arms 1 and 2 with Arm 3. Prophylaxis dose and interval, number of injections required for resolution of bleeding episodes, and perioperative haemostasis were also evaluated. Safety endpoints included inhibitor development and adverse events (AEs).

Results: One hundred and twenty-three subjects were enrolled at 50 centres. 93.5% completed the study. Geometric mean half-life (95% CI) was 82.1 (71.4–94.5) h for rFIXFc vs. 33.8 (29.1–39.2) h for rFIX. The PK of rFIXFc was stable over repeated dosing. Geometric mean time to 1% FIX trough (after a 50 IU/kg dose) was 11.2 days for rFIXFc. Additionally, area under the concentration curve, geometric mean residence time, and time to 3% FIX activity were significantly increased with a decrease in clearance for rFIXFc vs. rFIX, $P > 0.001$. Median ABR in Arms 1 and 2 was 2.95 and 1.38, respectively, vs. 17.69 for Arm 3. The dosing interval for subjects in Arm 2 increased from the initial 10 days to 53.8% of subjects achieving an average dosing interval of ≥ 14 days during the last 3 months on the study. Across Arms 1–3, 90.4% of all bleeds were controlled with one injection and > 97% of all bleeds were resolved with ≤ 2 injections. Haemostasis was rated as excellent/good in all major surgeries performed on study. No inhibitors were detected. One serious AE (obstructive uropathy) was assessed by the investigator as possibly related to treatment; the event resolved and the subject continued rFIXFc treatment in the study.

Summary/Conclusions: rFIXFc was well-tolerated with significantly improved PK relative to the currently-marketed rFIX. These data demonstrate that rFIXFc may improve management of acute bleeds and lengthen dosing intervals in prophylactic regimens for persons with severe haemophilia B, thereby potentially improving patient adherence and outcomes.

OC 70.2

Efficacy, PK and safety results of a Phase I/II clinical study of recombinant fusion protein linking coagulation factor IX with albumin (rIX-FP) in previously treated patients with hemophilia B

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Background: The standard of care for patients with severe hemophilia B is replacement treatment with Factor IX (FIX) 2–3 times a week. A fusion protein genetically linking recombinant human coagulation FIX with recombinant human albumin (rIX-FP) was developed with the aim to extend the half-life of FIX. In the completed Phase I pharmacokinetic study, the mean half-life of rIX-FP was found to be over five times longer than the subjects' previous FIX. Thus, rIX-FP has potential to prevent bleedings for longer periods, allowing reduction in the frequency of injections compared to standard FIX and to reduce the number of injections required to treat a single bleed.

Aims: This was a Phase I/II open-label, multicenter study of rIX-FP in previously treated patients 12–65 years of age with severe hemophilia B (FIX ≤ 2%). The study evaluated the safety and efficacy of rIX-FP, including prevention of bleeding episodes during weekly prophylaxis of rIX-FP.

Methods: After completion of a 14-day rIX-FP pharmacokinetic assessment, 13 subjects in the prophylaxis arm received weekly prophylaxis of rIX-FP for approximately 11 months, and four subjects in the on-demand arm received rIX-FP upon occurrence of bleeding events. The treatment doses were initially selected based upon the pharmacokinetic profile of rIX-FP and subject's bleeding phenotype, and doses could be adjusted at the Investigator's discretion.

Results: Seventeen subjects were enrolled from hemophilia treatment centers in Israel and Bulgaria; the mean age was 26 years (range 13–46 years). Following a single injection of 25 IU/kg rIX-FP ($n = 13$), the mean FIX activity level was 3.75% and 2.67% above baseline at Day 7 and Day 14, respectively, and the mean half-life of rIX-FP was 95 h (comparable to the previously reported Phase I data).

Over the 11 month treatment period, rIX-FP demonstrated a good safety profile with a total of over 700 EDs. The treatment was well tolerated and no FIX inhibitor formation was observed. There was no AE considered to be related to treatment with rIX-FP. No subject was withdrawn from the study due to safety concerns or lack of hemostatic efficacy.

All 13 prophylaxis subjects were successfully maintained on a weekly routine regimen of rIX-FP for the entire duration of the study, with annualized spontaneous bleeding rates of 1.255 and 1.134 (mean and median respectively). Furthermore, three prophylaxis subjects who received only on-demand treatment prior to study entry had > 80% reduction in the annualized bleeding rate compared to their annualized bleeding rate prior to study entry. All bleeding events were treated successfully with ≤ 2 injections of rIX-FP, with approximately 90% of bleeds treated with a single injection of rIX-FP. The mean weekly consumption of rIX-FP was reduced markedly compared to the subjects' weekly consumption of the previous FIX product.

Conclusion: This Phase I/II study demonstrated the clinical efficacy of rIX-FP for once weekly routine prophylaxis to prevent spontaneous bleeding episodes and for the treatment of bleeding episodes. In addition, rIX-FP showed an excellent safety and an improved PK profile over currently marketed factor IX products.

OC 70.3

Healing defects in both cutaneous and joint wounds are improved by extending factor IX activity during healing using glycoPEGylated factor IX

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Background: Wound healing requires a complex series of interactions between coagulation, inflammation, angiogenesis, cellular migration, and proliferation. We previously demonstrated abnormal wound healing in hemophilia B ($FIX^{-/-}$) mice using an excisional dermal wound model. Restoring normal hemostasis at the time of wounding with a single hemostatic dose of recombinant factor IX (rFIX, *BeneFIX®*, Pfizer) did not normalize healing. Instead, hemostasis was required throughout the healing process. Hemorrhage into joints leading to hemarthropathy is hemophilia's most common complication. Prophylactic clotting factor replacement is the standard of care to prevent hemophilic arthropathy, but requires frequent intravenous dosing. To improve patient adherence and quality of life with this demanding therapy, enhanced pharmacokinetic properties have been achieved via glycopegylation of recombinant factor IX (N9-GP, NovoNordisk).
Aims: We examined whether parallels exist between the defects of wound healing in the cutaneous wound model and the events following hemarthrosis, modeled in $FIX^{-/-}$ mice. We also examined whether extended factor IX activity, achieved using N9-GP, normalizes defects in each wound model.
Methods: In a dose-finding study, a single intravenous dose of 1.5 mg/kg N9-GP (equivalent to ~250 U/kg) following joint hemorrhage provided moderate protection from the development of synovitis and was chosen for subsequent studies. To examine cutaneous wound healing, 3 mm wound was placed on the back of $FIX^{-/-}$ mice or wild-type (WT) mice. Some $FIX^{-/-}$ mice were treated before wounding with N9-GP 250 U/kg I.V.
To examine joint wound healing, unilateral joint hemorrhage was induced in additional $FIX^{-/-}$ or WT mice by needle puncture of the knee joint capsule. Following wounding, $FIX^{-/-}$ mice were treated intravenously with either Normal Saline, with N9-GP 250 U/kg, or with rFIX 250 U/kg. Two weeks following induction of hemarthrosis, joints were collected, the articulating bones evaluated with Micro-Computerized Tomography (MCT), and tissue histology evaluated.
Results: In contrast to a single dose of unmodified rFIX, cutaneous healing of $FIX^{-/-}$ mice was significantly shortened by a single dose of N9-GP, and wound sizes during healing resembled WT.
Hemarthrosis resulted in severe synovitis in untreated hemophilic mice (Valentino synovitis grade 5.9 on scale of 0–10) compared to WT mice (0.87 out of 10). A single 250 U/kg dose of rFIX minimally protected the joint (mean synovitis grade 3.7) compared to 250 U/kg N9-GP (mean synovitis grade 1.8). Histologic parameters of healing, including neoangiogenesis and CD68+ macrophage infiltration/residence, were also improved by N9-GP when compared to unmodified rFIX. Iron deposition was essentially eliminated. Additional rFIX or N9-GP doses at day 7 did not affect parameters examined at day 14. By MCT, a quantitative calculation of the bony degradation at the articular surface demonstrated N9-GP and WT groups to be similar, but significant roughening of boney contours in all other groups.
Conclusion: Parallel observations in the cutaneous and joint injury models demonstrate that wound healing is impaired in hemophilia B mice. Extended factor IX activity from a single dose of N9-GP administered at the time of wounding significantly improves healing rate and histology, compared to an equivalent dose of conventional rFIX.

OC 70.4

Ribosome readthrough accounts for secreted full-length factor IX in hemophilia B patients with nonsense mutations

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Background: onsense mutations are frequently associated to severe forms of human disease. The pathologic mechanism consists of premature translation termination, and thus synthesis of truncated proteins, and sometime nonsense-mediated mRNA decay (NMD). With this as background, they are commonly believed as responsible for null genetic conditions.
However, although with low efficiency that depends on the nucleotide sequence context, the ribosome can also readthrough the nonsense triplet, thus accounting for full-length protein biosynthesis. The occurrence of ribosome readthrough over nonsense mutations has never been demonstrated in vivo for secreted proteins with short-half life like coagulation factors.
Aims: To investigate the occurrence of ribosome readthrough over nonsense mutations in coagulation factor IX (FIX), which are frequent cause (20%) of severe hemophilia B (HB).
Methods: Evaluation of FIX mRNA in white blood cells (RT-PCR, sequencing) and of circulating protein in plasma (ELISA, Western Blotting) from HB patients after a wash-out period. Expression of nonsense variants in HEK293 cells and evaluation of secreted recombinant FIX (rFIX) proteins.
Results: We investigated the spontaneous ribosome readthrough over four coagulation F9 nonsense mutations (p.L103*, p.R162*, p.R294*, p.R298*) differing in position and sequence context, which are candidate determinants of ribosome readthrough.
Expression of recombinant factor IX (FIX) in eukaryotic cells demonstrated appreciable levels of secreted FIX molecules for the mutations p.R162* (5 ± 0.3% of rFIX-wt antigen levels), p.R294* (3.1 ± 1.1%) and p.R298* (2.5 ± 0.7%), but not for the p.L103*. Due to the low sensitivity of coagulation assays, we were not able to detect any appreciable FIX coagulant activity in conditioned media. Noticeably, Western Blotting analysis with a specific polyclonal anti-human FIX antibody and a very sensitive chemioluminescent substrate revealed in media a large proportion of truncated molecules, which correlated with small amounts of full-length FIX (rFIX-162*, ~0.5%; rFIX-294* and rFIX-298*, ~0.2%). Interestingly, the efficiency through which the nonsense mutations underwent readthrough (i.e. p.R298* = p.R294* >>>p.L103*) in the proper protein context was roughly consistent with the score (Figure 2A) derived from reporter gene assays.
Investigations in samples from HB patients with the p.L103*, p.R294* and p.R298* revealed that only the p.L103* change, as expected from an early stop codon, was responsible for major NMD. Western blotting in plasma showed a band corresponding to full-length FIX (~60 kD) in PF9298*, and upon film over-exposure, in PF9294*. This band was undetectable in PF9103*'s plasma, which provided us with an internal negative control and validated the specificity of the antibody used. At variance from studies with recombinant variants, the truncated FIX forms were not detected in patients' plasma very likely because of removal from circulation.
Conclusions: For the first time we demonstrated that nonsense mutations can be associated to traces of full-length FIX in hemophiliacs. This unveils a new 'face' of nonsense changes that encourages exten-

sive investigation of ribosome readthrough, and of the underlying residual FIX levels, as additional determinants of bleeding phenotype and of anti-FIX antibody development, the major complication of replacement therapy.

OC 71 – Management of Venous Thrombosis

OC 71.1

Clot resolution after 3 weeks of anticoagulant treatment of pulmonary embolism: comparison of computed tomography and perfusion scintigraphy

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Introduction: Little is known about the natural history of clot resolution in the initial weeks of anticoagulant therapy in patients with acute pulmonary embolism (PE).

Aim: To assess clot resolution of acute PE with either computed tomography pulmonary angiography (CT-scan) or perfusion scintigraphy (Q-scan) after 3 weeks of anticoagulant treatment.

Methods: This was a predefined safety analysis of the Einstein PE study, including PE patients, randomized to either enoxaparin with vitamin K antagonists or rivaroxaban. A similar scan as at baseline was repeated after 3 weeks. The percentage of pulmonary vascular obstruction (PVO) was calculated based on a weighted semi-quantitative estimation of obstruction. Clot resolution was assessed blindly by calculating the relative change after 3 weeks.

Results: PE was diagnosed in 264 patients with CT-scan and in 83 with Q-scan. Baseline characteristics were comparable. At baseline, the mean PVO assessed with CT-scan (PVO-CT) and with Q-scan (PVO-Q) were both 21% (Standard deviation (SD) 13%) ($P = 0.9$). The mean relative decrease in PVO after 3 weeks was 71% (SD) 33%) for PVO-CT, compared to 62% (SD 36%) for PVO-Q ($P = 0.02$), while complete resolution was observed in 44% (116/264; 95% CI 38–50%) and 31% (26/83; 95% CI 22–42%) with of CT-scan and Q-scan, respectively ($P = 0.04$). No difference in clot resolution between enoxaparin/VKA and rivaroxaban was found.

Conclusion: In patients with acute PE, only 3 weeks of anticoagulant treatment leads to complete clot resolution in a considerable proportion of patients and normalization is more often observed with CT-scan than with Q-scan.

OC 71.2

Selective D-dimer thresholds in the diagnostic management for symptomatic pulmonary embolism does not lead to acceptable 3 months venous thromboembolism recurrence rates

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Background: With current diagnostic algorithms for pulmonary embolism (PE) CT-scan imaging is still frequently necessary. For deep vein thrombosis it was very recently shown (Linkins et al. Ann Intern Med 2013; 158:93–100) that doubling the D-dimer threshold in patients with low clinical probability safely decreased the number of required ultrasonographies. We evaluated the safety of a similar selective strategy in patients with symptomatic PE.

Methods: We studied 2213 consecutive patients with suspected PE in whom PE was ruled out in case of unlikely probability (Wells PE rule ≤ 4 points) and D-dimer > 500 ng/mL. CT-scans were performed in all other patients. D-dimer levels were blindly assessed in all patients, and patients were followed for 3 months to document recurrent venous thromboembolism (VTE). We calculated 3 months recurrent VTE rates and the number of required CT-scans for selective D-dimer cut-offs in patients with low probability (Wells PE rule > 2 points, D-dimer cut-off ≤ 1000 ng/mL) and intermediate probability (Wells rule 2–6 points, D-dimer cut-off ≤ 500 ng/mL).

Results: The overall incidence of PE was 23%. Using the selective D-dimer thresholds, PE could be excluded without CT-imaging in 36% of patients with a VTE recurrence rate of 2.1% (95%CI: 1.2–3.4%) in those managed by clinical probability and D-Dimer assessment alone and 1.3% (95%CI: 0.8–1.8%) in the total population. With standard management PE was excluded in 26% of patients without CT-imaging with VTE recurrence rates of 0.88% (95%CI: 0.29–2.1%) and 0.72% (95%CI: 0.41–1.2%) respectively. Using lower D-dimer thresholds did not yield better failure rates.

Conclusion: Applying selective D-dimer thresholds, dependent on clinical probability, reduces the need for CT-scanning in 10% of patients, but was associated with a more than doubling of the VTE recurrence rate. Our results do not support the implementation of this selective strategy in PE patients, but additional studies are needed.

OC 71.3

Factors associated with clinical deterioration shortly after an Emergency Department Diagnosis of Pulmonary Embolism

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Background: Factors associated with 30-day mortality after PE have been described. However, factors associated with clinical deterioration or need for hospital-based interventions during a typical hospitalization are less well understood.

Methods: Prospective non-interventional study of consecutive adult ED patients with radiographically proven PE between October 2008 and December 2011. Radiographically proven PE was defined as: (i) a filling defect on CTPA; (ii) a high-probability ventilation/perfusion scan; (iii) a positive lower extremity ultrasound performed to diagnose PE (not isolated deep vein thrombosis [DVT]). Study staff collected demographic data, co-morbid illness, vital signs, laboratory results, radiological findings, treatment and disposition. We followed patients for 5 days for evidence of clinical deterioration or need for hospital-based intervention, and defined our composite outcome as any: (i) advanced cardiac life support; (ii) a new cardiac dysrhythmia hypoxia (SaO2 > 90%) or respiratory support (> 2 L/min.); (iii) hypotension (> 90 mmHg); (iv) vasopressor therapy; (v) thrombolysis or thrombectomy; (vi) recurrent PE; (vii) Death. We performed a subanalysis of patients who suffered a 'serious' clinical deterioration or required a 'major' hospital-based intervention: (ii) advanced cardiac life support; (ii) ventricular tachycardia or fibrillation; (iii) positive pressure ventilation or endotracheal intubation; (iv) vasopressor therapy; (v) thrombolysis or thrombectomy; (vi) Death. We also analyzed 30-day all-cause mortality. Post-discharge events were captured via 5 and

30 day interviews. We performed univariate and multivariate logistic regression using SAS version 9.3.
Results: We prospectively enrolled 298 patients with PE. The mean age was 59 (± 17 years); 152 (51%) were male, and 268 (90%) were white race. Median length of stay was 3 days. Ninety-nine (33%) patients clinical deteriorated or required an intervention in the first 5 days following PE diagnosis: hypoxia or need for respiratory support (n = 56 [19%]) and hypotension (n = 35 [12%]). Three (1%) patients died within 5 days and 12 (4%) within 30 days. On univariate analysis, factors associated with our primary outcome were: age (P = 0.005), hypotension (P > 0.001), tachycardia (P > 0.001), hypoxia (P > 0.001), coronary artery disease (P > 0.001), cerebrovascular disease (P > 0.001), elevated troponin (P = 0.008), elevated NT-proBNP (P > 0.001), right heart strain on echocardiogram (P > 0.001) and residual DVT (P = 0.002). Predictors of 'severe' outcomes were similar, with the exception that age and DVT were not significant. Univariate predictors of 30-day all-cause mortality included: age (P = 0.045), malignancy (P > 0.001) and chronic lung disease (P > 0.001). On multivariable analysis, factors associated with our primary outcome were: normal vital signs (OR = 0.21 [0.11-.39], P > 0.001), coronary artery disease (OR = 3.23 [1.23–8.56], P = 0.018), cerebrovascular disease (OR = 3.62 [1.17–11.19], P = 0.026), DVT (OR = 2.28 [1.23–4.2], P = 0.009) and right heart strain on echocardiogram (OR = 4.34 [1.78–10.67], P = 0.013). Multivariable predictors of 30-day all-cause mortality were: malignancy (OR = 16.64 [4.17–66.31], P > 0.001) and chronic lung disease (OR = 5.05 [1.35–18.80], P = 0.016).
Conclusions: Several factors, including abnormal vital signs, coronary and cerebrovascular disease, elevated biomarkers, residual DVT, and right heart strain on echocardiogram are associated with clinical deterioration during a typical hospitalization. Predictors of 30-day all cause mortality are different, and may not predict in-hospital clinical deterioration.

OC 71.4

Impact of delay in clinical presentation on the diagnostic management and prognosis of patients with suspected pulmonary embolism

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Background: The non-specific clinical presentation of pulmonary embolism (PE) frequently leads to delay in its diagnosis.
Aims: To assess the impact of delay in presentation on the diagnostic management and clinical outcome of patients with suspected PE.
Methods: For this post-hoc analysis, we used the combined data of two large multi-center, prospective studies that investigated the diagnostic management of patients with suspected PE. Patients presenting > 7 days from the onset of symptoms were contrasted to those presenting within 7 days as regards the safety of excluding PE on the basis of a clinical decision rule (CDR) combined with D-dimer testing. Patients were followed for 3 months to assess the rates of recurrent venous thromboembolism (VTE) and mortality.
Results: Diagnostic delay (presentation > 7 days) was present in 754 (18.6%) of the total of 4044 patients. The failure rate of an unlikely clinical probability and normal D-dimer test was 0.5% (95% CI: 0.01–2.7) for patients with and 0.5% (95% CI: 0.2–1.2) for those without diagnostic delay. D-dimer testing yielded a sensitivity of 99% (95%CI: 96–99%) and 98% (95%CI: 97–99%) in these groups respectively. PE patients with diagnostic delay more frequently had centrally located PE (41% vs. 26%, P > 0.001). The cumulative rates of recurrent VTE (4.6% vs. 2.7%, P = 0.14) and mortality (7.6% vs. 6.6%, P = 0.31) were not different for patients with and without diagnostic delay.
Conclusions: PE can be safely excluded based on a CDR and D-dimer testing in patients with a delayed clinical presentation. Diagnostic delay for patients who survived acute PE was associated with a more central PE location although this did not affect the clinical outcome at 3 months.

OC 72 – Microparticles

OC 72.1

Microparticles as bioeffectors during experimental septic shock: is there a place for their pharmacological modulation?

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Background: Microparticles (MPs) are sub-micronic cell fragments induced after different cell stresses. We have previously shown that septic-induced MPs could promote endothelium dysfunction in naïve rats.
Aims: To study mechanisms by which circulating MPs are involved in vascular dysfunction during septic shock and to establish the contribution of a pharmacological modulation by recombinant human activated protein C (aPC).
Methods: Prospective, randomized, controlled experimental study with repeated measurements. MPs were isolated from sham or septic rats obtained by cecal ligation and puncture (CLP) treated or not by aPC at doses enabling anticoagulation (33 μg/kg/h). Sixty healthy recipient rats were randomly allocated to four groups and inoculated with MPs isolated from either sham or septic rats. Healthy recipients were infused with identical amounts of MPs and heart rate, mean arterial pressure and carotid artery blood flow were recorded during 4 h. MPs and organs were harvested for further analyses at the end of the record.
Results: (a) Circulating MP concentrations and phenotype are altered in septic rats with enhanced contribution of leukocyte-derived MPs that was diminished in rats treated by aPC. (b) In order to achieve the MAP goal in septic rats, aPC's treatment significantly decreased norepinephrine need. (c) In healthy recipients, MPs isolated from septic rats decreased MAP (−35 mmHg). Conversely, MPs from aPC treated septic rats increased MAP (+16 mmHg). (d) The hemodynamic effects of these MPs could be partly related to a significant increase in the MP thromboxane content in septic rats treated with aPC. (e) MPs modulated vascular inflammation, with the blunting of arterial activation of NF-κB, pIκB-α, COX-2 and iNOS after anticoagulant treatment. (g) Inoculation of MPs from aPC-treated septic rats pharmacologically modulates the phenotype of circulating microparticles in recipient rats, with increased platelet and endothelial MPs.
Summary/conclusions: Our septic shock model evidenced increased circulating procoagulant MPs originating from platelets but also from leucocytes and endothelial cells. aPC-induced MP modifications behave as cellular effectors conveying the anti-inflammatory message resulting in hemodynamic effects.

OC 72.2

Coagulant tissue factor in human wound blood and saliva is not associated with lipid rafts

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Background: Cell-derived vesicles expose coagulant tissue factor (TF). Whether this vesicle-associated coagulant TF is associated with lipid rafts, however, is unknown (Blood 2005; 106:1604–11).

Aim: To investigate whether coagulant TF is associated with lipid rafts in vesicles from human wound blood and saliva, and in vesicles and purified plasma membranes of cells constitutively expressing TF.
Methods: Vesicles were isolated from human wound blood, saliva and conditioned culture medium of human vascular smooth muscle cells. Vesicles were lysed in Triton X-100-containing buffer. Plasma membranes were purified by Percoll-gradient ultracentrifugation after cell disruption. Rafts were isolated by OptiPrep gradient ultracentrifugation, and fractions were analyzed for TF antigen (ELISA, Western blot), TF coagulant activity (fibrin generation assay), flotillin (planar raft marker), caveolin (caveolar raft marker) and TFPI (all Western blot).
Results: Vesicles from human wound blood contain a coagulant form and a non-coagulant form of TF. Coagulant TF is present in fractions 1–4, fractions which contain flotillin and caveolin, whereas non-coagulant TF is present in fractions 7–8, fractions containing flotillin, but in which caveolin is below the detection limit. Tissue factor pathway inhibitor was below the detection limit in all fractions. Because wound blood contains vesicles of various cellular origins (Circulation 1997; 96:3534–41), it is unclear whether TF and caveolin/flottilin originate from the same vesicles. Therefore, in saliva, which contains high levels of coagulant TF-exposing vesicles which are mainly of epithelial origin (Blood 2011; 117:3172–80), we performed similar studies. Again, isolated vesicles contain a coagulant and a non-coagulant form of TF. The coagulant form is present in fractions 3–5, fractions in which both flotillin and caveolin are absent. The non-coagulant form of TF is present in fractions 7–9, fractions which contain flotillin but lack caveolin. Because of the discrepancy in results between the vesicles from wound blood and saliva, we also investigated the TF distribution in vesicles and plasma membranes of smooth muscle cells. Vesicles and purified plasma membranes of human smooth muscle cells also contain a coagulant and a non-coagulant form of TF. The coagulant form is present in fractions 1–5 of both vesicles and plasma membranes, but these fractions lack detectable amounts of flotillin and caveolin. The non-coagulant form of TF is present in fractions 6–8, and is associated with flotillin in vesicles, and with both flotillin and caveolin in plasma membranes.
Conclusions: Our findings demonstrate that coagulant and non-coagulant TF co-exist in vesicles and plasma membranes. The coagulant form of TF is not associated with lipid rafts as defined by the presence of flotillin and/or caveolin.

OC 72.3
An improved method for quantitative and qualitative flow cytometric analysis of fluorescently labelled microvesicles

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Background: Investigations of microvesicles (MVs) as potential biomarkers by light scatter flow cytometry have been hampered by several issues including: size resolution using light scatter, differentiation between noise and biological events in small particles, separation of MVs from platelets, and lack of reproducible quantitation. Recently, Van der Vlist (Nature Protocols 7(7)) described a method that overcomes these issues using a custom flow cytometer; however this is impractical in multi-user facilities.
Aim: to adapt this method for qualitative and quantitative analysis of MVs to a commercially available light scatter-based flow cytometer.
Methods: The BD LSRFortessa with high performance photomultiplier tube small particle detector (FSC-PMT) was evaluated. Fluorescence thresholding was utilised to discriminate true events from background noise. Size resolution and quantitation limits were tested using yellow-green fluorescent beads, 100–1000 nm (FluoSperes, Invitrogen). Serial twofold dilutions (1–1024) of 200-nm beads were mixed with fixed number of 500-nm beads.
MVs from plasma, U251MG cell-culture supernatant and freeze-thawed pooled platelets were evaluated. MVs were separated from large particles and debris by sequential centrifugation. Microvesicles were stained after ultracentrifugation with membrane dye PKH67 to identify all vesicles, and fluorochrome-conjugated antibodies to identify origin of MVs (anti-CD41a-PE or anti-EGFR-B-PE). MVs were overlaid with linear sucrose gradient to separate MVs according to density and to remove unbound dye/antibodies and protein aggregates.
Quantitation was performed in 96-well plates by absolute count of events in a defined volume. Results are expressed as mean ± SD.
Results: Using the FSC-PMT and fluorescence thresholding, lower limit of detection for fluorescent nanobeads was 100 nm. 200 nm beads were clearly differentiated from 500 nm. Events measuring between 100 nm and 1000 nm by FSC-PMT were found in the gradient fractions expected to contain exosomes and microparticles with no contaminating large particles or platelets. Size distribution of MVs measured by FSC-PMT across all biological samples correlated well with predicted values based on the fraction density. Highest proportion (30–50%) of ≥ 500 nm particles was found in density range 1.23–1.28 g/mL while only 7% of particles in the 'exosome' fractions (density 1.11–1.20 g/mL) were ≥ 500 nm.
Fluorescence trigger on PKH67 signal eliminated 95% of events in the > 500 nm range compared with collection by a side scatter trigger differentiating true events from noise. This strategy allowed quantitation across the full 100–1000 nm range, including biologically active MVs > 500 nm that are normally obscured by noise.
Linear correlations with a slope of 1 were observed between the absolute number of 200-nm beads as well as the ratio of detected 200- vs. 500-nm beads and the dilution factor ($R^2 = 0.999$; $R^2 = 0.998$) indicating precise quantitation over a wide range. The absolute number of MVs in 100 μL deviated > 7% from the expected number (2 × absolute count in 50 μL) over a large concentration range 2976 ± 168–21 ± 2/μL, indicating satisfactory volumetric pump performance.
The intra- and inter-sample variations (CV) were 2.1% and 2.8% respectively. The sample carryover was 0.2%.
Summary/conclusions: Our fluorescence-based flow cytometric method for analysis of MVs utilising a commercially available platform combined with a specific sample preparation offers sensitivity, specificity and enumeration.

OC 72.4
Multicolor flow cytometry analysis of MPs in whole blood: comparison with analysis in platelet free plasma

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Background: The characterization of human circulating cell-derived Microparticles (MPs) is strictly dependent on sample preparation and analysis. For this reason the ISTH SSC have provided guidelines for standardization of the study of MP by flow cytometry, one of the most commonly used technique for their enumeration and characterization. Whether the total number and the relative abundance of the different cell-derived MPs present in plasma sample or in whole blood (WB), the most physiological 'environment', is, to the best of our knowledge, still unknown.
Aims: To characterize circulating MPs in WB, avoiding methodological manipulations, in order to have a complete picture of MPs in terms of number and phenotype.
Methods: Citrated WB from healthy volunteers ($n = 20$) was analysed with a multiparametric flow cytometry assay by using BD FACSAria II™, a four laser equipped cell sorter. Violet Proliferation Dye (VPD) was used to discriminate MPs (VPD^{dim}) from platelets (VPD^{bright}) and 7AAD to exclude apoptotic bodies. The cell origin of the different MPs was assessed by using the following antibodies: CD135 (erythrocyte), CD41 (platelet), CD144/CD31 (endothelial cell), CD45 (leukocyte), CD66 (granulocyte) and CD14 (monocyte). The procoagulant

phenotype of MPs was evaluated measuring tissue factor (TF, CD142) expression as well as assessing the presence of phosphatydilserine (PS) with Annexin V. Finally, MPs were counted by using BD Trucount tubes™. Results have been compared to those obtained with fresh Platelet Free Plasma (PFP) derived from the same donors and generated by conventional methods.

Results: WB contains a number of MPs 50-fold higher than that found in PFP (150.000 ± 40.000 vs. 3.000 ± 800 MPs/ml), evidencing that centrifugation steps induce a partial loss of them. Most of the MPs in WB originate from erythrocytes (80%), and the remaining are from endothelial cells and platelets (20%). By contrast 80% of MPs in PFP are equally distributed among those derived from erythrocytes, platelets and endothelial cells, the remaining coming from granulocytes and monocytes. The majority of MPs both in WB and in PFP are Annexin V negative (90% and 70% respectively). $TF^+/AnnexinV^+$ MPs are present in both type of samples representing 50% of the platelet-derived.

Conclusion: The analysis of MPs in WB and PFP revealed important differences between the two biological samples. The high number of erythrocyte-derived MPs found in WB emphasizes their potential physiological role in health and disease as documented by recent published studies. Similarly, the role of the Annexin V^- MPs, which represent the majority both in WB and in PFP, deserve further investigation.

OC 73 – Natural Anticoagulants

OC 73.1

The role of the different Kunitz domains of TFPI in the inhibition of TF-FVIIa catalysed FX and FIX activation

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Background: TFPI is a multi-Kunitz domain protease inhibitor that down-regulates the extrinsic coagulation pathway by inhibiting FXa and FVIIa. TFPI also blocks intrinsic FX activation by inhibiting TF-FVIIa catalysed FIX activation. Inhibition of TF-FVIIa can occur via two pathways >i.e. via direct inhibition and formation of a TF-FVIIa-TFPI complex or via inhibition of FXa by TFPI and subsequent formation of a quaternary TF-FVIIa-FXa-TFPI complex. Protein S enhances the formation of the binary FXa-TFPI complex and all three Kunitz domains (KD) of TFPI contribute to FXa and TF-FVIIa inhibition.

Aim: To investigate the role of protein S and the Kunitz domains of TFPI in the inhibition of TF-FVIIa catalysed FX and FIX activation.

Methods: Recombinant full length TFPI ($TFPI_{fl}$) and TFPI constructs (KD1, KD2, KD1-KD2 and $TFPI_{1\text{-}150}$ were purified from bacterial expression systems. Inhibition of TF-FVIIa catalysed FX- and FIX activation by TFPI (constructs) was quantified by measuring progress curves of FXa and FIXa generation using FXa- and FIXa-specific chromogenic substrates.

Results: $TFPI_{fl}$ inhibited TF-FVIIa catalyzed FIX activation with an IC_{50} of 4.9 nM. Protein S acted as co-factor of $TFPI_{fl}$ and reduced the IC_{50} value ~25-fold (0.2 nM). Truncated TFPI ($TFPI_{1\text{-}150}$ and KD1-KD2) lacking KD3 and *C*-terminus had 10-fold higher IC_{50} values and their inhibitory activity was not enhanced by protein S. Single Kunitz domains were poor TF-FVIIa inhibitors with IC_{50} values 847 nM (KD2) and 1394 nM (KD1).

FX activation was measured at 1) limiting FVIIa (1 pM) and excess TF (5 nM) and 2) limiting TF (3 pM) and excess FVIIa (0.25 nM) with/without phospholipids. Rates of FX activation at these conditions were the same. At both conditions, $TFPI_{fl}$ and truncated TFPI ($TFPI_{1\text{-}150}$ and KD1-KD2) showed similar inhibition of FX activation. However, when the phospholipid concentration was reduced by lowering TF or phospholipids, $TFPI_{fl}$ was 10–20 times more active as truncated TFPI. Single Kunitz domains were 1000-fold less active as $TFPI_{fl}$. Pre-formed FXa-$TFPI_{fl}$ or FXa-$TFPI_{1\text{-}150}$ complexes rapidly inhibited FX activation by TF-FVIIa in a stoichiometric manner. Protein S acted as co-factor of TFPI and enhanced quaternary complex formation at limited FVIIa and excess TF (5 nM). This effect was abolished by anti-protein S antibodies.

Summary and Conclusions: In the presence of protein S, $TFPI_{fl}$ is a good inhibitor of TF-FVIIa catalyzed FIX activation with an IC_{50} that is 0.2 nM which is in the range of the physiological $TFPI_{fl}$ concentration (0.2–0.5 nM). This shows that $TFPI_{fl}$ not only regulates the extrinsic coagulation pathway but also plays an important role in the inhibition of intrinsic FX activation even in the absence of FXa.

$TFPI_{fl}$ is a much better inhibitor than truncated TFPI of TF-FVIIa catalysed FX activation at low lipid concentrations. However, at high phospholipid concentrations differences between $TFPI_{fl}$ and truncated TFPI gradually disappeared, presumably because phospholipids act as a sink for $TFPI_{fl}$ but not for truncated TFPI. The different inhibitory activities of TFPI (constructs) and pre-formed complexes and the experiments with protein S indicate that binary TFPI-FXa complex formation is the limiting step in TF-FVIIa inhibition.

OC 73.2

Relevance of antithrombin A-sheet residues for the internalization of the RCL following interaction with target proteases

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Background: Antithrombin is a serpin that inhibits factor Xa (FXa) and thrombin, and to a lesser extent, other procoagulant serine proteases. The serpin inhibitory mechanism can be thought of as a race between two competing events once the protease has bound to the serpin reactive center loop (RCL) and undergone acylation: RCL incorporation into β-sheet A and deacylation of the protease. Indeed, the RCL is crucial in the first interactions with the target proteases and its full insertion into β-sheet A after its proteolytic cleavage. Under certain conditions and for certain serpin variants the rate of loop insertion relative to deacylation is decreased resulting in stoichiometries of inhibition significantly > 1, effectively rendering the serpin a substrate. To the best of our knowledge, all these variants affect RCL residues. Other residues of the serpin have been suggested that might also interfere with the RCL insertion, but the absence of crystal structures of intermediate RCL-inserted states has not allowed their identification.

Aims: To identify residues outside the RCL able to slow loop insertion and subsequent interactions with the protease.

Methods: We characterized two mutations present in subjects with type II antithrombin deficiency. Recombinant antithrombins were produced in HEK-EBNA and insect cell systems. Biochemical analysis included denaturation temperature, heparin affinity and kinetic and stoichiometric studies. The substrate behavior of these mutations also was verified by SDS gels under non-reducing conditions.

Results: The first mutation, S365L, was identified in a thrombophilic family with low anti-FXa (50%) but only moderately reduced antigen levels (75%) in carriers. The second mutation, I207T, was fortuitously identified in a young woman with low anti-FXa (66%) but nearly normal antigen values (80%). These two mutations behaved as substrates for FXa, as only cleaved antithrombin was observed when using recombinant molecules that were incubated with the protease. Both affected residues are conserved in antithrombin of different species, and located at or next to sheet A, S365 at the *N*-terminal end of s5A and I207 in the loop that connects helix F to s3A. Interestingly, both residues were very close structurally, and formed an opening for the internalized RCL to exit from the central A sheet. While the S365L

mutation also behaved as a substrate for thrombin, the I207T mutation only slightly impaired the reactivity with thrombin. Thus, the plasma anti-FIIa activity observed in the carrier was close to the antigen levels (85%). Moreover, the recombinant variant formed stable covalent inhibitory complexes with thrombin. Nevertheless, the I207T stoichiometry of thrombin inhibition was elevated, although much lower than that for FXa (~2 and > 4, respectively).
Conclusion: We identified a new domain in antithrombin, relevant for the efficient mechanism of inhibition of this anticoagulant serpin. The bottom of the central A sheet, plays a crucial role in the last steps of the RCL internalization. Mutations of these residues do not completely impair a correct folding, but slow RCL insertion, transforming the serpin into a substrate. Moreover, residues in this domain might also interact specifically with some proteases during its translocation.

OC 73.3

Low amounts of platelet TFPI and protein S are highly effective in regulating local thrombin generation which is not affected by proteolysis of protein S

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Introduction: Protein S is a cofactor for APC in the inactivation of FVa and FVIIIa, and a cofactor for TFPI in the inhibition of FXa. Protein S and TFPI are both present in platelets, and because platelets rapidly accumulate at sites of vascular injury, the platelet-dependent TFPI/protein S anticoagulant system might play an important role in regulating local hemostasis. Platelet TFPI by itself is active in the inhibition of FXa, however little is known about the cofactor contribution of platelet protein S. In addition, protein S is susceptible to platelet membrane proteases with subsequent loss of its APC-cofactor activity.
Objectives: Functional platelet TFPI/protein S release was studied to quantify and characterize the anticoagulant properties of the platelet TFPI/protein S system.
Methods: Whole blood samples were obtained from healthy volunteers after informed consent. Free TFPI and total protein S were determined by ELISA in platelet rich plasma (PRP) and in platelet isolates before and after stimulation of platelets with convulxin. In addition, fresh platelets were used to assess platelet TFPI/protein S activity using FXa and thrombin generation (TG) assays. Finally, protein S and thrombin-cleaved protein S were compared in TG-based APC sensitivity tests and FXa inhibition assays to assess the cofactor activity of cleaved protein S for APC and TFPI, respectively. Platelet count was set at 250–300 $\times$ 10^9/L in all experiments.
Results: Following convulxin stimulation of platelets in PRP, TFPI antigen levels in plasma increased significantly from 0.20 $\pm$ 0.09 to 0.27 $\pm$ 0.09 nM (p-value > 0.001). Mean TFPI and protein S antigen levels determined in supernatants from stimulated platelets were 0.11 $\pm$ 0.02 and 6.0 $\pm$ 1.0 nM, respectively. Convulxin-stimulated platelets effectively inhibited FXa generation by tissue factor/FVIIa complex (TF/FVIIa) in a reconstituted system with purified proteins. This inhibition was fully counteracted by anti-TFPI antibodies. Experiments performed in the absence and presence of anti-protein S antibodies showed that platelet protein S enhanced the anticoagulant function of platelet TFPI 3-fold. Reconstitution of TFPI/protein S-depleted plasma with platelets and subsequent TG showed that neutralization of platelet TFPI by anti-TFPI antibodies increased the thrombin peak height measured at low TF concentration (0.25 pM) by 70%. More than half of the TG inhibitory capacity of TFPI was contributed to the cofactor activity of protein S for TFPI. As protein S is readily cleaved by platelet proteases, the ability of cleaved protein S to act as a cofactor for TFPI was investigated. It was observed that the TFPI cofactor activity of cleaved protein S was fully retained, in contrast to its APC cofactor activity, which was completely abolished.
Conclusion: TFPI and protein S are released from stimulated platelets and potently inhibit FXa and TG. Although platelets contain > 2% of plasma protein S, platelet protein S plays a crucial role in TFPI-mediated FXa inhibition. As platelets localize at sites of vascular injury, the platelet-dependent TFPI/protein S system is likely to play an important role in modulating procoagulant activity at the growing thrombus.

OC 73.4

The serpin Protein C Inhibitor (PCI) interacts with phosphoinositides (PIs) and modulates the activity of the phosphoinositide-specific phosphatase SHIP2 in vitro

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Background: The serpin-type protease inhibitor protein C inhibitor (PCI) circulates at low levels in blood plasma. In addition to its various protease interaction partners, PCI is stimulated by non-protease ligands like heparin, DNA or phospholipids. It was shown that oxidized phosphatidylethanolamine (PE) and unoxidized as well as oxidized phosphatidylserine bind to PCI and stimulate the inhibition of different target proteases in a heparin like manner. Apart from the modulatory effect of phospholipids, it was shown that PCI acts as a lipid transferase mediating the incorporation of PE into cellular membranes. Furthermore PE supports the internalization of PCI itself, and the internalized serpin is detected in different cellular fractions including the nucleus.
Aims: The lipid binding property of internalized PCI might contribute to additional regulatory functions, influencing lipid signaling inside the cell. Therefore this study was performed to investigate the probable interaction of PCI with intracellular phosphoinositides (PIs) and to analyze the potential functional consequences.
Methods: To study the potential binding of PCI to PIs, commercially available protein overlay assays were used. The interaction of PCI with phospholipids that showed positive signals in dot blot assays were analyzed by native PAGE, Western blotting and ELISA. The influence of PCI on the phosphorylation status of AKT was studied in human cancer cells HEK293 as well as in primary HUVECs. Furthermore PIs are rapidly interconverted and serve as substrates for a variety of PI-specific enzymes like kinases, phosphatases or lipases. So the influence of preincubation of soluble lipid substrate with PCI on the activity of the PI-specific 5-phosphatase SHIP2 was analyzed.
Results: Dot blot analysis showed a specific binding pattern of PCI towards PIs. All mono- and bisphosphorylated PIs as well as phosphatidylinositol-3,4,5-trisphosphate bound to PCI. Furthermore PCI incubated with unsaturated phosphatidylinositol-4,5-bisphosphate exhibited different electrophoretic mobility of PCI antigen on native gels. As specific phospholipids modulate the inhibition of different proteases by PCI, we could show that also monophosphorylated PIs slightly stimulated APC inhibition by PCI. Some A-clade serpins like kallistatin or vaspin activate protein kinase B (AKT). Here we showed that the overexpression of PCI in HEK293 cells as well as the addition of PCI to the medium of primary HUVECs also led to an increase in AKT phosphorylation compared to control. Furthermore PCI stimulated the activity of the PI-specific phosphatase SHIP2 in vitro. The results showed that low concentrations of PCI had a stimulatory effect on the activity of SHIP2 while high concentrations did not affect the removal of the 5-phosphate from the inositol headgroup anymore.
Conclusion: We conclude that PCI is an additional intracellular interaction partner of PIs and affects intracellular lipid signaling. PCI stimulates the activity of the PI-specific phosphatase SHIP2 in vitro indicating that PCI might affect insulin signaling by modulating PI-mediated signaling pathways. In future experiments we will study the influence of PCI on intracellular PI-levels, the generation of IP_3 and diacylglycerol and its possible role in calcium signaling and protein kinase activation.

OC 74 – Novel Approaches in Vascular Biology

OC 74.1

Neutrophil histone modification by peptidylarginine deiminase 4 is crucial for deep vein thrombosis in mice

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Background: Neutrophil extracellular trap (NET) formation is an active cell death pathway in neutrophils, involving chromatin decondensation, nuclear membrane dissolution, and expulsion of chromatin lined with granule proteins into the extracellular environment. Recently, NETs were shown to be involved in thrombosis by promoting coagulation and platelet adhesion and are present in animal models of deep vein thrombosis (DVT). A critical step in NETosis is nuclear chromatin decondensation. Peptidylarginine deiminase 4 (PAD4) is an enzyme that converts specific arginine residues to citrulline on histone tails. Upon PAD4 activation, histones can become hypercitrullinated, resulting in the extensive chromatin decondensation that leads to nuclear swelling during NETosis.

Aims: Whether NETs are involved in the pathogenesis of DVT or whether they are merely a consequence of neutrophil recruitment to the thrombus is unknown. We wished to establish that PAD4$^{-/-}$ mice would not produce NETs upon venous stenosis and examine whether this may impact venous thrombosis.

Methods: We performed the inferior vena cava stenosis model of DVT in wild-type and PAD4$^{-/-}$ mice. Ability to form platelet plugs after injury was assessed using tail tip resection and $FeCl_3$-induced thrombosis in mesenteric venules. Functionality of PAD4$^{-/-}$ leukocytes and endothelial activation were evaluated by intravital microscopy.

Results: PAD4$^{-/-}$ mice were partially protected from producing venous thrombi early after stenosis, with only 28.6% of PAD4$^{-/-}$ mice forming thrombi at 6 h compared to 66.7% of wild-type (WT) mice ($P > 0.05$). To see if this early protection could be a result of delayed thrombus formation in PAD4$^{-/-}$ mice, we next maintained stenosis for 48 h and found that the protective phenotype became stronger. While 90% of WT stenotic vessels thrombosed, fewer than 10% of PAD4$^{-/-}$ mice had a thrombus at this time point ($P > 0.001$). Neutrophils were abundantly present in thrombi formed in both groups, while extracellular citrullinated histones (H3Cit) were seen only in thrombi from WT mice. Bone marrow chimera experiments indicated that PAD4 in hematopoietic cells was the source of the prothrombotic effect in DVT. Thrombosis could be rescued by infusion of wild-type neutrophils suggesting that neutrophil PAD4 was important and sufficient. After WT neutrophil infusion, extracellular H3Cit staining was present within both WT and PAD4$^{-/-}$ recipient thrombi, indicating that the WT neutrophils were incorporated and produced NETs in the thrombus scaffold in PAD4$^{-/-}$ recipient mice. Leukocyte adhesion and platelet aggregation were normal in PAD4$^{-/-}$ mice, as was hemostatic potential determined by bleeding time and platelet plug formation after $FeCl_3$-induced venous injury.

Conclusion: NETs comprise a crucial part of the pathologic thrombus scaffold, and the lack of NETs formation results in fewer thrombi early on which appear not to be sustained over time. The protection in PAD4$^{-/-}$ mice from DVT is likely due to the failure of neutrophils to form NETs as PAD4$^{-/-}$ neutrophils interact properly with the vessel wall and are present in the rare thrombi that form in PAD4$^{-/-}$ veins. Here we report that PAD4-mediated chromatin decondensation in the neutrophil is crucial for pathological venous thrombosis and unveil neutrophil activation and PAD4 as potential drug targets for deep vein thrombosis.

OC 74.2

Flow driven self-assembly of macroscopic proteins in microvessels

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Background: Thrombotic microangiopathy features a group of life-threatening disorders with characteristics of endothelial activation and microvascular thrombosis. Activated endothelial cells release von Willebrand factor (VWF), which can form long strands under flow and initiate microvascular thrombosis if not cleaved by metalloprotease ADAMTS13. Existing studies of thrombotic microangiopathy are limited in either animal models, which cannot distinguish individual contributions of various components of the blood and vessel walls, or flow chambers, which do not recapitulate the complex vessel geometry or flow.

Aim: We developed microvessels *in vitro* with different geometry and flow patterns to examine the formation of VWF strands and induced thrombi from the endothelium of stimulated vessels.

Methods: Soft lithographic method was used to generate microvessels with diameter of 30–1000 μm in collagen gel, and with bends or bifurcations along flow direction. After 7 days of culture under flow, the microvessels were treated with phorbol myristate acetate (PMA), shiga-like toxin-2, histamine, or TNF-a for 30 min, followed by perfusion of circulating VWF or whole blood. The adhesion of blood cells were monitored under fluorescence microscope. The microvessels were then washed, fixed, immunostained, and imaged under confocal microscope to 1) map the size and pattern of VWF solely from the endothelium, and 2) characterize the deposited fluid-phase VWF and blood cells with respect to vessel geometry and flow shear.

Results: VWF presented as fibers and web-like structures in agonist-treated microvessels, and their location and structure depend on vessel geometry and flow pattern. In microvessels with diameter of 500 μm or larger, VWF fibers attached tightly on the endothelial surfaces and followed the flow direction. In microvessels of 50–200 μm, VWF fibers extended across the vessel lumen and reached the opposite end of vessel walls at bends or bifurcations. Secondary flow at bends triggered the circular clump of VWF, and led to thick and continuous VWF fibers of 5 cm long through the center stream of vessel lumen. In microvessels smaller than 50 μm, VWF strands formed webs and partially blocked the flow in the microvessels, even in the absence of platelets. The structure and size of the VWF fibers also depended on the agonist employed on the endothelium. PMA and shigatoxin both produced thicker fibers than histamine did, and these were more resistant to ADAMTS13 cleavage.

When recombinant VWF was perfused over stimulated microvessels, the fluid-phase VWF associated with the vessel-bound fibers and further thickened them. When whole blood perfused into stimulated vessels, the transluminal VWF fibers caught flowing platelets and leukocytes to form aggregates in the middle of blood stream that sometimes occluded the vessels. The region of such occlusion also depends on vessel geometry.

Summary: Our data show that VWF secreted from activated endothelial cells can form transluminal fibers and webs in small vessels. Some VWF webs alone may occlude small vessels, some obstruct blood flow by binding to circulating platelets and leukocytes, and may also shred erythrocytes as they flow past. These findings provide insights for different characteristic signs of thrombotic microangiopathy.

OC 74.3

In vivo molecular ultrasound imaging for monitoring and efficacy testing of thrombolytic drugs using platelet-targeted microbubbles

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Introduction: Molecular ultrasound imaging offers a non-invasive technology widely available for rapid clinical diagnosis. We tested whether microbubbles (MBs), which are selectively targeted to activated platelets, provide a high-resolution, real-time imaging of thrombosis and monitoring of thrombolysis. We used this approach to evaluate a platelet targeted urokinase plasminogen activator (targ-scuPA) that is hypothesized to offer anti-thrombolytic potency without bleeding complications.

Methods and Results: MBs were conjugated to a single-chain antibody specific for an epitope called *L*igand *I*nduced *B*inding *S*ite on activated GPIIb/IIIa (LIBS-MB). LIBS-MBs strongly adhered to immobilized activated platelets and micro-thrombi under flow. Carotid artery thrombi in mice, induced by ferric chloride, were assessed with ultrasound before and after MB injection. Analysis of the thrombus area demonstrated a significant increase in decibel after LIBS-MB but not after MB injection ($P > 0.01$). After thrombolysis with 500 U/g BW of commercial urokinase (commUPA), LIBS-MB ultrasound imaging allows monitoring of the reduction in thrombus size ($P > 0.001$). Similar results were obtained when comparing the size to grayscale intensity reduction. In addition, 75 U/g BW of targ-scuPA is sufficient for thrombolysis, whereas 75 U/g BW of commUPA or non-targ-scuPA are not ($P > 0.01$). 500 U/g BW of commUPA, the concentration required to match the effectiveness of 75 U/g BW of targ-scuPA, resulted in prolonged tail bleeding time, whereas no increase in bleeding was observed when the equally effective but lower dose of 75 U/g BW scuPA ($P > 0.001$).

Conclusion: We are able to demonstrate that our targeted MB specifically bind to activated platelets enabling real-time molecular ultrasound imaging of thrombosis and monitoring of success or failure of thrombolysis in vivo. In an exemplary application a highly promising clot-targeted thrombolytic drug was shown to provide effective thrombolytic potential without compromising haemostasis.

OC 74.4

Cell painting with an engineered membrane-anchoring endothelial protein C receptor (EPCR) improves protein C activation and protease activated receptor-1 and -3 cleavage on EPCR-deprived cells

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The protein C pathway conveys beneficial antithrombotic and cytoprotective effects in numerous in vivo disease models, including ischemic stroke, thrombosis, and inflammatory diseases such as sepsis. The endothelial protein C receptor (EPCR) plays a central role in the protein C pathway, by acting as a cofactor with thrombomodulin in the activation of protein C on endothelial cells and by acting as a cofactor for activated protein C (APC)-mediated activation of protease-activated receptors (PAR) 1 and 3 that are required for APC's cytoprotective activities. Functional EPCR on endothelial cells is diminished during disease due to shedding of EPCR induced by proinflammatory cytokines and EPCR encryption induced by TNFα and secreted phospholipase A_2. This suggests that the bioavailability of EPCR may limit the efficacy of the protein C pathway's antithrombotic and cytoprotective effects. Consistent with this concept, $EPCR^{low}$ mice (expressing > 10% EPCR) challenged with endotoxin displayed profound plasma tissue extravasation, lung injury, severe renal hemorrhage and albuminuria compared to wild type mice. Thus, restoring EPCR's bioavailability via 'cell painting' with membrane-anchored EPCR could improve the efficacy of the protein C pathway beneficial effects and restore resistance to inflammatory disease.

To accommodate EPCR-restitution on the surface without a need for gene transfer, a glycosyl-phosphatidylinositol (GPI)-anchored derivative of EPCR was engineered. EPCR-shRNA stable transfected EA.hy926 endothelial cells ($EPCR^{KD}$) expressed > 25% EPCR and were defective in protein C activation, APC binding, and APC-mediated activation of PARs. Painting efficiency of EPCR-GPI on $EPCR^{KD}$ cells was time- and dose-dependent, and reached a plateau of 400% of wild type cells after 120 min. After removal of EPCR-GPI from the cells, there was a rapid decrease of surface EPCR during the first hour to approximately 250%, whereas after 24 h surface EPCR on painted $EPCR^{KD}$ cells was still 200% compared to wild-type cells. Thus, cell painting with GPI-anchored EPCR provided a long-lasting method to restore EPCR on the surface of endothelial cells. EPCR-GPI painting of $EPCR^{KD}$ cells also restored EPCR functionality. No appreciable APC binding was detected on $EPCR^{KD}$ cells but after EPCR-GPI painting, APC binding to the endothelial surface was normalized and the affinity for APC binding to EPCR was similar for EPCR-GPI compared to wild-type EPCR. EPCR-GPI painting also normalized endothelial PC activation. Knockdown of EPCR reduced PC activation on the endothelial cell surface with approximately 45%, while membrane-anchored EPCR completely normalized PC activation on $EPCR^{KD}$ cells. Finally, EPCR-GPI painting also improved APC's cytoprotective activities as EPCR-GPI painted on HEK293 cells expressing *N*-terminal-SEAP-PAR constructs permitted cleavage of PAR1 and PAR3.

In summary, our results suggest that cell painting with an engineered membrane-anchoring EPCR-GPI can be used to attain supra-normal EPCR levels on the endothelial cell surface, to restore APC binding to EPCR-deprived cells, and to enhance APC's anti-thrombotic and cytoprotective effects. Thus, EPCR-GPI provides a novel tool for both in vitro and in vivo cellular engineering approaches of both EPCR and APC to characterize, understand, and improve the beneficial effects of APC therapy and EPCR functional bioavailability on cells.

OC 75 – Pediatric Thrombosis

OC 75.1

Chylothorax in children with congenital heart disease: incidence of upper venous thrombosis

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Postoperative chylothorax is a frequently encountered pathology occurring in up to 4% of patients undergoing surgery for repair of congenital heart disease.The continuous loss of chyle is a challenging problem to treat and is associated with increased morbidity and mortality. It has been associated with prolonged ventilator dependence, increased length of hospital stay and associated nosocomial infections, malnutrition and central venous line related thrombosis.

Factors which increase patient risk for thrombosis include the presence of a central venous line, central venous hypertension in addition to an overall increased risk for thrombosis in children with congenital heart disease due to acquired coagulopathies. Symptomatic thrombosis is associated with chylothorax and may contribute to its severity and duration. Furthermore, vessel thrombosis resulting in persistent vessel occlusion may impede future treatments, diagnostic studies and cardio-surgical interventions. One retrospective review of 30 pediatric patients with chylothorax demonstrated that 26.7 percent of patients with chylothorax were symptomatic for thrombosis and confirmed by ultrasound. However, screening of all patients diagnosed with chylo-

thorax for VTE is necessary to understand the true incidence of thrombosis and its association with chylothorax.

Aim: To determine the incidence of upper system thrombosis in children with congenital heart disease diagnosed with chylothorax.

Methods: Stollery Children's Hospital, University of Alberta is one of two pediatric cardio-surgical centres for children across Western Canada. All pediatric patients with confirmed with chylothorax (fulfilling 3/4 criteria: white blood cell count > 1000 10^9/L, lymphocytes > 80%, triglycerides > 1.1 mM, and chest tube losses > 10 ml/kg/day for > 3 days) undergo ultrasound of the upper venous system as per hospital routine care. This cohort study enrolled all children between February 1, 2010-August 2012, post cardiac surgery with confirmed chylothorax. Data collected included variables that are reported to predict the development of chylothorax and potential thrombosis ARISTOTLE scores were calculated using underlying cardiac diagnosis and type of cardiac surgical procedure. This study was approved by the University of Alberta Health Ethics Board.

Results: One thousand three hundred and ninety-six cardio-surgical procedures with 760 undergoing cardiopulmonary bypass. Chylothorax occurred in 54 of 1396, 3.9% (95%CI 3.0; 5.0) procedures in all children, with 48/760, 6.3% (95%CI 4.8; 8.3) and 6/636, 0.9% (95%CI 0.4; 2.0) in the presence or absence of CPB, respectively. 28/54 children with chylothorax had thrombosis, 54% in the right internal jugular vein. Children with chylothorax and thrombosis had a significantly longer PICU stay, a longer CVL indwell time, and a higher CVP than those without thrombosis.

Conclusions: This is the first study to diagnostically assess children with confirmed chylothorax for the presence of upper venous system thrombosis. The incidence of thrombosis in this cohort is 51.8% compared with 26.7% reported by McCulloch et al. who described symptomatic thrombosis. The contribution of upper venous system thrombosis to chylothorax is unknown. Approaches to therapy either treatment of confirmed thrombosis or prevention of thrombosis in patients with chylothorax require formal evaluation.

OC 75.2

EmPoWarMent: five year evaluation of patient self management of vitamin K antagonist therapy

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Background: Close and frequent INR monitoring is required in children as VKAs are narrow therapeutic index drugs and in children are exceptionally difficult to manage. Time in target therapeutic range (TTR) is commonly used as a surrogate measure for safety and efficacy of VKA therapy. Capillary point of care (POC) International Normalized Ratio (INR) measurement is a feasible method of INR testing and is associated with non-inferior warfarin / INR control when compared to traditional approaches. With patient self-management (PSM) the patient takes an independent role in performing warfarin dose adjustments after receiving additional VKA education and training. However, KIDCLOT is available as a resource if necessary. Adult studies and one small randomized pediatric study demonstrate that PSM is safe and effective with improved adherence. However studies have only evaluated PSM over a period of > 12 months and no study has evaluated the sustainability of PSM over time.

Aims: To determine TTR, testing frequency, adherence to VKA dosing guideline, patient knowledge of VKAT for children and young adults performing long term PSM (1–5 years).

Methods: This prospective longitudinal comparative cohort enrolled all children and teens prescribed VKAT followed by the KIDCLOT© program, Stollery Children's Hospital. Patients performing PST for > 3 m were invited to participate in PSM. INRs were collected from PSM and PST groups and used to calculate TTR, frequency of INR testing frequency, and testing adherence (did they check INR per guideline). A validated knowledge test was completed by patients year one and will be again at study end (Spring 2013) and scores compared. Patients performing self-testing with the health provider adjusting doses was used a control for TTR, testing frequency, knowledge. The study was ethics approved and consents obtained.

Results: One hundred and twenty patients (median age 8 years, range 1–16 years) were enrolled in the study with 60 children and their families in the PSM group. Patient enrollment was evenly distributed over the years 2008–2012 for both groups with all patients performing PSM for > 1 year. There was no difference in mean age and underlying cardiac conditions necessitating VKAT between groups. There were 1385 (PSM) and 1339(PST) INR tests performed. TTR was 87% (PSM) and 80% (PST) ($P = 0.03$) and mean testing frequency was 2.0 (PSM) / and 2.2 (PST) weeks. Independent adherence was 90% in the PSM group. The KIDCLOT© health provider received 85/1365 calls for support from PSM patients. VKA knowledge and testing competency data is currently underway and will be reported. PSM results were not significantly different than previously reported for patients performing PSM for ≤ 1 year. There were no adverse events or hospital admissions related to VKAT in either group.

Conclusions: PSM for children may be a safe and effective management strategy for warfarinized children and appears sustainable over time. In addition, the PSM method is a process which relies on the application of the VKAT education/training, further integration of this knowledge into practical translation into their daily life and thus empowers for the future.

OC 75.3

Thrombosis in children: further evaluation of incidence and resolution

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Thrombosis in children most often occurs in children with life-threatening health conditions. The incidence of thrombosis in children is reported to be highest among infants ≤ 1 year of age and is associated with severe sequalae including loss of venous access. Thrombus resolution has not been adequately explored in children and is reported to be only 22% among adults. In these children central vascular access is essential to their survival. Consequently, anticoagulation is commonly used to prevent extension thereby allowing fibrinolysis. Determination of thrombus resolution may influence duration of therapy.

Aims: To evaluate the resolution of symptomatic line related thrombosis in children.

Methods: This single centre, quaternary care children's hospital, retrospective study included all pediatric patients objectively diagnosed with arterial or venous thrombosis from 2003 to 2012. Children with thrombosis, and available for follow-up, underwent repeat imaging to evaluate thrombus resolution at 2, 4 and 12 weeks post diagnosis. Intracerebral and intracardiac thrombi were excluded. Thrombosis was treated in 91% of this population. For analysis, children were divided into age groups consistent with developmental hemostasis (≤ 3 months 3.1–12 m, 1–5 years, 6–18 years). Thrombotic events were further subdivided into arterial and venous. Resolution was evaluated through objective diagnostic imaging to determine when complete resolution (CR) occurred. Medical records were reviewed to determine patient characteristics, first thrombotic event, and degree of resolution when available. Ethics approval was obtained.

Results: Five hundred and four children were diagnosed with arterial or venous thrombosis. 284(56%) were male. 234 (46%) had congenital heart disease. 232 (46%) infants were ≤ 3 months of age, 97 (19.2%) were aged > 3–12 months of age, 90 (17%) and 85 (17%) 1–5 years and ≥ 5–18 years respectively. Follow up imaging was available in 78% of all children. In infants ≤ 3 months of age full resolution occurred in 42/63(67%) and 57/117(49%) for arterial and venous thrombosis. Of

those, CR at 2, 4, 12 weeks was 14/42 (33%), 27/42(64%), 42/42 (100%) and 17/57 (30%), 29/57(51%), 52/57(91%), for arterial and venous thrombi respectively. In infants, > 3–12 months CR occurred in 18/22(82%) and 27/55(49%), for arterial and venous, respectively. Of those, CR at 2, 4, 12 weeks was 9/18(50%), 13/18(72%), 18/18 (100%) and 8/55 (15%), 12/55 (22%), 24/55 (44%) for arterial and venous thrombi, respectively. CR was not different between 1–5 and 6–17 years and was therefore combined. Of those, CR occurred in 15/21 (71%) and 63/116 (54%) by 12 weeks 14/15 (93%) and 49/63 (77%) for arterial and venous thrombosis, respectively.

Summary: This is the first report which defines the population with the highest incidence of thrombosis as infants' ≤ 3 months of age. Unlike older children and adults, CR occurred at 4 weeks for 64% and 41% of arterial and venous thrombosis for infants' ≤ 3 months, respectively. CR occurred at 4 weeks in 72% of arterial thrombi in infants' ≤ 12 months. Given this incidence of CR of thrombi at 4 weeks, imaging at 4 weeks should be considered if anticoagulation is ongoing. These findings validate the need for studies evaluating thromboprophylaxis. Well designed studies evaluating duration of therapy of are urgently needed.

OC 75.4

Traumatic lumbar punctures in children receiving low-molecular-weight heparin during treatment for acute lymphoblastic leukemia or lymphoma

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Background: Children with acute lymphoblastic leukemia (ALL) and lymphoma undergo numerous diagnostic and therapeutic lumbar punctures (LP). Traumatic LPs have decreased diagnostic value and may worsen clinical outcomes. Spinal hematoma is a rare complication, but can result in permanent neurologic injury. The risk of a traumatic LP and spinal hematoma may be increased in children who are on anticoagulation therapy after a treatment-related thrombotic event, but there is a paucity of data regarding optimal management of peri-procedural anticoagulation in children with leukemia or lymphoma undergoing LP.

Aims: To determine if peri-procedural anticoagulation utilizing a standard protocol increases the risk of traumatic LP or spinal hematoma in children undergoing treatment for ALL or lymphoma.

Methods: Following institutional ethics committee approval, the medical records of children younger than 19 years of age who were treated for ALL or lymphoma from 1/2004 through 10/2012 and who had a thrombosis treated with LMWH were reviewed. Standard institutional practice included holding low-molecular-weight heparin (LMWH) 24 h prior to LP, then resuming LMWH 24 h following the procedure. A traumatic LP was defined as at least 10 red blood cells per microliter of cerebrospinal fluid (CSF). Associations between categorical variables were evaluated via Fishers Exact test. Because multiple LPs per individual were analyzed, we accounted for the correlated nature of data via a statistical model with a binary response variable utilizing a binomial distribution (generalized estimating equation [GEE] model with PROC GENMOD in SAS v 9.2).

Results: A total of 139 children underwent 1794 LPs with valid CSF analysis, of which 19.0% (341/1794) were traumatic. Children were on therapeutic anticoagulation around the time of LP in 22.2% (398/1794) of the procedures. Traumatic LPs occurred in 12.1% (48/398) of procedures during LMWH therapy compared to 21.0% (293/1396) of procedures while not on anticoagulation ($P > 0.001$). After controlling for pre-procedural platelet count, age and accounting for intra-patient correlation, the incidence of traumatic LPs was still lower during LMWH therapy vs. LPs performed while patients were not on anticoagulation ($P = 0.02$). No spinal hematomas occurred in 398 LPs performed during LMWH therapy or in the 1396 LPs performed without anticoagulation.

Conclusions: In children with ALL or lymphoma, suspension of LMWH 24 h prior to and following lumbar punctures was safe. Adhering to this practice, children receiving therapeutic LMWH had no greater risk of traumatic LP or spinal hematoma compared to children not receiving anticoagulation. Whether a shorter interval without LMWH would be equally safe requires further study.

OC 76 – Platelet Signalling – II

OC 76.1

14-3-3? regulates the procoagulant function of platelets

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Background: The 14-3-3 family of signalling adaptor proteins regulate various important cellular functions including cell signalling, cell-cycle, proliferation and apoptosis through their interaction with proteins containing phosphoserine/threonine binding motifs. Numerous 14-3-3 family members have been identified in platelets, including the abundant 14-3-3ζ isoform, which has previously been implicated in regulating the adhesive and signalling function of Glycoprotein (GP)Ibα.

Aim: To investigate the role of 14-3-3ζ in regulating the haemostatic and prothrombotic function of platelets.

Methods: We have generated 14-3-3ζ-deficient mice and performed various platelet function studies in vitro, including platelet adhesion to von Willebrand factor (VWF), thrombus formation on collagen-coated surface and platelet aggregation. Also, we measured P-selectin expression, GPIIb-IIIa activation and phosphatidylserine (PS) exposure by flow cytometry following platelet stimulation by various agonists. Furthermore, we determined platelet life span by post-injection of sulpho-NHS biotin and quantified% CD41 & streptavidin-positive events after flow cytometry. To study arterial thrombosis in vivo, electrolytic injury of the carotid artery was induced in mice. Experiments were performed on 14-3-3ζ-deficient and Wild-type (WT) mice reconstituted with the bone marrow from WT and 14-3-3ζ-deficient mice, respectively. Additionally, venous thrombosis was investigated in the pulmonary embolism model, in which collagen plus epinephrine was injected intravenously into mice.

Results: Using a 14-3-3ζ-null mouse model, we have identified an important role for 14-3-3ζ in regulating the haemostatic and prothrombotic function of platelets in vivo. Unexpectantly, 14-3-3$\zeta^{-/-}$ platelets had no defects in GPIbα-mediated adhesion to VWF or thrombus formation at high shear on a collagen substrate in vitro. Also, the life span of platelets was unaffected by the deficiency in 14-3-3ζ. 14-3-3$\zeta^{-/-}$ platelets had subtle defects in platelet aggregation, P-selectin expression and integrin GPIIb-IIIaactivation by threshold concentrations of thrombin or PAR-4 peptide. However, platelet aggregation by ADP and collagen-related peptide (CRP) was unaffected by 14-3-3ζ deficiency. A major functional defect in 14-3-3$\zeta^{-/-}$ platelets was found in PS-exposure following stimulation with high concentrations of a combination of two potent platelet agonists, thrombin and CRP. This defect in agonist-induced PS-exposure was associated with decreased thrombin generation in vivo following collagen/epinephrine injection in 14-3-3ζ-null mice, leading to reduced pulmonary emboli and improved survival. 14-3-3ζ-deficient mice were also protected from arterial thrombotic occlusion following electrolytic injury to the carotid artery. This phenotype was reproduced in WT mice reconstituted with 14-3-3ζ-deficient haematopoietic cells, indicating that the thrombosis defect was likely to be related to altered platelet function.

Conclusion: These studies define an unexpected role for 14-3-3ζ in regulating the procoagulant function of platelets necessary for haemostasis and thrombosis.

OC 76.2

Kindlin-2 in platelets and in megakaryocytes: a possible role in β integrin activation

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Background: The kindlin family plays a crucial role by interacting with β subunits of integrins allowing conformational changes. The kindlin family is composed of three proteins, kindlin-1, -2, -3. Their importance in development and normal physiology is well established: kindlin-1 deficiency causes skin blistering (Kindler syndrome) and kindlin-3 deficiency causes the Leucocyte Adhesion Deficiency type III (LAD-III). The importance of kindlin-2 in adults is unknown since knockout mice die at early stage of embryogenesis most probably due to impaired cardiac development. The expression of kindlins was initially thought to be restricted to specific cell types and tissues. However, different kindlins have been described in the same cell type where they each have a specific function and cannot substitute for each other. In the hematopoietic lineage and more precisely in platelets and megakaryocytes, kindlin-3 is the predominant form. So far, kindlin-1 has not been described in platelets and evidence for the presence of kindlin-2 is limited.

Aim: To examine the presence and to determine the specific role of kindlin-2 in platelets and megakaryocytes.

Methods and Results: We first explored the presence of the three kindlins in platelets by western blot. As expected, kindlin-3 was found in platelets but to our surprise significant amounts of kindlin-2 was also detected. No expression of kindlin-1 was observed. To further confirm the presence of kindlin-2 and to avoid false positive due to cross reactivity of the kindlin antibodies, we analyzed platelet samples from a kindlin-3 deficient LAD-III patient. As expected, no kindlin-3 was detected whereas kindlin-2 was expressed as in control subjects. To test whether kindlin-2 in platelets could arise from synthesis in their precursors, we analyzed kindlin proteins and mRNA expression in three megakaryocytes cell lines (HEL, Meg-01 and CMK) after PMA-induced differentiation. Kindlin-2 and -3 mRNA and protein showed time-dependent increases as the cells differentiated. In contrast kindlin-1 was never detected.

To explore the subcellular localization of kindlin-2, permeabilized platelets and megakaryocytes cell lines adhering to fibrinogen were labeled with anti-kindlin-2 antibody. In undifferentiated megakaryocytes, kindlin-2 showed a diffuse cytoplasmic and nuclear localization. However, in differentiated cells, kindlin-2 appeared concentrated in focal adhesion plaques. The same kindlin-2 localization was observed in activated platelets. This suggests that upon activation, kindlin-2 most probably concentrate in areas where integrins are interacting with the fibrinogen matrix.

Using confocal imaging, we examined more closely whether kindlin-2 co-localized with integrins. Results obtained with fibrinogen-adhering Meg-01 showed that kindlin-2 indeed co-localize with the integrin ß3 subunit. This argues in favor of a possible role for kindlin-2 in either inside-out or outside-in signaling.

Conclusion: We have confirmed the presence of kindlin-2 in platelets and their precursors with an increased expression along megakaryocytic differentiation. We observed that upon cellular differentiation, kindlin-2 localizes in areas containing the integrin β subunit. To further investigate the precise function of kindlin-2 in platelet and megakaryocytes integrin activation, experiments of kindlin-2 overexpression and shRNA silencing are in progress.

OC 76.3

Protein kinase C mediates Rap1-dependent platelet aggregation in the absence of CalDAG-GEFI and P2Y12

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Background: The small-GTPase Rap1 is a crucial mediator of platelet activation. In previous studies we have demonstrated that Rap1-dependent platelet activation is controlled by two kinetically distinct pathways: a fast but reversible calcium- and CalDAG-GEFI (CDGI, RasGRP2)-dependent pathway and a slower but more sustained pathway that requires protein kinase C (PKC)-dependent granule release and signaling through the Gi-coupled ADP receptor, P2Y12. Simultaneous inactivation of CDGI and P2Y12 in mice completely blocked thrombus formation at sites of vascular injury and abolished Rap1-dependent integrin activation in platelets stimulated by various agonists *in vitro*. Aggregation of platelets lacking both CDGI and P2Y12 was only observed in samples stimulated with high doses of thrombin or PAR4 receptor activating peptide.

Aim: In this study we sought to determine if CalDAG-GEFIII (CDGIII, RasGRP3), the only other Rap1-GEF of the CalDAG-GEF family expressed in platelets, and/or PKCs can activate Rap1 independently of CDGI and P2Y12/Gi signaling.

Methods: To dissect the pathways upstream of Rap1, pharmacological inhibitors were used in combination with genetically engineered mouse models. Integrin activation and granule secretion (surface P-selectin) were assayed by flow cytometry and standard aggregometry. To compare the kinetics of Rap1 and integrin activation, washed platelets were stimulated in stirring conditions and Rap1 GTP-loading was measured by pull-down assay directly from the aggregometry samples.

Results: Stimulation with high doses of Par4-activating peptide led to delayed Rap1 signaling, integrin activation, and granule release in platelets from mice lacking both CDGI and P2Y12 (CDGI/P2Y12-DKO). All responses were not affected in platelets deficient in CDGIII. Furthermore, Rap1-dependent platelet activation was not significantly different in PAR4p-activated CDGI/III-DKO platelets when compared to CDGI−/− platelets, both in the presence or absence of the P2Y12 inhibitor 2-MeSAMP. Thus, CDGIII does not regulate Rap1 activation in platelets. In contrast, inhibition of PKC signaling (pan-PKC inhibitor Ro-31-8220) completely abolished Rap1 and integrin activation in PAR4p-activated CDGI−/− or CDGI/P2Y12-DKO platelets. Consistently, stimulation with the diacylglycerol-mimetic PMA, a direct activator of PKCs, led to delayed aggregation and Rap1 activation in CDGI/P2Y12-DKO platelets. Importantly, CDGI/P2Y12-DKO mice were only partially protected from thromboplastin-induced pulmonary thromboembolism, suggesting that this PKC-dependent third pathway of Rap1 activation may have patho-physiological significance.

Conclusions: In summary, we demonstrate a direct role for PKC, but not CDGIII, in the activation of Rap1 in platelets. This new pathway signals with slow kinetics and requires markedly higher agonist concentrations than CDGI- or P2Y12-dependent Rap1 activation, suggesting that it functions in patho-physiological situations other than platelet adhesion and aggregation at sites of vascular injury.

OC 76.4

Grb2 is essential for (hem)ITAM-mediated signalling in platelets

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Background: Platelet activation by GPVI is a prerequisite for platelet adhesion to collagen and the LAT signalosome, composed of different

signalling and adapter proteins, plays an important role in mediating this activation. Growth factor receptor-bound protein 2 (Grb2) is an ubiquitously expressed adapter protein and plays a prominent role in signalling processes of numerous receptors in different cell types by either amplifying or inhibiting signal transduction. Based on *in vitro* studies it has been proposed that the Grb2/SOS/MEK pathway is of central importance for megakaryocyte (MK) differentiation. In platelets, Grb2 is part of the LAT signalosome and becomes tyrosine-phosphorylated after GPVI ligation, indicating a possible role of the adapter in GPVI signalling. However, the exact role of Grb2 in platelet production and function has remained elusive.

Aims: We tested the hypothesis that Grb2 is an essential adapter protein in (hem) immunoreceptor tyrosine-based activation motif (ITAM) signalling in platelets.

Methods: Platelet function was assessed in mice with a conditional (PF4-Cre/loxP) deficiency of Grb2 in MKs and platelets by using flow cytometric analysis of cellular activation processes, aggregometry and flow adhesion systems. GPIIb/IIIa-mediated outside-in signalling was analysed by performing platelet spreading and measurement of clot retraction. *In vivo* function of platelets was studied in models of arterial thrombosis using intravital microscopy and haemostatic function was analysed by determination of tail bleeding times.

Results: We show that ablation of Grb2 in MKs did not interfere with MK differentiation or platelet production. However, Grb2 deficiency severely impaired GPVI-mediated platelet activation due to defective activation of phospholipase (PL) Cγ2 and Ca^{2+} mobilisation resulting in reduced adhesion, aggregation and coagulant activity on collagen *in vitro*. Similarly, CLEC-2-mediated signalling was impaired in Grb2-deficient platelets, whereas the cells responded normally to stimulation of G-protein coupled receptors (GPCR). Interestingly, in platelets, Grb2 acts downstream of Syk by linking the LAT signalosome to the GPVI receptor complex and Grb2-deficiency abrogated specifically ERK1/2 activation, whereas p38 and JNK activation were unaffected. *In vivo* this selective (hem)ITAM signalling defect resulted in prolonged bleeding times but affected arterial thrombus formation only after concomitant treatment with acetylsalicylic acid, indicating that defective GPVI signalling in the absence of Grb2 can be compensated through thromboxane A_2-induced GPCR signalling pathways.

Summary/Conclusion: These results reveal for the first time a crucial role of Grb2 in (hem)ITAM signalling in platelets in haemostasis and thrombosis, while it is dispensable for platelet production.

OC 77 – RNA and Coagulation

OC 77.1

Factor XI mRNA is spliced upon platelet activation

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Background: Platelets circulate in blood as anucleated cells yet contain spliceosome components and pre-mRNAs. Upon activation, platelets excise introns from pre-mRNAs yielding mature messages that are translated to proteins. Such signal-dependent splicing process has been demonstrated so far for three platelet proteins: IL-1β, cox-2 and tissue factor. Previous studies of platelet factor XI (FXI) yielded conflicting data regarding platelet FXI; some laboratories reported presence of mature FXI mRNA while others only found aberrant FXI mRNA that contained introns or skipped exons. Conceivably, these differences resulted from different degrees of platelets activation when mRNA was extracted.

Aim: To investigate whether platelet FXI pre-mRNA undergoes splicing upon platelet activation and yielding mature FXI mRNA that is translated to FXI.

Methods: Platelets were separated from blood of healthy volunteers in citrate and were washed gently in the presence of inhibitors such as PGE, Ibmax, and apyrase. Platelet mRNA was extracted from resting platelets and from platelets activated by thrombin or ADP as well as from partially activated platelets in which the fibrinogen receptor ($\alpha IIb\beta_3$) was activated without α-granules secretion. This partial activation was accomplished by using low concentrations of fibrinogen and manganese ions. The mRNA was reverse-transcribed to cDNA by random primers. Splicing patterns of the cDNA from activated, partially-activated or resting platelets were compared by PCR and agarose gel-electrophoresis. The presence of alpha-IIb message served as a positive control. FXI protein levels in activated or resting platelets were measured by ELISA using specific FXI antibodies.

Results: Both activated and resting platelets displayed FXI pre- as well as mature mRNA. Platelet activated by thrombin or ADP exhibited higher levels of mature FXI mRNA than resting platelets separated in the presence of inhibitors. The resting platelets contained increased levels of FXI pre-mRNA, including introns, compare to activated platelets. Levels of control alpha-IIb mature mRNA were similar in activated, partial-activated and resting platelets. The increased FXI mRNA splicing was not exhibited following partial platelet activation in which integrin $\alpha IIb\beta_3$ was activated without platelet α-granules secretion using low concentrations of fibrinogen and Manganese ions. FXI protein levels were also higher in activated platelets compared to inhibited platelets.

Summary: Mature mRNA of FXI as well as FXI protein are increased following platelet activation. Our data suggest that platelet FXI is the 4th protein whose pre-mRNA is spliced upon platelet activation. These results can explain previous publications in which different platelet FXI mRNAs were found. Conceivably, FXI that is produced in platelets upon their activation contributes to clot formation.

OC 77.2

The role of microRNA in the pathogenesis of venous thromboembolism

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Background: Microparticles (MPs) are small circulating membrane microvesicles which form via budding from surface of activated or apoptotic cells. MPs contain cytoplasmic content of parental cell, including regulatory RNA molecules, such as microRNA (miRNA), which are 20–25 nucleotide-long single-stranded RNAs that can induce degradation or inhibit the translation of one or several messenger RNA (mRNA) molecules. This will ultimately alter gene expression and modify cell functions. As part of inter-cellular communication, miRNAs could be transferred within MPs from the parental to target cell through adhesion and fusion of MPs to the specific cell type via specific receptors expressed on the surface of MPs. In plasma, miRNAs are located in MPs or bound to protein complexes (e.g., Ago2, NPM1). No study has so far investigated plasma levels of miRNAs in unprovoked venous thromboembolism (VTE).

Aims: The present study was undertaken to determine plasma levels of miRNAs in patients with unprovoked VTE.

Methods: A case-control study was performed in 20 patients with a history of incident unprovoked VTE 1–5 years prior to inclusion in the study, and 20 age- and sex-matched healthy controls recruited from the general population. RNA was isolated from citrated plasma using an improved variant of a column-based kit (miRNeasy kit, Qiagen, USA) including MS2 as a carrier RNA and thereafter successfully reverse transcribed (RT) into cDNA using miRCURY LNA™ Universal RT kit (Exiqon, Denmark). Profiling of miRNA from plasma was carried out by a SYBR-based quantitative PCR system assessing 743 miRNAs (The miRCURY LNA[TRADEMARK] Universal RT microRNA system, Exiqon, Denmark). For normalization of the data, we applied the average of the assays detected in all samples (n = 40 samples). Student's t test was used to evaluate differences between groups and a two-sided P-value below 0.05 was considered significant. The

study was approved by the regional committee for research ethics, and all participants gave their informed written consent to participate.

Results: VTE patients had higher BMI (28.3 ± 4.4 vs. 26.3 ± 3.9 kg/m^2, $P = 0.045$) and greater waist circumference (98.2 ± 12.5 vs. 93.4 ± 13.4 cm, $P = 0.041$) than age and sex matched controls. Signals of high quality from 73 miRNAs were identified in all samples, and several significant differences in levels of miRNAs were observed between the groups (p-values > 0.05). Interestingly, we found higher plasma levels of miR-10b ($P > 0.001$), miR-320b ($P = 0.002$) and miR-126 ($P = 0.02$) in plasma of patients with unprovoked VTE relative to healthy control individuals.

Conclusions: Our findings suggest that miR-10b, miR-320b and miR-126 are novel biomarkers of unprovoked VTE. Previous experimental studies have suggested that miR-10b and miR-126 are involved in regulation of endothelial cell functions and angiogenesis, whereas miR-320 has been associated with arterial cardiovascular risk factors and diseases. A nested case-cohort study will be performed to determine whether these miRNAs are a potential cause of unprovoked VTE rather than a consequence of the disease (reverse causation).

OC 77.3

Transfection of siRNA in human blood platelets: a high efficiency method for the horizontal transfer of siRNA from platelets to human monocytes and endothelial cells

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Background: Platelets contain messenger RNAs (mRNA) and are capable of protein synthesis. Platelets also contain a diverse array of microRNAs (miRNA) and its machinery (Dicer, Argonaute2). Preliminary data suggest that platelets and platelet-derived microparticles may transfer RNAs to other acceptor cells upon direct contact.

Aims: To develop an efficient method for the transfection of human platelets with siRNA and to assess whether the transfected siRNA can then be transfered to other cells.

Materials and Methods: We have developed a new method for the efficient transfection of siRNA to washed human platelets. siRNA transfection efficiency was assessed by using a fluorescent TYE563-labeled siRNA and flow cytometry. Platelet-derived microparticles (PMP) were generated by stimulating transfected platelets with Thrombin (1 U/mL) and TYE-563-labeled siRNA transfection in PMP was analysed by flow cytometry. mRNA expression in monocytes and in endothelial cells after co-incubation with siRNA transfected platelets was assessed by Real-Time PCR.

Fluorescent-labeled siRNA-transfected human platelets were resuspended in PBS at 10^9/ml and injected in NOD-SCID mice; 24 h later blood was sampled and siRNA fluorescence was assessed in murine monocytes by flow cytometry.

Results: We obtained a high siRNA transfection efficiency (97%) within 5 min of incubation with the siRNA of interest.

We also showed that the TYE563-labeled siRNA previously transfected into human platelets was transferred to platelet derived microparticles with high efficiency (96%).

We then co-incubated human monocytes with siRNA-transfected platelets for 4 h and observed that 15.8% of monocytes became positive for TYE563-labeled siRNA.

Similarly, human endothelial cells (HUVEC) co-incubated for 6 h with siRNA transfected PMPs resulted positive for TYE563 labeled-siRNAs (38%).

Human platelets transfected with fluorescent-labeled siRNA and injected in NOD-SCID mice persisted in the bloodstream for up to 48 h.

Mouse monocytes from mice injected 24 h earlier with siRNA-transfected human platelets showed siRNA fluorescence (7.1%).

Human monocytes coincubated for 24 h with platelets transfected with DICER-substrate siRNA directed against Tissue Factor mRNA show a strong downregulation of TF mRNA (more than 80%).

Conclusions: We have developed a novel, highly efficient method for the transfection of siRNAs into platelets.

We have also shown that siRNAs transfected in human platelets can be transferred to other cells (monocytes, HUVECs) with which platelets get into contact.

The persistence of transfected siRNAs within platelets transfused *in vivo* and the capacity of siRNA to pass into platelet-derived microparticles and then to be transfered to other cells in the bloodstream and/or in the vessel wall, may allow to interfere with genes of interest *in vivo* in various thrombotic or inflammatory diseases.

This new method allows to silence specific mRNAs involved in disease, and opens new possibilities for therapy.

OC 77.4

Downregulation of protein S by oestrogens and miR-494

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Background: Acquired Protein S (PS) deficiency is highly associated with elevated circulating oestrogen levels resulting from pregnancy, where levels of circulating oestradiol progressively increase to > 10-fold by the third trimester, and functional activities of PS also shown to decrease throughout pregnancy, contributing to an overall procoagulant shift. Increasing evidence indicate that activation of the oestrogen receptor (ER) signalling by oestrogens, modulates the expression of target genes indirectly by regulating the expression of miRNAs. We hypothesise that elevated circulating oestrogen levels result in the altered expression of specific microRNAs leading to acquired PS deficiency, and propose that oestrogen-regulated microRNAs are secreted from oestrogen-responsive tissues, transported in microparticles to the liver and endothelial cells to downregulate PS expression.

Methods: Computational analysis of *PROS1* 3′ untranslated region (UTR) identified multiple binding sites for miR-494, and direct targeting of *PROS1*-3′UTR by miR-494 was determined by dual luciferase reporter assays in HuH-7 and HeLa cells. Reporter vectors containing the *PROS1*-3′UTR sequence with deleted miR-494 binding sites were also analysed by luciferase reporter assays. Effects of oestrogen on miR-494 and *PROS1* mRNA levels in HuH-7 cells were determined by quantitative real-time PCR (RT-qPCR), and oestrogen-mediated changes to secreted PS levels in culture supernatant of HuH7 cells were measured using an enzyme-linked immunosorbent assay (ELISA). To analyse miR-494 expression in human plasma, miR-494 levels were determined by RT-qPCR from different fractions of ultracentrifuged human platelet-poor plasma samples.

Results: Research in the laboratory has demonstrated that miR-494 directly targets *PROS1* and miR-494 levels are upregulated following oestrogen treatment in Huh7 liver cells in association with downregulated *PROS1* mRNA and PS levels. In addition, the expression of miR-494 was also found to be concentrated in the microparticle-containing fraction of ultracentrifuged plasma.

Summary/conclusions: These results strongly suggest that oestrogen modulation of miR-494 expression and subsequent downregulation of PS expression by miR-494 is a mechanism contributing to oestrogen-mediated acquired PS deficiency. Ongoing work is investigating the clinical relevance of miR-494 expression, the mechanism of miR-494 transport by microparticles in circulation, as well as the characterisation of oestrogen-responsive miRNAs in pregnant women with elevated oestradiol levels and low PS levels, to establish the role of miRNAs in acquired PS deficiency.

OC 78 – Thrombophilia – I

OC 78.1

Reducing inpatient heritable thrombophilia testing using a clinical decision-making tool

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Background: The presence of heritable thrombophilia (HT), including factor V Leiden (FVL), prothrombin gene mutation, and deficiencies of antithrombin (AT), protein C (PC) and protein S (PS), does not have any impact on acute management of thrombotic diseases and the functional assay results can be unreliable during the acute event and while on anticoagulation. Consequently, testing for HT in patients admitted to hospital with an acute thrombotic event is of questionable clinical value. Prevalence of HT testing in the hospital setting has not been reported and a clinical decision-making tool to guide appropriate testing has not been developed.
Aims: To assess the effect of a clinical decision-making tool on the volume, pattern and cost of HT testing in a tertiary care teaching hospital.
Methods: We developed a clinical decision tool to guide HT testing in patients admitted to a tertiary care center and introduced it in a preprinted order (PPO) form. This form discouraged HT testing in patients with arterial or recent (i.e. $\leq$ 3 months) thrombosis and in those receiving anticoagulant therapy. HT testing was only performed if the PPO was completed by the ordering physician. Electronic laboratory records of FVL testing performed on inpatients were collected for 3 years prior to and 3 years following the PPO intervention. Results were compared with HT testing data from two other regional teaching hospitals affiliated with the same university. Records for AT, PC, and PS activity assays were also collected to confirm the FVL data trends. Cost savings was estimated based on reagent and staffing costs directly associated with HT testing.
Results: Between Jan 2007 and Dec 2009, 607 FVL tests were performed. Following the PPO introduction in Jan 2010 until Dec 2012, 257 FVL tests were performed and 80 FVL orders were cancelled due to the PPO not being completed. The FVL test volume reduction of 79.4% was significantly greater compared to those in the two control teaching hospitals over the same time periods (33.7% and 43.6%; both $P > 0.001$). There was a steep decline in FVL testing following the introduction of the PPO and the effect was maintained for the three subsequent years. Reductions in FVL testing post-intervention was observed amongst all specialists, including neurologists and general internists who together accounted for 64.3% of FVL testing overall. Hematologists accounted for only 2.8% of FVL testing. Similar post-intervention reductions in testing volumes were observed for AT (57.4%), PC (61.9%), and PS (62.2%) activity assays. The mean annual costs directly associated with HT testing decreased from $13,770.41 to $3635.80 after the implementation of the PPO, for a mean net savings of $10,134.60 annually.
Summary: In a large tertiary care hospital, the introduction of a clinical decision-making tool significantly reduced HT testing in inpatients by all clinician specialties, leading to considerable cost-savings. The impact on patient outcome should be assessed in further studies.

OC 78.2

New diagnostic considerations based on the experience of genetic analysis in Protein C deficiency

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Background: Protein C (PC) is a vitamin K-dependent glycoprotein, which inactivates active factor VIII and factor V (FV). PC deficiency confers a high thrombosis risk and it is classified as type I (quantitative) and type II (qualitative) disorder. A number of distinct mutations causing PC deficiencies have been discovered so far. Screening for PC deficiency in the hemostasis laboratory is executed by clotting or chromogenic functional tests. Both methods have several disadvantages. FV Leiden mutation (FVL) interferes with the clotting assay leading to low PC activity. Since the PC antigen determination (ELISA) is not influenced by FVL, patients carrying this mutation seem to have type II PC deficiency. Chromogenic tests do not suffer from this problem, however they may not detect some type II cases with certain mutations. Most of the guidelines recommend to use the chromogenic assay in order to bypass the interfering effect of FVL and other analytical errors caused by prolonged or shortened clotting time in certain cases.
Aims: To evaluate the functional clotting and chromogenic PC assays from the point of view of FVL interfering effect and to determine the mutation spectrum of PC deficiency in a high number of patients representing the Hungarian population.
Methods: Non-related individuals having 70% or lower PC activity measured by the clotting test were recruited (n = 109). PC activity was determined by both the clotting (Protein C reagent, Siemens) and the chromogenic (Berichrom Protein C kit, Siemens) method. PC antigen was measured by ELISA (Asserachrom Protein C, Diagnostica Stago). The gene coding PC (PROC) was analyzed by direct DNA sequencing. Second-line evaluation of PROC was performed by MLPA method in cases where the presence of larger genetic abnormality was assumed. FVL mutation was determined by real-time PCR and melting curve analysis.
Results: Most of the patients with low PC clotting activity were carriers of the FVL (n = 72). Among non-carriers 27 patients had causative mutation(s) in PROC identified by DNA sequencing (12 type I and 15 type II deficiency). Among type II deficients four patients showed normal result in the chromogenic test. In addition, two novel gross deletions were found by MLPA analysis (delE1-3 and delE1-9) leading to type I deficiency. It was observed that 78.4% of FVL negative patients had causative mutations in the PROC gene and only 12.5% of FVL carriers were positive for PROC mutation. In this latter group 2 patients with FVL and PROC mutation had normal PC activity in the chromogenic test. Altogether 28 different mutations were detected including 15 novel ones.
Conclusions: Based on our results three important considerations can be made: 1, Since chromogenic functional test gives normal result in some genetically confirmed PC deficient patients, clotting assay should not be bypassed. 2, DNA sequencing of PROC cannot be omitted in FVL carrier patients with low PC clotting activity and normal PC antigen. 3, MLPA method is required in 'sequencing negative' cases as second line genetic diagnostic tool.

OC 78.3

Antithrombin Debrecen (p.Leu173Pro): clinical and molecular characterization of a novel mutation associated with severe thrombotic tendency in a large pedigree

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Background: Hereditary antithrombin (AT) deficiency is a rare but major risk factor for venous thromboembolism. It is classified as type I (quantitative) and type II (qualitative) deficiency and caused by mutations in the SERPINC1 gene encoding AT. The genetic background is heterogeneous and different sub-types of type II AT deficiency are known according to the site of mutation.
Aims: The goal of this research was to characterize the molecular features of a novel mutation that was found in a populous family. We

wanted to determine the severity of the mutation's clinical consequences by conducting a detailed family investigation.
Methods: Thirty family members of four lineal generations were included in the family study. The age at the first thrombotic event, the type and location of the thrombosis, the recurrent thrombotic episodes, and the co-existing provoking factors and acquired conditions were determined in every family member. All the laboratory investigations for thrombophilia were carried out. AT activity was measured by a commercially available heparin cofactor activity assay, AT antigen was determined by immunonephelometry. The mutation was identified with fluorescent direct DNA sequencing of the SERPINC1 gene. HEK293 cells were transfected with constructs containing wild type and mutant SERPINC1 plasmids. Transfection efficiency was determined by beta-galactosidase co-transfection. Immunoprecipitation followed by Western blotting was used to visualize the presence or absence of the mutant AT in the cell lysates and in the conditioned media. The quantity of the expressed AT protein was measured by ELISA. Chromogenic test in the presence or absence of heparin using activated FX as substrate was used to measure the activity of the expressed AT.
Results: We found ten affected patients in the family and all of them suffered from venous thromboembolisms in their lives except two teenage boys in the youngest generation. In six cases the thrombosis was recurrent. Two of the patients died of pulmonary embolism, one of them at the age of 19. The average age at the time of the first thromboembolic episode was 36.5 years. The average AT activity of affected patients was 57% and the genetic analysis revealed a novel AT mutation (p.Leu173Pro). Classification of the new mutation was uncertain due to the inconclusive antigen results. Wild type and mutant AT were expressed in HEK293 cells and it was demonstrated that after synthesis a small amount of mutant AT (28% of wild type) was secreted by the cells. The majority of the mutant protein was trapped intracellularly. Activity of the secreted mutant AT was somewhat decreased.
Conclusion: Studying a large AT deficient family a novel mutation was found. The clinical data suggested that the mutation was associated with severe thrombotic tendency that manifested in early ages. The p.Leu173Pro mutation did not influence the synthesis of AT but led to severe secretion defect, probably because of major structural changes and deviation from the normal secretory pathway.

OC 78 – Thrombophilia – I

OC 78.4

A clinical laboratory test detecting antithrombin-resistance of the new thrombophilia

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Background: We have reported a novel mutation of the prothrombin gene (c.1787G>T, p.Arg596Leu: prothrombin Yukuhashi) conveying antithrombin-resistance in the patient with hereditary thrombosis. The antithrombin-resistant thrombin may have prolonged procoagulant activity *in vivo*, conferring a susceptibility to thrombosis. Current laboratory tests, however, cannot detect the pathological condition, such as antithrombin-resistance.
Aims: In order to detect an antithrombin-resistant condition in plasma, we developed a new clinical laboratory assay. We further aimed to identify and verify the frequency of antithrombin-resistance by analyzing plasma samples from the patients with venous thromboembolism of unknown cause.
Methods: We prepared reconstituted plasma by mixing prothrombin-deficient plasma with recombinant prothrombin (wild-type or mutant). The assay consists of three steps; thrombin generation, thrombin inactivation, and determination of residual thrombin activity. In the first thrombin generation step, we used *Oxyuranus scutellatus* venom or bovine Factor Xa (FXa) with factor Va (FVa) as prothrombin activator. In the second step, we inactivated thrombin by addition of antithrombin in the presence or absence of heparin. Inactivation times with heparin and without heparin were 0–5 and 0–30 min, respectively. In the final step, we measured the residual thrombin activity using chromogenic synthetic substrate (S-2238). We determined the optimum measurement conditions; pH, osmotic pressure, concentrations of antithrombin, chromogenic substrate and each component of the prothrombin activator (snake venom, FXa, FVa, phospholipids, $CaCl_2$), and reaction time of each step. We also verified normal and warfarinized patient's plasma samples by this optimized procedure.
Results: We diluted the samples 1:100 by the dilution buffer, and incubated with optimized concentrations of prothrombin activator components (phospholipid, calcium chloride, snake venom or FXa/FVa) at 37 °C for 2 min. After addition of antithrombin with or without heparin, we added sufficient amounts of S-2238 and measured the change in absorbance/min (delta A/min) at 405 nm. The relative residual activity of recombinant mutant thrombin after 5 min inactivation with heparin showed an obvious poor inactivation (40%) compared with that of recombinant wild-type thrombin (0.5%). After 30 min inactivation without heparin, the relative residual activity of recombinant wild-type thrombin in the reconstituted plasma was 18%, whereas that of recombinant mutant thrombin was 97%. These data indicated that this assay obviously detected the antithrombin resistance of mutant Arg596Leu prothrombin in plasma. Actually, the assay for 5 min inactivation with heparin demonstrated that the relative residual thrombin activity of the warfarinized patient's plasma with prothrombin Yukuhashi (14%) was clearly higher than that of the normal plasma (0.5%). So far, we further analyzed plasma samples of several unrelated Japanese patients with venous thromboembolism of unknown cause, but none of the patients showed antithrombin-resistance.
Summary/conclusions: We devised a method for analyzing thrombin inactivation kinetics in plasma. This assay was useful for detecting antithrombin-resistance of prothrombin Yukuhashi, even when taking warfarin.

OC 79 – Anticoagulant Agents – Clinical Studies II

OC 79.1

Safety, tolerability, and pharmacokinetics of edoxaban in end-stage renal disease subjects undergoing hemodialysis

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Background: Edoxaban, a direct factor Xa inhibitor, is currently under development as a once-daily oral anticoagulant for stroke prevention in atrial fibrillation, and for the treatment and prevention of recurrences of venous thromboembolism. Renal elimination accounts for approximately 50% of the total clearance of edoxaban.
Aims: Since subjects with end-stage renal disease (ESRD; with or without hemodialysis) may receive edoxaban, this study examined the safety, tolerability, and pharmacokinetics of edoxaban and its metabolites in subjects undergoing hemodialysis to provide dosing guidance.
Methods: This was an open-label, 2-treatment, 2-way crossover study in subjects with ESRD maintained on a stable regimen of thrice-weekly hemodialysis for at least 3 months. Subjects with recent major bleeding, trauma, any type of surgery ≤ 6 months prior, or with hemoglobin > 10.0 g/dL were excluded. Subjects were randomized to receive a single oral dose of edoxaban 15 mg on two occasions: edox-

aban dosed 2 h prior to a 4 h hemodialysis session (with hemodialysis), and edoxaban dosed on a day when subjects were not undergoing hemodialysis (without hemodialysis). There was a minimum washout of 7 days between doses. Serial blood samples were collected for quantification of edoxaban and four metabolites. Edoxaban was also measured in the dialysate. *In vivo* plasma protein binding was evaluated at 2 time points on each treatment day. Safety assessments included physical exam; vital signs; adverse events; and clinical labs, including coagulation parameters.

Results: Ten subjects (seven males, three females) were enrolled, and nine subjects completed both treatment periods. When administered without and with hemodialysis, edoxaban exposures were comparable ($AUC_{0\text{-inf}}$, 692 ± 150 and 676 ± 221 ng h/mL, respectively). Hemodialysis had minimal effects on the clearance of edoxaban, with total clearance values of 24 vs. 23 L/h with and without hemodialysis. Hemodialysis clearance was only 6 L/h. Thus, subjects undergoing hemodialysis may not need edoxaban dose adjustments to compensate for drug loss via hemodialysis. This also indicates that hemodialysis is not an effective mechanism for removal of edoxaban from the blood. Hemodialysis had minimal effect on the total exposure of the human-specific active metabolite, M-4, with metabolite-to-parent ratios of 25% vs. 22% with and without hemodialysis (compared to > 10% in healthy subjects). The plasma protein binding for edoxaban was similar with and without hemodialysis and ranged from 60% to 63%. This was similar to previously reported values in healthy subjects. Thus, renal impairment does not appear to affect plasma protein binding of edoxaban. In subjects with ESRD, the metabolite profile was different from healthy adults, with higher levels of M-4 and M-1, indicating that when renal clearance is compromised, edoxaban clearance through metabolism plays a larger role in the total clearance of edoxaban. Single oral doses of 15 mg of edoxaban were well-tolerated with only mild adverse events.

Conclusions: Data from this study indicate that additional dose adjustment is not necessary for edoxaban when ESRD subjects are undergoing hemodialysis, and hemodialysis is not an effective mechanism of removal of edoxaban from the blood.

OC 79.2

Apixaban: determination of its anticoagulant effects and influence on coagulation tests: a multicentre French GEHT study

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Background: Apixaban, a new oral anti-Xa inhibitor, was approved recently for venous thromboprophylaxis following major elective orthopaedic surgery and for stroke prevention in patients with non-valvular atrial fibrillation. While laboratory monitoring is not required, measurement of the anticoagulant activity may be useful in certain situations (urgent surgery, major bleeding, and frail patients). We have recently shown that PT (prothrombin time) and aPTT (activated partial prothrombin time) are not suitable to detect therapeutic plasmatic concentrations of apixaban. Therefore specific tests to measure apixaban concentrations are required.

Aims: The objective of this multicentre *in vitro* study was to determine the reliability and the variability of specific anti-Xa activity assays and of PT and aPTT measurements in plasmas samples containing various concentrations of apixaban.

Methods: Five blinded plasma samples (A, B, C, D, E) spiked with 0, 100, 200, 400 and 800 ng/mL apixaban were dry-ice shipped to 13 laboratories. The apixaban concentration in the samples was determined by HPLC tandem mass spectrometry. Each laboratory was supplied with anti-Xa and PT reagents and sets of apixaban calibrators and controls (Stago). Three chromogenic assays were assessed (Results ng/mL): Biophen heparin LRT® (Hyphen Biomed) in all laboratories, STA-Liquid-Anti-Xa® (Stago) in laboratories using a STAR instrument and HemosIL Heparin Liquid® (Instrumentation Laboratory [IL]) in those using an ACL-Top. Each assay was performed according to a standardized protocol. Plasma with apixaban concentration > 500 ng/mL was 1/3 diluted in normal pool plasma. PT were measured using RecombiPlastin 2G® (IL) and the local reagent, and aPTT using the local reagent (Results ratio of clotting times). The study was performed over five consecutive days. A calibration curve was generated every day for each anti-Xa assay and all measurements were in duplicate.The results are expressed as mean ± SD. Accuracy and precision of anti-Xa assay were assessed.

Results: The actual apixaban concentrations in the A-E plasmas were 0, 96, 209, 393 and 828 ng/mL. In samples A, B, C, D, E and E 1/3, the anti-Xa concentrations regardless of the assay used, were 7 ± 8, 90 ± 8, 197 ± 17, 393 ± 22, 695 ± 97 and 270 ± 24 ng/mL, respectively. Accuracy and precision for each assay were high for plasmas B to E. In plasma A, free of apixaban, mean anti-Xa were > 15 ng/mL in 10 laboratories; and over 15 ng/mL in three out of the four laboratories using the Biophen heparin® with ACL-Top. The PT and aPTT inter-laboratory reproducibility was high both with the centrally provided PT reagent and the local PT and aPTT reagents. As an example, in plasma B (96 ng/mL), the ratios were 1.15 ± 0.02 (RecombiPlastin 2G®), 1.10 ± 0.02 (local PT), 1.17 ± 0.05 (local aPTT).

Conclusions: We confirmed the low sensitivity of PT and aPTT reagents to apixaban. Apixaban plasma concentrations ≥ 100 ng/mL can be accurately and precisely quantified by the three anti-Xa assays tested. Accuracy for values > 100 ng/mL varied depending on the combination reagent/coagulometer used, and deserves further investigations.

OC 79.3

Comparing new oral anticoagulants (dabigatran, rivaroxaban and apixaban) to warfarin in patients with atrial fibrillation, a cost-effectiveness analysis from a German payer perspective

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The new oral anticoagulants (NOAC) dabigatran, rivaroxaban and apixaban are now approved for prevention of stroke and systemic embolism for patients with non-valvular atrial fibrillation (NVAF). In times where resource allocation is more and more important, thinking about cost-effectiveness of different therapy options is necessary, to obtain the best therapy at the lowest costs. This context explains how crucial the need of cost-effectiveness analysis (CEA) is.

Our aim was to determine which prices for NOACs in Germany are justified. We performed a CEA including direct and indirect costs regarding a set societal willingness to pay threshold.

We used the data form the RE-LY, ROCKET AF and ARISTOTLE trials, defining outcome events as ischemic stroke and systemic embolism, major bleeding, intracerebral hemorrhage, myocardial infarction, and mortality. The outcome of the CEA was defined as quality of life (QALYs), total costs, and incremental cost effectiveness ratios (ICER) for the NOACs comparing to dose-adjusted warfarin. Pricing of the clinical events and the outpatient care were taken from published data, form the institute for payment regulations in German hospitals (InEK) and German-Diagnosis Related Groups. Indirect costs for INR deter-

minations included non-productive time of a relative and/or transportation costs. For the drug costs of the NOACs, we used the current daily market costs of 3.20€. Performing a Markov model we did several deterministic and stochastic analyses, including cohort analyses, one- and two-way sensitivity analyses and Monte Carlo simulation. The time horizon was based on the life expectancy of the German population. The Markov decision model was adopted using the TreeAge Pro 2012 program.

The cohort analyses showed that using the RE-LY, ROCKET AF and ARISTOTLE trial data the results were the following. The QALYs were 11.53QALYs for dabigatran 110 mg bid and 11.66QALYs for dabigatran 150 mg bid comparing to warfarin with 11.41QALYs. The total costs were 42395€ for dabigatran 110 mg bid, 39392€ for dabigatran 150 mg bid and 38765€ for warfarin. In case of the ICER calculation dabigatran 110 mg had 29527€ per QALY and dabigatran 150mg bid had 2547€ per QALY compared to warfarin. Rivaroxaban showed QALYs of 11.05 comparing to 10.79QALYs for warfarin. Total costs were 44581€ for rivaroxaban and 43499e for warfarin. The ICER for the comparison of rivaroxaban with warfarin was 4158€ per QLAY. When calculating with the ARISTOTLE trial data the QALYs for apixaban comparing to warfarin were 11.38 compared to 11.04. Total costs were 37606e for apixaban compared to 36260€ for warfarin. The ICER for apixaban comparing with warfarin were 4047€ per QALY. The one- and two-way sensitivity analyses indicate that the model was highly sensitive to the daily costs for the drugs and to indirect costs but relatively insensitive to other model inputs. In the Monte Carlo simulation the model input parameters were validated.

When comparing the cost-effectiveness for the NOACs including indirect costs current German market prices are justified at a societal willingness to pay of a generally accepted amount of about 30,000 Euro. Indirect costs sensitively influence the outcome of the CEA of NOACs in NVAF.

OC 79.4

Incidence of recurrent venous thromboembolism in patients following completion of the EINSTEIN DVT and EINSTEIN PE studies

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Background: The EINSTEIN DVT and EINSTEIN PE studies compared rivaroxaban with enoxaparin/vitamin K antagonist (VKA) for the treatment of acute symptomatic deep vein thrombosis or pulmonary embolism for 3, 6 or 12 months of treatment (The EINSTEIN Investigators. *NEJM* 2010; 363:2499; The EINSTEIN-PE Investigators. *NEJM* 2012; 366:1287). Continued anticoagulation treatment was relatively common following discontinuation of the study medication.

Aims: This analysis compared the incidence of recurrent venous thromboembolism (VTE) in patients who continued anticoagulant treatment compared with those who stopped over the course of the 1-month post-study medication observational period.

Methods: Patients from the EINSTEIN DVT and EINSTEIN PE studies who entered the 1-month post-study medication observational period were eligible for the analysis. After discontinuing study medication, continuation of anticoagulant treatment was at the discretion of the treating physician. Two independent assessors validated the anticoagulant therapy that was provided during the 1-month post-study medication observational period as full anticoagulant treatment (i.e. VKA or full-dose low molecular weight heparin [LMWH]), including optimal and suboptimal use, or no anticoagulant treatment. In addition, patients randomized to rivaroxaban therapy were further divided into those who received bridging LMWH therapy (for the period in which patients had sub-therapeutic International Normalized Ratio values) and those who did not. Incidences of recurrent VTE, as assessed by the blinded adjudication committee, in the 1-month period after cessation of study drug, were compared between relevant groups.

Results: Full anticoagulant treatment was received by 861 of 3565 (24.2%) qualifying patients who had received rivaroxaban compared with 1422 of 3536 (40.2%) qualifying patients who had received VKA therapy ($P > 0.0001$). During the 1-month post-study medication observational period, 32 of 3587 (0.9%) patients who had received rivaroxaban compared with 22 of 3582 (0.6%) patients who had received enoxaparin/VKA developed recurrent VTE. The incidence of recurrent VTE was 0.4% (9/2283) among patients who received any full anticoagulant treatment, compared with 0.9% (45/4818) among those who did not (risk difference −0.5%, 95% confidence interval [CI] −0.8% to −0.1%). In patients who received any full anticoagulant treatment, the incidences among prior rivaroxaban recipients was 0.3% (3/861) and among prior enoxaparin/VKA recipients was 0.4% (6/1422) (risk difference −0.1%, 95% CI −0.5% to 0.5%). The incidence of recurrent VTE in patients who had received rivaroxaban and continued with VKA treatment was similar among those who did not receive LMWH bridging (0.4%; 2/498) and those who did receive LMWH bridging (0.3%; 1/363) (risk difference 0.1%, 95% CI −0.6% to 0.6%).

Summary/conclusions: The decision to continue anticoagulant treatment after cessation of study medication in the EINSTEIN DVT and EINSTEIN PE studies was influenced by knowledge of the randomized treatment received. The incidences of recurrent VTE during the 1-month post-study medication observational period were similar in patients who received any full anticoagulant treatment, independent of LMWH bridging in prior rivaroxaban recipients.

OC 79.5

Stopping oral anticoagulation and the risk of thrombotic events and mortality in patients with atrial fibrillation

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Oral anticoagulation (OAC) is highly effective preventing stroke and mortality in patients with atrial fibrillation (AF) compared to placebo/control. It has previously been described that the incidence of stroke and major hemorrhagic events is higher during the immediate period after initiation of Vitamin K antagonists (VKA), particularly amongst naïve patients. Stability of anticoagulant effect is often not achieved for several months after initiating VKA, as anticoagulation intensity fluctuations are influenced by diet, drug interactions, genetic variation and drug adherence. Bleeding complications usually lead to cessation of VKA, particularly amongst VKA-naïve patients. We evaluated clinical outcomes of a consecutive population of AF patients who were VKA naïve, and initiated on VKA therapy in our clinic.

Methods: We studied consecutive VKA-naïve patients with paroxysmal, persistent or permanent AF, who were initiated on VKA therapy in our anticoagulation outpatient clinic in 2009. During follow-up, adverse events (thrombotic and vascular episodes, major bleeding and death) were recorded as well as VKA treatment cessation. We also determined the time within therapeutic range (TTR), using the Rosendaal method. INR was performed by a point of care device.

Results: We included 529 patients (49% male, median age 76 [IQR 69–82]), who were followed-up for median 835 days (IQR 719–954), in this period 114 patients (21.6%) stopped VKA treatment. During follow-up, 63 patients suffered a thrombotic/cardiovascular event (annual rate 5.17%/year), of which 27 were strokes, 51 had a major

bleeding episode (annual rate 4.19%/year) and 48 died (annual rate 3.94%/year). Median TTR observed was 54% (34–57). On multivariate analysis, VKA cessation was independently associated with stroke (4.21 [1.87–9.47]; $P = 0.001$), the composite of thrombotic/cardiovascular events (HR 2.72 [1.56–4.72], $P > 0.001$), and death (HR 3.43 [1.86–6.32]; $P > 0.001$), which remained significant even after adjusting for CHA_2DS_2-VASc score. Independent risk factors for major bleeding were age [HR (95% CI): 1.08 (1.04–1.12); $P > 0.001$], previous stroke (1.85 [1.00–3.41]; 0.049), and TTR (0.97 [0.96–0.99]; $P = 0.001$).

Conclusions: In patients with AF, VKA cessation is independently associated with the risk of stroke and cardiovascular events, as well as mortality. Specifically, VKA cessation independently increased the risk of stroke, even after adjusting for CHA_2DS_2-VASc score. TTR constitutes a risk factor for major bleeding when VKA therapy is initiated.

OC 79.6

Venous thromboembolism: an annual European Union 27 cost-of-illness model for the burden of the disease

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Background: National costs for venous thromboembolism (VTE) have been defined for the United States (US). However, overall costs for the full 27 member European Union (EU-27) have not yet been derived.

Aims: To calculate the direct medical costs and indirect costs of VTE within the EU-27 as a cost-of-illness model. To identify total, hospital-acquired and preventable costs associated with VTE within the EU-27.

Methods: Investigators defined and conducted an a priori literature search to identify any diagnosis- or treatment-related costs for deep vein thrombosis (DVT), pulmonary embolism (PE), or VTE from 1994 to September 2012 within the EU-27. Identified publication references were also hand-searched to capture other applicable cost studies. Two investigators independently reviewed the literature search results. High and low inpatient, outpatient and recurrent DVT and PE costs were abstracted as well as high and low costs for minor and major bleeding, pulmonary hypertension, post-thrombotic syndrome, heparin-induced thrombocytopenia, VTE prophylaxis, and the cost of a premature death. Appropriate cost sources were available from 2006 to the end of the literature review.

All costs were categorized and converted to 2012 Euros taking into consideration country-specific inflation and purchasing power parities. Costs were then input into previously published US decision-analytic cost models that were pre-populated with accepted probabilities. Naess' et al. 2007 recent Norwegian PE and DVT incidence rates were used for lower estimates while the VTE Impact Assessment Group in Europe's (VITAE) (Cohen et al. 2007) prevalence (long-term attack) rates were used for high estimates.

The population utilized was 502.5 million focusing on the 424.11 million that were 15 years or older in the EU-27 as of January 1, 2011. Lower and higher median costs for each category were utilized to populate the base and sensitivity analysis. The average patient's cost, the expected value of all decision tree pathways', were multiplied by low and high annual PE and DVT incident events to determine cost ranges. Hospital-acquired costs were estimated at 67–75% of total costs, and preventable costs were estimated at 50–75% of the hospital-acquired costs based off of current literature.

Results: In the base model, annual total, hospital-acquired, and preventable VTE costs ranged from € 2.0 to € 3.6, € 1.4 to € 2.4, and € 0.7 to € 1.8 billion, respectively. In the sensitivity analysis utilizing higher costs and probabilities, annual total, hospital acquired and preventable VTE costs ranged from € 7.6 to € 13.7, € 5.5 to € 10.0, and € 2.8 to € 7.5 billion, respectively. Results of additional sensitivity analyses will also be presented.

Conclusions: VTE costs in the EU-27 are significant although these costs appear less than US costs primarily due to less expensive individual direct medical costs. Approximately € 0.7 to € 7.5 billion/year could be avoided if improved intervention systems were in place within the EU-27. The preventable costs compare to $4.5 to $39.3 Billion (2011 US$) in the US. Use of VTE prophylaxis for at-risk patients is cost-effective in both the EU-27 and US.

OC 80 – Basic Issues in Haemophilia A

OC 80.1

Genetic targeting of human coagulation factor VIII into platelet a-granules resulted in long-term improvement of hemostatic function in canine hemophilia A

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Background: It is essential to develop improved therapies for controlling excessive bleeding in patients with severe hemorrhagic disorders such as hemophilia A and B. Since activated blood platelets mediate the primary response to vascular injury by adhering to a wound site and secreting biologically active proteins, we hypothesized that synthesis and storage of recombinant Factor VIII (FVIII) within platelets may be an ideal strategy for providing a continuous, locally–inducible treatment for maintaining hemostasis.

Aims: Although FVIII is normally synthesized in the liver, here we show a clinically relevant strategy for hematopoietic stem cell gene therapy within a canine 'large animal' model for hemophilia A that permitted synthesis, trafficking, storage, and release of FVIII from activated platelet α-granules as a hemostatic response leading to reduced severe bleeding episodes.

Methods: This study was accomplished with sub–myeloablative conditioning with Bulsulfan (5–10 mg/kg i.v.) and autologous transplant (Tx) of cytokine mobilized CD34+ peripheral blood stem cells (PBC) transduced with a lentivirus encoding a fragment of the integrin αIIb (ITGA2B) gene promoter that permitted platelet–specific storage of human FVIII within three dogs affected with hemophilia A. One animal received a novel hybrid molecule consisting of FVIII fused to the human von Willebrand factor propeptide signal peptide and D2 domain (SPD2) that permitted optimal trafficking of FVIII directly into platelet α–granules. The three Tx recipients underwent periodic testing for expression of the FVIII transgene as well as immune tolerance and phenotypic correction of hemophilia A.

Results: PCR localized the lentivirus within genomic DNA isolated from leukocytes of each dog at 2.5 years after Tx. Immunofluorescence confocal microscopy detected FVIII in a subset of platelets. Immune electron microscopy revealed that FVIII trafficked and stored directly into platelet α-granules. Chromogenic analysis showed that biologically active FVIII (FVIII:C) was detected in all three dogs at 5–10 mU/mL/10^8 platelets for at least 2.5 years after Tx. In contrast, FVIII:C was not detected within the plasma of these animals. This result, coupled with the use of immunomodulation drugs at Tx, may help to explain why the dogs remained tolerant of human FVIII as supported by our inability to detect inhibitory antibodies to FVIII. The dogs showed clinical improvement of hemostatic function as evident by the measurement of reduced bleeding episodes that required the use of canine FVIII supplements. Specifically, the 1st Tx recipient had approximately three

bleeds/yr for 2.5 years (vs. expected five/yr in untreated hemophilia A control animals). Remarkably, the 2nd and 3rd dogs had zero severe bleeding episodes for at least 2.5 years after Tx.
Conclusion: Use of the canine model has confirmed and advanced our understanding of the requirements for viable synthesis, trafficking, storage and release of FVIII from platelet α-granules. These results demonstrate feasibility for translating this strategy for targeting biosynthesis of FVIII into platelets into a clinically relevant protocol. Thus, this work suggests great potential for platelet FVIII gene therapy to provide effective long–term control of bleeding in humans affected with hemophilia A.

OC 80.2

Allosteric modulation of the structurally related factor VIIa and IXa proteases is responsible for the procoagulant properties of a novel peptide family

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Background: Factor VIIa (FVIIa) and factor IXa (FIXa) are two structurally related serine proteases essential for the extrinsic initiation and intrinsic propagation of the coagulation cascade, respectively. The enzymatic cleavage of factor X (FX) into factor Xa (FXa) by these proteases is significantly increased by the presence of their native cofactors, tissue factor and factor VIIIa. We have discovered a novel procoagulant peptide family by phage display panning against FIXa that increased the catalytic activity of FIXa in FXa generation assays, but provided disproportionately greater ability to generate thrombin and clot FVIII-deficient blood in rotational thromboelastometry assays. This result indicated that the peptide family may also increase the catalytic activity of other enzymes in the clotting cascade.
Aims: These studies were designed to investigate the mechanism of action through which the discovered peptide family exerts its procoagulant activity and to further aid in the development of a potential novel drug candidate for hemophilia A bypass treatment.
Methods: A series of *in vitro* experiments was used to map the preferred selectivity and mechanism of action for the procoagulant peptide family. The first evidence of the peptides interacting with multiple enzymes in the coagulation cascade was revealed by plasma-based assays in the presence of neutralizing antibodies against FIX and/or FVIIa. The specificity was further uncovered by a modified thrombin generation assay with purified components allowing isolated steps of the clotting cascade to be studied. The degree of catalytic activity enhancement of FVIIa and FIXa for the conversion of FX into FXa in the presence of representative peptides was determined by a chromogenic FXa generation assay.
Results: The discovered procoagulant peptide family enhances the enzymatic activity of both FVIIa and FIXa as determined by FXa generation in the presence of phospholipids and calcium. The optimized peptides' ability to function as cofactors to FVIIa and FIXa and catalyze the zymogen-like to active protease transition is primarily reflected by increased catalytic activity. Lead peptides enhanced *kcat* of FVIIa by three orders of magnitude and FIXa a few 100-fold. These findings were confirmed by a modified thrombin generation assay where representative peptides stimulated both the extrinsic and intrinsic segregated pathways. Further analyses showed that the peptide family did not directly influence the enzymatic activity of the downstream serine proteases FXa and thrombin, mimicking physiological fibrin formation and regulation.
Conclusion: The FVIIa/FIXa dual specificity of this peptide family leads to stimulation of both the extrinsic and intrinsic coagulation pathways and results in correction of clotting in hemophilia A whole blood corresponding to > 50% of normal FVIII levels. These procoagulant peptides may lead to an alternative treatment for hemophilia A patients lacking a functional factor VIII molecule, whether in the presence or absence of neutralizing antibodies.

OC 80.3

Pharmacological modulation of the uptake of blood coagulation factor VIII by dendritic cells

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Background: The endocytosis of blood coagulation factor VIII (FVIII) by antigen-presenting cells (APCs) comprises the initial step leading to activation of FVIII-specific T and B cell responses in patients with hemophilia A. Although multiple receptors have been implicated in FVIII uptake, the detailed (intra)cellular mechanism of this process has not been investigated.
Aims: In this study we characterized in more detail endocytosis of FVIII by dendritic cells.
Methods: FVIII uptake experiments were performed using human monocyte-derived dendritic cells (moDCs). Immature moDCs were pre-treated with several drugs including cytochalasin D (cyto D), an established blocking agent for actin polymerization and dimethyl amiloride (DMA), which inhibits sodium channels and is thereby important for regulation of the intracellular pH and actin polymerization, and commonly used as a specific inhibitor of macropinocytosis. We also evaluated the effect of wortmannin, a specific inhibitor of phosphoinositide 3-kinase (PI3K) which has also been implicated in macropinocytosis. The role of heparan sulfate proteoglycans (HSPGs) in FVIII internalization was assessed using siRNA- and pharmacological approaches.
Results: Endocytosis of FVIII was compared to that of reference compounds such as transferrin, reported to be taken up via receptor-mediated mechanism, FITC-labeled high molecular weight dextran, a commonly used marker for macropinocytosis and Lucifer Yellow (LY), a small compound taken up via fluid-phase internalization. Endocytosis of both transferrin as well as dextran was strongly inhibited by cyto D indicating involvement of actin polymerization in the internalization of these antigens. Endocytosis of FVIII was blocked only partially, even when high concentrations (up to 25 mM) of cyto D were used. Uptake of LY was only marginally affected by cyto D. Similar effects were observed for DMA – a strong blockage was observed for transferrin and dextran, none for LY and only a partial effect was seen for FVIII. The PI3K inhibitor wortmannin, was able to partially block endocytosis of dextran, transferrin and FVIII, but not LY. These data suggest that FVIII is endocytosed in an actin-dependent, DMA-sensitive and PI3K-dependent manner however the pharmacological profile was clearly distinct from that observed for dextran. Blockage with dextran sulfate suggested that HSPGs could play a role in FVIII endocytosis by DCs. However, down-modulation of one of the components of the HS-copolymerase did not affect FVIII uptake. Monoclonal antibodies were used to dissect the contribution of the C1 and C2 domain to FVIII internalization. These experiments emphasized the importance of C1 domain but also suggest a potential modulatory role for the C2 domain in FVIII endocytosis by DCs.
Summary/conclusions: Altogether, our data suggest that C1 (and C2) domain mediated internalization of FVIII proceeds via an unusual endocytic pathway that is distinct from classical macropinocytosis pathways as described for high molecular weight dextran.

OC 80.4

An epitope-dependent increase in clearance of antigen-antibody complexes may increase the pathogenicity of a subset of anti-C2 factor fVIII antibodies in hemophilia A

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Background: Anti-factor VIII (fVIII) antibodies develop in about 25% of severe hemophilia A patients. There are both inhibitory and non-

inhibitory anti-fVIII antibodies. It remains unclear whether or not increased clearance of fVIII/antibody complexes plays a role in the pathogenicity of anti-fVIII antibodies.
Aim: The aim of this study was to investigate the clearance of fVIII in the presence of anti-fVIII antibodies using a panel of inhibitory and non-inhibitory anti-fVIII monoclonal antibodies (MAbs) of various IgG subclasses in $fVIII^{-/-}$ mice.
Methods: Eight to 12 weeks old $fVIII^{-/-}$ mice were injected (n = 2–5 per group) by tail vein with 8–10 μg anti-fVIII MAb, irrelevant anti-hen egg lysozyme (HEL) MAb, or saline control. Fifteen minutes later they were injected with 1 μg B domain-deleted fVIII. Plasma was obtained by cardiac puncture 30, 60, 120, 240 and 480 min following the second injection. Six MAbs were tested. 4A4 (IgG1) is an anti-A2 MAb with an inhibitory titer of 40,000 BU/mg. 3D12 (IgG2b) and I109 (IgG1) are classical anti-C2 MAbs that inhibit fVIII binding to phospholipid and von Willebrand factor (VWF). They have inhibitory titers of 2600 and 1500 BU/mg respectively. 2-77 (IgG2a) and 2-117 (IgG2a) are non-classical anti-C2 MAbs that do not interfere with binding of phospholipid and VWF. 2-77 has an inhibitory titer of 25,000 BU/mg and 2–117 is non-inhibitory. Clearance of FVIII antigen was determined by capture ELISA using anti-fVIII MAbs to the A1 and C1 domains of fVIII.
Results: There was no difference in the clearance of fVIII in the presence of saline or the irrelevant anti-HEL IgG. In the presence of the anti-A2 MAb 4A4 as well as the non-classical C2 MAbs 2-77 and 2-117, the fVIII/MAb complexes cleared at a similar rate to fVIII alone. In the presence of the classical C2 MAbs 3D12 and I109, the fVIII/MAb complex cleared more rapidly than fVIII alone. At 120 and 240 min In the 3D12 group had residual fVIII antigen levels of 46.5 and 16.7 ng/mL and the I109 group had levels of 66.7 and 27.2 ng/mL as compared to the control with 212 and 199 ng/mL ($P > 0.05$) for all comparisons, Student t test).
Summary: The classical C2 MAbs 3D12 and I109 increased the clearance of fVIII, while the anti-A2 and non-classical C2 MAbs did not. A potential mechanism for this increased clearance is that classical C2 MAbs block binding of fVIII to VWF, the carrier protein known to increase the half-life of fVIII in the absence of antibodies. We have identified a novel feature of classical anti-fVIII C2 MAbs that results in the clearance of fVIII and may result in increased antibody pathogenicity.

OC 80.5
Identification and characterization of deep intronic variations causing mild hemophilia A

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Background: In a small group of typical hemophilia A patients with reduced plasma factor VIII coagulant activity (FVIII:C) no mutations in F8 coding sequence (cDNA) can be found. In the current study, we performed a systematic screening of genetic and non-genetic parameters associated with reduced FVIII:C levels in patients with no detectable mutations in F8 cDNA.
Methods: For all the patients we determined FVIII and vWF activity and antigen levels. Moreover, we performed FVIII-vWF binding assay and collagen binding activity (CBA) as well as vWF multimer analysis. The exons 17-27 of vWF were sequenced to exclude vWF type 2 Normandy (2N). In addition the complete F8 locus including the introns was sequenced and the mRNA of F8 was analyzed quantitatively and qualitatively by real time PCR (qRT) and overlapping reverse transcription (RT) PCRs, respectively.
Results: We found that in both, patients and healthy controls, FVIII:C levels are largely assignable to FVIII antigen (FVIII:Ag) levels. However, the patients show considerably reduced FVIII:Ag levels that correlate well with both the severity and FVIII:C levels. On the other hand, all vWF tests were normal. Our overlapping Long-Range (LR) PCRs approach proved the integrity of F8 locus on DNA level in these patients. Interestingly, sequencing of the LR-PCRs, using NGS, showed the presence of several base changes; eight of them were found only in the patients and were absent in healthy individuals. Indeed, RT-PCRs analysis confirmed that two of these changes create new cryptic sites in the patients that result in introducing intronic DNA sequences into the mRNA. Our qRT PCR analysis showed reduced F8 mRNA expression over the exon boundaries flanking the intronic SNPs.
Conclusion: Deep intronic mutations in F8, although rare, could cause abnormal splicing that leads to hemophilia A.

OC 80.6
Polyphosphates corrected blood loss in a novel acute tail vein transection hemophilia A mouse bleeding model

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Background: Polyphosphates are linear chains of inorganic phosphate. In human platelets, polyphosphate polymers of 60–100 phosphate units ($polyP_{60-100}$) are stored in dense granules. Upon platelet activation, these platelet polyphosphates enhance coagulation by initiating the contact pathway, accelerating factor V activation, enhancing fibrin clot structure, and mediating factor XI activation by thrombin (Morrissey JH, et al. *Blood*. 2012; 119:5972–5979).
Aim: To evaluate the efficacy of purified $polyP_{70-85}$ in reducing blood loss in hemophilia A (hemA) mice, a novel acute tail vein transection (ATVT) bleeding model with sensitivity higher than the commonly used tail tip amputation model was developed.
Methods: In the ATVT model, hemA mice were anesthetized in a supine position. $PolyP_{70-85}$ or recombinant factor VIII (rFVIII) was administered via jugular vein 5 min before lateral tail vein transection injuries were performed to induce bleeding. Tails were then dipped vertically into warm saline tubes (37 °C) for blood collection. At the 30-min time point, the injury sites were disturbed to evaluate clot stability by removing the tails and placing them into new saline tubes for another 30 min of blood collection. The total blood loss collected over the 60-min period was measured gravimetrically.
Results: In this novel ATVT model, a 0.3-IU/kg dose of rFVIII reduced blood loss by 50%. In contrast, a dose of 30 IU/kg rFVIII was needed to reduce blood loss to the same extent in the tail tip amputation model. Using this sensitive ATVT model, the efficacy of $polyP_{70-85}$ (4 and 12 mg/kg) was evaluated. A 12-mg/kg dose of $polyP_{70-85}$ reduced blood loss to a level similar to the administration of 0.3 IU/kg rFVIII.
Conclusion: This study demonstrated that purified $polyP_{70-85}$ could potentially serve as a therapeutic for correcting the hemostatic defect in hemophilia.

OC 81 – Blood Coagulation Tests

OC 81.1
A next generation sequencing approach for genotyping patients with hemophilia

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Background: Genotyping in congenital hemophilia provides tangible clinical benefits including prediction of inhibitor risk and determina-

tion of carrier status in potentially affected females. Furthermore, genotyping will be critically important for the development of targeted therapies, including gene repair. However, at present, only about 20% of the approximately 20,000 patients with hemophilia in the United States have known genotypes.

Aims: Current approaches for genotyping patients with hemophilia are labor intensive and include long range and inverse PCR to screen for *F8* gene inversions and Sanger sequencing of PCR amplified regions including exons, intron/exon junctions, and 5′ and 3′ untranslated sequence. We sought to develop a genotyping approach based on a next generation sequencing platform for parallel screening of a large number of samples for the presence of *F8* inversions plus insertions, deletions, missense, and splice site changes in both *F8* and *F9*.

Methods: A simplified DNA digestion/ligation protocol using Ksp22i to generate circles of adjacent sequence, produced by intron 1 and intron 22 inversions, was developed. DNA capture was performed with molecular inversion probes (MIPs) to ligated circles (8 MIPs), and to exons, intron/exon junctions, and 5′ and 3′ untranslated sequence of both *F8* (349 MIPs) and *F9* (102 MIPs). The captured sequences were amplified with barcoded PCR primers, pooled together and then run on the Illumina HiSeq. Three 96 well plates containing 234 previously genotyped samples from patients with severe hemophilia A and B were sequenced simultaneously in a single lane. Individuals analyzing Illumina HiSeq results were blinded to the samples' previously determined genotypes.

Results: In hemophilia A samples the sensitivity of this new approach for identifying disease causing mutations was 96% (135/141), including 53/54 samples with inversion 22, 1/2 samples with inversion 1, 43/44 missense or nonsense mutations, 12/13 splice site mutations, and 26/28 samples with insertions or deletions. In hemophilia B the sensitivity was 89% (86/93), including 72/77 missense or nonsense mutations, 3/4 splice site mutations, and 11/12 samples with insertions or deletions. Furthermore, a mutation was identified in the one sample that had previously eluded detection by inversion analysis and Sanger sequencing. All other variants identified by our next generation approach matched those previously detected. Reasons that variants were missed included poor quality DNA in some samples, leading to insufficient MIP capture and sequence reads throughout both genes, and the poor performance of some MIPs that failed to generate an adequate number of reads in all samples tested.

Summary/Conclusion: Our results demonstrate a successful strategy to simultaneously genotype multiple patients with hemophilia A and B. To improve the performance of this assay we plan to evaluate methods to uniformly generate high quality DNA samples and plan to design new MIPs to improve DNA capture in regions that were not adequately covered. Optimization of our approach offers the promise of low-cost high-throughput mutational analysis for both hemophilia A and B that could be used for the large number of hemophilia patients in the US who have not yet been genotyped.

OC 81.2

Developmental hemostasis: preliminary results of a multicenter study aimed at defining the reference ranges for routine coagulation parameters in pediatric populations

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Background: Understanding of developmental hemostasis, a concept now universally accepted, is critical to ensure optimal prevention, diagnosis, and treatment of hemorrhagic and thrombotic diseases in children. However, coagulation test results are known to be dependent on the reagents and analyzers used. This is particularly critical for global assays such as prothrombin time (PT), activated partial thromboplastin time (aPTT). So, it is recommended for each laboratory to define the age-dependent reference ranges by using its own technical condition (J Thromb Haemostas 2012; 10: 298).

Methods: To address that issue, the present multicenter study was carried out in three centers using the same reagents i.e. RecombiPlasTin 2G for PT, SynthASil for aPTT, and HemosIL QFA for fibrinogen and the same instrument ACL TOP (all from Instrumentation Laboratory, Bedford, MA, USA).

Samples were obtained from the routine workload in all participating centers. Indication of the coagulation testing was pre-operative screening in most cases. There were 288 samples obtained from 288 pediatric patients:193 H and 95 F, with a mean age = 1.5 years (range: 2 weeks and 17 years).

Results: As the data obtained in the three centers were not significantly different, test results were pooled and further analyzed in the different age-groups. The data were found to be normally distributed, allowing expression as the mean values with SD. As the result, PT (ratio) was positively correlated with children age, with shorter clotting times in younger children: 0.94 + 0.08 in 14 children aged below 1 month, 0.98 + 0.07 in 99 children between 1 month and 1 year, 1.01 + 0.08 in 101 children aged between 1 and 5 years, 1.06 + 0.08 in 28 children aged between 6 and 10 years, and 1.05 + 0.07 in 46 children between 11 and 16 years. Conversely aPTT (ratio) was negatively correlated with age, with longer clotting times in younger children: 1.18 + 0.11 in children aged below 1 month, 1.12 + 0.12 in children between 1 month and 1 year, 1.06 + 0.12 in children aged between 1 and 5 years, 1.07 + 0.12 in children aged between 6 and 10 years, and 1.04 + 0.10 in children between 11 and 16 years. Fibrinogen levels (in g/L) were 2.08 + 0.53, 2.48 + 0.55, 2.92 + 0.64, 2.85 + 0.53 and 2.82 + 0.52, respectively in children pertaining to these five age groups.

Conclusions: These data suggest that, at least in the described technical conditions, routine coagulation test results are highly dependent on age and that age-specific reference ranges must be used to ensure proper evaluation of coagulation in children.

OC 81.3

Evidence that the effects of rivaroxaban are dependent on the degree of activation of the coagulation system

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Background: The effects of rivaroxaban can be assessed with variable responses using the Prothrombin Time (PT). When using Simplastin Excel S® (SES) the effects are much stronger than with any other PT reagent. PT prolongation with SES results in 2–4-fold increases, comparable to prolongations observed with vitK-antagonists.

Aims: We explored possible mechanisms explaining the strong response to rivaroxaban with the SES reagent and put this in context with findings using other global tests.

Methods: Clotting assays were performed on the Amax Destiny plus. TGA-RCH® (Technoclone) and Extem Rotem® were used. Effects of rivaroxaban were evaluated by spiking plasma. Effects of factor V were evaluated using mixtures of normal with factor V-depleted plasma, a factor V quenching monoclonal and a venom activating factor V (RVV-V). Contrasts were made between the most sensitive (SES) and the most insensitive reagent (Innovin®) for rivaroxaban.

Results: Using depleted plasmas showed that the strong rivaroxaban effect in SES was independent of coagulation factors XII, XI, X, IX and factor VIII. However, the effect using plasma with low FV levels was exaggerated. This was also noted when using Innovin when quenching antibodies to factor V were added. Conversely, addition of factor V activating venom (RVV-V) attenuated the rivaroxaban effect at low factor V levels and completely abolished the strong effect of rivaroxaban in SES.

Starting clotting with purified factor Xa, with or without RVV-V using factor VII deficient plasma to compare the lipids showed comparable IC50 for rivaroxaban for all combination (25–35 nM) excluding differences in lipid effect on the active components Xa and Xa-Va. This identified activation of FV in SES to be poor. We further explored if lipids could be involved in the poor activation of factor V with SES. Adding Innovin-lipids to SES completely abolished the strong sensitivity to rivaroxaban, identifying specific lipids in SES (currently unknown) to be responsible.

When the IC50 from SES (110 nM) and Innovin (385 nM) are converted (Cheng Prusoff formula) in amount of factor Xa participating in the inhibition it revealed that in Innovin 60% and in SES 17% of factor X is converted. The amount of factor Xa participating in other global tests was 8% in the thrombin generation test (TGA-RCH), 37% in the Extem Rotem alpha value, 46% in the APTT. The low participation of FXa in SES and the thrombin generation test apparently explains their sensitivity for measurement in the therapeutic range of rivaroxaban.

Conclusions: The lipid component in SES is responsible for a reduced activation of factor V resulting in limited FXa formation and a strong sensitivity to inhibition by rivaroxaban. Rivaroxaban inhibits more potently in tests with low factor Xa activation. For pharmacodynamic assessment in addition to SES other global tests with low factor Xa participation such as TGT-RCH may also be suitable. The physiological implication may be that rivaroxaban is primarily effective in situations with low grade coagulation activation and increasingly ineffective when coagulation activation is more massive.

OC 81.4

Development of a quantitative model of systemic procoagulant condition due to bacterial sepsis

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Background: Intravascular blood coagulation is common in patients with severe sepsis. The resulting coagulopathy is linked to the presence of bacterial cell components, possibly including polyphosphates. Polyphosphates (polyP) are linear polymers of inorganic phosphate that are abundant in the acidocalcisomes of prokaryotes and unicellular organisms as well as in the dense granules of platelets. PolyP has been shown to support the contact activation of coagulation factor XII (FXII), and accelerate the activation of factor XI (FXI), resulting in thrombin generation, complement system activation, and release of the inflammatory peptide bradykinin from high-molecular-weight kininogen. The contact proteases appear to play a significant prothrombotic role in response to bacteria as part of the innate immune response, which can lead to thrombotic complications in the setting of severe bacterial sepsis.

Aims: The aim of this study was to develop an assay to quantify the procoagulant phenotype associated with bacteria sepsis.

Methods: We have developed a quantitative *ex vivo* model of pathological thrombus formation. Our model takes advantage of gravity to provide a constant pressure gradient to drive recalcified blood that has been supplemented with coagulation activating and inhibiting agents through a capillary tube coated with collagen and tissue factor (TF).

Results: Our data demonstrate blood flow ceased after ~20 min in tubes that had been coated with collagen and TF. Experiments were designed to determine whether long polymers of polyP (~1000 phosphate units, such as those present in bacteria) were able to promote occlusive thrombus formation under a constant pressure gradient in a dose-dependent manner (time to occlusion = 19.3 ± 1.5, 15.8 ± 1.5, 12.8 ± 0.6, and 11.5 ± 1.5 min in the presence of vehicle vs. 25, 50 or 100 μM, respectively). Pretreatment of blood with corn trypsin inhibitor, which inhibits FXIIa, prolonged the time to occlusion in the presence of 100 μM long polyP to ~28 min, indicating a role for FXIIa in the ability of long polyP to promote occlusive thrombus formation. In contrast, a panel of function blocking anti-FXI antibodies failed to inhibit the prothrombotic effect of long polyP in our system.

Summary/Conclusion: We have developed a model of pathological occlusive thrombus formation to quantify the systemic procoagulant condition due to bacterial cell components that are present in sepsis. Our data show that polyP of the size present in bacteria promote occlusive thrombus formation in a FXII-dependent, yet FXI-independent, manner. Current efforts are focused on the calibration of thrombus formation in our model as a function of colony-forming units of bacteria. Future efforts will be focused on adapting this model for use as a point-of-care device for assessing patient procoagulant activity during bacterial sepsis.

OC 81.5

A high performance of Nijmegen-Bethesda assay in therapy to induce immune tolerance for hemophilia patients

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The Bethesda method originally described by Kasper in 1975 and later modified by Verbruggen in 1995 is the gold standard for assessment of inhibitory antibodies against factors (F) VIII and FIX. The method has some limitations, such as the presence of residual endogenous or exogenous factor in moderate and mild haemophilia or from replacement therapy, respectively. Some studies show that treatment of these samples with heat improves the sensitivity of this test. The objective of this study is to compare inhibitor quantification obtained of samples previously treated and untreated with heat from hemophilia patients with and without inhibitor. For each analysis the plasma samples were collected and processed equally and separated into two aliquots. In the moment of the analysis one of the aliquots was treated with heating at 56 °C for 30 min and then centrifuged at 15,000 rpm 5 min. The Nijmegen-Bethesda assay was performed for the two aliquots. Results > 0.6 BU were considered negative for the presence of inhibitor. The statistical analysis was performed using the Mann-Whitney test. One hundred nine analyses from 46 patients with severe hemophilia A (FVIII > 1.0 IU/dL) were performed. Patients were divided into three distinct groups: Group (I) 20 patients without history of inhibitor, exposed and not recently exposed to FVIII. Group (II) 21 patients with history of inhibitor not exposure to FVIII. Group (III) five patients (68 analysis) undergoing ITI protocol. For patients with no history of inhibitor the heating of samples did not modify results when compared with no heating sample ($P = 0.24$). However, a statistical significant difference between the samples was observed in patients with positive history of inhibitor. The difference found in the group II ($P > 0.05$) was evaluated against a ratio of FVIII:C/FVIII:Ag to obtain data about the FVIII exogenous to confirm the days without FVIII exposition. The higher difference was observed in the group III ($P > 0.001$), the title of inhibitor obtained with heat samples had a median of 7.1 times (2.0–16.1 times) higher when compared with samples without heat treatment and six of them shift the negative (0.19–0.39 BU) to positive (1.8–4.43 BU) results. In additional, the results obtained from each ITI patients show the same trend line obtained with the results of heating and no heating samples. The presence of FVIII in patient samples violates the principle of Bethesda method, where patient and control samples must be equivalent before incubation. Therefore, the additional presence of FVIII (exogenous) may modify the kinetics between antigen and antibody and provide results lower than the expected. In our experience, heating sample showed increased sensitivity of method and no shift from negative to positive was observed

in results of patients without history of inhibitor. Furthermore, this procedure appears to be an important application for samples from patients undergoing ITI protocol. In daily practice, when one cannot wait 72 h without infusion of FVIII containing products for analysis of quantification of inhibitor, heat treatment of samples may enable the determination of the test.

OC 81.6

A nationwide Belgian survey on the influence of the new oral anticoagulants dabigatran and rivaroxaban on commonly used coagulation assays

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Background: The new oral anticoagulants dabigatran etexilate (direct thrombin inhibitor, Pradaxa®, Boehringer Ingelheim) and rivaroxaban (direct factor Xa inhibitor, Xarelto®, Bayer) have been in clinical use for a few years for the prevention of thromboembolic events after elective hip- or knee-replacement surgery and for the prevention of stroke and systemic embolism in patients with atrial fibrillation. Although these agents do not require monitoring, their presence can significantly influence coagulation assays, potentially leading to incorrect interpretation of test results.

Aims: The Belgian national External Quality Assessment Scheme for blood coagulation organized a survey to investigate and illustrate to the clinical laboratories how these drugs affect their routine coagulation assays.

Methods: All Belgian clinical laboratories routinely performing coagulation testing (n = 192) received five lyophilized plasma samples in April 2012. These samples consisted of a normal plasma pool spiked with dabigatran or rivaroxaban to the following final concentrations: 0, 100 ng/mL dabigatran, 250 ng/mL dabigatran, 120 ng/mL rivaroxaban, and 290 ng/mL rivaroxaban. The samples were purchased from Hyphen BioMed (Neuville surOise, France). Participants were requested to determine the following coagulation assays: prothrombin time (PT, seconds,% and INR), activated partial thromboplastin time (aPTT, seconds and ratio), fibrinogen and antithrombin. They were also required to mention the reagent and analyser used.

Method-specific medians and robust standard deviations were calculated for all methods with ≥ 6 reported results (method of Tukey). Differences between methods were assessed by means of non parametric statistics (Wilcoxon test).

Results: All but three laboratories participated in the survey (response rate of 98.4%).

PT and aPTT: Both, dabigatran and rivaroxaban significantly prolonged the PT and aPTT in a concentration- and reagent-dependent manner. The PT was more influenced by rivaroxaban than by dabigatran, while the aPTT was more influenced by dabigatran than by rivaroxaban. There was a wide variation in responsiveness between reagent/instrument combinations. The PT reagents Neoplastin CI Plus and Neoplastin R (Diagnostica Stago, Asnières sur Seine, France) were the most sensitive to rivaroxaban and the reagents Innovin and Thromborel S (Siemens, Marburg, Germany) the least sensitive. The aPTT reagents most and least sensitive to dabigatran were CK Prest (Diagnostica Stago) and Actin FSL (Siemens). Converting PT results to INR did not reduce but even increased the variability between reagents.

Fibrinogen: Rivaroxaban did not influence the determination of fibrinogen but the presence of dabigatran led to a falsely reduced fibrinogen concentration when measured with a low thrombin concentration reagent.

Antithrombin: The presence of dabigatran caused an overestimation of the antithrombin level when measured with an assay based on thrombin inhibition while the presence of rivaroxaban caused an overestimation of the antithrombin level when measured with an assay based on FXa inhibition.

Summary/Conclusions: This study demonstrates that the influence of dabigatran and rivaroxaban on the routine coagulation assays used in Belgium largely depends on the reagent/analyser combination used and the drug concentration. All laboratories received a full report of the results in order to draw attention to the importance of careful interpretation of coagulation test results in patients taking dabigatran or rivaroxaban.

OC 82 – Cancer and Thrombosis

OC 82.1

The role of a prothrombinase – fibrinogen-like protein 2 in angiogenesis and tumorigenesis

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Background: Hematologic and solid tumors are associated with hypercoagulability the reason for which has not been delineated. Prothrombinase named fibrinogen-like protein 2 (FGL-2) is a 70 kD transmembrane protein that was found to have a quality of a serine protease capable of directly cleaving prothrombin to thrombin. FGL-2 is synthesized by monocytes, T-lymphocytes and endothelial cells. FGL-2 protein and its mRNA have been previously found within different tumor cells. In our previous work we found that FGL-2 activity was increased in peripheral blood mononuclear cells (PBMC) from patients with prostate, colon and lymphoproliferative malignancies. Our hypothesis is that upregulation of FGL-2 activity in cancer patients may contribute to angiogenesis and tumorigenesis.

Aim: To study the role of FGL-2 in angiogenesis and tumor development using *in vitro* and *in vivo* assays.

Methods: Thrombin generation reflecting FGL-2 activity was measured in different cells after addition of prothrombin. mRNA of FGL-2 was measured in PBMC, HUVEC and PC3 (human prostate carcinoma cell line) by quantitative RT-PCR analysis. *In vitro*, FGL-2 angiogenesis induction was evaluated by matrigel assay. *In vivo*, FGL-2-induced tumor development was tested in SCID mice after injection of PC3 cell line, which has increased FGL-2 activity or PC3 cell line where FGL-2 activity was inhibited by specific siRNA.

Results: Increased mRNA of FGL-2 was found in PBMC from patients with either lymphoma, prostate or colon cancer and in PC-3 cell line. Following treatment of HUVEC and PC-3 line with interferon-gamma the amount of mRNA increased even further. FGL-2 activity was knocked out in either HUVEC or PC3 by specific siRNA. *In vitro*, a significant angiogenesis was observed in matrigel assay after addition of PC3 cells with intact FGL-2 while addition of PC3 with knock out FGL-2 completely inhibited blood vessel formation. Addition of hirudin had no effect on angiogenesis indicating that FGL-2 mediated pro angiogenetic effect is not induced by thrombin.

PCR array of HUVEC with knock out FGL-2 using specific panel of human angiogenesis genes revealed a 500-fold decrease in the expression of Epidermal growth factor gene and Insulin-like growth factor 1. *In vivo*, tumor development in SCID mice after injection of PC3 cell line with or without FGL-2 activity was assessed. Nine out of ten mice injected with PC3 expressing FGL-2 developed tumors after 3 weeks. In contrast, out of five mice injected with blocked FGL-2 clone of PC3, three have not developed tumors at all, and the remaining two showed small undefined cluster of cells at the injection site. Precise his-

topathology analysis of all the mice will be done at week 6 after the injection.
Conclusions: The results of this research indicate that FGL-2 may induce angiogenesis and tumor development. In the future FGL-2 targeting may have a potential therapeutic value.

OC 82.2
Brain metastasis depends on tumor cell initiated coagulation

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Background: The incidence of brain metastasis exceeds that of primary brain tumors 10-fold and is most frequently associated with lung cancer, breast cancer and melanoma. Despite the dismal prognosis of only 6–9 months for patients with brain metastases, the mechanisms of tumor cell brain colonization from the blood stream are unknown. Understanding this step could enable effective therapeutic strategies to prevent the development of incurable metastatic brain disease.
We found that metastatic cancer cells reside within the cerebral microvasculature for several days and associate with platelets while penetrating the blood brain barrier. Intravascular survival and extravasation of the tumor cells during this time may critically limit the success of brain metastasis. The brain microvasculature may uniquely influence hemostatic system function in tumor cell colonization. Activation of coagulation, platelets and fibrin formation contribute to tumor progression, cancer-associated thrombosis and metastatic spread to peripheral organs, however the function of coagulation in the seeding of brain metastases is not known.
Aims: We evaluated whether tumor cell-expressed tissue factor, an activator of coagulation, promotes tumor cell seeding of brain metastases by initiating critical tumor cell-vascular interactions.
Methods: We developed models of experimental brain metastasis to study how intravascular tumor cells cooperate with the coagulation system during the early stages of colonization across the blood brain barrier. Human breast cancer cells are injected into the left cardiac ventricle of immune deficient mice. The tumor cells are then followed during the initial phase of brain metastasis and progressive metastatic brain disease using detailed and quantitative histological analyses. We address how coagulation contributes to brain metastasis by targeting tissue factor or platelets. This is accomplished by initial treatment with antibodies that specifically inhibit human tissue factor function or by depleting platelets. We use *ex-vivo* bioluminescence imaging and immunohistochemical analyses at later stages of brain metastasis to determine the extent to which an early, transient treatment will result in long-term, diminished macrometastatic burden as the disease progresses.
Results: We demonstrate that inhibition of tissue factor expressed by tumor cells reduces brain colonization, which results in diminished progression of breast cancer brain metastasis and extended animal survival. We find that tissue factor properties which initiate coagulation and promote cytoprotective signaling pathways differentially contribute to brain metastasis development. Our findings indicate that the inhibition of coagulation can prevent the seeding of breast cancer cells into the brain and result in overall reduction of brain metastatic burden.
Summary/Conclusions: Our studies provide mechanistic insights into the process of tumor cell brain colonization and may lead to the identification of targets and development of therapeutics to prevent cancer metastasis to the brain and enhance patient survival rates.

OC 82.3
The heparin/heparan-sulphate interactome of human breast cancer cells exerts a pro-tumourigenic role associated with activation of the PI3K/Akt and MAPK/ERK signalling pathways

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Background: Heparan sulphate (HS) interacting proteins form a tightly interconnected network – the heparin/HS interactome – that contributes to cell function in higher organisms. Heparin treatment which is able to modulate interactions between the heparin/HS interactome and HS proteoglycans (HSPGs) has been shown to prolong the survival of patients with cancer, but the fundamental mechanisms underlying this benefit are largely unknown.
Aim: To investigate the global effect of protein-heparin/HS interactions on the tumorigenicity of breast cancer cells.
Methods: Tow human breast cancer cells lines (MCF-7 and MDA-MB-231) were cultured under serum-free conditions in which the cellular response was maintained by secreted autocrine factors and other members of the heparin/HS interactome and in the presence of heparin to modulate HSPG interaction. Confirmatory observations were made using cells cultured in the presence of β-D-xyloside, which inhibits HSPG synthesis. The tumourigenicity of the cells was determined by measuring a wide range of indices including gene expression, and cell signalling through gene microarray, RT-PCR, Western blot and immunofluorescence analysis. Tumour cell properties were also evaluated by a series of cell function assays.
Results: Microarray analysis of MCF-7 cells cultured under these conditions showed that expression of 105 of 1357 genes potentially related to the pathogenesis of breast neoplasm was significantly altered by heparin treatment. The changes in gene expression correlated with a less tumourigenic phenotype, including reduction of cell adhesive, invasive and migratory properties. These effects were associated with an inhibition of the PI3K/Akt and Raf/MEK/ERK signalling pathways. The modulatory effect of heparin on HS-associated activity was confirmed with one member of the heparin/HS interactome: TGFβ, a key cytokine. The innate TGFβ activity of MCF-7 cells was reduced by heparin treatment, with specific interruption of the TGFβ–Smad signalling pathway. The pro-tumourigenic contribution of the heparin/HS interactome was verified in cells in which HSPG synthesis was blocked using β-xyloside.
Conclusion: Modulation of the HSPG-dependent action of the heparin/HS interactome induces a less tumourigenic genotype and phenotype associated with inhibition of the PI3K/Akt, Raf/MEK/ERK and TGFβ-Smad signalling pathways. The innate heparin/HS interactome of breast cancer cells makes a significant contribution to tumourigenicity, therefore establishing that the beneficial effect of heparin upon the survival of cancer patients is probably due to its ability to reduce the pro-tumourigenic activity of the heparin/HS interactome.

OC 82.4

Systemic venous thrombotic events are associated with significantly increased loss of central venous catheters in pediatric cancer patients: a Multicenter Study

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Background: Central venous catheters (CVCs) have greatly improved the care of pediatric oncology patients. Loss of CVC necessitates immediate replacement, requiring general anesthesia, surgical intervention and is a significant cost burden to the health care system. Convincing evidence shows that venous thrombotic events (VTE) occur frequently during childhood cancer and that VTE are associated with CVC use. However, clinical significance of VTE with respect to CVC management has never been studied. We wanted to explore our hypotheses that VTE in childhood cancer patients are significantly associated with increased CVC loss and that VTE maybe causal in some children, as indicated by an association of CVC loss with location of VTE.

Aim: (i) investigate the association of VTE with loss of CVCs and (ii) assess association of location of VTE with CVC loss.

Methods: In this multicenter case-control study, after ethics approval and informed consent, childhood cancer survivors with and without symptomatic VTE during their cancer therapy were recruited from 5 Canadian centers. Additionally, patients in whom an asymptomatic VTE was detected were assessed. Number and location of CVCs was compared between the three groups.

According to location, VTE were categorized into two groups (i) systemic VTE or (ii) non-systemic VTE. Systemic VTE included (i) central VTE: VTE in veins proximal to and including axillary and femoral veins and (ii) peripheral VTE: VTE in veins distal to axillary and femoral veins.

Results: Seventy-seven survivors (cases) with symptomatic VTE, 10 with asymptomatic VTE (systemic VTE $n = 7$, non-systemic $n = 3$) and 178 controls (no VTE) were recruited. The locations of symptomatic VTE were: systemic VTE ($n = 58$) (central VTE $n = 49$ and peripheral VTE $n = 9$) and non-systemic ($n = 19$) (sinovenous thrombosis $n = 7$ and other sites $n = 12$).

Among the controls, 11.8% required > 1 CVC. In comparison, > 1 CVC was required by a significantly increased proportion of children with (i) systemic VTE (53.4% $P = 0.0001$), (ii) central VTE (55.1%, $P = 0.0001$), (iii) peripheral VTE (44.4%, $P = 0.017$) and (iv) systemic asymptomatic VTE (57.1%, $P = 0.007$). Similar to controls, only 10.5% in the nonsystemic VTE group required > 1 CVC ($P = 0.592$).

The mean number of CVCs in the control group was 1.12 ± 0.37. In comparison, the mean number of CVC was significantly increased in the systemic VTE group (1.75 ± 0.872, $P = 0.0001$) and central VTE group (1.81 ± 0.86, $P = 0.0001$). In contrast, the mean number of CVC in nonsystemic VTE group (1.28 ± 0.958) was similar to controls ($P = 0.301$).

Central VTE and location of CVCs showed a concordance of 55% ($n = 27$) with probable concordance in additional 10.2% ($n = 5$) patients.

Summary: The current study is the first to demonstrate a significantly increased CVC loss as an adverse clinical outcome of both symptomatic and asymptomatic systemic, especially central, VTE. Non-systemic VTE do not contribute to increased CVC loss. A > 55% concordance in CVC location and central VTE suggests a significant contribution of CVCs in VTE development.

Given the clinical and economic implications of CVC loss, future trials need to focus on safety and efficacy of primary prophylaxis in VTE prevention in childhood cancer patients.

OC 82.5

Coagulant activity and cellular origin of circulating tissue factor exposing microparticles in cancer patients – two forms of TF-exposing microparticles

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Background: Because plasma of cancer patients presenting with venous thrombosis contains high numbers of tissue factor (TF)-exposing microparticles (TF-MP[1]), TF-MP have been causally linked to the occurrence of venous thrombosis in cancer patients. The relationship between numbers of TF-exposing MP and the TF-MP dependent coagulant activity, however, is unclear. In addition, to which extent TF-MP originate from cancer cells is also unknown.

Aim: We investigated the relationship between TF-MP numbers and coagulant activity, and the cellular origin of circulating TF-MP in cancer patients.

Methods: Citrate-anticoagulated blood was collected via a single blood withdrawal from cancer patients undergoing chemotherapy. The number of TF-MP was measured by flow cytometry and TF-MP coagulant activity was measured in a fibrin generation test (FGT) in the presence or absence of an inhibitory antibody to human factor VII. Patients were categorised as having a low or high number of TF-MP (≤ or > 95th percentile), and as having low or high TF-MP coagulant activity (≤ or > 13% TF-dependent prolongation of clotting time as determined by FGT). The cellular origin was determined by flow cytometry in those patients with number of TF-MP above the 95th percentile of the total cohort.

Results: Of the total cohort of 209 cancer patients, 98 had metastasis (47%). Overall, no correlation was present between the numbers of TF-MP and the coagulant activity ($r = 0.029$, $P = 0.69$). When comparing patients with a high and low number of TF-MP, respectively three of the 12 (25%) and 60 of the 192 (31%) patients had high TF-MP coagulant activity. Conversely, when comparing patients with high and low TF-MP coagulant activity, respectively only three of the 63 (4.8%) and nine of the 141 (6.4%) patients had a high number of TF-exposing MP.

There was a marked variation between patients with regard to the cellular origin of the TF-MP. Of the 13 patients with high TF-MP numbers, the cellular origin of TF in five patients was variable and added up to above 100%; including 67% and 44% staining for two independent tumor markers, 66% for a platelet marker, and 59% for a monocyte marker (all medians). In the other eight patients, the origin of > 25% of TF-MP could be established, and most of these TF-MP originated from platelets and no tumor-derived vesicles were detectable.

Summary/Conclusions: Because increased numbers of TF-MP and coagulant activity are almost mutually exclusive, we postulate that two forms of TF associated with MP circulate in cancer patients, a coagulant form which is present in minute quantities and below the detection limit of flow cytometry, and a non-coagulant form which is detectable by flow cytometry. Taken together, although at least part of the TF-MP can derive from tumor cells, the cellular origin of circulating and coagulant TF-exposing MP in cancer patients remains to be elucidated.

1. Zwicker et al., Clin Cancer Res 2009.
2. Davila et al., JTH 2008.

OC 82.6

Colon adenocarcinoma cell recruitment to platelets and thrombi under shear

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Background: Cancer metastasis involves separation of cancer cells from a primary tumor, entrance of tumor cells into the blood or lymphatic circulation, adhesion to or entrapment at a distal site, and proliferation to form of a secondary tumor. The anatomical site-specificity of cancer metastasis, the effect of antithrombotic treatments on metastasis, and the role of the vessel microenvironment in cancer metastasis remain to be explored.
Aims: We aimed to investigate the kinetics and molecular mechanisms of metastatic colon adenocarcinoma cell recruitment to fibrillar proteins and thrombi under shear flow, *ex vivo*.
Methods: SW620 cells (1×10^6 cells/mL; colon adenocarcinoma cells from lymph node) were perfused over immobilized fibrillar collagen, fibrinogen, laminin, fibronectin, or von Willebrand factor at physiologically relevant shear rates ranging from 25 to 200/s. Platelet aggregates were formed by perfusing citrate anticoagulated whole blood over fibrinogen or collagen. Thrombi were formed by perfusing recalcified whole blood over fibrinogen or collagen in the presence of coagulation. Tumor cells were perfused either during or following platelet aggregate or thrombus formation. The role of polymorphonuclear leukocytes (PMNs) in tumor cell recruitment was investigated by perfusing purified PMNs over both platelet aggregates and thrombi. The degree of transient tumor cell interactions (recruitment, rolling and release) and the number of firmly adhered tumor cells were quantified using fluorescence microscopy.
Results: The extracellular matrix proteins, fibrillar collagen, fibrinogen, laminin, fibronectin, and von Willebrand factor supported tumor cell recruitment and adhesion in a shear-dependent manner, with a maximal degree of binding observed at the lowest shear rate (25/s). The rate of transiently interacting SW620 cells varied from nearly 200 cells/mm^2/min on collagen to > 30 cells/mm^2/min on fibrinogen. Platelet aggregates and thrombi formed on either fibrinogen or collagen supported SW620 cell interactions and adhesion under shear. Increased SW620 cell interactions and binding was observed on platelet aggregates and thrombi formed on collagen as compared to fibrinogen. Moreover, thrombi supported a greater degree of SW620 cell interactions and adhesion as compared to platelet aggregates formed on either collagen or fibrinogen. Interestingly, in the absence of anticoagulation, we observed SW620 preferentially binding to clot-bound leukocytes. Along these lines, addition of purified leukocytes (1×10^6 PMNs/mL) to thrombi resulted in a doubling of the number of interacting and bound SW620 cells.
Conclusion: Our findings demonstrate that colon adenocarcinoma cell tethering, rolling and firm adhesion to extracellular matrix proteins, platelet aggregates, leukocytes, and thrombi under flow are enhanced in recalcified blood and reduced by shear. The results suggest that metastasizing cancer cells may preferentially home to sites of blood vessel injury and inflammation that induce local thrombin generation.

OC 83 – Coagulation Factor VII

OC 83.1

Novel insights into the therapeutic mode of action of recombinant FVIIa

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Background: Recombinant FVIIa (rFVIIa, NovoSeven®) is used as bypassing agent in haemophilia patients with inhibitory antibodies against factor VIII (FVIII) or IX. The need for supraphysiologic doses of rFVIIa has been explained in two ways; to efficiently compete with endogenous FVII for tissue factor (TF) or to obtain a sufficient amount of rFVIIa bound to activated platelets, that is, to allow rFVIIa to activate factor X in a TF-dependent and -independent manner, respectively.
Aims: To evaluate the influence of zymogen FVII competition for TF and FVII (auto)activation on thrombin generation induced by rFVIIa. To assess the relative contributions of the TF-dependent and -independent pathways under haemophilia A conditions.
Methods: Thrombin generation was quantified in FVII-deficient, FVIII-depleted platelet-rich plasma (PRP) (zymogen effect studies) or FVIII-depleted PRP (pathway studies) and initiated with TF (Innovin, 1 pM), Ca^{2+} and fluorogenic substrate. Recombinant FVII, $FVII_{R152A}$, FVIIa and $FVIIa_{DVQ}$ were produced in house. $FVII_{R152A}$ cannot be converted to FVIIa. $FVIIa_{DVQ}$ contains three amino acid changes (V158D/E296V/M298Q) resulting in enhanced TF-independent capability to activate factor X.
Results: Zymogen competition with FVIIa for TF and its influence on thrombin generation were studied using both FVII and $FVII_{R152A}$ in order to simultaneously assess the importance of FVII (auto)activation. In the presence of 6 nM FVIIa, a concentration-dependent lowering of thrombin generation was seen in the presence of $FVII_{R152A}$ (10–100 nM) but not with FVII. The reduction at 100 nM $FVIIa_{R152A}$ (15-fold excess over FVIIa) was the same as that obtained with a 15-fold reduction in TF in the absence of any FVII. In line with this, addition of $FVII_{R152A}$ and FVII at increasing $FVII_{R152A}$/FVII ratios (100 nM in total) resulted in gradually decreasing thrombin generation. A similar observation was made when lowering the FVIIa concentration in the presence of 100 nM FVII. To distinguish between the sites of FVIIa action, the effects of $FVIIa_{DVQ}$ and FVIIa in FVIII-depleted PRP were compared. At concentrations ≤ 100 pM, similar thrombin generation was observed. Above 100 pM, thrombin generation was higher with $FVIIa_{DVQ}$ than FVIIa, suggesting that the TF-independent contribution became evident and predominant above this threshold. In contrast, FVIIa and $FVIIa_{DVQ}$ behaved much more similarly in FVII-deficient PRP where they act as replacement agents.
Conclusions: Zymogen FVII competed with FVIIa for binding to TF but a subsequent rapid (auto)activation to FVIIa prevented a negative effect on thrombin generation. This rapid activation relied on a certain TF (TF:FVIIa complex) density. Because the thrombin generation potentials of FVIIa and $FVIIa_{DVQ}$ under haemophilia A conditions were indistinguishable at concentrations ≤ 100 pM the activity appears to be TF-dependent (saturation of membrane TF). Above 100 pM, the TF-independent activity became evident as seen by a much larger effect of $FVIIa_{DVQ}$. At therapeutic concentrations of rFVIIa (above 5 nM, typically 25 nM) the major component of its mode of action appears to be TF-independent.

OC 83.2

Multicentre, randomised, double-blinded, active-controlled, cross-over phase 3 trial on safety and efficacy of rFVIIa analogue (vatreptacog alfa) in haemophilia patients with inhibitors (adept™2)

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Background: Vatreptacog alfa is a recombinant factor VIIa (rFVIIa) analogue which was developed to provide an improved resolution of bleeds in patients with haemophilia and inhibitors. Its amino acid sequence is 99% identical to rFVIIa, with the only change being three

amino acid substitutions (V158D/E296V/M298Q). In a phase 2 dose-escalation trial, vatreptacog alfa demonstrated preliminary efficacy and safety, with no anti-drug antibodies (ADA) detected (JTH 10:81–89, 2012).

Aim: To evaluate the safety and efficacy of vatreptacog alfa in treating bleeding episodes in haemophilia patients with inhibitors.

Methods: This was a global, multi-centre, double-blind, cross-over, confirmatory trial in haemophilia A or B patients ≥ 12 years with inhibitors. Bleeding episodes were randomly assigned in a 3:2 ratio to treatment with vatreptacog alfa (1–3 doses at 80 μg/kg) or rFVIIa (1–3 doses at 90 μg/kg). If haemostasis was not achieved after three doses of trial product (TP), the patient was treated according to local standard of care. In addition to general safety assessments, ADA testing was performed prior to first drug exposure, at least every 3 months during the trial and at least 1 month after last TP administration.

Results: Seventy-two patients were enrolled, and 567 bleeds were treated with TP. Successful bleeding control (no use of additional haemostatic medication, other than TP, within 12 h; primary efficacy analysis) was similar for both vatreptacog alfa and rFVIIa, and was achieved in 93% of all treated bleeds with 1–3 doses. Vatreptacog alfa was superior to rFVIIa in several secondary efficacy analyses, including fewer doses used to treat a bleed and sustained bleeding control.

Incidence of serious adverse events reported after the treatment of bleeds was very low, with 0.9% for vatreptacog compared to 2.2% for rFVIIa, and general safety assessments revealed no safety concerns. However, 8/72 patients (11%) developed binding antibodies to vatreptacog alfa (including one with *in vitro* neutralising effect) following > 10 exposure days. In 4/8 patients, anti-vatreptacog alfa antibodies developed low titre cross-reactivity towards rFVIIa. The antibody binding to rFVIIa could, in all cases, be outcompeted by vatreptacog alfa, indicating that these antibodies are induced by vatreptacog alfa but cross-bound with lower affinity to rFVIIa.

Summary: The adept™ 2 trial, which is the largest of its kind ever conducted in inhibitor patients, confirmed the well-established efficacy and safety profile of rFVIIa and demonstrated that vatreptacog alfa is similar in primary efficacy outcome as rFVIIa and superior in secondary efficacy outcomes to rFVIIa. The high incidence of ADA development after vatreptacog alfa exposure indicates that even very small sequence changes can significantly alter the immunogenicity of rFVIIa compounds. Long term exposure would be needed to fully assess the immunogenicity of new rFVIIa compounds and to evaluate the risk that continued treatment could result in clinical inhibitor formation.

OC 83.3

EPCR binding to factor VIIa enhances its hemostatic function *in vivo*

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Background: In addition to tissue factor (TF), human FVIIa is a ligand for the endothelial protein C receptor (EPCR), but the functional consequences of EPCR binding on FVIIa activity are still unclear and may influence the therapeutic effects of FVIIa administration in hemophilic patients. Due to FVIIa-TF species incompatibilities and the fact that murine FVIIa (mFVIIa) does not interact with murine EPCR (mEPCR), mouse models are not appropriate to investigate the role of FVIIa-EPCR interaction *in vivo*. However, we have previously shown that mEPCR binding capacity can be engineered in mFVIIa by three modifications (L4F, L8M and W9R; FMR-mFVIIa). This molecule allows further studies into the functional consequence of EPCR-FVIIa interaction in a mouse model of hemophilia.

Aim: To investigate the effect of EPCR binding on FVIIa procoagulant activity *in vivo*.

Methods: Because of the sub-optimal species compatibility between clotting factors, we characterized the activity of FMR-mFVIIa or mFVIIa using homologous reagents that mimic the *in vivo* setting. Murine activated FX (mFXa) generation assays were performed on CHO-K1 cells expressing murine TF (mTF) only or mTF and mEPCR (CHO-K1-mTF and CHO-K1-mTF-mEPCR, respectively). Thrombin generation assays were conducted in mouse hemophilia B plasma supplemented with FMR- or mFVIIa. To investigate the hemostatic effects of EPCR-FVIIa interaction *in vivo*, we chose the $FeCl_3$ carotid artery injury model since EPCR is highly expressed in large arteries. A mild vessel injury was induced in hemophilic mice with a 2 min application of 7.5% $FeCl_3$ prior to FMR- or mFVIIa infusion, and blood flow was monitored for 30 min.

Results: A similar rate of murine FX activation was observed for mFVIIa and FMR-mFVIIa on CHO-K1-mTF (K_{cat} 0.16 ± 0.01 and 0.18 ± 0.01/s, respectively). The presence of mTF and mEPCR did not affect mFXa generation rates, which were similar to those observed on CHO-K1-mTF (K_{cat} 0.14 ± 0.01/s mFVIIa, 0.18 ± 0.02/s FMR-mFVIIa). In thrombin generation assays, FMR-mFVIIa showed a slightly reduced peak of thrombin (75% of mFVIIa), without affecting the endogenous thrombin potential between the two molecules. These results suggest that conferring mEPCR binding to mFVIIa does not impact proteolytic activity, therefore FMR-mFVIIa is an ideal molecule to investigate the role of EPCR in FVIIa-mediated hemostasis *in vivo*.

In a $FeCl_3$ carotid artery injury model in hemophilia B mice, FMR-mFVIIa infusion was more effective in forming occlusive thrombi compared to mFVIIa. Administration of 3 mg/kg FMR-mFVIIa resulted in complete vessel occlusion in 4.9 ± 0.3 min. In contrast, at the same dose, only 75% of mFVIIa treated mice exhibited stable occlusion, occurring after 12.9 ± 1.4 min. At a higher dose (5 mg/kg), FMR-mFVIIa was consistently more efficacious than mFVIIa (time to occlusion 2.6 ± 0.4 vs. 4.3 ± 0.6 min, respectively). Similar results were obtained in hemophila A mice, in which the infusion of 3 mg/kg FMR-mFVIIa resulted in complete occlusion in 2.8 ± 0.3 min compared to 13.8 ± 0.8 min for mFVIIa.

Conclusions: These results indicate that EPCR binding enhances the FVIIa procoagulant effects *in vivo*, suggesting a novel role of the endothelium in FVIIa-mediated hemostasis. The modulation of this interaction may suggest avenues for improved FVIIa-based therapeutics.

OC 83.4

Identification of novel platelet-targeting moieties that increase the activity of recombinant factor VIIa without affecting platelet function

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Background: Recombinant activated factor VII (rFVIIa) is approved for on-demand treatment of bleeding episodes in hemophilia patients with inhibitors. However, multiple doses of rFVIIa at high concentration are often required to control a bleeding episode in part due to its low affinity for platelets. We have previously shown that rFVIIa variants fused to antibody fragments or peptides that interact with platelet receptors display enhanced activity and affinity for platelets. Here we describe the generation of novel platelet-targeting moieties that interact with platelet receptor αIIbβ3 with high affinity. These moieties are single-chain fragment variable (scFv) antibodies selected on the basis of their affinity for the receptor and their inability to activate or inhibit platelet function.

Aims: To enhance the activity and efficacy of rFVIIa by increasing its affinity for platelets.

Methods: Monoclonal antibodies against human αIIbβ3 were raised in mice. Hybridoma supernatants were screened for αIIbβ3 and subunit specificity (αIIb vs. β3) by ELISA methods. Binding to human platelets, competition with fibrinogen, and platelet activation were mea-

sured by flow cytometry-based methods. In addition, purified rFVIIa variants containing αIIbβ3-specific scFv were tested for inhibition of platelet aggregation using a platelet aggregometer, and their effect on human platelet clearance was evaluated in human platelet transfused NOD/scid/gamma (NSG) mice. The activity of these platelet-targeted rFVIIa variants was measured by rotational thromboelastometry (ROTEM) and soluble tissue factor-dependent prothrombin time (sTF-PT) assays.

Results: A panel of hybridomas was categorized into six groups based on the following characteristics: affinity for αIIbβ3, subunit specificity, fibrinogen competition and platelet activation. Among these, only one group was found to activate platelets and thus excluded from further analysis. Sequencing of the variable regions revealed that all antibodies within each group shared a similar sequence. The variable regions of representative antibodies from each group were recombinantly fused as scFvs to the C-terminus of rFVIIa. The fusion proteins did not activate platelets, inhibit platelet aggregation in platelet-rich plasma or affect human platelet clearance in NSG mice. Binding assays revealed a broad range of affinities of the platelet-targeted rFVIIa variants for human and monkey platelets as a well as for purified human αIIbβ3 receptor. While the activity of these variants determined by the sTF-PT method was comparable to rFVIIa on an equal molar basis, their activity was enhanced by 10- to 50-fold relative to rFVIIa in ROTEM using whole-blood from hemophilia A donors. Furthermore, these platelet-targeted rFVIIa variants did not affect platelet clearance or demonstrate any evidence of thrombogenicity in monkeys administered with a dose equivalent in moles to 100 μg/kg of rFVIIa.

Summary/Conclusions: We have generated platelet-targeting moieties that do not affect platelet function and, when fused to rFVIIa, significantly increase its activity. These improvements in activity were only observed when tested in the presence of platelets, such as in whole-blood ROTEM assay. Moreover, these platelet-targeting rFVIIa variants were well tolerated in monkeys. Taken together these results suggest that platelet targeting rFVIIa has the potential to be a safe and effective alternative for the treatment of bleeds in hemophilia patients with inhibitors.

OC 83.5

Anti-drug antibody formation induced by recombinant activated FVII analogue (vatreptacog alfa) – results from the phase 3 adept™ 2 trial in haemophilia patients with inhibitors

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Aim: To evaluate the immunogenicity of vatreptacog alfa in haemophilia patients with inhibitors.

Methods: This multicentre, randomized, double-blinded, active-controlled, cross-over confirmatory trial evaluated the safety and efficacy of vatreptacog alfa in the treatment of bleed episodes in haemophilia patients with inhibitors. Patients ≥ 12 years of age with bleeding episodes were treated on demand in a blinded random sequence with either vatreptacog alfa or rFVIIa (NovoSeven®). Anti-drug antibody (ADA) testing was performed prior to drug exposure, at least every 3 months during the trial and at least 1 month after last trial product administration. A multi-tiered antibody analysis approach was applied and assays were validated according to international recommendations and guidelines. All samples were screened for binding antibodies towards vatreptacog alfa and rFVIIa in radioimmuno assays (RIA or RIPA) by a central laboratory. Samples above the specified cut point in the screening assays were subjected to a confirmatory radioimmuno precipitation assay to confirm that antibodies were binding to vatreptacog alfa and/or rFVIIa. Antibody positive samples were then tested for neutralizing activity in two neutralizing clot assays (measuring neutralization of vatreptacog alfa and of endogenous human FVII respectively).

Results: Seventy-two patients were enrolled in the trial, and a total of 567 bleeding episodes were treated with trial product (TP). The well-established efficacy and safety profile of rFVIIa was confirmed in this trial, but 8/72 patients (11%) developed non-inhibitory binding antibodies to vatreptacog alfa, including one patient with an *in-vitro* neutralising effect. In 4/8 patients the anti-vatreptacog alfa antibodies developed cross-reactivity towards rFVIIa after subsequent exposures. The antibody binding to rFVIIa could in all cases be competed out by vatreptacog alfa. This suggests that the antibodies were induced by vatreptacog alfa, but, due to the high amino acid sequence homology, cross-bound with lower affinity to rFVIIa. The antibody responses to vatreptacog alfa were observed in most cases following > 10 exposure days (EDs), but in one patient an antibody response was first detected after 28 EDs.

Summary: Immunogenicity is difficult to predict, and data from adept [TRADEMARK]2 indicate that even very small changes to the molecule can change the immunogenicity profile of rFVIIa products.

The high incidence of antibody development towards vatreptacog alfa (11%) observed in in this study population and the potential risks thereof should be taken into consideration for future development of rFVII-derived products. It is not possible to predict if the antibodies would disappear or develop further with continued vatreptacog alfa. Long term exposure will be needed to fully assess the immunogenicity of new rFVIIa compounds.

OC 83.6

A long-acting FVIIa -CTP proposing an improved prophylactic and on demand treatment for hemophilic patients following SC and IV administration – evaluation in animal models

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Introduction: Prolor Biotech Inc. is a clinical stage public company developing biobetter long acting versions of existing therapeutic proteins utilizing a technology termed CTP. The technology involves fusion of the C terminus peptide of hCG to one or both ends of the target protein. The technology was clinically validated and proven as a safe and efficient way for prolonging the half-lives of several therapeutic proteins while maintaining their biological activity. The aim of this study was to determine the pharmacokinetic (PK), pharmacodynamic (PD) and long term hemostatic effects of FVIIa-CTP in murine FVIII−/− mice following IV and SC administrations.

Methods: FVIIa-CTP was expressed in CHO cells, purified and activated utilizing a CTP specific purification process. FVIIa-CTP was administered to FVIII−/− mice, and following IV and SC injection the PK and PD profiles were determined. In addition, the long term hemostatic effect was evaluated following bleeding challenge by tail clip assay and tail vein trisection as compared to commercial rFVIIa. FVIIa-CTP *in-vitro* characteristics were also evaluated.

Results: FVIIa-CTP PK parameters following IV and SC administration, as assessed by a clotting assay, were superior to those of rFVIIa. Its half-life and AUC following IV administration were 5- and 3.5-fold higher, respectively and were also significantly superior following SC injection. In a IV and SC Tail Vein Transection study, FVIIa-CTP had a profound effect on survival rate, which was maintained for more

than 24 and 12 h respectively. Reduced duration and intensity of bleeding was also observed in the tail clip study in both routes. Following SC administration, FVIIa-CTP's bioavailability, was shown to be superior to commercial rFVIIa in both rats and hemophilic mice models. Correction of thromboelastography in FVIII −/− mice plasma was maintained longer with FVIIa-CTP than with rFVIIa. Finally, toxicological studies in rodents demonstrated that FVIIa-CTP is safe and tolerable at relatively high doses.

Conclusion: Attachments of CTP to FVIIa led to a markedly enhanced PK, increased exposure as reflected by AUC, improved recovery and a prolonged hemostatic effect in hemophilic mice with a comparable specific activity to rFVIIa. In addition, SC administration of FVIIa-CTP resulted in improved bioavailability and exposure was significantly prolonged relative to IV administration. Our data suggest that CTP fused FVIIa is safe and tolerable in rodents and has the potential to significantly improve the prophylactic and on demand treatment of hemophilic patients.

OC 84 – Fibrinolysis – II

OC 84.1

Characterization of knock-in mice harboring a variant of EPCR with impaired ability to bind protein C

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Background: The endothelial protein C receptor (EPCR) binds to protein C (PC) and increases the rate of activated protein C (APC) generation by the thrombin-thrombomodulin complex. APC exerts anticoagulant, anti-inflammatory, and cytoprotective effects, which are EPCR-dependent. Mutations and polymorphisms identified in the EPCR gene, which can affect the efficiency of protein C activation, have been associated with an increased risk of thrombosis. The physiologic importance of EPCR is also demonstrated in EPCR knockout mice, which show placental thrombosis and early embryonic lethality. Furthermore, overexpression of EPCR on endothelial cells protects animals against thrombotic and septic challenge.

Objectives: To study the role of EPCR *in vivo*, we have generated a knock-in mouse model harboring a variant of EPCR (R84A) with impaired ability to bind to PC. The objective of this study is to better understand the mechanism by which EPCR and its ligand(s) regulate coagulation and inflammation *in vivo*. We hypothesize that impaired PC/APC binding to EPCR will result in a procoagulant and pro-inflammatory phenotype.

Methods: Mice were given an intravenous injection of bovine thrombin (160 μg/kg) to activate PC, or factor (F) Xa (50 pmol/kg) and phospholipid vesicles (PCPS) (75 nmol/kg) to initiate thrombin generation. Blood was collected via the inferior vena cava 10 min post injection. PC antigen, APC, and thrombin-antithrombin complex (TAT) levels were measured by ELISA. Fibrin deposition in tissue sections was assessed by immunohistochemistry.

Results: EPCR R84A/R84A mice are viable, reproduce normally, and have a normal lifespan. EPCR expression at the endothelial surface of the aorta was not affected by the introduction of the R84A mutation in EPCR. Microscopic examination of the liver, heart, kidney, and lung from EPCR R84A/R84A mice did not reveal evidence of spontaneous thrombosis or morphological abnormalities. However, EPCR R84A/R84A mice developed splenomegaly, with spleen size 7-times higher than wild-type (WT) mice ($P > 0.05$). Circulating PC antigen concentration was higher in EPCR R84A/R84A mice (1.3 ± 0.12 μg/mL), compared to WT mice (1.0 ± 0.07 μg/mL) ($P > 0.05$). The APC levels of thrombin challenged EPCR R84A/R84A mice was only 8% ($P > 0.001$) of WT control mice. The circulating TAT complex levels, an indicator of thrombin generation, were not significantly increased in healthy EPCR R84A/R84A mice compared to WT mice, however TAT levels in FXa/PCPS challenged EPCR R84A/R84A mice increased by twofold compared to WT mice ($P > 0.01$). The elevated TAT levels in FXa/PCPS challenged EPCR R84A/R84A mice were also accompanied by an increase in fibrin deposition in the lungs (mean fluorescence intensity [MFI], 75.3 ± 20.3) compared to WT mice (MFI, 12.3 ± 5.2) ($P > 0.05$).

Conclusions: EPCR R84A/R84A mice are viable suggesting that defects in EPCR that impair PC binding do not affect embryogenesis or development. Introduction of the R84A mutation in EPCR results in impaired PC binding, PC activation, and upon thrombotic challenge, EPCR R84A/R84A mice develop a procoagulant phenotype. Enlargement of the spleen in EPCR R84A/R84A mice suggests that EPCR, which is expressed on endothelial cells as well as on hematopoietic stem cells, may play a biological role in the regulation of haematopoiesis.

OC 84.2

Inactivation of FVIIIa *in vivo* – insights into the physiological down-regulation of haemostasis

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Upon vascular injury, activated coagulation factor VIII (FVIIIa) plays a key role in propagation of the coagulation and subsequent clot formation. FVIIIa is unstable and quickly becomes inactive due to spontaneous dissociation of the A2 subunit with a half-life of approximately 2 min. Clinically, the importance of spontaneous dissociation is emphasised by the observation that patients with FVIII mutations such as the S298L mutation, giving rise to an increased dissociation of the A2 subunit, have mild haemophilia. Moreover, *in vitro* studies have shown that FVIIIa is inactivated by activated protein C (APC), but given the fast inactivation by dissociation it has been suggested that APC inactivation of FVIIIa may be of less relevance *in vivo*. However, until now the significance of either mechanism has only been explored *in vitro*.

To assess which inactivation pathway for FVIIIa that is predominant *in vivo,* we investigated the pro-coagulant effect of increasing doses of four different FVIII variants and compared to that of wt rFVIII in three different models of haemostasis in mice lacking FVIII. The FVIII variants were modified so that they either had (i) increased A2 domain dissociation (S289L) (ii), no A2 domain dissociation as the A2 and A3 domain was covalently linked by a disulphide bound (M662C-D1828C), (iii) were resistant to APC as the two APC cleavages site were mutated (R336/562A) or (iv) carried a combination of 2 and 3. All variants were purified to homogeneity following transient expression in CHO cells. The *in vivo* haemostasis models were (i) the tail bleeding model where blood loss over 30 min after amputation 4 mm of the tip of the tail was used as endpoint (ii) the $FeCl_3$ injury model where time to occlusion was determined after applying 10% $FeCl_3$ for 3 min to the carotid artery and (iii) vena saphenous bleeding model where the maximum bleeding time and number of clot formations was measured after vein puncture and repeated clot disruption.

Doses of the FVIII variants were selected to be on the linear part of the dose-response curves for wt rFVIII in the individual models, and accordingly wt rFVIII significantly and dose-dependently improved the coagulation in all three models. Destabilisation of the A2 domain (S289L) significantly reduced the haemostatic effect in the tail bleeding model and $FeCl_3$ injury model compared to wt rFVIII, whereas no significant difference between the A2 domain stabilised variant (M662C-D1828C) and wt rFVIII was observed. In contrast, APC-resistant FVIII variants (R336/562A and R336/562A/M662C-D1828C) showed superior haemostatic effect compared to wt rFVIII in all three animal models.

In conclusion, this is the first study to investigate the inactivation mechanism(s) of FVIIIa *in vivo* and our data suggest that APC mediated inactivation of FVIIIa may be the predominant inactivation mechanism *in vivo*. These data encourages for further investigations to increase our understanding of the inactivation mechanisms *in vivo*.

OC 84.3

Modulation of FXa zymogenicity yields variants that improve hemostasis in hemophilia

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There is tremendous interest in developing procoagulant hemostatic molecules to treat bleeding disorders. Recently, we generated a FXa variant (e.g. FXa^{I16L}) rendered partially inactive due to a defect in transitioning from zymogen to protease where residue 16 is critical to this process. While 'zymogen-like', the biologic activity of FXa^{I16L} is rescued when bound to its cofactor FVa. FXa^{I16L} is effective in correcting deficiencies in the intrinsic pathway; however the mutation site and general mechanism of action indicates that there is inherent flexibility in this new class of pro-hemostatic agent. To this end, we extended the mutational framework at position 16 to further alter the equilibrium position between zymogen and protease states to generate variants with a broad range of activities. Following screening and analysis, one mutant FXa^{I16T} showed high potential and had a markedly different zymogenicity compared to FXa^{I16L}. FXa^{I16T} active site function was severely impaired ($\ll$ 1% activity) compared to wild-type (wt)-FXa. Further it was less sensitive to physiologic inhibitors and exhibited a long half-life (~4 h) in human hemophilia B (HB) plasma vs. wt-FXa (> 1–2 min). Surprisingly, even though FXa^{I16T} had enhanced zymogen-like character, FVa was still able to rescue the variant as it exhibited only a 3–5-fold reduction in catalytic efficiency relative to wt-FXa. Intravenous administration of FXa^{I16T} (450 μg/kg; n = 6–9) in HB mice corrected thrombin generation over a 1 h period and the variant appears safe as platelet counts and D-dimer levels were not altered. In a tail clip assay, blood loss in HB mice (n = 7–10; 450 μg/kg) treated with FXa^{I16T} either 2 min after or 5, 15 or 30 min prior to injury was significantly reduced compared to PBS controls (n = 10). Moreover, FXa^{I16T} infusion in HB mice (n = 7–12) 5 min before injury reduced bleeding in a dose-dependent manner, where even 56 μg/kg was partially effective. Similar pro-hemostatic effects were found in a $FeCl_3$-induced injury model when the protein was infused either before or after injury in HB mice (n = 9). These data suggest that there must be sufficient amounts of FVa available at the site of injury in these models to at least partially restore the activity of FXa^{I16T}. To examine this further we evaluated whether addition of FVa with FXa^{I16T} would lower the effective dose range to correct the hemophilic phenotype. In a tail clip assay, no major reduction in blood loss was observed when FXa^{I16T} (28 μg/kg; n = 12) or FVa (400 μg/kg, n = 7) were infused individually. In contrast, co-infusion of FVa and FXa^{I16T} in HB mice (n = 12) significantly reduced bleeding. Collectively, our data indicate that the potential therapeutic advantage of the low activity zymogen-like FXa^{I16T} variant appears to be its ability to overcome the endogenous inhibitors and thus to impart a prolonged half-life and be rescued by FVa made available following coagulation activation. We conclude that the plasticity of this new class of FXa variants provides a useful and flexible platform to selectively bioengineer activity and half-life.

OC 84.4

Thrombosis and hemostasis in mice lacking factor VII activating protease (FSAP)

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Background: Imbalance between coagulation and fibrinolysis is a major factor in the development of thrombosis and hemostasis. Factor VII activating protease (FSAP) is a serine protease that activates FVII or pro-urokinase, and inhibits tissue factor pathway inhibitor (TFPI). The Marburg I single nucleotide polymorphism (SNP) of the FSAP-encoding gene (*HABP2*) gives rise to a protein which has reduced enzymatic activity and is associated with thrombotic disorders, atherosclerosis and stroke.

Aims: Identify the role of endogenous FSAP in thrombosis and hemostasis using $FSAP^{-/-}$ mice and define the mechanism of action of FSAP.

Methods: Wild type (WT) and littermate $FSAP^{-/-}$ mice were compared in various *in vivo* and *ex-vivo* model systems. Infusion of collagen/epinephrine was used to induce pulmonary thromboembolism. Topical application of $FeCl_3$ was employed to injure the carotid artery and time to thrombus formation/occlusion was determined. Similarly, the mesenteric arteries were injured and time to thrombus formation/occlusion was determined by intravital microscopy via observing the movement of labeled platelets. Venous thrombosis was induced by topical application of $FeCl_3$ and thrombus weight and total protein content was determined. Tail bleeding test was performed to assess physiological hemostasis.

Results: In pulmonary thromboembolism model mortality was reduced in $FSAP^{-/-}$ mice ($P > 0.01$). In the carotid artery thrombosis model, 41% of $FSAP^{-/-}$ mice showed no occlusion compared to 5% in the WT group. Intravital microscopy revealed that the occlusion time was significantly increased in $FSAP^{-/-}$ mice ($P > 0.01$) and a larger fraction of $FSAP^{-/-}$ mice did not occlude at all. Partial Venous Thrombosis (PVT) displayed a trend in the reduction of thrombus weight and total protein content in $FSAP^{-/-}$ mice. In the tail bleeding assay a re-bleeding pattern in $FSAP^{-/-}$ mice was observed. Western blot analysis of plasma samples showed a slightly higher TFPI expression in $FSAP^{-/-}$ mice than the WT mice; whereas plasminogen levels were unchanged and active urokinase levels were increased. Systematic analysis of various other factors of the coagulation system as well as platelet revealed no major differences between WT and $FSAP^{-/-}$ mice.

Conclusions: The clinical relevance of Marburg I polymorphism to thrombosis is not fully understood and the associated risk for thrombosis is under discussion. Interestingly, our mouse studies reveal that the endogenous FSAP plays a role in extrinsic coagulation cascade *via* inhibiting TFPI. Thus, in $FSAP^{-/-}$ mice the instability or delayed coagulation may be due to the lack of degradation of TFPI and this could shift the balance against clot formation. Our results indicate that Marburg I SNP is associated with lower levels of thrombosis, which may reflect the human pathophysiology.

OC 84.5

Characterization of two monoclonal antibodies that inhibit the antifibrinolytic but not the anti-inflammatory activity of activated TAFI

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Background and Aim: Thrombin activatable fibrinolysis inhibitor (TAFI), once activated by thrombin or plasmin (TAFIa), inhibits fibrinolysis by removing the plasminogen-binding sites from fibrin, and down-regulates inflammation by inactivating several mediators. The pharmacological inhibition of TAFIa is considered an attractive antithrombotic strategy, but carries with it the potential side effect of interfering with an anti-inflammatory pathway. We describe two anti-TAFI monoclonal antibodies (MA) that inhibit the antifibrinolytic but not the anti-inflammatory activity of TAFIa.

Methods and Results: Two anti-TAFI MA targeting plasmin-mediated activation (MA-TCK11A9 and MA-TCK26D6, referred to as *test-MA*), and two MA targeting either thrombin- or thrombin- and plasmin-mediated TAFI activation (MA-T12D11 and MA-TCK27A4, referred to as *reference-MA*), were studied. No MA

inhibited the cleavage of hippuryl-Arg by TAFIa. In two different plasma clot lysis models, *test-MA* (eightfold excess over TAFI) stimulated fibrinolysis (roughly doubling the efficiency of t-PA) through a TAFI-dependent mechanism as efficiently as PTCI (inhibitor of TAFIa) or *reference-MA*, regardless of whether the MA were incorporated into the clot (turbidimetric assay) or added to the plasma bathing fluid of a preformed clot (labeled-clot lysis assay). Surprisingly, *test-MA*, similarly to *reference-MA*, displayed an even stronger profibrinolytic activity in the presence of thrombomodulin (TM), a condition in which TAFIa is almost entirely generated by the thrombin-TM complex. Moreover, the assay of TAFIa, thrombin, and plasmin generation during clot formation and lysis revealed that plasmin-mediated TAFI activation occurred when fibrinolysis had already entered the exponential phase, and accounted for only 5–20% of total TAFIa. Accordingly, when thrombin-mediated TAFI activation was prevented by argatroban or by the use of a prothrombin-deficient plasma, the profibrinolytic activity of *test-MA* was reduced by > 80%, suggesting that *test-MA* stimulated fibrinolysis by mechanisms other than the inhibition of plasmin-mediated TAFI activation. In a functional TAFIa assay using fibrin as substrate, *test-MA*, but not *reference-MA,* inhibited TAFIa activity by > 95%. On the contrary, *test-MA,* at variance with PTCI, did not inhibit TAFIa activity when thrombin-activated osteopontin was used as substrate, as assessed by a Jurkat cell adhesion assay, suggesting that the inhibition of TAFIa by *test-MA* is substrate-specific, and depends on the size of the substrate. Because the other known pro-inflammatory substrates of TAFIa (bradykinin, C3a, C5a) are smaller than osteopontin, it can be anticipated that their inactivation will not be influenced by *test-MA*.

Conclusions: MA-TCK11A9 and MA-TCK26D6 inhibit the antifibrinolytic activity of TAFIa without interfering with its anti-inflammatory properties. These MA may represent the prototype of a new class of TAFI-inhibitors with improved pharmacological activity.

OC 84.6

Identification of heparin binding sites on TAFI that modulate plasmin-mediated activation, thermal stability and antifibrinolytic potential

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Thrombin activatable fibrinolysis inhibitor (TAFI) is a human plasma zymogen that provides a molecular connection between the coagulation and the fibrinolytic cascades. TAFI is activated through proteolytic cleavage by thrombin, thrombin in complex with the endothelial cell cofactor thrombomodulin (TM), or plasmin to generate the enzyme activated TAFI (TAFIa). TAFIa possesses basic carboxypeptidase activity which down-regulates fibrin clot lysis by removing carboxyl-terminal lysine residues from partially degraded fibrin thereby attenuating the positive feedback in the fibrinolytic cascade. Studies have shown that the presence of glycosaminoglycans (GAGs), such as heparin, is able to accelerate TAFI activation by plasmin and stabilize TAFIa causing a 2.3-fold increase in its half-life. However, the elements of TAFI structure that impart these characteristics are unknown. Potential GAG binding sites on TAFI have been suggested by homology to heparin-binding regions of other proteins as well as crystallographic data on bovine TAFI which revealed several bound sulfate molecules. To identify the GAG binding site(s) on TAFI we have expressed recombinant TAFI variants containing mutations in selected regions predicted to interact with heparin in order to determine the role of GAGs in regulating TAFI function. We examined the ability of the variants to bind GAGs by utilizing a heparin-agarose column. While wild-type TAFI bound quantitatively to the column and was specifically eluted by heparin, the variant R320A/R324A almost entirely eluted in the wash fractions. Variants K306A and H308F displayed similar elution profiles to wild-type whereas variants K211Q/K212Q and K327A/R330A exhibited a two peak elution profile where only a fraction of the total was specifically bound to the column. To determine the kinetics of activation the variants were activated by plasmin in the presence and absence of heparin. The variants H308F and K327A/R330A exhibited the greatest enhancement of catalytic efficiency in the presence of heparin of 2.3- and 2.4-fold, respectively, compared to 1.5-fold for wild-type. The variants K211Q/K212Q, K306A and R320A/K324A showed a lower degree of rate enhancement compared to wild-type. The intrinsic stability of each TAFIa variant in the presence and absence of heparin was determined. Variants R320A/K324A and K327A/R330A possessed substantially lower fold increases in half-life in the presence of heparin. We determined the antifibrinolytic potential of each variant in plasma clot lysis assays under conditions where (i) only plasmin was generated to activate TAFI or (ii) TAFI was quantitatively pre-activated such that only the effect of heparin on stability would be at play. Although not all variants with impaired heparin binding showed impaired stimulation of antifibrinolytic potential by heparin, in general, the effects on TAFIa stabilization were a better predictor of the properties of the variants in clot lysis assays than the effects on plasmin-mediated activation. Overall, the data show that connections can be made between impaired heparin binding, reduced effects on activation or stabilization of heparin and changes in antifibrinolytic potential. The data suggest that TAFI possesses multiple heparin binding sites which may contribute to the different functions of heparin in regulating TAFI activity.

OC 85 – Inflammation – Basic Studies

OC 85.1

Platelets promote immunopathology in *Plasmodium berghei* infection by inhibiting the development of interleukin-10 and interferon-? Expressing T-helper 1 cells

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Background: Malaria affects around 500 million people which result in 1 million fatalities mainly in children > 5 years. It has been shown that severe malaria pathology, especially cerebral malaria (CM) is inflammation driven. T cell cytokine interferon-ϒ has been shown to promote CM whilst T cell derived interleukin-10 has been shown to be protective. Thrombocytopenia is common in malaria pathology and studies have shown that platelet depletion can rescue mice from malaria-associated mortality, however, the underlying mechanism for survival remains incompletely understood.

Aims: We tested the hypothesis that platelet depletion in lethal *P. berghei* infection in mice leads to a more controlled inflammatory response leading to survival.

Methods: C57/BL6 mice were infected with 2×10^6 *P. berghei* via IP injection, or krebs saline as control. One day after inoculation, infected and naïve mice were split into three groups, one group receiving 100 μg of platelet-depleting GPIb antibody (Emfret, UK), one receiving 100 μg IgG control and the last group receiving PBS as control. Mice were either monitored for 10 days in the survival experiment, or were euthanized on day 5 after inoculation and spleens were harvested. 2×10^6 cells were added to anti-CD3 coated plates with addition of 4 μg/well of anti-CD28 (eBioscience, USA) and 20 μg/mL Brefeldin A (Sigma-Aldrich, USA). Cells were incubated at 37°C, 5% CO_2 and harvested after 6 h, and were surface stained with CD4-PerCP (eBioscience, USA) and CD8-FITC (eBioscience, USA), and stained for intra-cellular IL-10-APC (eBioscience, USA) and IFNϒ-PE (eBio-

science, USA). Using FlowJo software (Treestar) the CD4 and CD8 T cell populations were analysed for the expression of IL-10 and IFN-ϒ by flow cytometry.

Results: In line with previous reports our results showed that platelet depletion rescues mice from malaria associated pathology. Activation of malaria-specific CD4 T cells in *P. berghei*−-infected platelet-depleted mice ($n = 5$) lead to 1% CD4 T cells expressing IFNϒ, double that of *P. berghei* -infected IgG ($n = 5$) and *P. berghei*-infected PBS control ($n = 5$), 0.34% and 0.5% respectively. Similarly the population of IL-10 expressing CD4 T cells was also higher in infected platelet-depleted mice, 1%, compared to the IgG and PBS control, 0.43% and 0.63% respectively. Interestingly, there was an earlier emergence of self-regulating CD4 T cells expressing both IFNϒ and IL-10, which are critical for limiting immunopathology, in *P. berghei*-infected platelet-depleted mice compared to both IgG and PBS control indicating a more appropriate CD4 T cell response against *P. berghei*. CD8 T cell populations were unaffected by platelet depletion and both populations and the expansion of IFN-ϒ expressing CD8 T cells remained unaffected by platelet depletion.

Summary and Conclusions: It is becoming increasingly clear that platelets play an active role in the inflammatory mechanisms that lead to severe malaria pathology. Our results show that in lethal *P. berghei* infections platelets actively delay the development of Th1 cells leading to immunopathology and that CD8 T cells are unaffected by platelet depletion. Knowledge of this could lead to the development of treatments alleviating severe malaria pathology and reducing malaria associated fatalities.

OC 85.2
Histones and DNA in complex synergistically promote plasma coagulation

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Background: Nuclear structures released as nucleosomes from dying cells or secreted as extracellular traps by activated immune cells are newly recognized pro-thrombotic agents in the setting of infectious, inflammatory and neoplastic diseases. Although their main components, DNA and histones, have been shown to exert discrete procoagulant activities through interaction with platelets, coagulation proteins and anticoagulant systems, less information is available on the effects of histone-DNA complexes (H/DNA).

Aims: To evaluate the procoagulant activity of H/DNA as compared with that of the isolated components.

Methods: H/DNA (1:1 histones to DNA weight ratio), histones and DNA were tested at concentrations ranging from 5 to 40 μg/mL. Thrombin generation and fibrin formation were investigated in plasma by calibrated automated thrombinography (CAT) and turbidimetry, respectively. Clotting was induced by recalcification.

Results: H/DNA, but not the isolated components, dose-dependently shortened the time to fibrin formation (up to 40% reduction at 40 μg/mL). This effect was not recapitulated in a clotting assay employing purified thrombin and fibrinogen, thus pointing towards an effect of H/DNA on the coagulation cascade upstream thrombin formation. In the CAT assay, H/DNA enhanced thrombin formation in a dose-dependent manner: at 20 μg/mL H/DNA shortened the lag time from 20.5 ± 4.2 to 14.9 ± 1.4 min ($P = 0.02$) and the time to peak from 27.1 ± 5.8 to 17.9 ± 1.6 min ($P > 0.01$), and increased the endogenous thrombin potential from 861.7 ± 221.8 to 1446.9 ± 142.8 nM*min ($P = 0.002$), the thrombin peak from 81.5 ± 36.4 to 258.9 ± 26.2 mM ($P > 0.001$) and the velocity of thrombin formation from 16.1 ± 12.2 to 88.9 ± 13.7 nM/min ($P > 0.001$). In contrast, histones and DNA (up to 40 μg/mL) had a negligible influence on thrombin generation when tested alone or when their association was prevented by addition to plasma prior to complex formation or by mixing in a high ionic strength buffer. Replacement of histones with other cationic polypeptides known to interact with DNA (protamine and poly-lysine) failed to produce the same enhancement of coagulation, indicating that histones possess specific procoagulant features. H/DNA, but not isolated histones and DNA, stimulated thrombin formation also in the presence of the FXIIa inhibitor CTI and in FXII- or kininogen-deficient plasma, ruling out the involvement of the contact phase of coagulation. On the contrary, H/DNA failed to induce thrombin formation if plasma was depleted of FXI, or any other factor downstream, suggesting that H/DNA might promote the activation of FXI by mechanisms that remain to be elucidated.

Conclusions: Histones and DNA, when associated in complexes, are able to promote and sustain the activation of coagulation. This adds to the pathogenetic role that intranuclear material plays when released in the extracellular space.

OC 85.3
Gas6 promotes macrophage recruitment and activation in venous thrombosis

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Background: Gas6 is a member of the vitamin K-dependent protein family characterized by an enzymatic post-translational modification called g-carboxylation. Despite genetic data suggesting that Gas6 participates in vascular disease, a defined role for Gas6 in thrombus formation has remained elusive. However, we recently demonstrated the importance of Gas6 derived from the hematopoietic and the non-hematopoietic compartments in the pathophysiology of venous thrombosis. Interestingly, we found that gas6 induced the expression and activity of tissue factor in the vascular wall.

Aims: Venous thrombi are pervaded by a large numbers of leukocytes and it was recently shown that blood monocytes can provide the initiating stimulus for venous thrombus development by rolling and adhering to the venous endothelium. Studies have suggested a pro-inflammatory role for gas6 in the vasculature. Thus, we hypothesize that Gas6 may be involved in macrophage recruitment and activation in venous thrombosis.

Methods: Thrombosis was induced in the inferior vena cava of wild type (WT) and *Gas6* deficient (−/−) mice using 5% $FeCl_3$. Immunostaining were performed on thrombus sections for CD45 (leukocyte marker) and MOMA-2 (macrophage marker). Bone marrow derived macrophages (BM-DM) were also isolated from WT and *Gas6*−/− mice and differentiated for 7 days with M-CSF. Thereafter, macrophages were stimulated with thrombin for 4 h. RNA was isolated and the expression of integrins $alpha_M$, $alpha_L$, and $alpha_4$, as well as PSGL-1 (P-selectin glycoprotein ligand-1) and tissue factor were assessed by real time PCR.

Results: Immunohistochemistry showed a strong reduction in the number of leukocytes and macrophages within thombi of *Gas6*−/− compared to WT mice. The expression of the integrins $alpha_M$, $alpha_L$, and $alpha_4$ as well as PSGL-1 were increased in WT BM-DM treated with thrombin. However, in *Gas6*−/− BM-DM, incubation with thrombin did not affect the mRNA of these integrins or of PSGL-1. Finally, we found that tissue factor mRNA expression was up-regulated by thrombin in WT BM-DM but not *Gas6*−/− BM-DM.

Summary/Conclusions: In the present study, we show that Gas6 participates in macrophage recruitment and positively regulates the expression of tissue factor and several adhesion molecules well-known to interact with their countereceptors on endothelial cells or platelets. Thus, we provide significant insights in the understanding of the role of gas6 in macrophage involvement in venous thombus formation. Our study also reinforces the interest of targeting gas6 to develop new therapeutic strategies to treat thrombosis as loss of gas6 function results in reduced thrombus formation without excessive bleeding.

OC 85.4

Association of cell-free DNA with plasma von Willebrand factor levels in human and mouse models of inflammation

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Background: von Willebrand factor (VWF) is an acute phase protein and has been demonstrated to increase in concentration during chronic and acute inflammatory conditions such as aging and sepsis, respectively. In acute inflammation, cell-free DNA (CF-DNA) and histone proteins have been shown to activate the contact pathway and platelets. CF-DNA concentrations have also been used as a measure of disease activity in thrombotic thrombocytopenic purpura (TTP) patients, where acute inflammation is present. Additionally, studies have shown that unfractionated histones induce the release of VWF in a murine model. Therefore, a possible mechanistic link exists between the release of CF-DNA and VWF in inflammatory states.
Aims: To investigate the correlation and potential mechanistic association between CF-DNA and VWF plasma levels in acute and chronic inflammatory states in humans and mice.
Methods: CF-DNA in plasma was quantified using a PicoGreen assay. VWF antigen (VWF:Ag) levels were quantified in plasma samples through an ELISA. Lipopolysaccharide (LPS) was administered with an intraperitoneal injection (5 mg/kg) to 9- and 55- week old C57BL/6 normal and ADAMTS13 KO mice, and sampled at 3 and 6 h after injection. Hydrodynamic injections (HI) of 100 μg of a plasmid containing murine VWF cDNA were performed on 9-week old VWF KO and VWF/ADAMTS13 KO mice and sampled 1-, 2- and 6-weeks after injections.
Results: CF-DNA increased significantly in middle-age (30–49, $n = 42$) and old (55–87, $n = 78$) healthy human subjects compared to young (1–17, $n = 52$) individuals ($P > 0.05$). CF-DNA also increased significantly between 9 and 55 weeks old normal mice ($P > 0.0001$, $n = 27$). In humans, CF-DNA and VWF:Ag were not correlated in the young and middle-age groups, although a correlation was observed in the old age group ($P > 0.05$). This positive correlation was also observed in normal mice, regardless of age ($P > 0.0001$). The inflammatory state documented in acute exacerbations of TTP was also associated with correlations between CF-DNA and VWF:Ag ($P > 0.0001$, $n = 12$). In a murine LPS model of acute inflammation, there was a marked increase in CF-DNA at 3 and 6 h ($P > 0.05$) and an increase in VWF:Ag at 3 h ($P > 0.05$) after LPS administration ($n = 15$). In a mouse model of prolonged supraphysiological VWF expression (> 20 U/mL, $n = 32$) induced by HI, there was no increase in CF-DNA. These levels remained the same in the absence of proteolytic processing of supraphysiological VWF in VWF/ADAMTS13 KO mice, suggesting that an increase in VWF concentration and multimer size does not cause an increase in CF-DNA. Similarly, in the absence of VWF, as seen with type 3 von Willebrand disease patients, CF-DNA concentration was positively correlated with age ($P > 0.01$, $n = 17$) independent of any increase in VWF:Ag.
Summary/Conclusions: The release of CF-DNA in acute and chronic inflammation models was associated with VWF plasma levels; however, supraphysiological expression of VWF did not stimulate CF-DNA release. Activation of inflammation may simultaneously stimulate CF-DNA release and endothelial activation, leading to the coordinated release of VWF and CF-DNA. CF-DNA and histones may also directly trigger endothelial and platelet activation to increase plasma VWF levels.

OC 85.5

Soluble TREM LikeTranscript-1 (sTLT-1) enhances thrombin induced actin polymerization through a Rac1 and P38 mediated pathway

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Background: The TREM like transcript-1 (TLT-1) is a membrane receptor that plays an important role in inflammation derived hemostasis and/or thrombosis. To date TLT-1 has only been identified in the α-granules of megakaryocytes and platelets and is released to the platelet surface upon platelet activation. Activated platelets release a soluble form of TLT-1 (sTLT-1) that is found in serum but not in the plasma of healthy mice and humans. Patients diagnosed with sepsis have been found with plasma levels of sTLT-1 averaging more than 300 μg/mL, moreover high sTLT-1 levels correlate with the presence of disseminated intravascular coagulation (DIC). These studies are further supported by our investigations in *treml1−/−* mice. LPS induced sepsis in mice demonstrate that they have increases in plasma sTLT-1 levels during acute sepsis and that *treml1−/−* bleed where wild type mice do not in the Shwartzman model of vasculitis.
Aims: With this study it is our goal to elucidate the pathway that sTLT-1 initiates to enhance platelet function after low dose thrombin treatment in order to better understand TLT-1's role during sepsis and DIC.
Methods: Here we identify a multimeric form of sTLT-1 that is stored in resting platelets and released upon activation suggesting that sTLT-1 may play an important role during activation. Using a recombinant sTLT-1 (rsTLT-1), in *in vitro* static assays we show that rsTLT-1 enhances agonist induced actin polymerization. Subsequently, we developed a whole blood flow cytometry (WBF) assay to demonstrate sTLT-1 effects on thrombin induced secretion and gpIIb IIIa activation. Rac and Rho small GTPases play important roles in regulating actin polymerization, therefore we hypothesized that sTLT-1 may activate one of these pathways during activation to increase actin formation. Using the WBF assay we demonstrate that the Rac inhibitor NSC23766 and not the Rho inhibitor, C3, inhibits sTLT-1 (50 μg/mL) augmented activation, suggesting that sTLT-1 uses a Rac1 pathway to enhance platelet activation. To verify these results we evaluated the binding of activated Rac1 to the CRIB domain of PAK 21 at low doses of thrombin in the presence or absence of sTLT-1.
Results: We found that sTLT-1 induces a dose-dependent increase of Rac1 starting at doses of sTLT-1 as low as 5 μg/mL. The enhanced activation is reversed by the TLT-1 antibody, C10. Furthermore, we investigated p38 and p42 pathways as potential downstream mediators of the Rac1 signaling pathway. Consistent with current paradigms of Rac1 signaling and actin polymerization, sTLT-1 enhanced the phosphorylation of p38 and not p42.
Summary/Conclusion: This work is the first to demonstrate a pathway detailing how sTLT-1 leads to enhanced platelet activation as shown in our previous publications. Here we define a pathway of activation that is initiated with sTLT-1 and thrombin causing increased activation of both Rac1 and p38 leading to increased actin polymerization. Collectively our data outlines a mechanism of how high levels of sTLT-1 could mediate sepsis progression and propagation of DIC during sepsis.

OC 85.6

Platelets persistently enhance Treg responses, but biphasically regulate Th1/Th17 responses in platelet-CD4+ T cell co-cultures

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Background: Atherogenesis involves both inflammatory and thrombotic mechanisms, in which platelets and $CD4^+$ T cells play important

roles. Our recent study showed that platelets can simultaneously enhance differentiation and cytokine production of type 1 T helper (Th1), Th17, and regulatory T (Treg) cells via platelet derived chemokines in co-cultures of autologous human platelets and $CD4^+$ T cells.
Aims: To investigate if platelets persistently enhance $CD4^+$ T cell differentiation, of if platelet-enhanced $CD4^+$ T cell differentiation leads to an enhanced cross-talk among $CD4^+$ T cell subsets.
Methods: Human $CD4^+$ T cells were cultured without or with autologous platelets in the presence of anti-CD3/CD28 MAbs. Th1, Th17, and Treg cell differentiation were monitored during 5 days by intracellular staining of IFNg, IL-17, and FoxP3, respectively.
Results: We confirmed our previous findings that platelets can simultaneously enhance Th1, Th17, and Treg cell differentiation. At day 2, platelets increased Th1 cell phenotype from 14.9 ± 1.7% of $CD4^+$ T cells alone to 18.6 ± 2.1%, Th17 cells from 1.8 ± 0.4% to 2.4 ± 0.5%, and Treg cells from 13.0 ± 1.4% to 20.6 ± 1.7%. At day 3 and 5, platelets remained augmentative for Treg differentiation (29.3 ± 4.3% and 21.7 ± 4.8%, respectively), but turned suppressive for Th1 activation (11.0 ± 2.1% with vs. 13.1 ± 1.7% without platelets and 7.8 ± 2.2% vs. 11.8 ± 1.9%, respectiverly; $n = 9$, $P > 0.05$ for all). The latter was also seen with Th17 responses. In platelet-T cell co-cultures, the FoxP3-blocking peptide P60 mildly reduced Treg cell differentiation, but had no effect on Th1 cell activation. Transforming growth factor b (TGFb) and interleukin (IL)-10 blockade with neutralizing antibodies inhibited Treg cell differentiation, but did not reverse the suppression of Th1 cell differentiation. In $CD4^+$ T cells cultured alone, the same inhibitions of Treg differentiation had no influence on the dynamics of Th1 phenotyping. All evidence suggests that Treg cells had limited impact on, whilst platelets were likely accounted for Th1 suppression during the second phase. Using a BrdU incorporation assay, we showed that platelets attenuated aCD3/CD28-induced $CD4^+$ T cell proliferation, and that the inhibitory effect was more selective among FoxP3-negative cells.
Conclusion: Platelets constantly promote anti-CD3/CD28-stimulated Treg cell differentiation in human platelet-$CD4^+$ T cell co-cultures. However, platelets exert a biphasic regulation on Th1/Th17 cell differentiation, namely a transient enhancement that followed by a secondary suppression. Our findings suggest that platelets can foster more rapid and robust Th1/Th17 responses, but also cast a secondary inhibition on Th1/Th17 differentiation. This represents a novel mechanism of platelet-regulated $CD4^+$ effector cell responses, and may have a major significance in adaptive immunity.

OC 86 – Negative Regulation of Platelet Function

OC 86.1
Shedding'light on platelet CD84

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Background: CD84 is a type I transmembrane receptor of the *signalling lymphocyte activation molecule* (SLAM) family which is widely expressed in immune cells and platelets and undergoes homophilic interactions. Although members of the SLAM protein family have been shown to contribute to signalling during platelet aggregation, neither the role of CD84 in platelet physiology, nor its regulation is well explored. Acute ischaemic stroke is a 'thrombo-inflammatory' pathologic process where infarct progression strictly depends on early platelet adhesion/activation mechanisms and immune cell recruitment, but the exact mechanisms how these processes are linked are unknown.
Aims: We investigated the role of CD84 in thrombus formation, haemostasis and ischaemic stroke and further analysed the mechanisms regulating CD84 surface expression and degradation in platelets.
Methods: We generated CD84-deficient mice and analysed their platelets *in vitro* and *in vivo*. Further, *Cd84−/−* mice were subjected to *transient middle cerebral artery occlusion* (tMCAO), a highly standardised experimental model for ischaemic stroke. In addition, the regulation of platelet CD84 was studied by biochemical approaches (ELISA, Western blot) and use of three different genetically modified mouse lines.
Results: We demonstrate that CD84 is cleaved from the surface of human and murine platelets in response to platelet receptor agonists. Studies in transgenic mice identified ADAM10 as the principal sheddase responsible for CD84 cleavage, whereas ADAM17 was dispensable. Shedding of CD84 via ADAM10 occurs constitutively *in vivo* and also during blood clotting, since the robust plasma and serum levels of soluble CD84 of wild-type mice were reduced in ADAM10 deficient mice. Moreover, Western blot analysis revealed calpain-mediated cleavage of the intracellular CD84 *C*-terminus, occurring simultaneously with, but independently of ectodomain cleavage.
Studies on *Cd84−/−*mice showed that the lack of CD84 in platelets does not affect classic platelet functions such as integrin activation, granule release and aggregation in response to major agonists or spreading on fibrinogen. In contrast, mice lacking CD84 displayed significantly reduced brain infarctions and a better neurological outcome 24 h after tMCAO, revealing a previously unknown role of SLAM family receptors in ischaemic stroke.
Summary/conclusions: Our results reveal a tight regulation of platelet CD84 by simultaneous extra- and intracellular cleavage. The analysis of CD84 deficient animals indicates that CD84 may not serve an essential function in haemostasis and thrombosis. Instead, the protection of *Cd84−/−* mice in a model of ischaemic stroke implicates a functional importance of CD84 in thrombo-inflammatory processes.

OC 86.2
BMP and activin membrane bound inhibitor (BAMBI): A novel regulator of thrombus formation

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Background: *In vivo* thrombus formation after vascular injury requires a myriad of simultaneous events involving critical roles of components of the vessel wall, circulating platelets and other blood cells as well as numerous plasma proteins. By gene expression studies, we previously identified a novel platelet transmembrane receptor, BAMBI that is highly expressed in megakayocytes and endothelial cells. Moreover, using a zebrafish model of thrombosis we demonstrated that BAMBI acts as a positive regulator of thrombus formation.
Aims: This study aimed to characterise the role/contribution of BAMBI in platelet function, thrombus formation and haemostasis.
Methods: Bambi−/− mice were generated using Cre/Lox technology backcrossed onto a C57BL/6J background. Haematological parameters (including platelet counts) were assessed using an automated cell counter and by flow cytometry. Platelet function was assessed by measuring the expression of P-selectin and the activation of αIIbβ3 after stimulation with ADP, collagen-related peptide (CRP) or thrombin using flow cytometry. Thrombin generation in platelet poor plasma was investigated using calibrated automated thrombography. The tail bleeding time was performed on anaesthetised mice by cutting 2 mm of the tail and immersing it in PBS. The time needed to arrest bleeding was recorded as well as the volume of blood lost after 10 min. The thrombosis model was carried out by injuring the vessel wall of mesenteric arterioles with 10% $FeCl_3$-saturated filter paper after intravenous administration of $DIOC_6$. Thrombus formation was recorded in real time, and images analysed offline using Slidebook software. Genotypes of the mice were blinded.
Results: Although Bambi−/− mice were born at the expected Mendelian ratio, female Bambi−/− mice exhibited a small reduction in body

weight, and increased mortality at 5 weeks than littermates (15% of female Bambi−/− pups). Despite normal platelet counts, the bleeding time in Bambi−/− and Bambi+/− mice was significantly increased compared to Bambi+/+ littermates (587 ± 58, 553 ± 43 and 453 ± 42 s, respectively; $P = 0.02$) and was accompanied with a significant increase in blood loss at 10 min (70 ± 12, 67 ± 8 and 41 ± 5 μL, respectively; $P = 0.04$). In addition, using the $FeCl_3$ thrombosis model, a significant increase ($P = 0.003$) in the time to occlusion was observed in Bambi−/− and Bambi+/− mice compared to control littermates (1687 ± 190, 1500 ± 147 and 850 ± 156 s, respectively). No defects in thrombin generation in Bambi−/− plasma, expression of major platelet receptors and platelet activation after ADP, CRP and thrombin were detected that might account for the haemostatic defect.

Conclusions: Together, these data provide compelling evidence for an important role for BAMBI not only in haemostasis but also in thrombus formation. These results are in agreement with the phenotype observed in zebrafish and also confirm the role of BAMBI as a positive regulator of thrombus growth. *Ex vivo* and additional *in vivo* thrombosis models are currently under way to further elucidate the role of BAMBI in thrombosis.

OC 86.3

Paxillin is an intrinsic negative regulator of platelet activation in mice

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Background: Paxillin is a LIM domain protein that is localized at integrin-mediated focal adhesions. Considering the multiple interaction motifs located within its structure, paxillin seems to serve as a signaling platform for the recruitment of numerous regulatory proteins near integrins. Although direct interactions of paxillin with the cytoplasmic tail of integrins are believed to be involved in a number of cell processes by modulating integrin functions, little is known about the contribution of this LIM domain protein to integrin signaling pathways for platelet activation.

Aims: The aim of this research is to clarify the role of paxillin in integrin signaling pathways for platelet activation in mice.

Methods and Results: To inhibit the expression of paxillin in anucleate platelets, we transplanted bone marrow cells transduced with a lentiviral vector that simultaneously expressed EGFP and short hairpin RNA (shRNA) sequences for paxillin into lethally-irradiated recipient mice. The Pxn-KD platelets slightly increased in size, and showed augmented integrin αIIbβ3 activation following stimulation of multiple receptors, including GPVI and G protein-coupled receptors. TXA_2 biosynthesis and the release of α-granules and dense granules in response to agonist stimulation were also enhanced in Pxn-KD platelets. Conversely, talin-dependent conformational changes in integrin αIIbβ3 expressed in Chinese hamster ovary cells were not affected by Pxn-KD. Although Pxn-KD did not affect intracellular calcium mobilization in platelets, Pxn-KD significantly enhanced Rap1b activation. Intravital imaging confirmed that Pxn-KD enhanced platelet adhesion to the endothelium and thrombus formation.

Conclusions: Our findings suggest that paxillin negatively regulates several common platelet signaling pathways, resulting in the activation of integrin αIIbβ3 and release reactions, possibly through altered Rap1b activation.

OC 86.4

Apelin acts as an endogenous antithrombotic factor

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Background: Apelin is a bioactive peptide identified as the endogenous ligand of the G protein-coupled receptor APJ. This peptide displays a number of physiological roles, including regulation of cardiovascular functions, fluid and energy homeostasis, and blood vessel formation. In contrast, among its pleiotropic functions, the effects of apelin/APJ system in platelets are unknown.

Aims: The aim of this study is to investigate the role of apelin on platelet function.

Methods: The expression of apelin/APJ in platelets was detected by RT-PCR, western blotting and immunofluorescence. The involvement of apelin in platelet function was investigated (i) *in vivo* in a tail bleeding assay and in a mouse model of arterial and venular thrombosis after intravenous injection of apelin, and (ii) *in vitro* in platelet aggregation induced by thrombin, collagen and ADP, and in platelet adhesion and thrombus formation over a collagen matrix under blood flow conditions.

Results: RT-PCR, immunoblot analysis and immunofluorescence studies revealed that human and mouse platelets express apelin and its receptor APJ. In tail bleeding assays, the intravenous injection of apelin in wild-type (WT) mice exhibited significantly increased bleeding times (70% of mice injected with apelin had a bleeding time > 10 min). Interestingly, the bleeding time of apelin-deficient mice ($apelin^{-/-}$) is significantly reduced compared to WT mice.

In addition, the role of apelin in thrombus formation was assessed *in vivo* in a ferric chloride-induced thrombosis model, followed by intravital microscopy in mice. The injection of apelin induced a significant delay of vessel occlusion. This observation of reduction of thrombus formation by apelin was confirmed *in vitro* in a whole-blood perfusion assay over a collagen matrix. Altogether, these *in vivo* and *in vitro* studies provide evidences that apelin plays a role for normal hemostasis and thrombus formation.

As thrombus formation and platelet function, including integrin αIIbβ3 engagement, are closely associated, the effect of apelin was studied in platelet aggregation induced by various agonists. In the presence of apelin, platelet aggregations induced by suboptimal concentration of thrombin or collagen were inhibited by 67 ± 3% and 37 ± 5%, respectively for human platelets. Interestingly, no effect of apelin was noticed in ADP-induced platelet aggregation. Flow cytometry analysis of integrin αIIbβ3 activation and P-selectin exposure revealed an inhibitory effect of apelin, suggesting that apelin is able to modulate platelet activation.

Conclusion: In the current study, we reveal for the first time the functional importance of apelin in hemostasis. Indeed, we have identified that this hormone, and its receptor (APJ), are present in platelets and that apelin can inhibit platelet functions. Thereby, besides to its previously reported effects, our study revealed the potential antithrombotic function of apelin, offering new therapeutic opportunities for patients with high thrombotic risk.

OC 86.5

The role of NADPH oxidase (NOX) 1 and 2 in GPVI-dependent platelet activation and thrombus formation

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Background: Glycoprotein (GP) VI is an essential platelet receptor regulating thrombosis and haemostasis. Collagen, the most thrombogenic constituent of the extracellular matrix, signals primarily through GPVI, inducing a signaling pathway culminating in platelet activation,

granule secretion, spreading and thromboxane A_2 production. Additionally, GPVI activation regulates the production of reactive oxygen species (ROS), which have been implicated as important secondary messengers mediating various platelet functions. Previous studies have implicated NADPH Oxidase-2 (NOX2) as the isoform of the NOX complex catalytic subunit regulating ROS production in platelets, whilst a recent study identified a functional role for NOX1. One major disadvantage of these studies is the overreliance on non-specific NOX inhibitors, especially those that can also act as radical scavengers (antioxidants), which may confound interpretation of the origin of platelet-derived ROS.

Aim: The aim of this study was to explore the role of NOX1 and NOX2 in GPVI-dependent platelet activation and collagen-induced thrombus formation using a pharmacological inhibitor specific for NOX1 (ML171) and a NOX2-deficient mouse model.

Methods: Washed platelets from wildtype (WT) or NOX2 knockout (KO) mice were incubated with vehicle control or ML171. Platelets were then stimulated with the GPVI-specific agonist, CRP, and monitored in multiple platelet function tests including platelet aggregation, intracellular ROS production (using the oxidation-sensitive dye, H_2DCFDA), integrin $\alpha_{IIb}\beta_3$ activation, dense body and α-granule release (ATP and P-selectin, respectively), platelet spreading, thromboxane (Tx) B_2 production and activation of intracellular kinases (ERK1/2, Akt and p38) by western blot. Blood from WT or NOX2 KO mice, pretreated with vehicle control, ML171 or aspirin (as positive control) was also perfused over a collagen-coated surface at arterial shear (1500/s) and monitored for thrombus formation.

Results: ML171, a specific NOX1 inhibitor with no ROS scavenging activity, significantly reduced CRP-dependent ROS production, while NOX2 KO mice showed similar ROS formation to that of WT platelets following CRP stimulation. The absence of functional NOX1 and NOX2 did not significantly affect CRP-mediated platelet aggregation, $\alpha_{IIb}\beta_3$ activation, platelet spreading, and dense body and α-granule release, whereas TxA_2 production required NOX1-derived ROS. NOX1, but not NOX2, was essential for early (1 min) activation of p38 following GPVI stimulation, but was not involved in ERK1/2 or Akt activation. Both NOX1 and NOX2 were crucial for collagen-mediated thrombus formation under arterial shear as quantified by thrombus volume analysis, whereas platelet surface coverage was similar between experimental conditions.

Conclusions: NOX1 is the key NOX homologue regulating GPVI-dependent ROS production, which is necessary for p38 activation and TxA_2 formation. Both NOX homologues are crucial for collagen-mediated thrombus formation, but the mechanism pertaining to the defective thrombus phenotype observed with the NOX2 KO remains unclear.

OC 86.6

An essential role of the inhibitory Fc gamma receptor IIb in antibody-induced glycoprotein VI ectodomain shedding *in vivo*

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Background: Glycoprotein VI (GPVI) mediates platelet activation on exposed subendothelial collagens at sites of vascular injury and thereby contributes to normal haemostasis, but also to the occlusion of diseased vessels. GPVI is an attractive target for antithrombotic therapy, particularly because previous studies have shown that anti-GPVI antibodies induce irreversible down-regulation of the receptor in circulating platelets. GPVI down-regulation, which occurs through ectodomain shedding and/or internalisation, is accompanied by a transient thrombocytopenia. We have recently shown that GPVI ectodomain shedding *in vitro* depends on the presence of the two metalloproteinases ADAM10 and ADAM17. However, *in vivo* anti-GPVI antibody induced effects were unaltered in mice with a platelet-specific ADAM10 deficiency and mice lacking functional ADAM17 in the haematopoietic system. Fc gamma receptors (FcγR) are capable of binding antibodies and might thus contribute to the *in vivo* effects of anti-GPVI antibodies. Mice bear three different activating FcγRs (FcγRI, FcγRIII and FcγRIV), which are expressed on different immune cells. FcγRIIb, the only inhibitory FcγR in mice, is present on various immune cells and some endothelial cells, but not platelets.

Aims: We speculated that another cell-type might be involved in mediating anti-GPVI antibody triggered effects *in vivo* via FcγRs.

Methods: We studied the *in vivo* effects of the rat anti-GPVI antibody JAQ1 in mice pretreated with the FcγRIIb / FcγRIII blocking antibody, 2.4G2, and in mice genetically modified to lack either FcγRIII or FcγRIIb.

Results: In contrast to control mice, 2.4G2 pre-treated mice developed no thrombocytopenia upon JAQ1-injection and the FcγR blockade abolished GPVI ectodomain shedding. Studies in FcγRIII-deficient mice unexpectedly revealed that this FcγR was dispensable for both JAQ1-induced thrombocytopenia and GPVI ectodomain shedding. In contrast, both processes were completely blocked in mice lacking the inhibitory FcγRIIb. Histological analyses revealed that the transient thrombocytopenia of JAQ1-opsonized platelets was caused by platelet sequestration to the liver through a FcγRIIb-dependent mechanism. Remarkably, Kupffer cell depletion with clodronate-liposomes did not prevent the JAQ1-induced transient thrombocytopenia or the down-regulation of GPVI, indicating a contribution of endothelial cells in this process.

Summary/Conclusion: Our data demonstrate that anti-GPVI antibody triggered transient thrombocytopenia and GPVI ectodomain shedding *in vivo* occur through an unprecedented FcγRIIb-dependent mechanism. We speculate that FcγRIIb positions JAQ1-bound GPVI for shedding *in trans*, however, further studies are needed to verify this hypothesis.

OC 87 – Platelet Adhesion and Function

OC 87.1

Beta3 integrin PSI domain has thiol isomerase function: new insights into integrin function and for anti-thrombotic agent development

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Background: Platelets are critical for maintaining hemostasis, but inappropriate platelet activation can lead to pathogenic thrombosis. Integrin αIIbβ3, the most abundant protein on the platelet surface, is a key molecule for platelet aggregation and thrombus formation. The PSI domain of β3 integrin is highly conserved among different species; however, the function of the PSI domain in the integrin family and in hemostasis and thrombosis is poorly understood. It has been reported that β3 integrin possesses PDI activity, which may play a role in integrin activation and platelet aggregation. However, whether the PSI domain of β3 integrin has PDI function is currently unknown.

Aims: To investigate whether the PSI domain of β3 integrin has thiol isomerase function and the effect of anti-PSI domain antibody on platelet function, specifically aggregation and thrombus formation.

Methods: We generated recombinant protein of the PSI domain of mouse β3 integrin. Mouse anti-mouse PSI domain monoclonal antibodies (mAbs) were generated from β3-deficient mice ($\beta3^{-/-}$) immunized with the recombinant protein. Antibody specificity was

determined by flow cytometry and western blotting. A PDI activity assay of mouse PSI domain and native human β3 integrin was performed using reduced and denatured RNase (rdRNase). The effects of the mAbs on platelet function were measured *in vitro* using aggregometry and *in vivo* using an intravital microscopy laser-induced thrombosis model.

Results: Analysis of the PSI domain of β3 integrin revealed that it contains two CXXC amino acid sequences (the active site motif of PDI), which are exclusively conserved among different integrins (β1-β8 integrin) and species. Refolding of rdRNase showed that the PSI recombinant protein has endogenous PDI activity. Bacitracin, a well-known PDI inhibitor, inhibited PSI domain PDI function in a dose dependent manner. However we did not see any inhibitory function of Quercetin-3-rutinoside on the thiol isomerase function of the PSI domain of β3 integrin. Four anti-PSI domain mAbs were generated and showed different inhibitory effects on PDI function of the recombinant PSI domain and purified human platelet β3 integrin. *In vitro* and *ex vivo* studies showed that anti-PSI antibodies inhibited mouse and human platelet aggregation. Using intravital microscopy we demonstrated that anti-PSI mAbs inhibited mouse platelet aggregation and thrombus formation *in vivo*.

Conclusions: To the best of our knowledge, this is the first study to demonstrate that the PSI domain of β3 integrin has endogenous PDI activity, which may play important roles in cell biology of platelets and other cells. Our data suggest that the PSI domain of β3 may be a new target in controlling platelet function and our mAbs may have potential in anti-thrombotic therapy.

OC 87.2

A role for CD40L signalling in platelet activation and thrombus formation that is independent of CD40

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Background: The glycoprotein CD40 (TNFRSF5) is a member of the tumour necrosis factor receptor (TNFR) superfamily, while its counter-receptor or ligand CD40L (TNFSF5, CD154) is a membrane protein of the TNF superfamily. The latter can be cleaved from the membrane surface by extracellular proteases, yielding a soluble fragment (sCD40L). CD40 and CD40L are both highly expressed on megakaryocytes and platelets, and have been proposed to play a role in platelet activation, but the mechanisms are not well understood. The current view is that CD40L-CD40 interaction signals to cell activation via TNFR associated factors (TRAFs), phosphoinositide 3-kinase (PI3K) isoforms and NFkB.

Aims: Determine the role of the CD40L-CD40 axis in platelet activation and thrombus formation.

Methods: Human and mouse platelet activation was determined by flow cytometry (integrin αIIbβ3 activation and P-selectin expression) and by light transmission aggregometry. Phosphorylation of Akt at Ser-473 was measured by western blot analysis. Thrombus formation was measured *in vitro* by whole blood perfusion over collagen type I or plaque material (rich in collagen) at a shear rate of 1000/s. Thrombi were assessed for size, stability and for platelet activation using fluorescently labeled probes. All mice had a C57BL/6 Apoe$^{-/-}$ genetic background. Mice were deficient in CD40 (Cd40), CD40L (Cd40lg).

Results: Whereas mouse platelets lacking CD40 (Cd40$^{-/-}$) showed increased αIIbβ3 activation and secretion in response to collagen receptor (GPVI) stimulation, mouse platelets deficient in CD40L (Cd40lg$^{-/-}$) showed diminished integrin activation and secretion in response to GPVI stimulation. Response to ADP, thromboxane or thrombin receptor agonists was not affected. On the other hand, addition of sCD40L potentiated the activation and aggregation responses of mouse and human platelets at low GPVI stimulation, even in the absence of CD40. This potentiation was antagonized by inhibition of PI3Kα/β, but not of PI3Kγ/δ isoforms. Whereas sCD40L alone did not cause Akt phosphorylation at Ser-473, it markedly enhanced the phosphorylation at low GPVI stimulation, which effect again was antagonized by PI3Kα/β inhibition. Whole blood thrombus formation was decreased in Apoe$^{-/-}$Cd40lg$^{-/-}$ mice, but unchanged in Apoe$^{-/-}$Cd40$^{-/-}$, in comparison to Apoe$^{-/-}$ controls. In Apoe$^{-/-}$ blood, perfused over collagen or atherosclerotic plaque material, thrombus formation was enhanced, platelet aggregates were denser, and platelet activation was increased by addition of sCD40L; effects that were antagonized by inhibition of PI3Kα/β.

Conclusions: These results indicate a role of released sCD40L to enforce collagen-dependent platelet activation, by supporting integrin αIIbβ3 activation, secretion and dense thrombus formation, via an activation pathway involving PI3Kα/β. This role of CD40L is not phenocopied by CD40, indicating that CD40 is not a main counter-receptor of CD40L and not the signal transmitter to PI3K.

Funding: Netherlands Heart Foundation 2011T6.

OC 87.3

Integrin a5β1 and glycoprotein VI play important roles in mediating activation and aggregation of platelets adhering to fibrillar fibronectin

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Background: Fibronectin (Fn) is a high molecular weight glycoprotein (440 kDa) present in the plasma as a dimer and in the subendothelium in a fibrillar form. A pioneer study using a flow-based assay, reported that plasma Fn allows recruitment of platelets through a mechanism involving integrins $\alpha_5\beta_1$ and $\alpha_{IIb}\beta_3$ (Beumer et al., *Blood* 1994). More recently, *in vitro* and *in vivo* experiments have suggested that plasma Fn also participates in thrombus growth upon binding to the surface of activated platelets, thereby enhancing platelet recruitment and thrombus stability (Cho et al., *Blood* 2006; Ni et al., *PNAS* 2003). To date, the ability of fibrillar fibronectin, which is exposed after vessel injury, to promote platelet adhesion and activation remains unknown.

Aims: The aim of this study was to generate fibrillar fibronectin *in vitro* and investigate its ability to support platelet adhesion, activation and aggregation.

Methods: Dimeric fibronectin was immobilized onto glass microcapillaries and multimerized through a mechanical stretch applied by aspiration with a vacuum pump (Robert et al., *Scientific Reports* 2011). Anticoagulated (hirudin 100 U/mL) human or mouse whole blood was perfused through these capillaries at various shear rates. Platelet adhesion was monitored by video-microscopy using differential interference contrast microscopy. Platelet activation was evaluated by observing morphological changes and variations in intracellular Ca^{2+} levels. Thrombus volumes were measured by confocal microscopy.

Results: The mechanical forces applied to immobilized dimeric Fn allowed the formation of a fibrillar network. Blood perfusion at low shear rates (300 s) showed that fibrillar Fn, contrary to dimeric Fn, was able to support efficient platelet adhesion in a process which relied on integrin $\alpha_5\beta_1$ and $\alpha_{IIb}\beta_3$. The glycoprotein (GP)Ib-IX complex did not play a crucial role in the initial steps of platelet recruitment, despite evidence that von Willebrand factor (VWF) binds to Fn in a purified system using an ELISA. Activation following platelet adhesion was evidenced by: (i) morphological changes comprising cell body contraction and extension of numerous filopodia; (ii) strong increases in intracellular Ca^{2+} concentrations above basal levels (872 ±

117 nM). Interestingly, the use of inhibitory peptides and blocking antibodies showed that this activation was dependent on integrins $\alpha_5\beta_1$ and $\alpha_{IIb}\beta_3$. Fn also led to the formation of thrombi at low (150/s) and intermediate wall shear rates (750/s) in a process relying on the interplay of various receptors including integrins $\alpha_5\beta_1$ and $\alpha_{IIb}\beta_3$, the GPIb-IX complex and GPVI.

Conclusions: This study provides evidence that fibrillar Fn supports efficient platelet adhesion, activation and aggregation through the interplay of a series of receptors including the known receptors for the soluble form, $\alpha_5\beta_1$ and $\alpha_{IIb}\beta_3$ integrins, but also the collagen receptor GPVI and the GPIb-IX/VWF axis.

OC 87.4

CD40L deficiency protects from a pro-thrombotic phenotype induced by angiotensin-II in mice

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Background: CD40 ligand (CD40L), a transmembrane protein belonging to the tumor necrosis factor family, is an important player in inflammatory-thrombotic disorders, such as atherosclerosis and cardiovascular disease. In addition to leukocytes and vascular cells platelets express functional CD40L on the surface which facilitates platelet aggregation and thrombus stability. However, the contribution of CD40L in haemostasis under conditions of inflammatory arterial hypertension is not known so far.

Aims: Investigating CD40L-deficient ($CD40L^{-/-}$) mice we hypothesized that CD40L may modulate platelet-dependent haemostatic function in mice treated with the vasoactive peptide angiotensin-II.

Methods: Angiotensin-II (1 mg/kg/d) was infused in wild-type (C57/Bl6) littermates and $CD40^{-/-}$ mice for 7 days using osmotic minipumps. The tail vein bleeding time was determined according to standardized protocols. Citrate-anticoagulated whole blood was taken by retro-orbital vein puncture. Platelet-monocyte association was analysed in diluted and fixed whole blood by flow cytometry. Platelets were identified by anti-CD41-FITC antibody and monocytes by anti-F4/80-PE antibody staining. CD41-positive and F4/80-positive leukocytes were defined as platelet bound monocytes / platelet-monocyte associates. Platelet-dependent thrombin generation in recalcified platelet rich plasma was triggered with 0.6 pM active recombinant tissue factor and assessed by calibrated automated thrombography.

Results: Angiotensin-II treated wild-type mice showed a significant ($P > 0.05$) shortening of the tail bleeding time accompanied by a significant ($P > 0.05$) increase of the platelet-dependent endogenous thrombin potential (ETP) triggered by tissue factor and significantly ($P > 0.05$) increased blood levels of platelet-monocytes associates compared to untreated wild-type mice. Although, untreated $CD40L^{-/-}$ mice exhibited prolonged bleeding times, as expected, platelet-dependent thrombin generation (ETP) as well as platelet-monocyte association did not differ significantly compared to untreated wild-type mice. However, angiotensin-II reduced significantly ($P > 0.05$) the prolonged tail bleeding time in $CD40L^{-/-}$ mice. Moreover, elevated levels of platelet-dependent ETP and blood platelet-monocyte associates observed in angiotensin-II treated wild-type mice were reversed in angiotensin-II treated $CD40L^{-/-}$ mice.

Summary/Conclusion: Angiotensin-II induces platelet hyperreactivity and hypercoagulability in wild-type but not in $CD40L^{-/-}$ mice. These results indicate that CD40L contributes crucially to a pro-thrombotic tendency in mice with angiotensin-II mediated arterial hypertension.

OC 87.5

Differential effects of platelet-derived MMPs on thrombus formation and collagen matrix degradation

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Background: Platelet thrombus formation is essential in the acute prevention of excessive blood loss at sites of vascular injury. However, platelets have also been implicated in longer term processes, such as wound healing and vascular remodelling. Platelets contain a pool of bioactive proteins, among which the matrix metalloproteinases (MMPs)-1, -2, -3 and -14. The platelet-derived MMPs may regulate platelet activation, but also remodelling of the extracellular matrix.

Aims: I) To assess the roles of MMP isoforms on thrombus formation. II) To determine the collagenolytic/gelatinolytic activity and extracellular matrix degradation capacity of platelet and thrombus-derived MMPs.

Methods: Human blood or blood from MMP-deficient mice was flowed over a fibrillar collagen matrix at high shear rate. Confocal microscopy and scanning electron microscopy was used to measure thrombus formation, thrombus surface localisation of MMP-1, -2, -9 and -14 isoforms, and collagen matrix degradation by the thrombus. Activity of MMP isoforms was measured in membranes and releasate of activated platelets, employing a panel of fluorogenic MMP substrates.

Results: Pharmacological inhibition of MMP-1 or -2 (human) or deficiency in MMP-2 (mouse) markedly reduced platelet activation and secretion in thrombus formation, whereas MMP-9 deficiency increased these processes. Deficiency in MMP-3 was without effect. Immuno-fluorescence staining of human thrombi showed that both the membrane type MMP-14 and the soluble MMP-1 and -9 isoforms accumulated at the thrombus surface. Fluorogenic substrate measurements with activated platelets in suspension indicated high collagenolytic and high gelatinolytic activity (Omni-MMP substrate, DQ-collagen, DQ-gelatin), which was predominantly present in the platelet membrane fraction. Strikingly, platelet-associated MMP activity was not induced by stimulation with ADP or thrombin, but only by stimulation with potent Ca^{2+}-rising agonists, convulxin or ionomycin. This suggested that collagen stimulation can provoke the exposure of proteolytically active MMPs. In agreement with this, thrombi formed on a collagen surface actively degraded the underlying collagen fibers. The characteristic triple-helical structure of type I collagen fibers disappeared within 2 h, while the fibers were resolved within 4 h. This pointed to a role of platelet-derived MMP-1 and -2 activity, since the collagen degradation: a) was not affected by the absence of blood or plasma; b) was blocked by general MMP inhibition, but not by serine/cysteine/threonine protease inhibition; c) was decreased by specific inhibition of MMP-1 (FN439) or MMP-2 (MMP-2 inhibitor I), but not by inhibition of MMP-9 (MMP-9 inhibitor-I).

Conclusions: MMP-1 and -2, surface-exposed after collagen-induced activation, support thrombus formation as well as collagen matrix degradation. MMP-9 does not phenocopy the MMP- and -2 effects. These data suggest a key role of platelet-derived MMP isoforms in matrix remodelling.

OC 87.6

Role of EphB2-Ephrin signalling in platelet function

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Background: Eph receptors are the largest family of cell surface receptor tyrosine kinases and with their counter receptors, the Ephrins, form a vital cell communication system that controls tissue patterning, axonal guidance and cardiovascular development. Little is known of

the mechanisms that regulate stable and sustained interactions between platelets within a thrombus, although members of the Eph receptor family have been proposed to play a role. Human platelets are known to express the Eph receptors EphA4 and EphB1 as well as the ligand EphrinB1.

Aims: In this study we report the expression of a second B family Eph receptor (EphB2) in human and mouse platelets, and through the use of transgenic mouse models (EphB2-deficient (EphB2 −/−) and kinase-defective EphB2) and recombinant proteins which interfere with EphB2-Ephrin interactions, we demonstrate the importance of the EphB2 receptor for platelet function.

Methods: Platelet aggregation and dense-granule secretion were measured in platelet rich plasma (PRP) by optical aggregometry. Fibrinogen binding assays and alpha-granule secretion (P-selectin exposure) measurements were performed on whole blood using flow cytometry. The spreading of gel-filtered platelets on fibrinogen and collagen related peptide (CRP-XL) was assessed using light microscopy. *In-vitro* and *in-vivo* thrombus formation was measured using Vena8 biochips and laser-induced injury to cremaster muscle arterioles, respectively.

Results: EphB2-deficient platelets exhibited enhanced aggregation, fibrinogen binding and granule secretion (alpha and dense) in response to either collagen or the GPVI selective agonist CRP-XL. This was not accompanied by an equivalent increase in early signalling by receptor-proximal components within the GPVI signalling pathway, suggesting that the defect was mediated further downstream. Conversely, EphB2 kinase-defective platelets displayed negligible change in these assays when compared to sibling-matched control platelets, suggesting that EphB2 kinase activity may not be critical for these functions. The absence of EphB2 on platelets was also associated with enhanced spreading on fibrinogen or CRP-XL. Interestingly, kinase defective EphB2 platelets adhered well to the matrix and began to form filopodia, but very few spread fully. In spreading assays, platelets attached directly to the matrix where conventional Eph-Ephrin interactions are unlikely to occur, suggesting a role for EphB2 in the modulation of other molecules at the platelet membrane. Recombinant external domains of EphB2, EphrinB1 and EphrinB2 were used in separate assays with human platelets to investigate the effect on platelets of disrupting Eph-Ephrin signalling (aggregation, fibrinogen binding, alpha-granule secretion). Exposure to recombinant EphB2 reduced platelet function, while recombinant EphrinB1/B2 enhanced platelet function. The size of thrombi formed (*in-vitro* and *in-vivo*) were similar in both EphB2-deficient and control mice, although deficiency was associated with reduced thrombus stability.

Summary/Conclusion: Our data are consistent with the EphB2 receptor performing a negative regulatory role in platelets. Data suggest that the EphB2-Ephrin interactions modulate platelet function following the assembly of a platelet aggregate/thrombus. EphB2 deficiency also resulted in defective function in the absence of platelet-platelet contact (spreading assays) suggesting a role for EphB2 in Ephrin-independent functions.

OC 88 – Platelet Collagen Receptors

OC 88.1

Activated STIM1 controls GPVI signaling and enhances calcium store release in platelets

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Background: *Stromal interaction molecule 1 (*STIM1) is a major Ca^{2+} sensor that activates store operated Ca^{2+} entry (SOCE) in platelets. Constitutively active STIM1 mutation in Saxcoburgski mice (*Stim1-Sax/+*) induced a severe macrothrombocytopenia associated with megakaryocyte hyperplasia and splenomegaly. Additionally, increased Ca^{2+} levels in the cytoplasm and permanently opened Ca^{2+} channels in the plasma membrane of unstimulated platelets were found. Interestingly, GPVI mediated Ca^{2+} responses, integrin activation and P-selectin exposure were strongly altered in *Stim1Sax/+* platelets while G-protein coupled receptor (GPCR) induced platelet activation was normal.

Aims: The Ca^{2+} overload phenotype in mutant platelets indicated that STIM1 might regulate Ca^{2+} release and/or the refilling of the Ca^{2+} store. The selective GPVI signalling defect in *Stim1Sax/+* platelets indicated that activated STIM1 might induce a negative feedback mechanism to GPVI signalosome which could down-regulate Ca^{2+} store release during SOCE.

Methods: The physiological function of the constitutively active STIM1 in store depletion was studied in *Stim1Sax/+/Orai1−/−* double mutant mice to exclude Orai1 mediated Ca^{2+} responses and its signaling mechanism. Due to the severe developmental complication and early lethality of *Stim1Sax/+/Orai1−/−* mutant mice, irradiated recipient C57Bl6 mice were transplanted with fetal liver cells isolated from *Stim1Sax/+/Orai1−/−* and control embryos (E13.5).

Results: The pathological phenotype described in *Stim1Sax/+* mice was almost completely reversed in the *Orai1−/−* knockout background. *Stim1Sax/+/Orai1−/−* mice displayed nearly normal platelet counts, size and absence of splenomegaly. In unstimulated *Stim1Sax/+/Orai1−/−* platelets cytoplasmic Ca^{2+} concentration was reduced whereas abnormal Ca^{2+} entry was absent in comparison to *Stim1Sax/+* platelets. Moreover, activated STIM1 strongly enhanced PAR-phospholipase C (PLC) β induced Ca^{2+} store release, but in sharp contrast, GPVI-PLCγ2 mediated Ca^{2+} store depletion was severely reduced. The defective GPVI mediated Ca^{2+} response was based on the nearly abolished inositol triphosphate (IP_3) production which could be attributed to a strongly reduced phosphorylation of PLCγ2 at Tyr759 while phosphorylation of Linker of activated T cells (LAT) and other components in the GPVI signalosome were normal.

Conclusions: The rescue of the *Stim1Sax/+* phenotype by deleting Orai1 function suggests that STIM1 regulates Ca^{2+} influx only through Orai1 in murine platelets. The leaky Ca^{2+} store and the accelerated store release after thrombin stimulation indicates that activated STIM1 positively influences store depletion during SOCE by a yet undefined mechanism.

OC 88.2

Novel loci associated with glycoprotein VI expression identified by a genome-wide association study and locus-specific fine-mapping

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Background: The *GP6* gene on 19q13.4 encodes glycoprotein (GP) VI, which is complexed with the common Fc receptor gamma chain (encoded by *FCER1G*) on the platelet membrane to form the major collagen receptor. Common variants in *GP6* explain a significant proportion of the population variation in GPVI expression, particularly rs1613662 (encoding S219P). Its major allele is associated with a higher platelet response to collagen than the minor allele and with an increased risk of deep venous thrombosis (DVT)[1].

Aims: We postulated that a further proportion of the sequence variation underlying GPVI expression would be revealed by a genome-wide association study (GWAS), with rare variants being identified by fine-mapping of the *GP6* locus.

Methods: The Cambridge Platelet Function Cohort (PFC) comprising almost 1000 individuals with known platelet GPVI expression levels

and functional responses were genotyped for the GWAS. Individuals with extreme phenotypes were sequenced at the *GP6* locus.

Results: The GWAS confirmed the previous observation that variant rs1613662 accounts for about 14% of the population variation in GPVI expression ($P = 2.7 \times 10^{-24}$). In addition, 46 unique *GP6* haplotypes were inferred by analysing 39 SNPs with a Minor Allele Frequency (MAF) range from 0.024 to 0.496, in 500 PFC samples. Their association with GPVI expression was explored in the five most frequent haplotypes. GPVI expression levels in five individuals homozygous for the 5th haplotype (5/5) were significantly higher than in those for haplotypes 1 and 2 ($P = 0.012$). Typing 8000 individuals from the Cambridge BioResource identified another 18 homozygous (5/5) individuals. Further testing of an additional three haplotype 5/5 individuals replicated the original association with increased GPVI expression.

In addition to the *GP6* variants, the GWAS identified two novel common variants associated with GPVI expression; at 2q37.2 (MAF = 0.318) and at 1q23.3 (MAF = 0.388) with $P = 2.87 \times 10^{-8}$ and $P = 2.16 \times 10^{-7}$, respectively, being below and just above the genome-wide significance cut-off. The rare variant rs3557 (MAF = 0.095) in the *FCER1G* gene showed a weak signal of $P = 2.1 \times 10^{-4}$, in line with observations in our earlier candidate gene study[2].

Two PFC individuals with approximately 50% normal GPVI surface expression and low P-selectin expression (> 12%) and fibrinogen binding (> 5%) after activation through GPVI were selected for targeted Sanger sequencing of the *GP6* exons. In one case this revealed a novel non-synonymous SNP in exon 4 encoding the 2nd Ig-domain, leading to a P194T substitution. In the second case a S195N substitution was observed.

Summary: An association study using haplotype and fine-mapping analysis of the *GP6* locus revealed novel rare variants with larger effect sizes on GPVI expression than the common variants. Furthermore, the GWAS identified novel, potential *trans*-acting common variants at 2q37.2 and 1q23.3 exerting smaller effects. These newly identified features explain more of the population variation in GPVI expression and their discovery is of possible clinical relevance for the prediction of the risk of DVT.

1. Austin,H. et al. *J Thromb Haemost*. (2011) 9:489–95.
2. Jones,C. et al. *Blood*. (2009) 114:1405–16.

OC 88.3

Disturbed glycoprotein VI-mediated signaling in platelets from hypercholesterolemic mice and humans

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Background: We recently showed that high plasma HDL-cholesterol levels are associated with platelet hyperreactivity in mice and humans.

Aim: The aim of this study was to elucidate platelet reactivity under conditions of elevated plasma LDL-cholesterol levels in mice and humans.

Methods: Experiments were performed with platelets from LDL receptor knockout (LDLr KO) mice, a commonly used hypercholesterolemic mouse model. When fed regular chow diet (5.7% (w/w) fat and no added cholesterol), LDLr KO mice show an elevation of cholesterol in the LDL fraction, which is further amplified after a Western-type diet (WTD; 15% (w/w) cacao butter and 0.25% (w/w) cholesterol) challenge. To verify the relevance for human physiology, platelets from four familial hypercholesterolemia (FH) patients suffering from reduced LDLr functionality due to a defect in the *ldlr* gene, were studied. At the time of blood collection, patients were not taking cholesterol-lowering medication. The study was approved by the institutional review board of the Academic Medical Center, Amsterdam. All participants provided written informed consent.

Results: The elevated LDL-cholesterol levels in LDLr KO mice were associated with significantly lower platelet counts ($1024 \pm 63 \times 10^9$ LDLr KO platelets/L vs. $1244 \pm 143 \times 10^9$ WT platelets/L; $P > 0.001$), which were further reduced when LDLr KO mice were fed WTD ($876 \pm 68 \times 10^9$ platelets/L; $P > 0.001$). In agreement with earlier studies, platelets from LDLr KO mice on WTD showed 1.3-fold more activated $\alpha_{IIb}\beta_3$ ($P > 0.05$) upon stimulation by ADP. Surprisingly, the collagen-related peptide CRP-XL showed a reduced ability to activate $\alpha_{IIb}\beta_3$ on these platelets as compared to wild-type platelets (chow: 0.9-fold, $P > 0.01$, WTD: 0.7-fold, $P > 0.001$). Similarly, surface-expression of P-selectin was attenuated after CRP-XL stimulation on platelets of LDLr KO mice on chow (0.4-fold, $P > 0.05$), and was almost completely blocked when mice were fed WTD ($P > 0.01$). ADP also enhanced fibrinogen binding to and P-selectin expression on platelets from FH patients as compared to platelets from healthy subjects. In contrast, CRP-XL was less effective in activating FH platelets, resulting in reduced GPVI-mediated tyrosine phosphorylation, diminished fibrinogen binding, lower P-selectin expression, and a reduced ability to adhere to collagen under conditions of flow. Since GPVI-mediated platelet signaling depends on its translocation to lipid rafts, Förster Resonance Energy Transfer (FRET) by time-gated fluorescence lifetime imaging microscopy (FLIM) was used to determine the amount of GPVI in lipid rafts of FH and control platelets. FRET/FLIM analysis revealed that although more GPVI resided in rafts of unstimulated FH platelets (FRET efficiency of $4.00 \pm 0.46\%$ for FH platelets vs. $1.68 \pm 0.45\%$ for control platelets; $P > 0.01$), significant translocation of GPVI into lipid rafts upon stimulation by collagen was only observed in control platelets (FRET efficiency of $3.28 \pm 0.22\%$ for FH platelets vs. $7.24 \pm 0.68\%$ for control platelets; $P > 0.01$).

Summary/conclusions: Hypercholesterolemia due to elevated plasma LDL-cholesterol impairs translocation of GPVI to lipid rafts, which could explain the diminished platelet responsiveness to collagen. These findings reflect an important protective mechanism to prevent early platelet activation in hypercholesterolemic mice and humans.

OC 88.4

Deletion of GPVI and CLEC-2 partially rescues macrothrombocytopenia in G6b-B-deficient mice

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Background: G6b-B is a unique immunoreceptor tyrosine-based inhibition motif (ITIM)-containing receptor that is highly expressed in mature megakaryocytes and platelets. Unlike other megakaryocyte and platelet ITIM-containing receptors, G6b-B is constitutively tyrosine phosphorylated and associated with the protein-tyrosine phosphatases Shp1 and Shp2. One of the functions of G6b-B is to inhibit signalling from the immunoreceptor tyrosine-based activation motif (ITAM)-containing collagen receptor GPVI-FcR γ-chain and the hemITAM-containing podoplanin receptor CLEC-2. Mice lacking G6b-B exhibit a complex phenotype that includes macrothrombocytopenia, enhanced GPVI shedding and platelet hyper-reactivity to antibody-mediated cross-linking of CLEC-2 (Mazharian et al. Sci Signal. 2012).

Aim: In this study, we investigated whether the phenotype of *G6b* knockout mice is due to tonic GPVI and CLEC-2 signalling. Our hypothesis is that deletion of GPVI and CLEC-2 will rescue the macrothrombocytopenia in G6b-B-deficient mice.

Method: To test our hypothesis, we generated mice lacking both GPVI and G6b-B, and used an anti-CLEC-2 antibody to deplete CLEC-2 levels in these mice. *G6b* knockout mice were treated with the Src family kinase (SFK) inhibitor Dasatinib to inhibit tonic signalling. Platelet counts, aggregation and secretion, and tyrosine phosphorylation of SFKs and Syk were measured.

Results: Targeted deletion of both *Gp6* and *G6b* resulted in a 54% increase in platelet counts compared with deletion of *G6b* alone. Platelets from *Gp6*/*G6b* double-heterozygous mice were hyper-responsive to the GPVI-specific agonist collagen-related peptide compared with platelets from *Gp6* heterozygous mice, which express comparable levels of surface GPVI. This provides evidence that G6b-B is an inhibitor of GPVI-mediated functional responses in platelets. An additional 87% increase in platelet counts was observed in *Gp6*/*G6b* double-knockout mice following antibody-mediated depletion of CLEC-2 compared with *Gp6*/*G6b* double-knockout mice treated with an isotype control antibody. However, treatment of *G6b* knockout mice with the SFK inhibitor Dasatinib did not rescue platelet counts in these mice. Biochemical data demonstrated that Syk tyrosine kinase was hyper-phosphorylated on its activation loop (Syk Tyr 519/520) in resting G6b-B-deficient platelets, whereas SFKs were phosphorylated to the same extent on their activation loop and *C*-terminal inhibitory tyrosine residues compared with control platelets.
Conclusion: Findings from this study suggest that tonic GPVI and CLEC-2 signalling contribute to the phenotype of *G6b* knockout mice. The defect appears to be at the level of Syk tyrosine kinase rather than SFKs. This study provides important mechanistic insights into how G6b-B regulates platelet homeostasis in mice.

OC 88.5

Regulation of the platelet collagen receptor GPVI by the tetraspanin Tspan9

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Background: The platelet-activating collagen receptor GPVI recognises collagen as a GPVI-GPVI dimer and initiates signalling via its associated Fc receptor γ-chain. GPVI-deficient mice are protected from arterial thrombosis and ischaemic stroke but have no major bleeding complications, suggesting GPVI as a potential target for anti-thrombotic therapy. The tetraspanins comprise 33 transmembrane proteins in humans, and at least 10 are expressed by platelets. Advanced imaging techniques, such as single particle tracking in combination with total internal reflection fluorescence (TIRF) microscopy, have shown tetraspanins to regulate the lateral mobility of other proteins with which they associate. We have previously identified GPVI as a tetraspanin-associated protein, but it is unclear how this contributes to GPVI function and dynamics. We have also previously identified Tspan9 as a relatively platelet specific tetraspanin, but have not addressed Tspan9 function and whether it could regulate GPVI.
Aims: The aims are to functionally characterise Tspan9-deficient platelets and to investigate whether the absence of Tspan9 affects GPVI lateral mobility using single particle tracking.
Methods: The Tspan9-deficient mouse was generated by gene trapping and platelet function assessed using aggregometry, spreading and flow adhesion studies. Single particle tracking was used to assess the dynamics of GPVI in wild-type and Tspan9-deficient platelets. The single particle tracking used single fluor labelled anti-GPVI Fab fragments to label single GPVI molecules on the platelet surface. Live cell imaging using TIRF microscopy allowed various parameters of GPVI dynamics to be measured.
Results: The Tspan9-deficient mouse was viable and healthy. The platelet count, size and expression levels of major surface proteins, including GPVI, were normal. Additionally, Tspan9-deficient mice had no tail bleeding phenotype. Aggregation studies highlighted a delay in response to the GPVI agonists collagen related peptide and collagen in the Tspan9-deficient platelets, which was overcome at high agonist concentrations. Aggregation was normal in response to thrombin and CLEC-2 stimulation, suggesting a GPVI-specific defect. Static platelet spreading on collagen related peptide was reduced in the absence of Tspan9, and aggregate formation on collagen under flow was delayed. These findings suggested that the absence of Tspan9 might be delaying GPVI signalling by affecting GPVI lateral mobility, which could impact on its dimerisation and response to agonists. Therefore single particle tracking was employed to assess the dynamics of GPVI in the platelet membrane. In wild-type platelets GPVI molecules followed trajectories that had elements of both Brownian and confined motion. The Brownian diffusion coefficient was ~0.11 $\mu m^2/s$ and the area of confinement was ~250 nm in diameter. We hypothesise that future analysis of Tspan9-deficient platelets will reveal defective GPVI dynamics.
Summary/conclusions: The functionally uncharacterised tetraspanin Tspan9 appears important for the fine tuning of GPVI function in platelets, potentially by regulating GPVI membrane dynamics. Additionally, for the first time, the membrane dynamics of a platelet receptor have been studied at the single molecule level.

OC 88.6

Combined *in vivo* depletion of GPVI and CLEC-2 severely compromises hemostasis and abrogates arterial thrombosis in mice

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Background: Platelet inhibition is a major strategy to prevent acute ischemic cardiovascular and cerebrovascular events which may, however, be associated with an increased bleeding risk. The (hem)ITAM-bearing platelet receptors, GPVI and CLEC-2, might be promising antithrombotic targets as they can be depleted from circulating platelets by antibody treatment leading to sustained antithrombotic protection but only moderately increased bleeding times in mice.
Aim: We investigated whether both (hem)ITAM-bearing receptors can be targeted simultaneously, and what the *in vivo* consequences of such a combined therapeutic GPVI/CLEC-2 deficiency are.
Methods: For this, we induced single- or double-receptor depletion of GPVI and CLEC-2 by the administration of the anti-GPVI JAQ1 and/or anti-CLEC-2 antibody, respectively. Moreover, we verified our results by the generation of double-genetically deficient *Gp6−/−/Clec-2 fl/fl, Pf4-Cre*mice.
Results: We demonstrate that isolated targeting of either GPVI or CLEC-2 *in vivo* does not affect expression or function of the respective other receptor. Moreover, simultaneous treatment with both antibodies resulted in the specific and sustained loss of both GPVI and CLEC-2 receptors while leaving other activation pathways such as G protein-coupled receptor signaling and GPIb function intact. In addition, neither the coagulation system (aPTT and PT) was impaired nor relevant cytokine levels were released in double depleted mice further excluding off-target effects of antibody treatment. However, GPVI/CLEC-2-depleted mice displayed surprisingly a dramatic hemostatic defect and profound impairment of arterial thrombus formation. Furthermore, a strongly diminished hemostatic response could also be reproduced in mice genetically lacking GPVI and CLEC-2.
Conclusion: These results demonstrate that GPVI and CLEC-2 can be simultaneously downregulated in platelets *in vivo* and reveal an unexpected functional redundancy of the two receptors in hemostasis and thrombosis. These findings may have important implications of the potential use of anti-GPVI and/or anti-CLEC-2 based agents in the prevention of thrombotic diseases.

OC 89 – Platelet Disorders – II

OC 89.1

Non-myeloablative conditioning with busulfan prior to hematopoietic stem cell transplantation leads to phenotypic correction of murine Bernard Soulier Syndrome

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Background: Bernard Soulier Syndrome (BSS) is an autosomal recessive disease characterized by macrothrombocytopenia and bleeding symptoms. BSS is caused by a defect in the platelet glycoprotein(GP) Ib/IX complex. The primary treatment for BSS is platelet transfusion but repeated platelet transfusion often results in allo-immunization. We recently showed that following myeloablative total body irradiation (TBI) conditioning, transplantation of hematopoietic stem cells (HSCs) transduced with a lentivirus that expresses hGPIbα under integrin αIIb promoter control could rescue the macrothrombocytopenia and prolonged bleeding of murine BSS ($GPIb\alpha^{null}$).
Aims: For application of gene therapy to treatment of human patients, it is important to minimize treatment-related side effects. We aimed to model clinically relevant nonmyeloablative HSC transplantation using murine BSS and busulfan conditioning.
Methods: Using transplantation of bone marrow mononuclear cells (BMMNCs) from transgenic mice that express hGPIbα ($hGPIb\alpha^{tg+}$), 1) determine the percentage of $hGPIb\alpha^{tg+}$ HSCs required for therapeutic benefit, 2) evaluate the efficacy of non-myeloablative conditioning, and 3) test the effect of anti-thymocyte globulin (ATG) on prevention of immune reactions.
Results: (i) To determine the percentage of HSCs required for phenotypic correction of murine BSS, mixed BM chimeras were generated by reconstituting lethally irradiated $GPIb\alpha^{null}$ mice with variable mixtures (5–100%) of BMMNCs isolated from $hGPIb\alpha^{tg+}$ and $GPIb\alpha^{null}$ mice. Tail bleeding time assays performed after BM reconstitution demonstrated that 10% $hGPIb\alpha^{tg+}$ BMMNCs mixed with 90% $GPIb\alpha^{null}$ BMMNCs were sufficient to correct the tail bleeding time ($n = 5$). Platelet analysis showed that the bleeding phenotype was rescued in recipients having higher than $40 \times 10^3/\mu L$ of $hGPIb\alpha^{tg+}$ platelets, which corresponds to 6.5% of wild type mouse platelet counts ($630 \pm 57 \times 10^3/\mu L$, $n = 8$). These results suggest therapeutic potential for non-myeloablative conditioning regimens for treatment of BSS. (ii) Transplantation of $hGPIb\alpha^{tg+}$ mouse BMMNCs into $GPIb\alpha^{null}$ recipients conditioned with a non-myeloablative dose of busulfan (50 mg/kg total) showed that that tail bleeding times of busulfan-conditioned recipients were corrected and were not significantly different from lethally irradiated recipients (3.8 ± 3.8 min, $n = 23$ vs. 2.0 ± 1.3 min, $n = 12$). Antibody response to hGPIbα and immune-mediated thrombocytopenia was documented in eight of 29 recipient mice, suggesting immunogenicity of hGPIbα transgene protein in $GPIb\alpha^{null}$ mice preconditioned with busulfan. However, these antibodies disappeared without treatment in seven of eight mice 3–7 months after BM transplantation. (iii) When $GPIb\alpha^{null}$ recipients were conditioned with ATG and busulfan, all recipients showed a high percentage of hGPIbα-positive platelets ($93.3 \pm 3.3\%$, $n = 11$) and no antibody reactions were documented.
Conclusions: Non-myeloablative doses of busulfan conditioning prior to transplantation could achieve phenotypic correction of murine BSS. Lower immunotoxicity of busulfan led to higher incidence of humoral immune response compared to TBI, but pretreatment with ATG was shown to effectively prevent such immune reaction. A conditioning regimen of busulfan in combination with ATG could be utilized in non-myeloablative autologous gene therapy in human BSS.

OC 89.2

MYH9-related disease in France: a growing cohort among inherited macrothrombocytopenias on behalf of the French network on MYH9 related disorders

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Background: Inherited macrothrombocytopenias (IMTs) represent a group of heterogeneous disorders. Among the twelve genetically identified IMTs, MYH9-Related disease (MYH9-RD) is now considered to be the more frequent after Bernard Soulier Syndrome (Balduini C et al., Hämostaseologie, 2012, 32: 259–70). MYH9-RD represents a group of autosomal dominant macrothrombocytopenias associated with mutations in the *MYH9* gene that encodes the non muscle myosin heavy chain IIA (NMMHC-IIA). Macrothrombocytopenia döhle bodies and leukocyte myosin aggregates are common features; they are associated in many cases with various combinations of extra-hematological symptoms: deafness, nephropathy, cataracts. The other IMTs are quite rare or very rare. Due to the high frequency of sporadic cases, the frequent underestimation of platelet macrocytosis by the analyzers and the difficulty, at least for some patients, to detect the leukocyte inclusion bodies, the diagnosis of MYH9-RD may be difficult when not confirmed by the genotype. In addition, a suspicion of MYH9-RD is not infrequent in the presence of an isolated macrothrombocytopenia especially if familial cases are known.
Aim: To confirm or exclude the diagnosis of MYH9-RD in patients with macrothrombocytopenias and to further characterize the phenotype and genotype of new isolated or familial cases of MYH9-RD.
Methods: Patients with macrothrombocytopenia and a hypothesis of MYH9-RD were included in the study. The diagnosis of Bernard-Soulier and gray platelet syndromes, Paris Trousseau thrombocytopenia and Platelet-type von Willebrand disease was initially excluded. The 40 exons, exon A and exon-intron boundaries of the *MYH9* gene were PCR amplified and sequenced.
Results: A total of 188 propositi and 147 family members were screened. Direct sequencing could confirm the diagnosis of MYH9-RD in 115 (61%) propositi and 75/147 (51%) family members. All were heterozygous. Mutations affected 17 different exons. The majority were missense mutations affecting 31 different residues. We found also five deletions, one duplication, one insertion and three nonsense or frameshift alterations. Seven patients had two mutations. No mutation in the *MYH9* gene was identified in 73 propositi. Among them, we could distinguish two groups of cases. One ($n = 8$) presented with a 'MYH9-like' phenotype associating macrothrombocytopenia, deafness, nephropathy and eventually cataracts but without leukocyte inclusion bodies. Another group ($n = 65$) included patients with macrothrombocyopenia alone ($n = 44$) or associated with deafness ($n = 6$), nephropathy ($n = 1$), hematuria ($n = 1$), leukocyte inclusion bodies ($n = 10$, with one positive by immunofluorescence using an anti-myosin antibody). Familial cases of thrombocytopenia were reported in 27/65 cases. Finally, a mutation in the *TUBB1* gene was identified in 3/65 and will be reported separately.
Conclusion: These results confirm the high frequency of MYH9-RD among inherited macrothrombocytopenias and the importance of gene analysis for the confirmation of the diagnosis. The identification of a group of patients with a 'MYH9-like' phenotype suggests an abnormality in the regulation of MYH9 biosynthesis or the role of other genes. The discovery of *TUBB1* mutations shows that the role of beta-tubulin 1 should also be considered in the diagnosis of inherited macrothrombocytopenias. The definition of homogeneous groups of patients will also be of help in the search for new genes involved in the development of macrothrombocytopenias.

OC 89.3

Identification of a patient with bleeding diathesis, associated with dysfunctional platelet P2Y12 receptor

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Background: P2Y12, the Gi-coupled receptor for adenosine diphosphate (ADP), plays a central role in platelet function. Inherited and drug-induced defects of P2Y12 are associated with an increased risk of bleeding.

Aims: To describe a patient with lifelong bleeding diathesis, associated with dysfunctional P2Y12, due to a previously underscribed missense mutation in the encoding gene. Patient and Methods. The patient is a 45 year old man of Turkish origin, living in Germany who presented with recurrent epistaxis, postoperative bleedings after tooth extraction, thyroid explantation and spleen explantation. Platelet aggregation and secretion were measured by lumiaggregomety in 10.9 mM citrate-anticoagulated platelet-rich plasma (PRP). The platelet content of adenine nucleotides (luminometry) and serotonin (o-phthaldialdehyde, fluorometry) was measured. The platelet production of cyclic AMP was measured by a radioisotopic assay in the presence and absence of prostaglandin E1 (PGE1) (1 μM), ADP (0.1 and 1.0 μM) and epinephrine (0.1 and 1.0 μM). The platelet phosphorylation of the vasodilator stimulated phosphoprotein (VASP) was measured by flow cytometry, using a commercially available kit (BioCytex). The number of platelet P2Y12 receptors was measured in binding studies of the radiolabeled selective P2Y12 antagonist [3H]PSB-0413 to washed platelets. DNA was isolated from peripheral leukocytes: the entire coding sequence of the P2Y12 gene was amplified by PCR and subjected to DNA sequence analysis.

Results: The platelets from the patient changed shape normally after stimulation with 2–20 μM ADP, but their aggregation was markedly lower than normal and rapidly reversible. ADP did not induce platelet secretion. Platelet aggregation and secretion induced by other agonists was normal. The platelet content of nucleotides and serotonin was normal. Inhibition of VASP phosphorylation by ADP was impaired (PRI 30%). Inhibition of PGE1-induced increase in cAMP by ADP was impaired, but normal by epinephrine. The number of binding sites (Bmax) for [3H]PSB-0413, which was measured in two separate experiments, was normal (285 and 436 sites/platelet, normal values in 10 healthy subjects, 425 ± 50), but its affinity was reduced (KD 8.4 and 33 nM, normal values 3.3 ± 0.6). DNA from the patient showed a homozygous missense mutation, a T-to-A transition at nucleotide 847 that changed the codon for His-187 to Gln. Molecular modeling studies predicted that the mutated residue in the $P2Y_{12}$ Gln187 variant does not directly interact with the ligand in the binding site, but appeared to have effects on the trans-membrane (TM)5-TM6 contact region of the receptor, which could affect the conformational changes needed for receptor activation. In fact, the close proximity and the different interactions pattern of the H187Q mutation in TM5 with residues of the TM6-extracellular loop 3 region critical for the activation process in GPCRs, can explain its importance in influencing the function of the P2Y12 receptor.

Conclusion: These studies delineate a region of P2Y12 required for normal function after ADP binding.

OC 89.4

Platelet and megakaryocyte abnormalities in the Gray platelet syndrome

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Background: The Gray Platelet Syndrome (GPS) is a rare congenital platelet disorder characterized by mild to moderate bleeding symptoms, macrothrombocytopenia and gray appearance of peripheral platelets due to lack of α-granules. The genetic defect responsible for GPS was recently identified in biallelic mutations in the *NBEAL2* gene.

Aims: Although the GPS is considered a disease with a heterogenous phenotype, aim of the present study was the identification of bone marrow features and of platelet defects that could be considered specific for the disease.

Methods: Five GPS patients from four unrelated families were recruited in four different institutions to study the platelet function and the bone marrow features. All subjects or their legal guardians gave written informed consent according to the Declaration of Helsinky. Protocols were approved by the Ethics review Board at the Institution that enrolled the patients.

Four patients underwent extensive study of the platelet function and four patients underwent bone marrow (BM) biopsy. Patients underwent platelet studies at their Institutions. BM samples were obtained using standard procedures and embeded in paraffin. BM samples were shipped to the Catholic University of Rome for morphological and immunhistochemical analysis.

Platelet studies included: (i) platelet aggregation according to standard Born method, (ii) flow cytometric analysis of platelet activation, by measurement of anti-P-selectin and PAC-1 antibodies binding to platelets activated by several agonists (iii) binding of FITC-fibrinogen to washed platelets activated by thrombin (iv) measurement of PAR1 expression on platelet membrane by flow cytometry and anti-PAR1 monoclonal antibodies (v) immunofluorescence quantitative analysis of the thrombospondin-1 content of alpha-granules on peripheral blood slides.

BM studies included hematoxylin-eosin staining, reticulin staining and immunohystochemical analysis with the avidin-biotin-peroxidase complex method for the following proteins: (i) Platelet factor 4, (ii) P-selectin, (iii) CD61, (iv) c-MPL, (v) PAR1, (vi) PAR4.

Results: All GPS patients who underwent platelet studies had a severe defect of platelet response to PAR1-activating peptide. Specifically, the PAR1-mediated platelet aggregation and activation, as measured by exposure of p-selectin and PAC1 binding sites, were severely affected in 4 GPS patients. The platelet response to other agonists was slightly or moderately affected in two patients, normal in one. Expression of PAR1 on platelet membrane was reduced in all four patients (50–70% of control values). The binding of fibrinogen to GPS platelets activated by thrombin was severely reduced.

All four patients who underwent BM biopsy had marked fibrosis (grade 2-3) and marked emperipolesis. Fifthy to 80% of megakaryocytes contained up to four leukocytes engulfed within the cytoplasm. BM immunohistochemistry in two patients showed increased immunolabeling for P-selectin and decreased immunolabeling for PAR1, PAR4, thrombospondin and c-MPL on MKs.

Conclusion: Defective PAR1-mediated platelet responses and marked emperipolesis are typical features of GPS, as they were found in all patients studied. The defect of PAR1-mediated platelet responses can be used as a screening test for the diagnosis of the disease, which can be underdiagnosed. In fact, on the basis of the high number of compound heterozygous described so far, the disease might be more frequent than expected.

OC 89.5

A new autosomal dominant macrothrombocytopenia maps to chromosome 9

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Background: We report a family with a new autosomal dominant bleeding disorder associated with red cell anisopoikilocytosis, macrothrombocytopenia and platelet dysfunction which is different from all reported α-granule defects and /or hereditary macrothrombocytopenia.
Case report: Affected family members report excessive bruising from childhood, epistaxes and prolonged bleeding from cuts and superficial injuries. Symptomatic bleedings occurred after tooth extraction, adenoidectomy, circumcision and vasectomy. The severity of bleeding is variable with some affected individuals developing spontaneous bleeding while other family members only experiencing abnormal bleeding with surgery.
Methods: The study protocol was approved by our local Ethics Committee and written informed consent was obtained to prepare and store proteins and DNA. Platelet function testing, proteomics and genome-wide association studies were used to characterise the disease and locate the genetic abnormality.
Results: In a four generation Caucasian Australian family, we have identified 14 affected individuals (10 males and four females). Blood film show macrothrombocytopenia with occasional giant platelets as well as anisocytosis and poikilocytosis. Using an automated platelet function analyzer (PFA-100™), all affected family members demonstrated prolonged closure times with the collagen/epinephrine cartridge (294 vs. 125 s, $P > 0.001$); collagen/ADP closure times were increased but still within the normal range (110 vs. 88 s, P:0.024). Platelet aggregation by light transmission aggregometry was markedly impaired in response to ADP, collagen, adrenaline, arachidonic acid and TRAP. By flow cytometry, surface glycoproteins were all within normal ranges but platelets failed to express P-selectin following ADP activation. Platelet proteomics revealed abnormal cytoskeletal and α-granule contents. By electron microscopy, platelets from affected individuals were larger and had reduced number of α-granules. Linkage analysis performed on family members genotyped by SNP array mapped the genetic abnormality to chromosome (chr) 9q.
Summary: We have identified a new genetic abnormality mapped to chromosome 9q and responsible for an autosomal dominant macrothrombocytopenia which differs from the previously reported mutated genes on chr 3 (GPIX, NBEAL2), chr 7 (GPIV), chr 17 (ITGB3, GPIbA), chr 22 (GPIbB, MYH9) or X chr (GATA1).

OC 89.6

Thrombotic tendencies and bleeding in a thrombocytosis mouse model

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Background: Essential thrombocythemia (ET) is a myeloproliferative disorder presenting with thrombocytosis. Hemorrhages and arterial and venous thrombosis are major complications. The role of platelets in this disorder, in particular the contribution of their abnormal number and/or function, is still unclear.
Aims: The purpose was to evaluate the impact of abnormally increased platelet counts in hemostasis and thrombosis.
Methods: A thrombocytosis mouse model developed in an $Mpl^{-/-}$ background (Yall;$Mpl^{-/-}$) was used and thrombosis and bleeding tendencies (*in vitro and in vivo* models) have been evaluated.
Results: Thrombus formation evaluated *in vitro* by perfusing hirudin-anticoagulated whole blood over a collagen matrix (1500/s) showed a sevenfold increase in thrombus volume in Yall;$Mpl^{-/-}$ as compared to to wild-type (WT) mice (311 ± 43 vs. 43 ± 3 μm^3). The thrombotic tendency was also observed *in vivo* in a systemic thromboembolism model of collagen-adrenaline injection. In contrast, thrombosis was only modestly increased in a model of $FeCl_3$-induced lesion of carotid arteries (791 ± 128 mm^2 peak thrombus surface vs. 289 ± 41 mm^2) and was even less stable in a model of mechanical injury of the aorta when compared to WT. Yall;Mpl−/− mice exhibited severely prolonged tail bleeding times when compared WT (1452 ± 120 vs. 490 ± 149 s). The hemorrhagic trend was not caused by intrinsic platelet dysfunction, as normal aggregation and only slightly decreased [H^3]-serotonin secretion responses were observed in response to several agonists (ADP, collagen, U46619 and thrombin) after normalizing platelet counts to WT levels. Similarly, normal responses were observed in washed platelets from a series of patients with ET ($n = 5$). As reported in patients, a decreased proportion of VWF high molecular weight forms was observed in the plasma of Yall;$Mpl^{-/-}$ mice which could contribute to the hemostatic defect. A similar thrombo-hemorrhagic phenotype was recorded in two mouse models with the JAK2V617F mutation reproducing ET and PV.
Conclusion: these murine models recapitulate the bleeding tendency observed in certain ET patients, but only partially the thrombotic phenotype. Failure to reproduce this more prevalent complication in the patients could be linked to missing factors such as conditions of inflammation, leukocytosis and atherosclerosis.

OC 90 – Thrombophilia – II

OC 90.1

Risk assessment of venous thrombosis in families with known hereditary thrombophilia: the MARseilles-Nimes (MARNI) prediction model

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Background: Predicting the risk of venous thrombosis (VT) in a subject belonging to a family with inherited thrombophilia is often not feasi-

ble. We aimed to develop a simple risk assessment model that improves prediction of the risk of VT within these families.

Methods and Results: One thousand two hundred and one relatives from 430 families with inherited thrombophilia (deficiencies of antithrombin, protein C, or protein S, factor V leiden or *F2* 20210A mutations) were included. 122 had a personal history of VT. 16 variables known as VT risk factors were preselected and used for scoring: seven clinical variables (family history score of VT, number of VT episodes, age and triggering circumstances at first VT of the proband; gender, age and number of triggering circumstances without anticoagulant prophylaxis in relatives) and nine laboratory variables (severity of thrombophilia, ABO blood group and number of allele O or A2, fibrinogen, factor VIII and von Willebrand factor plasma levels, two single nucleotide polymorphisms in *F11* gene (rs2289252 and rs2036914) and 1 in *FGG* coding for the fibrinogen gamma chain [rs2066865]). The score based on the 16 variables or on the five most predictive variables performed similarly (areas under receiver-operating characteristic curves [AUC] of 0.85 and 0.83, respectively). For the 5-variables score (including family history score of VT, von Willebrand factor antigen levels, age of relative, severity of thrombophilia and *FGG* rs2066865), the risk of VT increased from 0.2% for individuals with a score of 0 (n = 186) to more than 70.25% for individuals with a score higher than 6 (n = 27). The model was validated using bootstrap method.

Conclusions: By use of a simple scoring system, the assessment of the risk of VT in subjects from families with inherited thrombophilia can be greatly improved.

OC 90.2

Antithrombin (AT) type II deficiency in Finland caused by a single point mutation – difficulties in detecting AT activity using commercially available assays

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Background: AT deficiency is a rare thrombophilia. The method of screening for AT deficiency is measuring AT activity. We have observed that some patients receive conflicting results according to assay used.

Aim: The aim of the study was to determine, whether all commercially available AT activity assays give congruent results in known patients with type II AT deficiency. We also investigated the genetic background of AT type II deficiency in Finns, a population genetically characterized by a strong founder effect.

Methods: 1. All patients in Finland with prior AT deficiency diagnosis were approached with a questionnaire and new blood samples were drawn for analysis. 245 of 374 patients gave informed consent and participated in the study. Of these 109 had type II AT deficiency and belonged to 49 families. The study was approved by the medical ethical committee.

2. AT activity was measured using five commercially available assays, either thrombin- or FXa-based (A1 STA-Stachrom ATIII®, A2 STA-Stachrom ATIII® with prolonged incubation, B Berichrom Antitrombin®, C Innovance Antithrombin®, D HemosIL®).

3. One person in each family with either type I or II AT deficiency was sequenced using Sanger-sequencing for mutations in the exonal regions in the AT coding gene *SERPINC1*. All other family members were then studied for these detected mutations.

Results: 1. The AT activity assays gave conflicting results in 92 of 109 patients with known AT type II deficiency. Assays A1 and C were able to detect all AT deficient patients, whereas assays A2, B and D gave normal results in these patients. In the rest of the patients the activity results were congruent in all assays.

2. In all patients with AT deficiency regardless of subtype, 17 different point mutations were observed. In type II AT deficiency the large majority of cases (45 families of 49) was explained by a single point mutation Pro73Leu. In the remaining four families three other single point mutations (Val30Glu, Arg220Cys, Pro234Ala) were detected, and in one family no mutation was seen using our approach.

3. This founder mutation Pro73Leu explains the varying results in AT activity assays in type II AT deficient patients. Assays A1 and C were able to detect these patients reliably, but other tested assays missed the majority of cases.

Summary and Conclusions:

1 Type II AT deficiency in Finland is caused almost exclusively by a single point mutation Pro73Leu.

2 In carriers of Pro73Leu mutation, some AT activity measuring assays are unable to detect the change in AT function and therefore miss the correct diagnosis. This must be taken into account in the screening of this thrombophilia, at least in Finnish patients.

OC 90.3

Mutation spectrum of antithrombin deficiency in Hungary; prevalence of antithrombin Budapest 3 mutation in patients with venous and arterial thrombosis

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Background: Antitrombin (AT) is the major inhibitor of thrombin and activated factor X (FXa). It is a progressive inhibitor, but in the presence of heparin the rate of inhibition is highly accelerated. AT deficiency can be classified as type I (quantitative), and type II (qualitative) deficiency. In type II deficiency the defect may involve the reactive site (RS), the heparin-binding site (HBS) or may be pleiotropic (PE). Both RS and PE deficiencies confer a high thrombosis risk in heterozygous form; homozygosity is lethal. Type II HBS deficiency seems to be less severe. Homozygous patients are alive, although they often suffer from premature thrombosis. AT Budapest 3 (p.L99F), a missense mutation leads to type II HBS deficiency. Only sporadic data – mainly case-reports – exist concerning this mutation; no population-based studies have been conducted, yet. Presence of a founder effect was suggested, however only five probands were genotyped for DNA markers.

Aims: Description of the mutation spectrum of AT deficiency in the Hungarian population. Determination of the frequency of AT Budapest 3 variant in the general Hungarian population and in patients with venous thrombosis (VT) and with myocardial infarction (MI). Investigation of a founder effect.

Methods: Consecutive patients suffering from VT with the laboratory diagnosis of AT deficiency (n = 97), young adults, i.e. below the age of 40 with the history of MI (n = 81) and their age-matched controls (n = 320) were recruited. DNA samples of 1000 healthy individuals representing the general Hungarian population were kindly provided by the Department of Preventive Medicine, University of Debrecen, Medical and Health Science Center. Mutations within SERPINC1 were detected by direct DNA sequencing. The presence of AT Budapest 3 and rs2227596, rs941989, rs2227612, rs5877 and rs5878 intragenic SNP's were examined by real time PCR using melting point analysis and FRET detection on a LightCycler 480 instrument. Allelic frequency distribution of the above mentioned SNP's was determined in AT Budapest 3 carriers and healthy controls, then haplotype and – where possible – family tree analysis were performed.

Results: Among the 97 patients with low AT activity AT Budapest 3 was the most frequent (74%), while AT Basel (p.P41L) and Padua (p.R47H) were detected in 5–5% of the patients. Other mutations (p.L173P+, p.E237K, p.G392R, p.P407T, IVS5-14G>A, p.N418I+, p.R132X, p.T85K+) were identified only in one family each. (Marked mutations are novel or not yet characterized.) AT Budapest 3 was

associated with the same haplotype in all carriers. Family tree analysis also suggested founder effect. AT Budapest 3 mutation was absent in 1000 individuals representing the general population and in the age-matched controls of MI patients, while two patients with the mutation was identified among MI subjects.
Conclusions: The high frequency of AT Budapest 3 in Hungary can be explained by a founder effect. Our results indicate the importance of searching for AT Budapest 3 (and type II HBS deficiency) not only in VT patients but also in the background of MI especially in young individuals without 'classical' risk factors.

OC 90.4

The influence of ABO, Lewis and Secretor genotypes on von Willebrand factor and lipid levels in two population cohorts

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Background: Non-O blood groups are associated with an increased risk of arterial (AT) and venous (VTE) thrombosis that has been attributed to higher von-Willebrand factor (VWF) levels and possibly alterations in lipid profiles. However, the exact nature of these relationships in those potentially higher thrombotic risk genotypes associated with the least O(H) expression, in those with no history of thrombosis, and the parallel influence of Lewis and Secretor genotypes has not been defined in studies large enough to investigate less frequent genotypes.
Aims and Methods: We investigated whether there is a consistent relationship of blood group with VWF antigen and lipid levels in two population cohorts from Orkney and Croatia ('ORCADES' [n = 882] and 'CROATIA-Viz' [n = 924]). All participants gave informed consent and the studies were approved by the ethics committee of the medical faculty of Zagreb and the Multi-centre Research Ethics Committee for Scotland.
Results: In both cohorts (excluding those with a history of thrombosis, vascular disease or diabetes and coding A^2 as O), a stepwise increase in VWF from *OO* (ORCADES median 91 IU/dl, IQR 74–116, CROATIA-Vis median 106 IU/dl, IQR 85–134) to the combined group *A1O*/*A1A2*/*A2B*/*BO* (ORCADES median 124 IU/dl, IQR 99–151, MWU $P > 0.001$, CROATIA-Vis median 136 IU/dl, IQR 114–161, $P > 0.001$) and from this to the combined group of *A1B*/*BB*/*A1A1* (ORCADES median 137 IU/dl, IQR 112–161, $P = 0.01$, CROATIA-Vis median 149 IU/dl, IQR 121–182, $P = 0.02$) was seen. In both, VWF levels were significantly higher in those carrying A^1 compared with A^2 (difference in medians 24 and 28 IU/dl, respectively, $P = 0.001$). Although, as expected, VWF increased with age, the difference in VWF between non-O and O remained relatively constant. Of interest, however, a median VWF of 125 IU/dl (with its attendant ~twofold VTE risk and increased AT risk), was only achieved by *OO* Croatians at 61–70 years and *OO* Orcadians over 70, but reached at least 20 years earlier in non-O subjects. In this regard, VWF levels ≥ 150 IU/dl identify those at highest VTE risk and were found in 16 and 30% of the cohorts, respectively. By contrast, levels of this magnitude were 2–3 times more likely in *A1B*/*BB*/*A1A1* groups. No consistent influence of Secretor genotypes or Lewis status on VWF was observed.
Excepting higher HDL levels in non-O (median 1.7 mM vs. *OO* median 1.6 mM, $P = 0.002$) and particularly group B (median 1.7 mM vs. OO $P = 0.006$) Orcadians, no consistent influence of ABO on lipids was seen. Non-*Secretors* were, however, associated with significantly higher cholesterol and LDL in both cohorts, with, as expected by obligatory Secretor status, lower levels confirmed in Croatian *Le(b)* subjects.
Summary/Conclusions: Those ABO genotypes which may carry the highest thrombotic risk (*A1B*/*BB*/*A1A1*) also carry the highest levels of VWF. Levels which rise proportionately with increasing age. By contrast, Secretor/Lewis status does not consistently influence VWF, but does alter lipid profiles with higher LDL observed in non-Secretors. Routine consideration of ABO genotype may therefore assist in identifying those with increased VTE and AT risk.

OC 90.5

Identification of dominant thrombosis modifier loci using a sensitized ENU mutagenesis screen

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Background: Pathologic blood clotting (thrombosis) is a leading cause of morbidity and mortality in the developed world. While a number of genetic risk factors have been identified for venous thrombosis, > 30% of recognized individuals will ever sustain an event, due to the presence of unidentified modifier genes.
Aims: Identification of such modifier loci would facilitate more accurate prediction of individuals at risk for thrombosis, but is difficult to achieve in human populations due to limited pedigree sizes. The zebrafish is a powerful genetic model in which the hemostatic system is nearly entirely conserved with mammals, and its ability to generate thousands of offspring makes it a powerful system in which to screen for modifier genes.
Methods: We have developed a zebrafish model of lethal thrombosis by targeted mutagenesis of the antithrombin III gene (*at3*) using zinc finger nucleases.
Results: Genotyping of offspring from heterozygous incrosses of 22 and 90 base pair deletions ($at3^{\Delta 22}$ and $at3^{\Delta 90}$) revealed a significant reduction of homozygotes compared to the expected Mendelian frequency as early as 1–2 months post fertilization (mpf), and no homozygotes have survived beyond 7 mpf. This lethality was rescued by an *at3* cDNA transgene, enabling survival to at least 15 months of age. Examination of homozygous mutants revealed large intravascular thrombi in the heart and liver. A sensitized ENU (ethylnitrosourea) mutagenesis screen was conducted for suppression of lethal thrombosis, and has produced 4017 total G1 offspring from intercrosses of $at3^{\Delta 90}$ mutants, corresponding to an expected ~1250 homozygotes (~2× genome coverage). Genotyping at 2–3 months of age identified 267 $at3^{\Delta 90/\Delta 90}$ mutants, 15 have survived beyond 7 mpf, and six of those are now 1 year old. These harbor potential suppressor mutants, and we are in the process of positionally cloning the underlying modifier genes.
Summary/Conclusions: This approach will identify novel pathways in the regulation of blood clotting beyond the canonical coagulation cascade. Zebrafish genes identified in this screen will be candidates for common variants that modify thrombosis in human populations, and may direct diagnosis and treatment for patients with venous thrombosis and other thrombotic disorders.

OC 90.6

Antithrombin defects in thromboembolism are underestimated when calculated only by Antithrombin activity measurement

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Background: We previously (Blood Coagulation and Fibrinolysis, in press) were able to demonstrate Antithrombin (AT) defects determined by gene sequencing to be more common than AT deficiency calculated upon AT activity measurement in patients with thromboembolism. AT is the major inhibitor of blood coagulation, inactivating thrombin and factor Xa. Individuals with inherited AT deficiency have a markedly increased risk for thromboembolic events. The prevalence of inherited AT deficiency is estimated to be about 0.2% in normal population.
Aim: Aim of this study was to calculate prevalence of AT deficiency calculated by AT activity measurement in a large cohort of thrombosis patients. In total, 4259 subjects were examined: 2983 patients (1038 male, 1945 female) with thromboembolic events (average age at first thromboembolic event: 39 years, median 37 years, range 0–87 years) and 1276 (304 male, 972 female) family members.
Methods: Thrombophilia screening (Factor V Leiden mutation, Prothrombin (G20210A) polymorphism, protein C, protein S, Factor VIII:c, D-Dimer, antiphospholipid antibodies and AT) was done in all patients. Additionally, AT gene sequencing was determined in AT deficient patients.
Results: AT deficiency was diagnosed in 37 (11 male, 26 female) out of 4259 subjects. 24 out of these 37 AT deficient patients presented with thromboembolic events. Average age at first thromboembolic event was 26.1 years (median 24 years, range 0.1–61 years). 13 persons (average age at examination 29 years, median 20 years, range 11–49 years) were family members. Average AT level (n = 24) was 54% of normal (median 54%, range 19–78% of normal). AT gene sequencing in AT deficient persons predominantly showed heterozygous missense mutations.
Conclusion: Prevalence of AT deficiency in patients with thromboembolic events is about 0.8%. Patients with AT deficiency experience their first thrombotic event at a very young age – this demonstrates clinical severity of AT deficiency.
We previously demonstrated (Blood Coagulation and Fibrinolysis, in press) prevalence of AT Mutations in thrombosis patients calculated by AT gene sequencing to be up to 3%. Thus, using only AT activity measurement, prevalence of AT defects is severely underestimated in thrombosis patients.

OC 91 – Von Willebrand Factor – II

OC 91.1

Syntaxin binding protein 1 (STXBP1) modulates release of Weibel-Palade bodies from endothelial cells

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Background: Vascular endothelial cells contain unique large rod-shaped granules, called Weibel-Palade bodies (WPBs). These granules function as storage organelles for various haemostatic and inflammatory components, such as von Willebrand factor (VWF), chemokines and P-selectin. Controlled release of these components into the vascular lumen through WPB exocytosis is essential for maintaining vascular homeostasis in response to vascular trauma or stress. Secretory granule exocytosis is tightly controlled by signalling pathways that are linked to G-protein coupled receptors. One of the final steps in exocytosis involves the assembly of the so-called SNARE-complex. SNARE-complexes are formed by zipper-like proteins, VAMPs (or v-SNAREs) on vesicles and syntaxins (or t-SNAREs) on the plasma-membrane. Formation of the SNARE-complex promotes fusion of secretory vesicles with the plasma membrane and subsequent release of granule content into the lumen. SNARE-complex formation is controlled by proteins belonging to the syntaxin binding protein (STXBP) family.
Aim – In this study we investigate the possible role of syntaxin binding protein 1 (STXBP1), also known as Munc18-1, in WPB exocytosis.
Methods: A proteomic screen for downstream effectors of Slp4-a was performed using a GST-fusion protein coupled to a Slp4-a-variant lacking theSHD-domain. Candidate proteins were identified using mass spectrometry. Expression of STXBP1 in endothelial cells was monitored by immunoprecipitation followed by Western blot analysis. Functional involvement of STXBP1 in endothelial cells was monitored by siRNA mediated knockdown of gene expression.
Results: STXBP1 was found in a proteomic screen for downstream effectors of the Rab27A effector synaptotagmin-like protein 4-a (Slp4-a), a positive regulator of WPB release. We assessed functional involvement of STXBP1 in WPB release by siRNA mediated knockdown of gene expression. Histamine-induced WPBs release was impaired in STXBP1 depleted cells indicating that STXBP1 is involved in Ca^{2+}-mediated release of WPBs. Depletion of STXBP1 also abolished cAMP-mediated release of WPBs as shown by the reduced forskolin-induced VWF release in STXBP1-depleted cells. We used an immunoprecipation approach to look for interactive partners of STXBP1 in endothelial cells. Both syntaxin-2 and -3 where found to associate with STXBP1 in endothelial cells. In contrast syntaxin-4 did not associate with STXBP1.
Summary/Conclusion: Our findings suggest that STXBP1 regulates WPB release through its interaction with Slp4-a, thereby docking WPBs to the plasma membrane and facilitating the release of haemostatic, inflammatory and angiogenic cargo from these organelles.

OC 91.2

A novel cleavage-resistant and highly prothrombotic von Willebrand factor mutant

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Background: Thrombus formation, whether in the context of hemostasis or thrombosis, requires von Willebrand factor (vWf) recruitment of platelets at the site of vascular injury. In patients suffering from thrombotic thrombocytopenic *purpura* (TTP), cleavage of large vWf multimers into smaller units by metalloproteinase ADAMTS13 is impaired, resulting in pervasive blood clot formation, platelet depletion and red blood cell shredding. The cleavage site of vWf is located within its A2 domain, and only becomes accessible to ADAMTS13 when elongational forces cause the A2 domain to unfold. Structural evidence suggests that the susceptibility of the A2 domain to unfolding is due, in part, to the absence of a *N*- to *C*-terminal disulfide bond present in other vWA domains. Another feature that may account for the susceptibility of A2 to unfolding is a disulfide bond shared by Cys_{1669} and Cys_{1670} in the *C*-terminal end of the domain.
Aims: Would the introduction of cysteine residues in the *N*-terminal end of the A2 domain of vWf allow for the simultaneous disruption of the Cys_{1669}-to-Cys_{1670} bond and the formation of an A2 *N*-terminal to *C*-terminal disulfide bond? In the event that it does, would such a mutation render vWf multimers resilient to deformation forces and proteolysis? Lastly, what would be the impact of one such mutation on platelet-vWf interactions *ex* and *in vivo*?
Methods: We have created 12 human and 11 matching murine vWf mutants, sporting various cysteine substitution combinations through-

out the A2 domain. All mutants were subjected to multimer analysis, proteolysis assays and hydrodynamic injection-driven expression in vWf-null mice. Complementary studies were also performed on isolated recombinant WT and mutant A2 domains.
Results: Mutant expression: all 12 mutants and WT control express at comparable levels in HEK293T cells. vWF S1494C showed impaired multimerisation and vWF S1494, 1534, 1671C an abnormal SDS-agarose electrophoretic migration profile. All other mutants showed multimerisation patterns similar to WT vWf. ADAMTS13 cleavage: Flow assay studies on recombinant and live cell-expressed vWf revealed that only vWf S1494, 1534C was resistant to proteolysis (shear rates up to 2500/s). DTT or b-mercaptoethanol pretreatment of recombinant vWf S1494, 1534C and isolated A2 S1494, 1534C was found restore their susceptibility to cleavage. Prothrombotic properties: Mutant 4, when expressed in HEK293T cells, formed long and highly pro-thrombotic strings. Mouse studies: Preliminary hydrodynamic studies performed in vWf-deficient mice suggest that expression of vWf S1494, 1534C, in itself, is sufficient to promote the formation of circulating microthrombi.
Conclusion: We have engineered a vWf mutant (S1494, 1534C in humans, S1494C,A1534C in mice) that shows complete resilience to proteolysis by ADAMTS13, even when subjected to very high flow rates. Treatment of this mutant by reducing agents was shown to restore its susceptibility to cleavage by ADAMTS13, suggesting that the presence of these two cysteine residues was sufficient to cause at least one novel disulfide bound to form. Lastly, vWf S1494, 1534C was found to support strong thrombotic responses in flow chamber assays and live animals.

OC 91.3
CTL-2 is a new von Willebrand receptor: role in antibody mediated neutrophil aggregation induced by anti-CTL-2 antibodies

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Background: Recruitment of neutrophils to endothelial cells lining the blood vessels is the key stage in inflammation. This process is controlled by a series of molecular interaction between difference adhesive molecule expressed on neutrophils and endothelial cells. Subsequently, neutrophils migrate into surrounding tissue. Recent evidence indicated that antibodies against neutrophils or/and endothelial cell could contribute as active players in neutrophil recruitment (Williams et al., 2011). Antibodies against choline transporter like protein-2 (CTL-2) are capable to induce stable neutrophil aggregation *in vitro*; a phenomenon that may associate with severe acute lung injury in patients who received anti-CTL-2 antibodies containing blood products (known as TRALI; Transfusion Related Acute Lung Injury). CTL-2 is a multiple membrane spanning glycoprotein, expressed on neutrophils and platelets as well on endothelial cells, abundantly found in human lung. To date, little is known about the function of CTL-2.
Aims: In this study, we sought to analyse the mechanism of neutrophil aggregation mediated by anti-CTL-2 antibodies.
Methods: Purified IgG fractions containing anti-CTL-2 antibodies were tested in granulocyte agglutination test (GAT) in the absence or presence of different purified plasma proteins (albumin, vWF, fibrinogen). ROS production was measured by the use of ferricytochrome-c reduction method. Stable transfected cells expressing CTL-2 and CD11b/CD18 were established and their interaction with purified plasma proteins was evaluated by adhesion assay and surface plasmon resonance (SPR) technology.
Results: Our results showed that neutrophil aggregation mediated by anti-CTL-2 antibodies depends on vWF. Washed neutrophils in PBS primed with IgG fraction of anti-CTL-2 antibodies did not form cell aggregates. In the presence of recombinant vWF-A1-A2-A3, vWF-A1 but not vWF-A3 domain, neutrophil aggregates could be observed. This phenomenon is Fc-independent; F(ab)2 fragment of anti-CTL-2 antibodies is capable to induce neutrophil aggregation. This aggregation could be inhibited by mab against vWF-A1 domain and mabs against CD11b or CD18. Cross-linking studies showed that CTL-2 on neutrophils could interact with CD11b/CD18 integrin. Interestingly, neutrophils incubated with anti-CTL-2 antibodies in the presence of soluble vWF did not lead to ROS production. In contrast, significant ROS production was detected when neutrophils interact with immobilized vWF. In cell adhesion assay, CTL-2 transfected HEK cells adhere strongly onto vWF immobilized on microtiterwell, but not on fibrinogen. This interaction, CTL-2 and vWF, was significantly stronger when compared to the adhesion capability of CD11b/CD18 transfected cells on vWF. In addition, CD11b/CD18 transfected HEK cells bound equally to fibrinogen compared to vWF. These results could be confirmed by real time protein-protein interaction analysis by SPR; CTL-2 bounds more efficiently to vWF when compared to CD11b/CD18.
Summary and Conclusions: Altogether, our findings indicated the important role of vWF for anti-CTL-2 mediated neutrophil aggregation. Furthermore, this study introduced CTL-2 as new binding partner for vWF (via A1-Domain). Together with CD11b/CD18 integrin, this molecular cross talk may open new insights on the mechanism of neutrophil recruitment during inflammation.

OC 91.4
Impact of ADAMTS13-mediated regulation of von Willebrand factor multimer (VWF) profile on hemostasis and VWF clearance

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Background: An essential biosynthetic processing step for von Willebrand Factor (VWF) is the formation of disulfide linked multimers that result in molecules of up to 20 mDa. Newly released VWF multimers undergo limited proteolytic processing by ADAMTS13 to generate molecules of an optimal size for the primary hemostatic function. While there is a consensus that the largest VWF multimers optimally support the protein's primary hemostatic function, there has been no systematic assessment of the influence of multimer size on hemostatic efficacy and clearance of this protein.
Aims: To evaluate the effect that VWF multimer size has on its role in hemostasis and rate of clearance from the circulation.
Methods: Full-length murine VWF and ADAMTS13 cDNAs were cloned into pCIneo and pcDNA3.1 plasmids, respectively and recombinant mouse VWF (rmVWF) and ADAMTS13 (rmADAMTS13) were produced. To reduce multimer size, rmVWF was treated with either rmADAMTS13 or N-acetylcystine (NAC). NAC reduces VWF multimer size by reduction of the intersubunit disulfide bonds. The loss of HMW multimers was confirmed by 1% SDS agarose gel electrophoresis and function of the VWF multimer subpopulations was assessed by an *in vitro* flow chamber system. Briefly, whole blood from ADAMTS13/VWF double knockout mice was perfused over a collagen/digested VWF coated surface at 2500/s and platelet accumulation was quantified by the Quorum WaveFX- X1 spinning disk confocal system. For the clearance study, digested VWF was injected into ADAMTS13/VWF double knockout mice and blood was collected by retro-orbital sampling and VWF:Ag was monitored.
Results: Treatment of VWF with 25 and 35 mM NAC resulted in a respective 62% and 78% reduction in HMW multimers, while cleavage with 0.5, 2.0 and 3.0 U/mL ADAMTS13 reduced HMW multimers by 65%, 75% and 81%, respectively. In the flow chamber experiment, surface coverage and thrombus volume were evaluated and showed very similar trends. 80.8%, 81.8% and 91.1% reduction of thrombus volume were observed when VWF was digested with 0.5, 2.0, 3.0 U/mL ADAMTS13, respectively. However, there was no difference in these parameters of thrombus generation between undigested or NAC-treated VWF. The loss of platelet adhesion to ADAMTS13-

cleaved VWF was partially mitigated with additional exposure to 35 mM NAC resulting in 25.0%, 18.2% and 30.2% increases in thrombus volume. Clearance of infused ADAMTS13 cleaved VWF was significantly faster than either the undigested or NAC-treated VWF. The half-life for infused undigested VWF was 27.8 min while the half-lives for NAC and ADAMTS13-treated VWF were 17.9 and 8.5 min, respectively.

Summary/Conclusion: Our data highlights the impact of VWF secondary structure on both hemostasis and protein clearance. Notably, the loss of HMW multimers through reduction of disulfide interactions does not alter thrombus formation and has only a moderate effect on VWF clearance. These effects are presumably the result of the reduction of intra-subunit disulfide linkages. In contrast, cleavage with ADAMTS13 is associated with significant loss of VWF function and rapid clearance from the circulation. These data reveal the efficient regulation of VWF activity by ADAMTS13 and document the preferential clearance of smaller, less functional VWF molecules.

OC 91.5

Analysis of ADAMTS13 proteolysis of Von Willebrand Factor under physiological shear stress conditions

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Background: Within the circulation the multimeric size and thus activity of Von Willebrand Factor (VWF) is controlled by ADAMTS13 that cleaves VWF in its A2 domain. *In vivo* the cleavage site becomes available under shear stress when VWF is anchored to endothelial cells or the exposed sub-endothelial matrix. The analysis of VWF proteolysis is hampered by the need to use small fragments of VWF that are readily cleaved, but do not represent the full length molecule or the use of denaturants to artificially expose the ADAMTS13 cleavage site. Alternatively cultured HUVECs can be stimulated under flow to release VWF, and in the presence of platelets these can be visualised as long strings that can be cleaved by ADAMTS13. However, this assay cannot be used to investigate different VWF variants. Several studies have used a vortex mixer to generate unfolding of the VWF molecule, however the mechanism behind this is not fully defined.

Aims: To develop a physiological flow based assay to investigate ADAMTS13 proteolysis of VWF and its variants under conditions of shear stress.

Methods: Collagen coated flow slides were perfused with washed erythrocytes and labelled platelets supplemented with plasma derived or recombinant VWF in the presence or absence of recombinant ADAMTS13. Real time movies were recorded and the extent of platelet capture determined. Alternatively, VWF and ADAMTS13 were perfused over collagen surfaces in the absence of platelets, the flow through and stripped surface proteins were analysed by multimer gels.

Results: ADAMTS13 reduced the extent of VWF mediated platelet capture in a concentration dependent manner, with 20 nM ADAMTS13 reducing surface coverage by ~45% after 5mins at 1500/s. The loss of platelet capture was specific to ADAMTS13 cleavage of VWF since the use of an inactive ADAMTS13 mutant or an anti-ADAMTS13-MP domain antibody prevented loss of platelet capture. Furthermore, analysis of the flow through and stripped collagen bound VWF demonstrated loss of VWF multimers only in the presence of ADAMTS13. Extent of cleavage was also correlated with increasing shear rate, with proteolysis most effective at the highest shear rates. Finally, time course experiments demonstrated that ADAMTS13 mediated both the initial rate of platelet capture and the final extent of surface coverage. Application of the assay to investigate different VWF variants demonstrated that as expected the type 2A variants G1629R and E1638K were proteolysed faster than wtVWF. In keeping with data obtained under static conditions, Asialo-VWF lacking terminal sialic acid residues was proteolysed slower under shear stress. Intriguingly, the VWF variant S1468A which we have recently shown to be proteolysed *slower* under static conditions was proteolysed *faster* under shear stress. Moreover, the ADAMTS13 P3 variant with a mutation at L1603, which has been shown to abolish cleavage of VWF under static conditions, was able to proteolyse VWF under flow to a similar extent to wtADAMTS13.

OC 91.6

Early stages of Weibel-Palade body biogenesis revealed by light and electron microscopy

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Background: The hemostatic protein Von Willebrand factor (VWF) is stored by the vascular endothelium in specialized organelles called Weibel-Palade bodies (WPBs). WPBs have a typical elongated shape which is the result of many densely packed helical tubules of VWF. Correct packaging of VWF in these tubular structures is thought to be a hallmark for proper VWF functioning during hemostasis. Understanding the formation of WPBs could therefore provide new insights in the mechanisms that are disturbed in patients with the bleeding disorder Von Willebrand disease (VWD). It is known that the WPBs originate from the Trans Golgi Network (TGN). Upon formation of high molecular weight VWF multimers, VWF tubules are formed which are stored in WPBs. Previous electron microscopy studies have described several structures, including WPB-like vesicles with one or two tubules, most probably representing immature WPBs formed at the TGN. However it is not known how and when these structures are formed in time.

Aims: The aim of our study is to obtain new insights in WPB biogenesis using both light and electron microscopic techniques.

Methods: Identification of WPBs emerging from the TGN was achieved by Correlative Light and Electron Microscopy (CLEM). Human Umbilical Vein Endothelial Cells (HUVEC) were transfected with VWF propeptide-EGFP, to label the WPBs, and we also co-transfected cells with Rab1a-mCherry to label the TGN. Cells expressing fluorescently labeled WPBs at the Golgi region were imaged and afterwards prepared for TEM using conventional resin embedding. Previously imaged fluorescent WPBs were then correlated to the ultrastructure in serial TEM sections. In addition, we prepared cells using high pressure freezing and freeze substitution. Cells were embedded in Lowicryl HM-20. Immuno-gold labeling on TEM sections was performed in order to detect structures corresponding to VWF in newly forming WPBs.

Results: Using CLEM, we could correlate fluorescently labeled immature WPBs to TEM sections. We observed WPBs with loosely packed VWF tubules which showed clathrin coats around their membrane. These immature WPB contained between 4 and 8 tubules that were already aligned creating elongated organelles. With this technique we did not detect vesicles containing tubule-like VWF subunits which were previously hypothesized to be involved in WPB formation. However when we performed a VWF specific immuno-gold labeling on cells prepared by high pressure freezing and freeze substitution we did find VWF positive structures that seem to contain subunits of VWF tubules. The diameters of these structures ranged between 250 and 400 nm which would be rather large in case they represented cross sectioned WPB. Further analysis of Lowicryl embedded and immunolabeled TEM samples is required to obtain more information about WPB biogenesis, especially regarding the order in which the morphological changes occur.

Summary/Conclusions: Correlative light and electron microscopy shows that tubule containing, clathrin-coated vesicles in proximity to the TGN correspond to newly forming WPBs. Using immuno-gold labeling a population of VWF immunoreactive small vesicles is identified that contains building blocks for assembling tubules. Together our findings yields novel insight into the early stages of the biogenesis of WPBs.

EPOSTER ORAL PRESENTATIONS

PA1.01 – Antiplatelet Agents: ADP Receptors – I

PA 1.01-1

Comparison of a new ELISA-based with the flow cytometric assay for vasodilator-associated stimulated phosphoprotein (VASP) phosphorylation to assess P2Y12-inhibition after ticagrelor intake

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Background: Ticagrelor is a $P2Y_{12}$ receptor antagonist, with superior effects but also ensuing enhanced bleeding risk as compared to clopidogrel. Determination of platelet inhibition may be useful to confirm efficient platelet inhibition on an individual patient level, and to identify patients at risk for bleeding, particularly in a pre-operative setting. The vasodilator-associated stimulated phosphoprotein (VASP) phosphorylation assay specifically measures platelet $P2Y_{12}$ inhibition, but has so far required special flow cytometric equipment and individual sample processing. A new ELISA-based VASP assay has been developed which allows batch analysis after initial platelet activation. Due to the reversible binding of ticagrelor it is unclear if the ELISA and flow cytometric assays provide comparable results.

Aims: The aim of the study was to compare the performance and reliability of a newly developed ELISA-based VASP assay with the currently available flow cytometric approach. We hypothesized that the conventional and new methods may be comparable when the reversible $P2Y_{12}$ inhibitor ticagrelor is used.

Methods: The clinical trial was approved by the Ethics Committee of the Medical University of Vienna and performed in accordance with Good Clinical Practice guidelines and ethical principles that have their origin in the Declaration of Helsinki. Written informed consent was obtained from all healthy volunteers.

Healthy volunteers received a single 180 mg loading dose of ticagrelor. Blood samples were drawn into non-wettable plastic tubes containing 3.8% sodium citrate (0.129 M) as anticoagulant. Overall, 84 data points were evaluated. To directly compare the performance of the new ELISA-based technique to the currently used flow cytometric assay, VASP phosphorylation was determined by the new ELISA VASP-P and the commercially available test (PLT VASP/$P2Y_{12}$, both BioCytex, Marseille, France). Venous blood samples were stored unopened at room temperature (RT, 18–25 °C) and were processed within 24 h after collection. All samples were handled following the manufacturer's instructions enclosed in the assay kits.

We pair-wise compared the platelet reactivity index (PRI) between. For correlation of the two different assays, the Pearson's coefficient was used and a linear regression analysis was performed. By means of the Bland-Altman analysis, a direct comparison of the ELISA and the flow cytometric method was possible and the agreements as well as the systematic error between the different assays were evaluated. The limits of agreement were specified as mean difference ± 1.96 SD.

Results: PRI-values of the two methods correlated well ($r = 0.97$, $P < 0.001$). Ticagrelor rapidly decreased PRI values on average after 50 min, but nadir levels 2–6 h after ticagrelor intake were 15% higher when PRI% was measured with the flow cytometric method. Bland-Altman analysis showed that the flow cytometric assay measured markedly higher PRI levels than the new ELISA-based technique (mean difference 13%).

Summary/Conclusion: The new ELISA-based VASP assay offers an alternative to the currently used flow cytometric method, but measures lower PRI levels, particularly when PRI falls below 20% after ticagrelor intake.

PA 1.01-2

Impact of body mass index on response to thienopyridines in patients treated after acute coronary syndrome

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Background: Variability of response to thienopyridine has been described for both clopidogrel and prasugrel and the underlying factors are multiple.

Aims: The aim of the present study was to analyze the impact of body mass index (BMI) and different type of obesities on response to clopidogrel and prasugrel in ACS patients.

Methods and Results: One thousand five hundred and forty-two ACS consecutive patients undergoing coronary stenting were included: 287 with clopidogrel 75 mg, 868 patients with clopidogrel 150 mg and 387 patients with prasugrel 10 mg. Platelet reactivity was assessed 1 month after discharge with PRI VASP. Three hundred and thirty-six patients (21.8%) were obese (BMI > 30) including 264 obesity type 1 (3040). Among, 1206 non obese patients, we identified 1.6% ($n = 24$) underweight patients (BMI < 18.5), 33.4 ($n = 515$) patients with normal BMI and 42.9% ($n = 429$) overweight patients. We observed a stepwise increase of on-treatment platelet reactivity according to the different groups with higher platelet reactivity in patients with higher BMI with all thienopyridine regimens ($P < 0.0001$). Using the predefined cut-off of VASP > 50% to define the HTPR, incidence of HTPR was higher in obese patients than in non obese patients with all regimens (OR[95%CI]: clopi. Seventy-five milligram; clopi.150 mg; prasu. Ten milligram: 2.7[1.4–5]; 1.4[1.1–2]; 10[4.7–21.2] respectively); $P < 0.05$ for all). Using PRI VASP < 20% to define LTPR on prasugrel linked with higher risk of bleeding, the incidence of LTPR was lower in obese patients: 13% (12/93) vs. 33% (97/294), OR [95%CI]: 0.30 [0.16–0.58], $P < 0.001$.

Conclusion: BMI has a strong impact on response to thienopyridine with a linear relationship between BMI and on treatment platelet reactivity. We also reported higher incidence of HTPR and lower incidence of LTPR in obese patients. These data suggest the potential value of tailored antiplatelet therapy based on BMI to improve prognosis of this high risk population.

PA 1.01-3

Evaluation of non-specific agents to control prasugrel-related bleeding in a rabbit model

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Background: Prasugrel is a thienopyridine that provides a more rapid and more effective inhibition of platelet aggregation compared to clopidogrel, thus leading to an increased bleeding risk.

Aims: The aim of the study was to evaluate the efficacy and safety of non-specific agents such as recombinant activated factor VII (rFVIIa), tranexamic acid (TA) and desmopressin (DDAVP) to limit blood loss after a prasugrel loading dose in a rabbit model of bleeding and thrombosis.

Methods: Anesthetized and ventilated rabbits ($n = 49$) were randomly assigned into five groups: control (placebo-placebo), placebo (prasugrel 4 mg/kg-placebo), rFVIIa (prasugrel 4 mg/kg-rFVIIa 150 μg/kg),

TA (prasugrel 4 mg/kg-TA 20 mg/kg), DDAVP (prasugrel 4 mg/kg-DDAVP 1 μg/kg). Two hours after a prasugrel loading dose, a stenosis and a vessel injury were performed on the carotid artery that induced cyclic thrombotic events detected as flow reductions. Non-specific agents were given during the following observation period (1 h before inducing bleeding for DDAVP, 30 min for TA and rFVIIa). Then, haemorrhage was induced by standardized hepatosplenic sections and blood loss was monitored for 15 min.

Results: Prasugrel decreased mean ADP-induced platelet aggregation from 66 ± 4% (mean ± SD) in the control group to 41 ± 8% in the prasugrel-treated rabbits ($P < 0.001$) and doubled the blood loss (median and interquartile range: 10.6 g [9.7–12.7] in the control group vs. 20.0 g [16.7–25.1] in the placebo group, $P = 0.004$).

Blood loss in the prasugrel-rFVIIa group did not differ from blood loss in the placebo group (17.9 g [11.2–26.5] vs. 20 g [16.7–25.1], $P = 0.43$). TA and DDAVP had no impact on hepatosplenic bleeding neither. Regarding safety, rVIIa induced more than three cyclic flow reductions for 33% of rabbits, vs. 0% in the placebo group ($P = 0.031$). TA and DDAVP were not associated with an increase of thrombotic events.

Conclusions: Neither rFVIIa, nor TA or DDAVP were able to significantly decrease prasugrel-related bleeding. rFVIIa-treated rabbits were more prone to thrombotic events.

PA 1.01-4

How to test the effect of aspirin and clopidogrel in patients on dual antiplatelet therapy?

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Background: Dual antiplatelet therapy with clopidogrel and aspirin is currently the standard therapy in high-risk patients for the prevention of recurrent ischemic events. Monitoring antiplatelet therapy is an important issue in this patient group. However, some frequently used laboratory tests are clearly affected by both antiplatelet agents what makes interpretation of the results ambiguous.

Aims: To determine which laboratory tests, used for the detection of clopidogrel, are affected by aspirin and which laboratory tests, used for the detection of aspirin are influenced by clopidogrel.

Patients and Methods: Study population included 111 patients with the history of ischemic stroke being on clopidogrel monotherapy for at least 1 month. Among these patients, 62 showed good response to the drug as demonstrated by flow cytometric analysis of vasodilator stimulated phosphoprotein (VASP) phosphorylation and a newly developed P2Y12 receptor specific aggregation method in which ADP-induced aggregation is performed on prostaglandin E1 treated platelets (ADP [PGE1]). Laboratory tests routinely used for the detection of aspirin effect (ADP-, collagen-, epinephrine-, and arachidonic acid (AA) induced platelet aggregation and secretion, PFA-100 assay with collagen/epinephrine cartridge, VerifyNow ASA test and AA-induced thromboxane B2 (TXB2) production in platelet rich plasma) were carried out on samples obtained from these patients. The other arm of the study involved 52 patients with coronary artery disease being on aspirin monotherapy for at least 1 month. These patients showed good response to aspirin as demonstrated by the AA-induced platelet aggregation and TXB2 production. Methods used for testing the effect clopidogrel (ADP induced platelet aggregation and secretion, flow cytometric analysis of vasodilator stimulated phosphoprotein (VASP) phosphorylation and ADP[PGE1] aggregation) were performed on samples obtained from these patients. Besides, all tests were carried out on samples from 140 healthy volunteers. For each method, diagnostic cut-offs were determined according to the guidelines of Clinical and Laboratory Standards Institute. All patients and controls gave informed consent and the study was approved by the Regional Ethics Committee.

Results: Of the methods used for detecting the effect of aspirin resistance, clopidogrel monotherapy significantly inhibited all aggregation and secretion tests including tests using AA as agonist. AA-induced TXB2 production was also decreased ($P < 0.0001$). VASP phosphorylation and arachidonic acid induced platelet aggregation showed a surprisingly fair correlation in patients taking clopidogrel only (Spearman $r = 0.49$, 95% CI 0.23–0.67, $P < 0.001$). Clopidogrel therapy did not inhibit the VerifyNow ASA test. Of the methods used for testing clopidogrel resistance, aspirin monotherapy influenced ADP induced platelet aggregation and secretion ($P < 0.0001$), but did not have an effect on VASP phosphorylation and on the P2Y12 specific platelet aggregation test.

Conclusions: Interestingly, AA-induced platelet aggregation and TXB2 production are influenced by clopidogrel therapy and they are not recommended for testing the effect of aspirin in patients on dual therapy. For clopidogrel testing in patients on combined therapy the VASP phosphorylation test or the P2Y12 specific ADP[PGE1] aggregation test could be recommended. As ADP-induced platelet aggregation is affected by both antiplatelet agents, it should not be used to test the effect of either clopidogrel or aspirin in patients on dual therapy.

PA 1.01-5

Reversal strategy in antagonizing the P2Y12-inhibitor ticagrelor

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Background: Patients on anti-platelet therapy have a higher incidence of bleeding complications and reversal of anti-platelet drug effects is an important issue at trauma or emergency departments. For old and conventional anticoagulants, reversal strategies are established. However, there is no experience or recommendation how to antagonize the reversible and highly effective $P2Y_{12}$-inhibitor ticagrelor and how to restore platelet function following ticagrelor dosing.

Aims: The aim of the study was to describe an *ex vivo* model to reverse the effects of ticagrelor and to estimate the optimal quantity of platelet transfusions required to normalize platelet aggregation.

Methods: The clinical trial was approved by the Ethics Committee of the Medical University of Vienna and performed in accordance with Good Clinical Practice guidelines and ethical principles that have their origin in the Declaration of Helsinki. Written informed consent was obtained from all healthy volunteers.

To normalize platelet aggregation, increasing amounts of autologous platelet rich plasma (PRP) were added *ex vivo* to hirudin-anticoagulated blood which was obtained 3 h after the administration of ticagrelor, by spiking PRP into blood at ratios of 1:10, 1:5 and 1:3.

Platelet aggregation was assessed by whole blood multiple electrode aggregometry (MEA; Multiplate®; Dynabyte, Munich, Germany). For interpretation of aggregation, we defined a cut-off level of 40 A.U. as the lower limit of the range. Volunteers above this level were considered to exhibit normal platelet reactivity.

Nonparametric tests were used and statistical comparisons were performed with the Friedman ANOVA, and the Wilcoxon test for post-hoc comparisons. A two-tailed P-value of < 0.05 was considered significant.

Results: The strategy to reverse the effect of ticagrelor was tested in 20 healthy volunteers. Basal ADP induced platelet aggregation averaged 71 ± 16 A.U. Ticagrelor decreased ADP induced platelet aggregation to 16 ± 8 A.U.

A clear dose-response was obtained after spiking whole blood with increasing amounts of PRP. After addition of PRP at a ratio of 1:10, platelet aggregation increased to 31 ± 14 A.U. When assuming that one apheresis platelet concentrate (200 mL) typically contains a mini-

mum of 2×10^{11} platelets, the ratio of 1:10 corresponds to 0.5 units of apheresis platelet concentrates.
A ratio of 1:5 – equivalent to 1 unit of platelet concentrates – increased ADP induced platelet aggregation to 41 ± 14 A.U. Platelet aggregation increased further to 48 ± 18 A.U. following the addition of PRP at a ratio of 1:3, which corresponds to 1.5 units of platelet concentrates. All comparisons were significant at $P < 0.01$.
Summary/Conclusion: Platelets dose-dependently improve *ex vivo* platelet aggregation of subjects after a loading dose of 180 mg of ticagrelor. It is estimated that > 2 units of apheresis platelet concentrates will be necessary to completely restore baseline platelet aggregation in the majority of patients. Point-of-care platelet function tests may be suitable tools to verify this concept in emergency patients and to estimate the extent of the reversal and de-risk on an individual patient's level.

PA 1.01-6
Ticagrelor and endothelial dysfunction: platelet-independent effects

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Background: Platelets play a crucial role in the pathogenesis of acute coronary syndromes (ACS) and in line with this, anti-platelet therapy is a key area of research in primary and secondary prevention of major adverse cardiovascular events (MACE). P2Y12 receptor inhibitors, such as clopidogrel, prasugrel and ticagrelor prevent ADP-induced platelet aggregation; different P2Y12 receptor blockers have shown different spectra of beneficial effects on mortality due to myocardial infarction (MI), stroke and bleeding. Since P2Y12 receptors are not only expressed in platelets but also in vascular smooth muscle cells, neurons and other cells, the reported data may also be the result of effects on other cells than platelets.
Aims: To investigate platelet-independent effects of P2Y12 receptor inhibitors with a special focus on endothelial dysfunction, an early mediator of cardio- and cerebrovascular disease.
Methods: Experiments were performed on human primary endothelial cells of aortic, cerebral and cardiac origin. To investigate the effect of ticagrelor on the production of free radicals, endothelial cells incubated with vehicle or tumor necrosis factor alpha (TNFalpha, 10 ng/mL) were treated with increasing concentrations of ticagrelor (10^{-7} M, 10^{-6} M and 10^{-5} M). ROS production was measured by electron spin resonance (ESR) and expressed as 0_2ˆ-/min/10^5 cells. Additionally, activation of endothelial nitric oxide (eNOS) as well as cell toxicity were assessed by western blotting and LDH assay, respectively. Finally, to confirm the expression of P2Y12 receptors at the protein and mRNA level, western blot analysis and qrt-PCR were performed. Data is expressed as mean ± SEM.
Results: Ticagrelor reduced TNFalpha-induced ROS production in human aortic endothelial cells (TNFalpha, 7.043 ± 1.632, 0_2ˆ-/min/10^5 cells; TNFalpha + ticagrelor 10^{-7} M, 2.647 ± 1.048, 0_2ˆ-/min/10^5 cells; TNFalpha + ticagrelor 10^{-6} M, 4.053 ± 1.662, 0_2ˆ-/min/10^5 cells, TNFalpha + ticagrelor 10^{-5} M, 5.233 ± 1.716, 0_2ˆ-/min/10^5 cells). Additionally, ticagrelor augmented activation of eNOS at Serine 1177 and increased cell survival.
Interestingly, P2Y12 receptor expression could not be detected neither at the protein nor at the mRNA level in either human aortic, cardiac or cerebral endothelial cells.
Conclusion: Ticagrelor treatment in human aortic endothelial cells
1 reduces generation of ROS in response to TNFalpha
2 increases eNOS activation and
3 reduces cell death
Lack of expression of P2Y12 receptor in endothelial cells not only confirms platelet-independent effects but also supports the concept of P2Y12 receptor-independent effects by ticagrelor.

PA1.02 – Platelet Activation: Novel Proteins – I

PA 1.02-1
Disabled-2 is required for efficient platelet activation by thrombin in mouse

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Background: During blood vessel wall damage, platelets adhere on injury site to form a stable plug and prevent excessive blood loss. Platelet activation is a complex process that is regulated delicately. Disabled-2 (Dab2) has been reported to regulate platelet aggregation. Recently, megakaryocyte lineage-restricted Dab2 knockout ($Dab2^{-/-}$) mice were generated to assess the role of Dab2 in platelet function *in vivo*. $Dab2^{-/-}$ mice are normal in size and platelet production while bleeding time is prolonged and thrombus formation is impaired. However, the mechanistic insight leading to the impaired haemostatic phenotype in $Dab2^{-/-}$ mice is not clear.
Aims: In this study, the effects of Dab2 deficiency on platelet activation and integrin signaling are investigated with an aim to delineate the underlying mechanism of Dab2 function in haemostasis.
Methods: Washed platelets were isolated from wild type ($Dab2^{fl/fl}$) and $Dab2^{-/-}$ mice for agonist-induced platelet aggregation, granule secretion and integrin alpha(IIb)beta(3) activation analyses. In addition, the spreading of platelets on fibrinogen and the clot retraction assays were performed to investigate the role of Dab2 in cytoskeleton reorganization and outside-in signaling. The activation status of platelet signaling proteins in the assay conditions was analyzed by Western blot to delineate the signaling pathways regulated by Dab2.
Results: Agonist-induced platelet aggregation assays revealed that $Dab2^{-/-}$ platelets responded normally to collagen, U46619, ADP, and 1 U/mL of thrombin. However, the responsiveness of $Dab2^{-/-}$ platelets to low concentration of thrombin (0.05 U/mL) was significantly decreased. The percentage of light transmission was 49.4 ± 7.4% and 13.3 ± 8.5% for $Dab2^{fl/fl}$ and $Dab2^{-/-}$ platelets, respectively. In accordance with this notion, the activation of integrin alpha(IIb)beta(3) for $Dab2^{-/-}$ platelets was 64.6% of $Dab2^{fl/fl}$ platelets after thrombin (0.05 U/mL) stimulation. The percentage of clot retraction was 49.2 ± 6.3% for $Dab2^{-/-}$ platelets that was significantly different to 73.5 ± 6.0% for $Dab2^{fl/fl}$ platelets. Moreover, the increase in the surface area of $Dab2^{-/-}$ platelet induced by low concentration of thrombin was 56.7 ± 13.7% of $Dab2^{fl/fl}$ platelets. In contrast, the spreading of $Dab2^{-/-}$ and $Dab2^{fl/fl}$ platelets on fibrinogen induced by $MnCl_2$ was similar, implying that Dab2 is mainly involved in thrombin-induced inside-out but not outside-in signaling of integrin alpha(IIb)beta(3). Analyses of the thrombin-stimulated signaling revealed that the expression levels of thrombin receptors (PAR3 and PAR4) and PDK1-Ser241 phosphorylation were similar for $Dab2^{fl/fl}$ and $Dab2^{-/-}$ platelets. However, thrombin-stimulated phosphorylations of Akt-Ser473 and mTOR-Ser2448 in $Dab2^{-/-}$ platelets were significantly decreased, implying that Dab2-deficient platelets were impaired in thrombin-stimulated inside-out signaling.
Summary: Dab2 is a key haemostatic regulator by playing a selective role in cytoskeleton reorganization and mTOR pathway underlying thrombin-stimulated inside-out signaling and is required for efficient platelet activation by thrombin in mouse.

PA 1.02-2

Human platelet aminophospholipid translocase (APLT) ATP8A1 and TMEM16F are calpain substrates

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Background: Resting platelets have an asymmetrical distribution of phospholipids across their plasma membrane, with the anionic aminophospholipid phosphatidylserine (PS) residing mainly in the inner, cytoplasmic leaflet. APLT maintains membrane phospholipid asymmetry by rapidly and specifically transporting aminophospholipids from the outer to the inner membrane leaflet in a Ca^{2+}-sensitive manner; APLT is likely the P-type ATPase ATP8A1. Activated platelets express a procoagulant surface on a distinct platelet subpopulation due to Ca^{2+}-dependent scrambling of membrane phospholipids, exposing PS on the platelet surface. The Ca^{2+}-activated cation channel TMEM16F has recently been shown to be important in this scrambling. Calpain, a Ca^{2+}-dependent intracellular protease, is involved in many regulatory processes in platelets via limited proteolysis of its substrates.
Aim: To determine whether ATP8A1 and TMEM16F are substrates of calpain in activated human platelets.
Methods: Washed platelets were stimulated with the combination of 1 U/mL thrombin and 10 μg/mL collagen (T + C), which yields approximately 20% PS-exposing platelets, or 3 μM A23187, which yields approximately 95% PS-exposing platelets. We have previously shown that, under these conditions, calpain is not activated in T + C-stimulated platelets, but is activated in A23187-stimulated platelets (Gwozdz et al, Thromb Haemost 2010;103:1218). To inhibit calpain, platelets were pretreated with the cell-permeable calpain inhibitor calpeptin (300 μM) before stimulation. Western blot analysis of platelet lysates was done using a polyclonal antibody specific for the cytoplasmic C-terminus of ATP8A1 (Ding et al, J Biol Chem 2000;275:23378). Alternatively, to detect TMEM16F, commercial polyclonal antibodies reported to be specific for either the cytoplasmic N-terminus or an extracellular domain of the protein were used.
Results: ATP8A1: A single band of molecular weight approximately 120 kDa was observed by immunoblotting lysates of resting platelets and of platelets stimulated with T + C. This band was not detected in A23187-stimulated platelets, however pretreatment with calpeptin prevented the loss of the band following A23187 stimulation.
TMEM16F: Recognition of either cytoplasmic or extracellular domains of TMEM16F by the 2 commercial antibodies was confirmed by flow cytometry and immunofluorescence. Immunoblotting with either antibody demonstrated a single broad band of approximately 125 kDa in lysates of resting platelets and platelets stimulated with T + C. In A23187-stimulated platelets, this band was not detected with the cytoplasmic N-terminus specific antibody, and with the extracellular-domain specific antibody, the molecular weight of the band was reduced by approximately 15 kDa. Pretreatment with calpeptin before stimulation with A23187 prevented these changes, preserving the approximately 125 kDa band observed in control or T + C-stimulated platelets.
Summary/Conclusions: We have demonstrated that two membrane proteins involved in transport of phospholipids between the inner and outer bilayer leaflets, APLT ATP8A1 and TMEM16F, are substrates of calpain. ATP8A1 is cleaved at the C-terminus; the inhibition of APLT activity that we have previously observed in PS-exposing A23187-stimulated platelets (Leung et al, JTH 2007;5:560) may be due, at least in part, to the cleavage of ATP8A1 that is associated with calpain activation. TMEM16F is cleaved at the N-terminus; whether this cleavage plays a role in the regulation of PS exposure on the surface of activated platelets remains to be determined.

PA 1.02-3

Platelet Toll-like receptor 9 stimulation enhances ATP- and ADP-dependent platelet activation and aggregation

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Background: Damage-associated molecular patterns (DAMPs), released upon cell necrosis, act as endogenous danger signals to exacerbate the inflammatory response. Among these DAMPs, ATP and ADP are key players in platelet activation and aggregation as well as in immune cell functions. High mobility group box-1 (HMGB1) is a central mediator of Toll-like receptor 9 (TLR9) activation in immune cells, and increased levels of this protein have been associated with inflammatory diseases, including sepsis. However, the role of TLR9 and HMGB1 in platelet function remains unclear.
Aims: Since platelet hyper-activation and microvessel thrombosis is a hallmark of severe sepsis, we wondered whether ATP/ADP and HMGB1 cooperate with TLR9 activation by bacterial DNA to promote platelet activation and aggregation.
Methods: We used oligonucleotides bearing unmethylated CpG motifs (CpG ODN), as TLR9 agonists, control non-CpG ODN, recombinant HMGB1, platelet aggregometry, thromboxane B_2 enzyme immunoassay, and flow cytometric analyses of platelet activation in healthy human and mouse PRP, washed platelet suspensions and in whole blood. Responses of platelets isolated from $P2X_1^{-/-}$ mice were also studied.
Results: We found that a 30 min-preincubation with CpG ODN causes irreversible $\alpha_{IIb}\beta_3$-dependent platelet aggregation in human and mouse PRP, and in washed mouse platelet suspensions. CpG ODN-induced platelet aggregation was completely inhibited by the ATP/ADP scavenger, apyrase and by $P2Y_{12}$ antagonism with cangrelor. In human hirudinized-PRP, inhibition of $P2X_1$ receptors for ATP with NF449 significantly reduced platelet aggregation. Accordingly, platelets from $P2X_1^{-/-}$ mice displayed impaired CpG ODN-induced aggregation. Platelet activation analyses revealed increased exposure of P-selectin and fibrinogen binding on platelet surface upon incubation with CpG ODN, which involved released ATP/ADP and thromboxane A_2 production. In washed human platelets, CpG ODN promoted granule secretion, thromboxane A_2 production and aggregation triggered by low concentrations of collagen. Interestingly, CpG ODN-induced platelet aggregation was potentiated by addition of recombinant HMGB1 in human PRP. Furthermore, both CpG ODN and HMGB1 significantly augmented the number of platelet-leukocyte aggregates in whole blood.
Conclusion: Thus, platelet TLR9 stimulation may contribute to sepsis-associated platelet hyperactivation and aggregation by enhancing the release reaction and thromboxane A_2 production.

PA 1.02-4

Regulation of platelet function by diacylglycerol kinase

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Background: Platelets play a key role in preventing blood loss through haemostasis, but the uncontrolled platelet activation at the site of atherosclerotic plaque rupture can lead to occlusive thrombi. One of the key events in platelet activation is transient increase in levels of cellular diacylglycerol (DAG) levels through hydrolysis of phosphoinositides by the enzyme phospholipase C. DAG is a critical second messenger and is best known as an allosteric activator of protein kinase C (PKC), a central signalling molecule in platelets. Diacylglycerol kinase (DGK) phosphorylates DAG to produce phosphatidic acid and therefore controls DAG signalling and through this, PKC activation.

Aim: To investigate the role of DGK in functional responses in platelets and on PKC activation.
Methods: Optical aggregometry and ELISA-based 5-HT release were used to assess agonist-stimulated responses of isolated human platelets. The levels of protein expression were analysed by Western blotting.
Results: Immunoblotting of platelet lysates for class II DGK isoforms demonstrated for the first time the expression of DGKδ in both human and murine platelets, which is consistent with platelet transcriptome data indicating DGKδ as a predominant DGK family isoform (1). Incubation of human platelets with class II DGK-specific inhibitor R59949 caused a concentration-dependent increase in collagen-related peptide (CRP) (1.25 μg/mL) and PAR1 (1 μM) receptor activating peptide (PAR4 AP)-stimulated aggregation. The increased aggregation was paralleled by a concomitant increase in dense granule secretion (measured by 5-HT release) from CRP and PAR1 treated platelets. The observed events were associated with a time-dependent augmentation in pleckstrin phosphorylation and PKC translocation to the plasma membrane. Moreover, immunoprecipitation identified DGKd association with all main platelet PKCs -PKCα, β, θ and δ, suggesting cross talk between DGK and PKC isoforms.
Conclusion: This investigation demonstrates that class II DGK enzymes, specifically DGKδ, contributes to the modulation of platelet activity potentially through the regulation of PKC isoforms.
Reference: 1. Rowley JW, Oler AJ, et al. Blood 2011; 118: 101–11.

PA 1.02-5
A role for histone deacetylases in the regulation of platelet function

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Background and Aims: Eighteen human histone deacetylase (HDAC) genes have been described which are divided into four classes. HDACs deacetylate acetyl-lysine residues in histone and non-histone proteins. It has recently be proposed that acetylation of non-histone proteins may modulate non-genomic processes including cell signalling. This led us to ask question: Are HDACs present in anucleate platelets, and if so do they modulate platelet function?
Methods: The presence of platelet HDACs was investigated by immunoblot analysis. Platelets were prepared from human citrated blood and aggregation, in response to collagen, collagen-related peptide (CRP), was measured by optical aggregometry in the presence of selective HDAC inhibitors. Dense granule secretion was measured by changes in the adenosine triphosphate (ATP) concentration using the luciferin/luciferase system kit. Flow cytometry was used to examine fibrinogen binding and P-Selectin exposure. Thrombus formation was studied *in vitro* also using whole citrated blood with 3,3-dihexyloxacarbocyanine iodide labeled cells. Blood was perfused through collagen-coated Vena8 Biochips at a shear rate of 20 dyn/cm^2, and stacks of Z-images were recorded. Analysis of the acetylated proteins in platelets and study of early GPVI signalling events was investigated by immunoblot analysis.
Results: Seven HDACs were identified in human platelets. Using an inhibitor for HDAC classes I, II and IV (SAHA), and a class III Sirtuin inhibitor (splitomicin), the potential involvement of HDACs in the regulation of platelet aggregation, dense and α-granule secretion were explored. Both inhibitors caused a reduction in collagen- and CRP-stimulated platelet aggregation, implicating HDACs in GPVI-stimulated signalling mechanisms. Splitomicin and SAHA were accompanied by inhibition of fibrinogen and P-Selectin. Accordingly, thrombus formation *in vitro* also inhibited.
Early GPVI signalling events were studied; neither inhibitor affected CRP-stimulated SYK, LAT, BTK and PLCγ2 phosphorylation. Tubulin can modulate cell motility and shape change, and is indeed important in the regulation of platelet activation. We found that a-tubulin is acetylated in unstimulated platelets, but is rapidly deacylated upon collagen, or CRP stimulation. Stimulation of platelets in the presence of SAHA or splitomicin blocked deacetylation of α-tubulin.
Conclusions: Our data provide evidence of possible roles for protein acetylation/deacetylation in the regulation of platelet signalling and function.

PA 1.02-6
Regulation of platelet GPCR receptor function by NHERF1 in mouse platelets

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Background: Recent studies investigating P2Y receptor (P2YR) function in cell lines have revealed a key regulatory role for Na^+/H^+ Exchange Regulatory Factor (NHERF) proteins (Nisar et al., 2012). ADP signaling via $P2Y_1$ and $P2Y_{12}$ plays a key role in platelet activation and haemostasis. We sought to investigate P2YR function in NHERF1 (−/−) mouse platelets and investigate the consequences of any changes in P2Y receptor traffic on platelet function both in isolated platelet preparations and in thrombus formation assays.
Aims: To characterize platelet responsiveness and thrombus formation in NHERF1 (−/−) mouse platelets.
Methods: Mouse platelets were isolated and prepared in either Tyrode's buffer as 'washed platelets' or in plasma as platelet rich plasma (PRP). Ligand binding was employed to determine P2YR surface expression and traffic. Aggregation responses, Ca^{2+} signaling and GTPase activity were measured in response to a number of platelet agonists and concentrations. Thrombosis formation *in vitro* (flow of blood over a collagen-coated surface) and *in vivo* (FeCl3-injured carotid arteries) were also measured.
Results: Ligand binding studies revealed that NHERF1 (−/−) mouse platelets have an increase in surface P2YR levels, a marked reduction in ADP-dependent P2YR internalization and a virtual absence of P2YR recycling following ADP removal. However these changes in receptor expression and traffic did not significantly alter ADP induced aggregation or ADP induced Ca^{2+} signaling.
Interestingly NHERF1 (−/−) platelets displayed reduced aggregatory responses to agonists at the PAR4 receptor (thrombin and PAR4 peptide) and the TP thromboxane receptor (arachidonic acid and U46619). No significant differences were observed however in Ca^{2+} signaling or GTPase activity following PAR4 receptor activation. The reductions in aggregation responses following PAR4 or TP thromboxane receptor activation did not however translate into significant changes in thrombus formation either in flow models or in whole animal intravital microscopy studies.
Summary: This study supports our recent findings in cell lines which show that NHERF1 proteins regulate P2YR traffic. However, alterations in P2YR traffic do not translate into significant changes in ADP-dependent platelet aggregation/activity in NHERF1 (−/−) mice.
Unexpectedly, we observed a reduction in TP thromboxane and PAR4 receptor responsiveness although PAR4-dependent Ca^{2+} signaling and GTPase activity remained unaltered. These findings suggest novel interactions between NHERF1 and PAR3/4 and TP thromboxane receptors in murine platelets Further studies investigating the association of PAR4 receptors and NHERF1 and other NHERF isoforms are currently underway.
Reference: 1. Nisar SP, Cunningham M, Saxena K, Pope RJ, Kelly E, Mundell SJ. Arrestin scaffolds NHERF1 to the P2Y12 receptor to regulate receptor internalization. J Biol Chem. 2012 Jul 13;287 (29):24505–15. doi: 10.1074/jbc.M112.347104. Epub 2012 May 18.

PA1.03 – Platelet Hyperfunction

PA 1.03-1

Podoplanin overexpressed rat C6 glioma cells enhances platelet aggregation and lymphatic metastasis

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Background: Tumor metastasis was considered as the major causes of death for cancer patients. Cancer cells acquire the ability to penetrate the walls of lymphatic and/or blood vessels for escape from the primary site. Recent studies demonstrated that lymphatic metastatic routes were controlled by specific gene alternation of tumor cells. Furthermore, podoplanin (pdpn), a membrane protein in tumor cells, is an endogenous ligand for platelet activation receptor CLEC-2 and facilitates tumor metastasis by inducing platelet aggregation. Recent studies indicated pdpn was involved in cancer development, lymphatic metastasis and poor prognosis a wide variety of cancer types. Our previous study, genetically modified rat C6 glioma cells (C6-LG) that elicit high procoagulant activity and express luciferase/GFP reporter gene were used for xenograft injection into nude mice to establish a metastatic model. The cancer cells colonized at lung were isolated for establishing a C6 subline (C6-lung). When the C6-lung cells were inoculated into nude mice subcutaneously, we found that it's enhancing *in vivo* metastatic potential and activity. Interestingly, we also noticed that the pdpn mRNA and protein was up-regulation in metastatic C6-lung cells. Therefore, we hypothesize the C6 glioma cell which overexpressed pdpn has potential of lymphatic metastasis.
Aims: To investigate whether pdpn overexpressed C6 glioma cells enhances platelet aggregation and facilitates lymphatic metastasis.
Results: Our results shown that compared with parental cells, the C6-lung cells exhibited significant decreasing in cell motility and cell growth rate, but had a greater migratory capacity. Furthermore, C6-lung cells activated platelet and induced platelet aggregation that is closely associated with their *in vivo* metastatic ability. The C6-Lung cells provided a strong chemoattractant signal which together induced the directional migration of human lymphatic endothelial cells (HLEC) through the micropores compared to the parental C6 cells. Furthermore, the real-time RT-PCR and Western blot results shown that endogenous pdpn mRNA and protein expression was significantly inhibited from stably pdpn shRNA expressed C6-lung clones. We also found that the podoplanin knockdown C6-LG subline reduced HLEC cell migration, and reduced metastatic ability *in vivo*.
Conclusion: We demonstrated that pdpn overexpression in C6-lung cells was associated with tumour lymphatic metastasis.

PA 1.03-2

Does loss of insulin signalling lead to a hyperactive platelet phenotype? Studies on Pf4-Cre insulin receptor knock out mice

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Platelets from patients with obesity and type 2 diabetes mellitus (T2DM) have a hyperactive phenotype, which may contribute to the increased risk of cardiovascular events observed in these patients. Circulating insulin levels in obesity and T2DM are often increased due to peripheral insulin resistance. Previous work by other groups suggested that insulin inhibits platelet function and that the loss of insulin responsiveness of megakaryocytes/platelets from patients with diabetes contributes to a platelet hyperactive phenotype. In contrast, our group found that insulin and insulin-like growth factor do not inhibit, but increase platelet function.

To determine the role of the insulin receptor, insulin receptor signalling and platelet insulin resistance in platelet function, we used the *Pf4-Cre* system to generate megakaryocytic lineage specific insulin receptor knock out mice (*Insr*−/−). Western blot analysis of platelet lysate confirmed that the insulin receptor was present in wild type platelets, but not in *Insr*−/− platelets, whereas IGF receptor expression levels were unchanged. Insulin stimulation resulted in phosphorylation of the insulin receptor and the PI3K substrate Akt in wild type, but not *Insr*−/− platelets, demonstrating that this receptor is essential for insulin signalling.
PAR-4-mediated integrin activation and α-granule secretion were unchanged in *Insr*−/− platelets, indicating that 'insulin resistance' of megakaryocytes/platelets alone does not increase platelet function. Furthermore, both ATP secretion and PAR-4-mediated aggregation were slightly reduced in *Insr*−/− platelets, suggesting that insulin receptor activation may contribute to platelet function. Consistent with this, insulin induced an enhancement of PAR-4 mediated integrin activation and platelet aggregation, which was absent in *Insr*−/− platelets.
Together, these results demonstrate that insulin resistance of platelets/megakaryocytes does not lead to a platelet hyperactive phenotype. Furthermore, insulin enhances platelet aggregation and integrin activation, with the effect lost when the insulin receptor is absent.

PA 1.03-3

Hemostasis and thrombosis in JAK2V617F-KI mice

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Background: Hemostasis disorders represent a leading cause of mortality and morbidity in patients suffering from myeloproliferative neoplasms (MPN) with a high incidence of thrombosis and a lower incidence of hemorrhagic disorders.
The pathophysiology of these anomalies is not known but some risk factors have been identified including the V617F mutation of JAK2 found in 95% and 60% of PV and TE patients, respectively. The specific effect of $JAK2^{V617F}$ on hemostasis deregulation is difficult to characterize in patients because of their heterogeneity in $JAK2^{V617F}$ allele burden and prophylactic treatments. Recently, animal models of MPN were developed as $JAK2^{V617F}$ transgenic and knock-in mice.
Aims: Our objective was to determine, the effect of the $JAK2^{V617F}$ mutation on the *in vitro* and *in vivo* hemostatic responses using $JAK2^{V617F}$mice.
Methods: We used two KI models, the Vav-Cre and Scl-CreERt/$JAK2^{V617F}$ KI that constitutively and upon tamoxifen induction express the mutation in hematopoietic cells, respectively. Both models mimic a PV-like disease with its evolution into secondary myelofibrosis. The effect of the mutation on platelets was analyzed *in vitro* by flow cytometry, and by measuring adhesion and aggregation in flow conditions on collagen-coated area. Hemostatic responses were assessed *in vivo* by measuring the tail bleeding time and FeCl3-induced thrombosis on mesenteric vessels.
Results: Platelets from Vav-Cre/$JAK2^{V617F}$ mice did not exhibit elevated CD62P level and had normal surface expressions of the major glycoproteins to the exception of GPVI that was significantly decreased ($P < 0.01$). Platelets adhesion and aggregation, in arterial flow conditions (1200/s) on immobilized collagen was profoundly impaired with a platelet surface decreased by up to 80% ($P < 0.01$). *In vivo*, Vav-Cre/$JAK2^{V617F}$ mice exhibited an enlargement of mesenteric vessels and have a prolonged tail bleeding time ($P < 0.01$). After FeCl3-induced injury, platelet aggregates formed rapidly but were highly unstable.
Interestingly, using Scl-CreERt/$JAK2^{V617F}$ mice, platelet GPVI deficiency, morphological vessel abnormalities, the increased tail bleeding time, and the *in vivo* accelerated rate of thrombosis with low thrombus stability, only appeared 2 months after induction of the mutation by the tamoxifen suggesting no direct link with it.

Secondary polycythemia induced by EPO-treatment of WT mice occurred without hemostasis disturbance; Thrombocytemia induced by retroviral expression of TPO in WT mice reproduced the GPVI deficiency, the prolonged bleeding time and the absence of *in vivo* occluding thrombi.

Conclusions: Our results in $JAK2^{V617F}$ mice are consistent with the complex hemostasis disorders observed in MPN patients i.e. thrombosis, embolism and hemorrhages. They seemed not directly associated with the mutation but rather with the development of MPN. Further work is needed to evaluate the cause and consequence of the GPVI and collagen adhesion deficit.

PA 1.03-5

Platelet activation and function during dengue virus infection

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Background: Dengue is the most prevalent mosquito-borne viral disease and is characterized by thrombocytopenia. Bleeding and plasma leakage the most frequent complications and they typically occur around the time of defervescence which corresponds with platelet nadir. Platelets are important for preserving vascular integrity. Not only thrombocytopenia but also thrombocytopathy may contribute to plasma leakage. Because complications are often observed with platelet numbers above the level for spontaneous bleeding, we hypothesized that platelet hypo-responsiveness and storage pool disease may occur as consequence of prolonged platelet activation and/or megakaryocyte infection.

Aims: To investigate the prevalence of platelet activation, secondary platelet hypo-responsiveness and storage pool disease in Indonesian patients with dengue.

Methods: We performed a prospective cohort study on 75 adult dengue patients admitted to a referral hospital in Bandung, Indonesia and 30 healthy local controls. Whole-blood flow cytometry was performed in the four phases of dengue: febrile (n = 11, early stage of illness), critical (n = 63, time at which most complications occur), early recovery (n = 61, time at which platelet number starts to recover) and convalescence phase (n = 43, 2 weeks after hospital discharge). The expression of CD62P (alpha granule marker), CD63 (lysosomal marker) and changes in $\alpha_{II}\beta_3$ activation were measured without stimulation and following maximal platelet activation with the PAR-1 agonist TRAP. Furthermore, CD62P expression to increasing concentrations of TRAP was evaluated. Finally, intra-platelet and plasma concentrations of serotonin were measured to determine storage pool disease of dense bodies.

Results: The platelet count in the febrile and critical phase was 69 and 40 $\times$ 10^9/L, respectively. Increased CD62P, CD63 expression and $\alpha_{II}\beta_3$ activation was only found in a minority of dengue patients. There was no difference in marker expression between patients with severe (n = 11) and non-severe dengue (n = 64) or between patients with and without bleeding or plasma leakage. Expression of CD63 and $\alpha_{II}\beta_3$ activation after maximal platelet stimulation was, however, reduced in the critical phase. There was also a trend for reduced CD62P expression in response to increasing TRAP concentrations in the febrile and critical phase. While median serotonin plasma concentrations were below the detection limit of 0.3 ng/mL during dengue, intra-platelet serotonin concentrations (median, interquartile range) were significantly increased in the febrile (4.9; 4.2–7.7 μg/mL) and critical phase (5.0; 4.1–6.9 μg/mL) vs. convalescence (2.5; 2.3–2.7 μg/mL), all $P < 0.001$) and correlated inversely with platelet counts ($R_s = -0.65$, $P < 0.001$) in early dengue.

Summary/Conclusion: Increased expression of activation markers of unstimulated platelets was only found in a subset of dengue patients. However, maximally stimulated platelets show a reduced expression of CD63 and $\alpha_{II}\beta_3$ activation, while there was a trend for lower CD62P expression in response to increasing TRAP concentrations in the febrile and critical phase. These findings indicate that platelet activation with secondary hypo-responsiveness occurs in dengue. There was no secondary storage pool disease as platelet serotonin concentrations were higher during dengue. Increased uptake of serotonin per platelet in dengue is required to prevent serotonin-induced plasma leakage. Although significant changes in circulating platelets were noticed during dengue infection, no relation with severity of disease was observed.

PA 1.03-6

Platelet-mediated angiogenesis is independent of VEGF and fully inhibited by aspirin

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Background: Platelets are major players in every step of vessel development through the local delivery of angiogenesis-modulating factors. Among these factors, the proangiogenic vascular endothelial growth factor (VEGF) and the antiangiogenic endostatin have been the most studied and can be differentially released from alpha-granules upon platelet activation by different stimuli. Although thrombin is the most potent agonist and is highly elevated in angiogenesis-related diseases, studies regarding its action on the release of platelet angiogenic factors are scarce and controversial.

Aim: To investigate the role of thrombin not only in VEGF and endostatin release but also in the net platelet angiogenic activity.

Methods: Washed human platelets were stimulated with thrombin (5 min) in the presence of inhibitors of the main signaling pathways involved in platelet activation and supernatants/releasates were used to determine the levels of angiogenic molecules and to induce angiogenic responses (ANOVA. n = 4–5, $^*P < 0.05$).

Results: We found that thrombin (EC_{50}, 0.051 ± 0.005 U/mL) induced the secretion of both VEGF and endostatin (7.9 ± 0.5 and 2.5 ± 0.3 ng/mL, respectively) (ELISA); however, the overall effect of the releasates was proangiogenic as they promoted the formation of tubule-like structures (matrigel) and increased endothelial proliferation (2.3 ± 0.3* fold) (MTT). Both processes were independent of VEGF as they were only slightly suppressed by a VEGF receptor-neutralizing antibody (15 ± 2*% of inhibition). Pharmacological studies revealed that the formation of tubule-like structures and endothelial cell growth were partially inhibited by pretreatment of platelets with inhibitors of PKC (Gö6983, 1 μM), p38 (SB203580, 25 μM), ERK1/2 (U0126, 10 μM), Src kinases (PP1, 5 μM), or PI3K/Akt (Ly-294002, 10 μM) (60 ± 6*, 63 ± 4*, 60 ± 5*, 33 ± 7*, and 15 ± 9*% of inhibition, respectively) but fully suppressed by the blockade of the cyclooxygenase pathway with aspirin (96 ± 4*% of inhibition) (500 μM). Protein array analysis revealed that the pretreatment of platelets with the different drugs inhibited the release of IL-1-beta, IL-2, Angiopoietin-1, Angiopoietin-2, G-CSF, GM-CSF and TNF-alpha release, but not VEGF, with a similar inhibition pattern observed in the angiogenic functional responses.

Conclusions: Our data indicate that the proangiogenic activity of platelets is independent of VEGF and appears to be the result of the combined action of several molecules. Aspirin could be a promising therapeutic agent to treat angiogenesis-related diseases.

PA1.04 – Circulating Microparticles

PA 1.04-1

Increased levels of P-selectin glycoprotein ligand-1 positive microparticles in patients with unprovoked venous thromboembolism

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Background: Microparticles (MPs) are small circulating membrane vesicles, less that 1 μm in diameter, released from activated or apoptotic cells, and reported to play an important role in pathogenesis of thrombotic diseases. MPs are known to express the P-Selectin Glycoprotein Ligand 1 (PSGL-1) receptor. Experimental studies have shown that accumulation of active tissue factor (TF) in the developing thrombi *in vivo* is dependent upon interactions between PSGL-1 and P-selectin (P-sel). P-sel inhibitors have also been shown to be equally efficient as low molecular weight heparins to reopen venous occlusions in animal models.

Aims: The present study was undertaken to investigate whether patients with unprovoked venous thromboembolism (VTE) had higher circulating levels of MPs baring PSGL-1, and to explore the role of PSGL-1/P-sel interaction in thrombogenicity of these MPs.

Methods: A case-control study was performed in 20 patients with a history of incident unprovoked VTE 1–5 years prior of the study, and 20 age- and sex-matched healthy controls recruited from the general population. Plasma levels of PSGL-1-positive MPs were measured in total MPs preparations by immunostaining with phycoerythrine (PE)-conjugated monoclonal antibody (clone KLP-1, Santa Cruz Biotechnology) using a FACS Aria instrument (BD Biosciences). Cellular origins of PSGL-1-positive MPs were determined by immunostaining of total MP fraction with cyanine (PerCP-CY5.5)- conjugated anti-PSGL-1 antibody, coupled with immunostaining using PE-conjugated monoclonal antibodies specific to parental cell, namely CD14 for monocytes, CD41 for platelets and CD62E for endothelial cells (all from BD Biosciences). The presence and expression levels of PSGL-1 on circulating MPs were confirmed by confocal microscopy and western blotting. The impact of interaction between P-sel and PSGL-1-positive MPs on thrombogenicity of MPs was assessed by levels of TF protein expression and functional activity on the surface of MPs, along with the assessment of presentation of negatively charged phospholipids. Differences between groups were tested by a nonparametric test for paired samples (Wilcoxon). Values are medians with 25% and 75% percentiles in brackets. The study was approved by the regional committee for research ethics, and all participants gave their informed written consent to participate.

Results: VTE patients had significantly higher body mass index (BMI) (28.2 kg/m^2 (24.1, 31.6 kg/m^2) vs. 26.1 kg/m^2 (22.6, 30.3 kg/m^2), $P = 0.045$) and circulating levels of PSGL-1-positive MPs (94×10^3/mL (72, 137×10^3/mL) vs. 66×10^3/mL (55, 78×10^3/mL), $P = 0.002$) compared to healthy controls. PSGL-1-positive MPs were found positive for CD14 and CD62E, but not for CD41. The data from confocal microscopy and western blotting confirmed expression of PSGL-1 at the surface and lysates of isolated MPs. Interaction between PSGL-1-positive MPs and P-sel boosted TF functional procoagulant activity, as well as higher surface presentation of negatively charged phospholipids on MPs and several cell types.

Conclusions: We found elevated levels of PSGL-1 positive circulating MPs in patients with unprovoked VTE originating from monocytes and endothelial cells. Our findings indicate that the interaction between PSGL-1-positive MPs and P-sel augmented the thrombogenicity of MPs, suggesting that this receptor-ligand interaction is important for the role of MPs in the pathogenesis of VTE.

PA 1.04-2

Microparticles from monocytes and CFTR?F508 mutated exocrine cells are deleterious for insulin secreting cells

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Background: Cystic fibrosis (CF) is a genetic disorder targeting CFTR channel, the most frequent mutation being CFTRΔF508. Cystic fibrosis-related diabetes is a particular form of diabetes detected in 50% of patients in the adult age. It is characterized by an early exocrine pancreas alteration followed by endocrine dysfunction. Chronic infections leading to major cytokine generation contribute to endocrine dysfunction by promoting insulin resistance. Microparticles (MPs) are plasma membrane fragments that are reliable markers of cell stress and act as cellular effectors.

Aims: Our aim was to study the role of MPs as effectors of cellular cross-talk between monocytes or exocrine cells and endocrine insulin secreting β-cells, in the context of CF and LPS infection.

Methods: Normal exocrine pancreatic cells (PANC-1) or with CFTRΔF508 mutation (CFPAC-1) and human monocytes (THP-1) submitted to CFTR inhibitor (CFTR(Inh)-172), were stimulated by LPS from *P. aeruginosa* to produce MPs. RIN-m5F rat ß-cells were targeted by 10 nM exocrine or monocyte MPs (18 h) or submitted to 25 μM CFTR(Inh)-172 thereby limiting expression of active CFTR at cell surface. PANC-1 and CFPAC-1 were targeted by monocyte MPs. NFkB activation induced by MPs was detected in HEK-Blue [TRADEMARK] reporter cells. Immunological labeling and Tenase activity probed the presence of Tissue Factor (TF) on MPs and cells. Cell apoptosis was quantified by cytometry and lysosomal activity by Neutral Red; MPs integration into target cell membrane was assessed by lipid PKH26 probe.

Results: LPS was increased apoptosis in the CFTR-inhibited RIN-m5F. LPS challenge in CFPAC-1 resulted in exaggerated apoptosis and enhanced release of TF-bearing MPs (13-fold increase compared to normal exocrine cells). After 18 h incubation, 98% of normal RIN-m5F target cells had captured PKH26-probed MPs. By restoring the decreased lysosomal activity caused by LPS ($n = 5$; $P < 0.01$), monocyte MPs exerted a cytoprotective effect on exocrine cells but were deleterious to endocrine cells by increasing apoptosis (12.5% vs. 3.8%; $n = 6$; $P < 0.0001$) and lowering lysosomal activity (−13%; $n = 5$; $P < 0.01$). MPs from THP-1 and CFPAC-1 delivered proinflammatory signal as demonstrated by NFkB activation in HEK reporter-cells, while MPs from PANC-1 did not. Addition of MPs from THP-1 and CFPAC-1 respectively generated a 33% and 40% decrease in insulin secretion ($n = 6$; $P < 0.0001$). Conversely, MPs from PANC-1 cells did not alter insulin secretion or lysosomal activity of RIN-m5F.

Conclusions: The exaggerated LPS susceptibility of CFTRΔF508 exocrine cells favors the shedding of MPs with high TF content, that are deleterious to normal endocrine cells. Similarly, MPs from monocyte and exocrine cells with defective membrane CFTR activity both deliver a proinflammatory and proapoptotic signal. In CF, TF-bearing MPs of exocrine and monocyte origin may contribute to early exocrine and endocrine pancreas dysfunction by reducing insulin secretion and ß cell survival.

PA 1.04-3

Circulating MPs show a prothrombotic phenotype in patients with long-life exposure to high LDL levels and directly associate with lipid-rich atherosclerotic plaque burden

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High plasma cholesterol levels are a risk factor for atherothrombosis and cardiovascular disease. Familial hypercholesterolemia (FH) is a monogenic disorder characterized by a reduced expression of the LDL receptor that leads to life-time exposure to high levels of LDL and the development of premature coronary artery disease. Circulating microparticles (cMPs), released from various cell types of the vascular compartment, may play important roles in vascular function. High levels of cMPs have been associated with a variety of pathological conditions, including thrombosis, inflammation, and metabolic disorders. We hypothesize that cMPs could contribute to the increased atherothrombotic risk in heterozygous FH patients.

The present study aimed to investigate cell-associated thrombogenic markers in cMPs of patients with genetic diagnosis of FH and subclinical atherosclerosis detected by aortic magnetic resonance imaging (MRI) under lipid-lowering therapy (LLT). The control was an age/gender/treatment-matched group of non-FH patients treated with LLT for secondary hypercholesterolemia.

Clinically and genetically characterized FH patients and control subjects (n = 37/group) were from the SpAnish Familial hypErcHolEsterolaemiA cohoRT (SAFEHEART). Patients were treated as per guideline indication. This study was approved by the local ethics committee, was conducted according to the Declaration of Helsinki and a written informed consent was obtained from all participants prior to the study. cMPs were obtained by high speed centrifugation from citrated platelet-free plasma (PFP) and quantitatively analyzed by flow cytometry for a) annexin V (AV) binding; b) specific blood cell activation surface markers; and c) tissue factor. cMP-associated TF activity was measured by a functional assay determining the FVII-dependent FXa generation.

At the moment of blood collection LDL cholesterol levels were 145 and 134 mg/dL in FH and control patients, respectively. The total number of cMPs was significantly increased in FH patients compared with controls (2390 [1890–3416] vs. 1872 [1418–2274] cMPs/μL PFP, $P < 0.005$). cMPs positive for platelet activation markers ($PAC1^+$ and $PAC1^+/CD62P^+$) increased 2- and 4-fold, respectively in FH patients ($P < 0.0001$ for both markers). FH patients had significantly higher overall numbers of tissue factor-rich ($CD142^+$) cMPs than controls ($P < 0.0001$). TF-exposing cMPs exerted procoagulant activity showing that cMPs carry functional active TF. Furthermore, the presence of lipid-rich atherosclerotic plaques in the FH patients was significantly associated with total AV^+-cMP levels, activated AV^+-pMPs ($TSP1^+$) and TF-bearing AV^+-pMPs ($CD142^+/TSP1^+$) ($P < 0.05$ in all cases). Taking one time-point LDL level as co-variate gave similar results, indicating that long-term exposure to high LDL in FH increases activation of the cells of the vascular compartment. Levels of AV^+-cMPs, activated AV^+-pMPs ($TSP1^+$) and TF-bearing AV^+-cMPs ($CD142^+$) were significantly lower in FH patients with calcified plaques.

Circulating MPs showed a prothrombotic phenotype in patients with FH and directly associated with lipid-rich atherosclerotic plaque burden. Thus, the specific increase in $\alpha_{IIb}\beta_3$-integrin$^+$ and TF^+ microparticles indicate that cMPs are active contributors in the on-going atherothrombotic process in FH patients and highlights the potential of TF-rich cMPs as a biomarker of thrombotic risk and as attractive therapeutic target in FH patients.

PA 1.04-4

Plasmatic levels of leukocyte-derived microparticles predict unstable plaque in asymptomatic patients with high-grade carotid stenosis

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Background: Preventive carotid surgery in asymptomatic patients is currently debated given the improvement of medical therapy. Therefore, non-invasive biomarkers that can predict plaque instability are needed. LMPs, originating from activated or apoptotic leukocytes, are the major microparticle subset in human carotid plaque extracts.

Aim: To analyze whether plasmatic levels of leukocyte-derived microparticles (LMPs) associate with unstable plaques in patients with high-grade carotid stenosis.

Methods: Forty-two patients with greater than 70% carotid stenosis were enrolled. Using a new standardized high-sensitivity flow cytometry assay allowing to measure low size microparticles, LMPs were measured before thromboendarterectomy. The removed plaques were characterized as stable or unstable using histological analysis according to the AHA criteria. LMP levels were analyzed according to the plaque morphology.

Results: The median LMP levels were significantly higher in patients with unstable plaque (n = 28, CD11bCD66b+MP/μl: 240 [147–394], and CD15+ MP/μl: 147 [60–335]) compared to those with stable plaque (16 [0–234] and 55 [36–157], $P < 0.001$ and $P < 0.01$, respectively). The increase in LMP levels was also significant when considering only the group of asymptomatic patients with unstable plaque (n = 10; CD11bCD66b+ MP/μl: 199 [153–410] and CD15+ MP/μl: 78 [56–258] compared to those with stable plaque (n = 14, 20 [0–251] and 55 [34–102], $P < 0.05$ and $P < 0.05$, respectively). After logistic regression, the neurologic symptoms (OR 48.7, 95% CI 3.0–788, $P < 0.01$) and the level of CD11bCD66b+ MPs (OR 24.4, 95% CI 2.4–245, $P < 0.01$) independently predicted plaque instability.

Conclusions: LMPs constitute a promising biomarker predicting plaque vulnerability in patients with high-grade carotid stenosis. These data provide clues for identifying asymptomatic subjects who may benefit the most from carotid surgery.

PA 1.04-5

Circulating microparticle plasma levels in obese patients

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Background: Arterial and venous thromboembolic risks have been shown to increase in obese subjects. Obesity is associated with a hypercoagulable state with either increased levels of clotting factors or inhibition of the fibrinolytic pathways. The procoagulant state involves several plasma proteins such as tissue factor (TF), fibrinogen, FVII, FVIII and PAI. Obesity is further characterized by enhanced platelet response predisposing to thrombi formation. Adipose tissue may play a significant role in stimulating the up-regulation of TF production and platelet function by the production of proinflammatory cytokines (TNF-α) and adipokines (eg leptin and adiponectin).

Aim: To elucidate the hypercoagulable state that characterizes obesity, we evaluated procoagulant microparticles (MP), both type and activity, in patients with different degree of obesity.

Methods: We measured the plasma levels of annexin V-microparticles (AMP), platelet-MP (PMP), P-Selectin-bearing MP (P-Selectin+), endothelial activated MP (E-Selectin+), leukocyte-MP (LMP) and tissue factor-bearing MP (TF+), and the MP procoagulant activity (PPL) in 80 patients (of whom 20 were overweight [BMI = 25–29.9 kg/m^2], 20 with 1st degree obesity [BMI = 30–34.9 kg/m^2], 20 with 2nd degree obesity [BMI = 35–39.9 kg/m^2] and 20 with 3rd degree [BMI > 40 kg/m^2]), and in 80 age and gender-matched normal weight healthy individuals. MPs levels were assessed by flow-cytometry (Beckman Coulter, USA) and MPs procoagulant activity was measured using a FXa based clotting assay (Diagnostica Stago, France). The study was approved by the Ethical Committee of the University Hospital of Padua.
Results: A significant increase of MP levels (mean ± SD) was found in obese patients, with a gradual rise according to the degree of obesity. In particular, AMP and PMP levels (MPs/μL) were higher in patients with 2nd obesity degree (4029 ± 2465 and 2881 ± 926, respectively) and 3rd obesity degree (4948 ± 2254 and 3098 ± 1426, respectively) than in controls (AMP 1635 ± 897, $P < 0.01$ for both comparisons; PMP 402 ± 225, $P < 0.0001$ for both comparisons). P-Selectin+ and E-Selectin+ MP showed the same pattern. LMP and TF+ were higher in overweight patients (273 ± 83, P vs. controls < 0.01 and 162 ± 36, P vs. controls < 0.0001, respectively), 1st degree obesity (238 ± 98, P vs. controls < 0.01 and 163 ± 57, $P < 0.0001$, respectively), 2nd degree obesity (305 ± 63 and 127 ± 27, $P < 0.0001$ for both comparisons, respectively) and 3rd degree obesity (393 ± 183 and 182 ± 66, $P < 0.0001$ for both comparisons, respectively) than in controls (LMP 106 ± 54; TF+ 50 ± 35). The phospholipids clotting time was significantly shorter in 2nd obesity degree (51 ± 16 s) and in 3rd obesity degree (56 ± 13 s) than in controls (68 ± 9 s, $P < 0.05$ for both comparisons).
MP levels are found to correlate to BMI (r 0.41, $P < 0.001$), waist circumference (0.43, $P < 0.001$), HDL (r −0.27, P 0.004), TNFa (r 0.37, P 0.02), leptin (r 0.34, P 0.04). Diabetes and thyroid diseases were associated with increased MP levels.
Conclusions: Our study indicates that excessive adipose tissue may induce overproduction of different types of MP. These MP levels significantly correlated with degree of obesity and with proinflammatory cytokines. Further studies are needed to clarify the role of increased MP levels, if any, in thrombosis risk in obese patients.

PA 1.04-6
Variations of procoagulant microparticles during disseminated intravascular coagulopathy and septic shock: a prospective multicentre study

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Background: Septic shock remains a leading cause of death in intensive care units (ICU) despite care improvement during the past 20 years. Alteration in haemostatic balance and disseminated intravascular coagulopathy (DIC) contribute to multiple organ failure. Microparticles (MPs) are sub-micronic plasma membrane fragments released from stressed cells that expose procoagulant phospholipids (almost phosphatidylserine – PhtdSer).
Aims: Study of circulating MP variations as biomarkers of vascular competence and dysfuntion during septic shock.
Methods: One hundred patients with septic shock were enrolled and their haemostatic status evaluated at admission (D1), and at D2, D3 and D7. Routine and specific haemostasis parameters were measured. DIC was diagnosed according to JAAM 2006 criteria. To assess cellular injuries, total microparticles (PhtdSer-MPs), GPIb-MPs (platelets), CD11a-MPs (leucocytes), CD31− MPs (endothelial cells) were quantified by prothrombinase assay. Soluble (s)E-selectin, sP-selectin, t-PA, PAI-1, platelet glycoprotein V (sGPV) and prothrombin fragments 1 + 2 (F1 + 2) were quantified by ELISA. Repeated measures were analysed with ANOVA, linear mixed model and two-way ANOVA with post hoc analysis when recommended.
Results: Ninety-two patients were investigated. Early DIC (first 24 h after ICU admission) was diagnosed in 40 (43.5%) patients, 30 were diagnosed at D1, and 10 at D2. Early DIC was characterised by higher SAPS2 and SOFA scores (65 ± 20 vs. 52 ± 14 and 11.8 ± 3.0 vs. 8.5 ± 2.1 respectively, $P < 0.05$). Acute renal failure (82.5% vs. 36.5%) and renal replacement therapy (55.0% vs. 25.0%) were significantly more frequent. Bacteraemia was associated with a greater DIC occurrence (20.0% vs. 1.9%, $P = 0.01$) whereas pneumonia seemed to be protective (40.0% vs. 63.5%, $P = 0.03$).
PhtdSer-MPs were in the same range (15 nM eq. PhtdSer) in all patients and remained at high level on D7. GPIb-MPs were strongly correlated to platelet count ($r^2 = 0.24$, $P < 0.001$). Leucocyte count remained unchanged but leucocyte CD11a-MPs were increased in DIC, a higher MPs/leucocyte ratio suggesting leucocyte activation. In DIC CD11a-MPs/leucocytes returned to baseline overtime, with concomitant secondary hyperleucocytosis contributing to still important CD11a-MPs at D7 compared to patients without DIC. At D1, endothelial cell activation was evidenced in all patients with elevated CD31-MP that were at higher level in patients with no DIC. Antithrombin, protein C, F1 + 2, fibrin monomers, sE-selectin, sP-selectin, sGPV, t-PA and PAI-1 were not associated with DIC diagnosis in our study.
Summary/Conclusion: During septic shock, haemostasis is greatly activated and procoagulant MPs are thrice as high as in control. If MPs originates from platelets and endothelial cells in no DIC patients, DIC is associated with a phenotypic change where MPs originate from leucocytes and endothelial cells.

PA1.05 – Immune Thrombocytopenia Purpura – I

PA 1.05-1
CD8+ regulatory/suppressor T cells in primary immune thrombocytopenia

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Background: Primary immune thrombocytopenia (ITP) is an acquired disorder characterized by autoantibody-mediated platelet destruction and insufficient platelet production. Multiple factors have been implicated in ITP pathogenesis, including breakdown of immune tolerance. Regulatory/suppressor T cells, which have inhibitory functions on inflammation, play important roles in maintenance of peripheral immune tolerance. They include $CD4^+$ regulatory T cells, CD8+ regulatory T cells, $CD8^+$ suppressor cells, natural killer T cells and so on. It is reported that $CD4^+CD25^+$ regulatory T cells were reduced in active ITP patients with poor functions, but whether $CD8^+$ regulatory/suppressor T cells were involved in ITP is still unknown.
Aim: To elucidate the role of $CD8^+CD25^+CD127^-$ regulatory and $CD8^+CD28^-CD127^{low}$ suppressor T cells in pathogenesis of ITP.
Method: A total of 27 untreated active ITP patients, 23 ITP patients in remission and 34 age- and sex-matched healthy donors were enrolled in this study. The mRNA expression of transforming growth factor-β1 (TGF-β1), membrane cytotoxic T lymphocyte-associated antigen-4 (mCTLA-4), indoleamine-2,3-dioxygenase (IDO) and forkhead box protein 3 (FoxP3) in peripheral blood mononuclear cells (PBMCs) were detected by real-time PCR. The serum concentration of TGF-β1 and soluble CTLA-4 were measured by ELISA. In addition, flow cytometric analysis was carried out to detect the percent of $CD8^+CD25^+CD127^-$ regulatory and $CD8^+CD28^-CD127^{low}$ suppressor T cells in peripheral blood from ITP patients and healthy controls. This study was approved

by the hospital-based ethics committee and informed consents were obtained from the patients or their parents as appreciate.
Results: (i) The mRNA expressions of mCTLA-4 and TGF-β1 were significantly decreased in PBMCs from ITP patients compared with those of normal controls ($P < 0.05$). In addition, although the mRNA expressions of IDO and FoxP3 were decreased in PBMCs from ITP patients, it did not reach the statistic difference ($P = 0.126$, $P = 0.175$ respectively). (ii) The serum concentration of TGF-β1 was down-regulated but sCTLA-4 was up-regulated in ITP patients than those of healthy controls ($P < 0.01$). We speculated that these increased sCTLA-4 can act as a competitor of mCTLA4 to bind CD80 or CD86, thereby interfering the inhibitory function of regulatory T cells. In addition, the down-expression of multiple inhibitory molecules indicated that the dysfunctions of immune negative regulations played an important role in ITP etiology. (iii) The percent of $CD8^+CD25^+CD127^-$ regulatory and $CD8^+CD28^-CD127^{low}$ suppressor T cells in peripheral blood from active ITP patients were lower than those of ITP patients in remission and healthy controls, but there was no difference between ITP patients in remission and healthy controls.
Conclusion: The down-regulation of $CD8^+CD25^+CD127^-$ regulatory cells, $CD8^+CD28^-CD127^{low}$ suppressor T cells and multiple inhibitory molecules indicated that breakdown of immune tolerance is a key defect in ITP patients. In addition, considering the association between the number of $CD8^+$ regulatory/suppressor T cells and disease progression, the treatment targeted to $CD8^+$ regulatory/suppressor T cells will be a candidate strategy for ITP therapy.

PA 1.05-2
Defective proliferation and the immunosuppressive function of bone marrow-derived mesenchymal stem cells in patients with primary immune thrombocytopenia

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Background: Primary immune thrombocytopenia (ITP) is an acquired autoimmune disease characterized by decreased platelet count resulting from increased platelet destruction and insufficient platelet production. Both the development of autoantibodies against platelet glycoproteins and the cellular mechanisms of immune modulation are involved in the pathogenesis of ITP. Bone marrow-derived mesenchymal stem cells (BM-MSCs) are key components of the hematopoietic microenvironment and provide support to hematopoiesis. At the same time, BM-MSCs are also being considered as potential therapeutic agents in various inflammatory autoimmune diseases for their tissue-repair and anti-inflammatory properties.
Aims: The qualitative properties and immune modulation capacity of MSCs derived from ITP patients in Chinese population is unclear. The aim of this study is to further characterize phenotypically and functionally BM-MSCs from ITP patients in Chinese population.
Methods: *In vitro* cultures of BM-MSCs from patients with ITP ($n = 12$) and age-and sex-matched healthy individuals ($n = 9$) were established and characterized by their differentiation potential into adipocytes and osteoblasts, and phenotype by flow cytometry. BM-MSCs (irradiated) from healthy and ITP patients were tested for their ability to suppress the *in vitro* proliferation of allogeneic peripheral blood mononuclear cells (PBMC) (from healthy donors) stimulated with PHA.
Results: Our results showed that BM-MSCs from ITP patients and normal controls can be successfully culture-expanded, but the BM-MSCs from ITP grew more slowly than those of normal controls ($P < 0.05$). Cells from both groups were positive for CD73, CD90 and CD105, and negative for CD11b, CD19, CD14, CD34, CD45 and HLA-DR. ITP BM-MSCs were shown to have a similar capacities to differentiate along adipogenic and osteogenic lineages as those of healthy donor BM-MSCs. Both the BM-MSCs from healthy donors and ITP patients could reduce the proliferation of allogeneic PBMCs, inhibit the secretion of IFN-γ and TNF-α, and promote the secretion of IL-10 in a cell dose-dependent fashion. MSC-mediated suppression of PHA-induced PBMCs proliferation was significantly lower in the ITP group than the healthy cohort, when the ratio of PBMC/MSC was 10:1 ($P = 0.003$).
Summary/Conclusions: These results showed that BM-MSCs from patients with ITP under the described culture conditions exhibited the same phenotype and differentiation potential as healthy counterparts. The proliferation of MSC and the ability to downregulate PHA-induced PBMC priming is deficient in patients with ITP. BM-MSCs may play an important role in the ITP pathogenesis.

PA 1.05-3
Detection of circulating B cells producing anti-GPIb autoantibodies in patients with immune thrombocytopenia

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Background: Immune thrombocytopenia (ITP) is an autoimmune disease caused by IgG anti-platelet autoantibodies. This condition is seen in patients with various diseases, such as systemic lupus erythematosus (SLE), and can also occur without an underlying disease. Production of IgG autoantibodies to platelet surface glycoproteins, such as GPIIb/IIIa and GPIb, is the hallmark of the disease. We previously reported that an enzyme-linked immunospot (ELISPOT) assay for detection of B cells secreting anti-GPIIb/IIIa antibodies was a sensitive method for identifying ITP patients.
Aims: To assess potential usefulness of measuring circulating anti-GPIb antibody-producing B cells for diagnosis of ITP and evaluation of anti-platelet autoantibody profiles.
Methods: We examined 114 patients with primary ITP, 94 with various conditions potentially causing secondary ITP, including 25 with SLE, 30 with liver cirrhosis (LC), and 39 with post-hematopoietic stem cell transplantation (post-HSCT), and 18 with aplastic anemia or myelodysplastic syndrome (AA/MDS). All patients had a platelet count $< 50 \times 10^9$/L at blood collection. Thirty-two healthy individuals without thrombocytopenia were used as a control. All samples were obtained after the subjects gave their written informed consent as approved by the Institutional Review Board. B cells producing IgG anti-GPIIb/IIIa and anti-GPIb antibodies were simultaneously measured using ELISPOT assay. The antigens used included GPIIb/IIIa affinity-purified from human platelets and a recombinant protein encoding an extracellular domain of GPIb. Results represented the mean of 10 values and were expressed as the number per 10^5 peripheral blood mononuclear cells.
Results: Circulating anti-GPIb antibody-producing B cells were significantly greater in patients with primary ITP, SLE, LC, and post-HSCT than in healthy controls (3.0 ± 3.3, 10.5 ± 25.6, 4.8 ± 5.2, and 3.4 ± 6.2 vs. 0.4 ± 0.4; $P < 0.01$ for all comparisons). When a cutoff level was set at five standard deviations above the mean of healthy controls, a positive result was detected in 44% of primary ITP, 48% of SLE, 50% of LC, 44% of post-HSCT, 17% of AA/MDS, and none of healthy controls. Anti-GPIb ELISPOT assay for the diagnosis of primary ITP had sensitivity of 44% and specificity of 83%, whereas anti-GPIIb/IIIa ELISPOT assay had sensitivity of 86% and specificity of 72%. When two tests were combined together, sensitivity was improved to 90%. Interestingly, frequencies of anti-GPIb and anti-GPIIb/IIIa antibody-producing B cells in circulation were correlated each other. The frequency of anti-GPIIb/IIIa antibody-producing cells exceeded the frequency of anti-GPIb antibody-producing B cells in patients with primary ITP, LC, and post-HSCT, but anti-GPIb antibody-producing B cell frequency predominated in SLE patients. Serial measurement revealed decrease of anti-GPIb and anti-GPIIb/IIIa antibody-producing B cells in parallel with platelet count recovery after therapeutic intervention, including corticosteroids, splenectomy, and eradication of *Helicobacter pylori*.

Conclusions: The ELISPOT assay for detection of anti-GPIb antibody-secreting B cells is useful for identifying patients with primary ITP and various forms of secondary ITP. In addition, anti-platelet autoantibody repertoire may be different among conditions accounting for ITP.

PA 1.05-4

Is the total 2 g/kg of intravenous immunoglobulin G optimal dose for acute immune thrombocytopenic purpura in childhood?

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Background: Immune thrombocytopenic purpura (ITP) is the most common bleeding disorder in children. High dose intravenous immunoglobulin G (IVIG) has been used for treatment of ITP since 1981 by Imbach, and now several methods of IVIG infusion are used. But total doses were 2 g/kg for almost patients, uniformly. Since 1983, we have done short-term IVIG therapy according to the individual patient's response.

Aims: Through this study, we showed that low dose IVIG therapy according to the individual patient's response is reasonable and cost-effective in childhood acute ITP.

Methods: Daily 200 mg/kg of IVIG was given until platelet count reached over 50,000/mm^3 in 75 childhood acute ITP who were newly diagnosed at the Department of Pediatrics, Kyungpook National University Hospital from September, 2006 to December, 2012. We evaluated the time to reach desired platelet counts (50,000/mm3), relapse rate and changes of neutrophil count after treatment of IVIG.

Results: The median age was 2.7 years (1 month–15 years), male to female ratio was 1.5:1 and median follow up duration was 14.8 months (0.5–67 months). The median platelet count was 10,787/mm^3 (2000–37,000/mm^3) at diagnosis. The platelet count increased to 22,920/mm^3 (3000–103,000/mm^3) after one dose, 46,507/mm^3 (3000–185,000/mm^3) after two doses, 51,155/mm^3 (2000–282,000/mm^3) after three doses and 52,833/mm^3 (4000–294,000/mm^3) after four doses of IVIG therapy. The median duration of IVIG therapy was 3.8 days and median total dose of IVIG therapy was 900 mg/kg. Sixteen patients (21.3%) were relapsed (< 50,000and median interval to relapse was 26 days (15–44 days) after first diagnosis. Among 49 patients followed up over 6 months, nine patients (18.4%) were eventually diagnosed as chronic ITP. All chronic ITP was observed only in relapse patients. Forty-one patients (54.7%) showed neutropenia after IVIG treatment, and the neutropenia had resolved spontaneously in all patient after median 31.1 days (2–135 days). No serious complications were observed.

Summary/Conclusion: Compared to the previous our data (Low-dose and short term therapy of IVIG for childhood acute ITP: in Korean Journal of Hematology, 2001, 36:3241–6, 247–52), this very low-dose and short term therapy of IVIG (200 mg/kg/day) has similar effect on treating acute ITP in response and relapse rate. Total dosage of IVIG has no association with neutropenia in frequency and severity. The short-term very-low dose IVIG therapy (200 mg/kg/day) according to the individual response is resonable for effect, cost-effectiveness and safety in childhood ITP.

PA 1.05-5

Successfully sparing splenectomy in immune thrombocytopenia purpura (ITP)

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The hallmark of primary ITP is the development of an isolated thrombocytopenia in the absence of a recognised secondary cause. Some of the earliest experiments to elucidate the pathogenesis of ITP demonstrated that an asplenic state could abrogate the platelet destruction seen with ITP serum. Since then, the role of splenectomy has been inextricably linked with the management of this condition. However, the recent analysis of our single centre experience with the management of ITP demonstrated a significant disparity between most accepted international guidelines and our current practice: splenectomy has not been performed for newly diagnosed patients with primary ITP over the last 8 years.

We aimed to investigate the reasons why our current practices were so divergent from conventional guidelines on the role of splenectomy.

We performed a subanalysis on a recent retrospective observational study on thrombocytopenia in a single Australian centre. All patients with newly diagnosed primary ITP between 2005 and June 2010 were investigated after obtaining local ethics approval. Updated information was then obtained from clinical files and via correspondence with treating physicians to elaborate their decision making processes.

Fifty-one adult patients were newly diagnosed with primary ITP during the study period. Seven have been reclassified due to clinical evolution as secondary ITP (autoimmune pancreatitis, SLE, drug-induced), congenital thrombocytopenia in two, myelodysplasia and cirrhosis. One patient was lost to follow-up abroad. One other patient developed pancreatic carcinoma and was treated with chemotherapy.

Forty-two patients were therefore evaluable for the potential role of splenectomy. 19/42 required no treatment at any point. Of the 23 who did require treatment, 10 eventually required second-line therapy. Four patients were successfully re-treated with first-line therapy for relapsed but responsive disease.

4/10 patients requiring second-line therapy were unfit for surgery: three with primarily refractory ITP and one elderly male with multiple medical co-morbidities. Six patients were potential candidates for splenectomy: four patients refused and two were not offered by the clinician as steroid-sparing agents were successfully introduced within 4 weeks of initial presentation (azathioprine and danazol).

Of the four patients who refused splenectomy, three received thrombopoietin agonist therapy. All three have maintained a response to this therapy, one of whom is in an enduring complete remission off all therapy. One patient who refused splenectomy was unable to obtain compassionate access to thrombopoietin agonists and intermittently requires steroids and danazol without significant adverse effects.

This small analysis of consecutive patients newly diagnosed with primary ITP between 2005 and 2010 demonstrates several salient features. Despite recent refinements in the classification of ITP, the clinical diagnosis still encompasses a broad clinicopathological range of patients with diverse outcomes. As a diagnosis of exclusion, primary ITP is reclassified in at least approximately 15% of cases after a median follow-up of 18 months. Owing to patient preferences and the dearth of steroid-sparing and splenectomy-sparing options, surgery is infrequently required in newly diagnosed patients with primary ITP. Although splenectomy is not obsolete, it is an unpopular option in this Australian tertiary referral centre.

PA 1.05-6

Cell-based haemostasis assessment to improve disseminated intravascular coagulopathy (DIC) diagnosis during septic shock

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Background: Cell-free plasma routine tests failed to predict thrombotic or haemorrhagic tendency during septic shock and large randomised clinical trials aiming at anticoagulation were not relevant. *Post-hoc* tests highlighted patients' survival improvement only when DIC was present.

Aims: To improve DIC diagnosis during septic shock time-course and assess vessel cells activation using circulating microparticles (MPs) and truly soluble membrane proteins as vascular cell surrogates.

Methods: One hundred patients with septic shock were enrolled and their haemostatic status evaluated at admission (D1), D2, D3 and D7. Circulating MPs were isolated, quantified by prothrombinase assay and their cellular origin determined after capture onto specific antibodies. Soluble proteins were measured by ELISA. Cell markers of endothelial cells were sE-selectin, sP-selectin, t-PA, PAI-1, CD31-MPs (apoptosis) and CD105-MPs (activation); sP-selectin, sGPV and GPIb-MPs accounted for platelets; IL-6, IL-10, TNFa, MCP-1 and CD11a-MPs for leucocytes. Thrombin formation was assessed by prothrombin fragments 1 + 2 (F1 + 2), fibrin formation by fibrin monomers (FM) and fibrinolysis by D-dimers (DDi). DIC score was established according to JAAM 2006 criteria. (NCT #01604551)

Results: Forty patients had DIC during the first 24 h. Compared to no DIC, they were characterised by higher multiple organ failure especially acute renal failure (82.5% vs. 36.5%). Haemostasis activation was obvious in all patients with sustained thrombin generation and fibrinolysis over time. Delayed AT and PC restoration was observed in DIC patients ($P < 0.05$ for time-course D1–D7).

Regardless DIC diagnosis, septic shock induced high level of total MPs. CD31-MPs, t-PA and PAI-1 were increased but not differently in DIC vs. no DIC patients. t-PA and PAI-1 returned to baseline at D7 ($P = 0.04$, $P < 0.01$, respectively) with delayed improvement when DIC was present while CD31-MPs were stable. CD105-MPs were significantly increased at D1 only in patients having early DIC. CD105-MPs and sE-selectin were greatly increased prior DIC diagnosis and significantly tended to return to baseline at the end of the follow-up period ($P < 0.05$). Leucocyte activation was enhanced in all DIC patients (CD11a-MPs/leucocyte ratio, MCP-1 and IL-10) whereas platelet activation (sGPV/platelet and sP-selectin/platelet ratios) was increased only once DIC was present. Interestingly, sGPV/platelet and sP-selectin/platelet ratios were increased at D3 then returned to baseline. GPIb-MPs mirrored platelet count. In multiple logistic regression analysis, CD105-MPs (OR 6.55 [2.54–16.92], $P < 0.01$), CD31-MPs (OR 0.49 [0.26–0.94], $P = 0.03$) and FV (OR 0.97 [0.95–0.99], $P = 0.02$) were strongly associated to DIC, while FM ($P = 0.06$) and prothrombin F1 + 2 ($P = 0.09$) were tightly associated.

Using our global approach of individual vascular competence, we identified three different profiles: (i) no DIC – low grade, controlled injury, (ii) pre DIC – obvious vascular injury but still-controlled thrombin generation, and (iii) DIC – uncontrolled thrombin and fibrin formation.

Summary/conclusion: According to cell-based haemostasis assessment, the initial activation of endothelial cells and leucocytes in septic shock prompts thrombin generation followed by platelet activation. MPs and soluble selectins are vascular cells activation surrogates that could improve early stratification for anticoagulant therapy during septic shock.

Key words: disseminated intravascular coagulopathy, microparticles, septic shock, cell-based haemostasis.

Acknowledgements: This work was supported by French Ministry of Health (PHRC HUS #4649), Laboratoire Français de Fractionnement et des Biotechnologies (LFB SA, Les Ullis, France), the Société de Réanimation de Langue Française (SRLF, Paris, France) and the Association pour le Développement et la Recherche en Réanimation (ADRER, Strasbourg, France).

PA1.06 – Fibrinolytic System: Basic – I

PA 1.06-1

Identification of a novel, nanobody-induced, mechanism of TAFI inactivation and its *in vivo* application

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Background: Down regulation of fibrinolysis due to cleavage of C-terminal lysine residues from partially degraded fibrin is mainly exerted by the carboxypeptidase activity of activated thrombin-activatable fibrinolysis inhibitor (TAFIa). Some intrinsic carboxypeptidase activity (= zymogen activity) for the pro-enzyme TAFI, has been reported.

Aims: The aim is to identify and characterize *in vitro* nanobodies (single-domain fragments derived from heavy-chain-only antibodies) that affect the pro-enzyme properties of mouse TAFI (mTAFI). Then, their applicability will be evaluated in an *in vivo* mouse thromboembolism model.

Methods and Results: Screening of a library of nanobodies towards mTAFI revealed one nanobody (VHH-mTAFI-i49) significantly stimulating the zymogen activity of mTAFI to 4.4 U/mg (at a 16-fold molar ratio over mTAFI, $P < 0.0001$ compared to no addition of nanobody). The stimulated zymogen activity is unstable at 37 °C, with a half-life of 8.1 ± 0.8 min. More importantly, incubation of mTAFI with VHH-mTAFI-i49 reveals a time-dependent reduced activatability (by thrombin/thrombomodulin) of mTAFI: 47%, 71% and 89% reduced activatability after incubation at 37 °C for 15, 30 and 60 min, respectively. *In vitro* clot lysis experiments were performed by pre-incubation of mouse plasma with VHH-mTAFI-i49 for 60 min followed by $CaCl_2$ triggered clot formation and t-PA induced lysis. VHH-mTAFI-i49 induced a dose-dependent reduction of the 50% clot lysis time, < i.e. 28 ± 7 min in the presence of a 16-fold molar excess of the nanobody over TAFI vs. 80 ± 4 min in the absence of the nanobody. Extensive analysis during *in vitro* clot lysis experiments revealed that the enhanced lysis was associated with a reduced activatability of TAFI to TAFIa, through transient zymogen stimulation. The affinity (K_A) of VHH-mTAFI-i49 for TAFI, as determined by surface plasmon resonance (Biacore®), is $4.2 \pm 1.2 \times 10^7$/M. *In vivo* application of VHH-mTAFI-i49 (2.6 mg/kg) in a tissue factor-induced mouse thromboembolism model significantly decreases the fibrin deposition in the lungs (20 ± 6.9 and 114 ± 20 μg/mL for VHH-mTAFI-i49 and buffer respectively). Epitope mapping discloses that Arg^{227} and Lys^{212} are important for the nanobody/mTAFI interaction and suggests that the zymogen stimulation and subsequent destabilization of TAFI may be the result of translocation of the activation peptide, thereby disrupting the stabilizing interaction between activation peptide and the dynamic flap region.

Conclusion: The nanobody-induced, reduced activatability of mTAFI, results in a very potent enhancement of *in vitro* clot lysis. Evaluation

of this nanobody in an *in vivo* mouse thromboembolism model illustrates that this novel way of interfering with the generation of TAFIa results in a strong profibrinolytic effect.

PA 1.06-2

Alpha2-antiplasmin is a potential regulator of neuronal morphology

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Background: α2-Antiplasmin (α2AP), a member of the serine protease inhibitor (serpin) family, is a principal physiological plasmin inhibitor. On the other hand, α2AP regulates cell differentiation without the mediation of plasmin, suggesting that α2AP is a possible regulatory factor of cellular functions in a plasmin-dependent or plasmin-independent manner. α2AP is mainly produced by the liver and kidneys, but it is also expressed in the brain, particularly at a higher level in the hippocampus than in the cortex, hypothalamus and cerebellum. Hence α2AP might play an important role in hippocampal neuronal functions. However, no study thus far has addressed the role of α2AP in the neurons.

Neurons have a unique morphology characterized by dendrites and a long axon. During neuronal maturation, MAP2 and tau, which are abundant microtubule-associated proteins in neurons, segregate into dendrites and axon, respectively, and form cross-bridges between microtubules to regulate the development and stabilization of axon and dendrites. Transforming growth factor (TGF-β) is a key factor to promote neuronal maturation, neurites sprouging and axonal development. α2AP regulates the expression of TGF-β, thereby suggesting that α2AP could be a potential regulator of neuronal morphology.

Aims: This study investigated whether α2AP regulated the neuronal morphology, including dendritic and axonal growth, and also whether TGF-β was involved in the regulation. In addition, the mechanisms underlying the morphological regulation by α2AP were determined.

Methods: Primary hippocampal neurons were obtained from $\alpha 2AP^{+/+}$ [wild-type (WT)], $\alpha 2AP^{-/-}$mice and plasminogen$^{-/-}$ mice, and treated with α2AP, TGF-β or aprotinin. Immunocytostaining and phalloidin staining were performed, and then the length, the number of tips, and the branching of the neurites were measured. The expression of MAP2, tau1 and TGF-β in the neurons was analyzed by Western blotting.

Results: α2AP was intensely expressed in mature neurons, but not in immature neurons. Exogenous treatment with α2AP increased the length, the number of tips, and the branching of the neurites, and enhanced the expression of MAP2 and tau1 in both WT and $\alpha 2AP^{-/-}$ mice. Aprotinin, another plasmin inhibitor, had little effect on the expression of MAP2 and tau1 in the neurons from WT mice, and furthermore, α2AP induced the expression of MAP2 and tau1 in the neurons from plasminogen$^{-/-}$ mice. In addition, the activation of p38 MAPK, but not ERK and JNK, was involved in the induction. α2AP also induced the TGF-β expression in the neurons, and TGF-β signaling mediated the α2AP-induced expression of MAP2 and tau1.

Conclusion: α2AP induces TGF-β expression to regulate dendritic and axonal growth through activation of p38 MAPK without the mediation of plasmin, thus providing new insights into the role of α2AP in the neuron.

PA 1.06-3

Generation and characterization of homozygous plasminogen-Tochigi mutant mice bearing reduced fibrinolytic activity

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Plasminogen (Plg) is a precursor of a serine protease, plasmin that degrades fibrin fiber composed of blood clot. Fibrinolysis is an important event to dissolve unfavorable clot in blood vessel. Deficiency of Plg causes thrombosis, fibrin deposition, and delay of wound healing. Plg-A620T mutation previously named as Plg-Tochigi mutation is positioned close to the enzymatic active site and decreases the proteolytic activity of plasmin. Plg-Tochigi mutation has not been found in Caucasian but observed in Chinese and Japanese with an estimated prevalence of 4% in the general Japanese population. We suspected that Plg-Tochigi mutation is a potential risk factor for Asian to cause thrombosis because of the low fibrinolytic activity. In fact, Plg-Tochigi mutation was originally found in Japanese patients of deep vein thrombosis (DVT). However, our recent analysis proved that heterozygous Plg-Tochigi mutation is not a genetic risk factor for DVT. Herein, we focus on homozygotes of Plg-Tochigi mutation and investigate whether the homozygous Plg-Tochigi mutation in mice causes serious thrombosis.

We generated the Plg-A622T (Plg-Tochigi) knock-in mice corresponding to human Plg-A620T. The homozygous Plg-Tochigi mutant mice did not show any abnormality in birth, growth, and fertility. The urokinase-induced amidolytic activity of Plg in homozygous Plg-Tochigi mutant mice was reduced by about 25% of those in wild-type mice. Unexpectedly, delay of wound healing was not observed in Plg-Tochigi mutant mice unlike Plg-knockout mice previously reported. We compared artery and vein thrombogenesis between Plg-Tochigi mutant and wild-type mice using three different experimental thrombosis models. In a middle cerebral artery occlusion model of ischemia-reperfusion injury, we did not find any difference, indicating that the Plg-Tochigi mutation did not significantly affect the severity of ischemic artery diseases. Next, we induced acute pulmonary embolism by injection of recombinant human tissue factor via inferior vena cava (IVC). The survival rate of Plg-Tochigi mutant mice during 20 min after the injection was as same as wild-type mice although the degree of vascular occlusion in the lung of Plg-Tochigi mutant mice was a little severer than wild-type mice. We also induced IVC thrombosis by electrolytic stimulation of IVC at a current of 250 μAm for 15 min. Plg-Tochigi mutant mice tended to form slightly larger thrombi than wild-type mice but there were no significant differences in the thrombus weight.

In conclusion, homozygous Plg-Tochigi mutation showed reduced enzymatic activity of plasmin but did not cause severe thrombosis in mice. The potential of Plg-Tochigi mutation to modify thrombosis triggered by other genetic risk factors needs to be investigated.

PA 1.06-4

Enhanced t-PA-mediated fibrinolysis through co-administration of a TAFI-inhibiting nanobody

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Background: One of the main disadvantages of current t-PA thrombolytic treatment is the increased bleeding risk. Upon activation, Thrombin Activatable Fibrinolysis Inhibitor (TAFI) is a very powerful antifibrinolytic enzyme. Therefore, co-administration of a TAFI inhibitor during thrombolysis could reduce the t-PA dose, thereby decreasing bleeding risks without compromising the efficacy.

Aim: The aim is to generate and characterize inhibitory nanobodies (single-domain fragments derived from heavy-chain-only antibodies) towards rat TAFI (rTAFI) and evaluate their profibrinolytic properties *in vitro* and *in vivo*.
Methods and Results: Nanobody VHH-rTAFI-i81 inhibits (at a 16-fold molar ratio nanobody over TAFI) the thrombin/thrombomodulin (T/TM) mediated activation of rTAFI by 83 ± 1.8% with an IC_{50} of 0.46 (molar ratio nanobody over TAFI). The affinity (K_A) of VHH-rTAFI-i81 for rTAFI, as determined by surface plasmon resonance (Biacore®), is 2.5 ± 0.2 × 10^{10}/M and illustrates a very strong binding. VHH-rTAFI-81 was also found to interact with mTAFI: 78 ± 2.6% inhibition of T/TM mediated mTAFI activation with an IC_{50} of 0.59 and an affinity (K_A) of 6.9 ± 1.2 × 10^9/M. *In vitro,* a dose-response curve of the profibrinolytic effect of VHH-rTAFI-i81 in rat plasma, in the presence of 1000 pM t-PA, shows a strong, maximal profibrinolytic effect already at an equimolar ratio of nanobody over rTAFI. A dose-response curve of the fibrinolytic effect of t-PA, in the presence and absence of VHH-rTAFI-i81 at a two-fold molar ratio over rTAFI, indicates that (i) in the absence of VHH-rTAFI-i81 lysis remains limited even at the highest concentration of t-PA (900 pM), whereas in the presence of VHH-rTAFI-i81, full lysis is achieved and (ii) in the presence of VHH-rTAFI-i81 the time to reach full lysis is 25 min whereas in the absence of VHH-rTAFI-i81, no full lysis is reached at 180 min. Epitope mapping discloses that Lys^{380} and Lys^{392} are of primary importance for the nanobody/rTAFI interaction besides minor contributions of Tyr^{175} and Glu^{183}. *In vivo* application of VHH-rTAFI-i81 (1.3 mg/kg) in a tissue factor-induced mouse thromboembolism model significantly decreases fibrin deposition in the lungs (8.0 ± 2.2 and 180 ± 27 μg/mL for VHH-rTAFI-i81 and buffer, respectively).
Conclusion: Nanobody VHH-rTAFI-i81 is a very potent inhibitor of TAFI activation. Co-administration of this nanobody and t-PA enhances the fibrinolytic efficacy. In an *in vivo* mouse thromboembolism, application of VHH-rTAFI-i81 reduces fibrin deposition in the lungs.

PA 1.06-5
Clots formed from y′-fibrinogen are more resistant to lysis than those formed from? A-fibrinogen because of delayed plasminogen activation by tissue plasminogen activator

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Background: About 15% of circulating fibrinogen (Fg) contains a γ-chain variant with an extended C-terminus and is designated γ'-Fg. Fibrin (Fn) clots formed from γ'-Fg are more resistant to lysis by tissue plasminogen activator (tPA) than those prepared from the predominant γA-Fg. Because release of fibrinopeptide (FP) B is purported to expose a cryptic plasminogen (Pg) binding site, we speculated that the resistance to lysis may reflect delayed release of FPB from γ'-Fg.
Aim: To determine whether resistance of γ'-Fn clots to lysis relative to γA-Fn clots reflects impaired FPB release.
Methods: Samples containing γA- or γ'-Fg at various concentrations, Pg, tPA, and a_2-antiplasmin were incubated at 37 °C with thrombin, which releases FPA and FPB, or with batroxobin, which only releases FPA. Turbidity was monitored at 400 nm and the time to half maximal decrease was designated as the lysis time. To compare the cofactor activities of γA- and γ'-Fn, experiments were repeated (a) in the presence of S2251 so that kinetic parameters for Pg activation by tPA and the time to initiation of Pg activation relative to that of clot formation could be determined, and (b) using plasmin in place of Pg and tPA so that the Pg activation step could be bypassed. Finally, to explore the impact of FPB release on the Pg/Fn interaction, immobilized γA- or γ'-Fg was converted to Fn with thrombin or with batroxobin and surface plasmon resonance (SPR) was used to quantify Pg binding.
Results: With tPA, lysis times of γ'-Fn clots were longer than those of γA-Fn clots; a difference that reached 30% with increasing Fg concentrations. Substitution of batroxobin for thrombin or plasmin for Pg/tPA abolished the difference, suggesting that it is related to an effect of FPB release on Pg activation. Although Fg isoform and the use of thrombin vs. batroxobin to clot the Fg had no effect on the individual kinetic parameters for Pg activation by tPA, the time to initiation of Pg activation with γ'-Fn clots was delayed by 95.0 ± 15.6 s compared with γA-Fn when clots were formed with thrombin, but not with batroxobin; findings that also implicate FPB release. In support of this concept, based on the SPR data, 2-fold more Pg bound to Fn lacking both FPA and FPB compared with Fn lacking only FPA.
Conclusion: Our findings suggest that the capacity of Fn to promote Pg activation by tPA is accelerated by FPB release, which promotes Pg binding. Therefore, slower lysis of γ'-Fn likely reflects delayed FPB release rather than impaired plasminogen activation or resistance to plasmin-mediated Fn degradation. Thus, these studies provide additional insights into the mechanism by which Fn promotes Pg activation by tPA, and why incorporation of γ'-Fg into Fn clots may render them resistant to lysis.

PA 1.06-6
Structural and biochemical studies of naturally occurring antiplasmin variants

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Antiplasmin(AP) is a highly effective inhibitor of plasmin and its deficiency often results in a variable, often severe, bleeding disorder. Two AP mutations that cause bleeding disorders have been identified. AP *Enschede* involves an insertion of an alanine residue in the RCL (353A-A-A-A357). Although the variant is normally expressed, it is a substrate rather than an inhibitor of plasmin. A second mutation, V372M is located just C-terminal to the RCL on the first strand of the C-sheet and results in deficiency through an as yet uncharacterized mechanism. To understand how these mutations alter AP function we generated recombinant forms of human and mouse AP *Enschede* and mouse V372M variant and investigated their structural and biophysical properties. The X-ray crystal structures of murine AP *Enschede* and V372M were solved at 2.8 Å and 4.0 Å resolution, respectively. The structural data reveals that both variants are in a native conformation and that the serpin fold is not perturbed by the mutations. No electron density was observed for the RCL of either protein. The biophysical properties of recombinant human and mouse AP *Enschede* were unaltered by the mutation despite both proteins demonstrating substrate like inhibitory activity. In contrast, the recombinant mouse V372M variant had inhibitory properties similar to wild-type mouse AP and no differences in polymerization and biophysical characteristics were observed. This suggests that the V372M mutation alone does not alter the native fold of the serpin or its inhibitory efficiency. The human V372M variant could not be expressed in *E. coli* and may indicate malfolding or structural instability in the human V372M protein.

PA1.07 – Haemophilia A: Basic – I

PA 1.07-1

Antiplasmin, but not amiloride, prevents synovitis and cartilage destruction following hemarthrosis in hemophilic mice

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Background and Aims: Blood-induced joint damage in hemophilia is characterized by synovitis and cartilage destruction. Recently, we demonstrated that a joint bleeding in hemophilic mice results in alterations in the fibrinolytic system with an increase in synovial cells expressing urokinase-type plasminogen activator (uPA), an increase in synovial levels of uPA and plasmin, and in plasmin-mediated cartilage destruction. In this study, we evaluate the alterations in synovitis and cartilage destruction following the induction of a joint bleeding in hemophilic mice treated with placebo, amiloride (a specific inhibitor of uPA), or antiplasmin.

Methods: The right knees of hemophilic mice were punctured to induce hemarthrosis. Subsequently, mice were randomized between daily oral treatment with amiloride or control, while other mice were randomized between weekly intra-articular treatment of the right knee joint with amiloride, antiplasmin, or control. After 5 weeks of treatment, the mice were sacrificed, knee joints were isolated, sectioned for histology, and stained with hematoxylin-eosin (synovitis) or safranin O (cartilage destruction). Hemophilic synovitis and cartilage destruction were determined by two blinded observers according to Valentino and Glasson. An increase in Valentino and Glasson score represents an increase in hemophilic synovitis and cartilage destruction, respectively. Treatment with amiloride or antiplasmin was compared with control. Categorical data were analyzed by loglinear analysis and Pearson Chi-Square.

Results: No significant alterations in synovitis and cartilage destruction were found when comparing the oral amiloride group with the oral control group, and when comparing the intra-articular amiloride group with the intra-articular control group. In contrast, intra-articular treatment with antiplasmin resulted in a statistical significant ($P < 0.01$) reduction in synovitis, as assessed by the Valentino score, when comparing the intra-articular control group to the intra-articular antiplasmin group: 1 (0% vs. 11.1%), 2 (4.2% vs. 11.1%), 3 (16.7% vs. 61.1%), 4 (29.2% vs. 5.6%), 5 (20.8% vs. 11.1%), 6 (8.3% vs. 7.7%), 7 (8.3% vs. 0%), and 8 (12.5% vs. 0%). In addition, treatment with intra-articular antiplasmin resulted in a statistically significant ($P < 0.01$) reduction in cartilage destruction, as assessed by the Glasson score, when comparing the intra-articular control group to the intra-articular antiplasmin group: 2 (8.3% vs. 10%), 3 (12.5% vs. 50%), 4 (33.3% vs. 30%), 5 (33.3% vs. 10%), 6 (12.5% vs. 0%), and 7 (4.2% vs. 0%).

Conclusions: Intra-articular treatment with antiplasmin following the induction of a joint bleeding prevented synovitis and cartilage destruction in hemophilic mice. Oral and intra-articular treatment with amiloride failed to attenuate synovitis and cartilage destruction. Given that complete prevention of joint bleeds in hemophilia is not feasible at the moment despite the prophylactic use of factor replacement, the data presented herein offer promise for the use of antiplasmin as a new therapeutic intervention.

PA 1.07-2

A single intra-articular injection with IL-4 plus IL-10 ameliorates blood-induced cartilage degeneration in haemophilic mice

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Background: Blood-induced joint damage is still a major cause of morbidity amongst haemophilia patients, despite the increased use of prophylaxis. IL-4 and IL-10 are modulatory cytokines and candidates for stopping the progress of this blood-induced joint damage. It has been shown previously that combination of interleukin (IL)-4 and IL-10 protects against blood-induced cartilage damage *in vitro*.

Aims: The present study investigated whether a single intra-articular injection of IL-4 plus IL-10 immediately after a joint bleed limits cartilage damage in an *in vivo* haemophilia mouse model of blood-induced joint damage.

Methods: Factor VIII knockout mice with severe haemophilia A were punctured once with a needle below the patella to induce a joint haemorrhage. Subsequently IL-4 plus IL-10 ($n = 24$) or vehicle ($n = 24$) was injected intra-articularly. After 35 days, the time needed for development of detectable joint degeneration, knee joints were examined for cartilage damage by macroscopic and microscopic evaluation.

Results: A single intra-articular injection of IL-4 plus IL-10 ameliorated progression of cartilage degeneration caused by a single joint bleed to a certain extent. No effect on inflammation was observed at this time point.

Summary/Conclusions: A single intra-articular injection of IL-4 plus IL-10 directly after a single joint bleed limits progression of cartilage degeneration over time. Improved bioavailability (half-life) of both cytokines might improve their protective ability in the development of cartilage degeneration, and probably also inflammation.

PA 1.07-3

Structure of a cyclic peptide binding to Kunitz domain 1 and 2 inhibiting tissue factor pathway inhibitor (TFPI)

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Background: TFPI is a potent inhibitor of tissue factor (TF)-induced blood coagulation. It is composed of three consecutive and flexible linked Kunitz-type domains (K) where K1 and K2 are involved in efficient inhibition of TF/FVIIa and FXa. TFPI inhibition has been shown to promote coagulation and hemostasis in hemophilia models *in vitro* and *in vivo*.

Aim: We solved the co-crystal structure of a TFPI construct comprising K1-K2 in complex with a cyclic TFPI inhibitory peptide (JBT-B5) to reveal molecular details of its inhibitory mode of action.

Methods: Recombinant K1-K2 (residues 22–150) produced by *E. coli* and complexed to JBT-B5, a cyclic peptide composed of 23 amino acids, was co-crystalized in 20% w/v PEG6000 and 50 mM imidazole, pH8.0. JBT-B5 binding to TFPI was verified by biomolecular interaction analysis (BiaCore). Functional inhibition of TFPI by JBT-B5 was tested in model assays including TFPI inhibition of FXa and FX activation by TF/FVIIa, and by global hemostatic assays including calibrated automated thrombography in FVIII-inhibited plasma and rotational thromboelastometry using FVIII-inhibited whole blood.

Inhibition of cell surface TFPI was analyzed in a FX activation assay performed on HUVECs.

Results: A co-crystal structure of TFPI K1-K2 bound to JBT-B5 was solved at 1.95 Å resolution. This is the first structure of TFPI consisting of K1, K2 and their linker. The K1-K2 structure is defined in the electron density from Met22 through Gly150. Both domains show a Kunitz-type structure, where only approximately 1/3 of the structure is engaged in secondary structure elements. These elements form the framework that is stabilized by the three canonical disulfide bonds in each of the Ks. The 23mer JBT-B5 assumes a β-hairpin-like structure which can be segmented into (i) a two-stranded β sheet comprising Tyr2-Ala8 and Thr17-Phe23; (ii) and a β-turn comprising Asp11-Thr15. The β-sheet is stabilized by a disulfide bridge (Cys7 and Cys18) and a hydrophobic zipper comprising the side chains of Tyr3, Trp5 and Trp20. JBT-B5 is sandwiched between K1 and K2 and locks them in a distinct conformational state bringing the reactive center loops (RCL) to opposite sides. The interaction between K1-K2 and JBT-B5 is mediated by a hydrophobic anchor in JBT-B5, forming more than 2/3 of the interaction surface (1340 Å2). In addition, several polar interactions stabilize the K1-K2/JBT-B5 complex, explaining JBT-B5′s exlusive binding to human TFPI. Interaction studies revealed high affinity binding to TFPI (K_D: 0.5 nM). The highly complex and extensive interaction of JBT-B5 with TFPI translates to a highly efficient inhibition of TFPI as demonstrated in model assays on FXa (EC_{50}: 1.3 nM), FXa generation by TF/FVIIa (EC_{50}: 0.2 nM), HUVE cell-based FX activation and global hemostasis assays, such as thrombin generation in hemophilia plasma (EC_{50}: 4 nM) and ROTEM in FVIII-inhibited whole blood.

Summary/Conclusions: For the first time, a structure of TFPI comprising K1, K2 and their linker in complex with a TFPI- inhibitory cyclic peptide was solved. This structure provides atomic details explaining the inhibitory mode of action of this highly efficient TFPI antagonist, and will guide its improvement for use as a hemophilia therapeutic.

PA 1.07-4

The effect of a novel TFPI inhibitory fusion peptide on TFPI clearance

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Recently, a hemophilia phase 1 clinical trial with the aptamer BAX 499 targeting human full length (hfl)-TFPI was stopped due to an increase in bleeding events – a consequence of considerably elevated hfl-TFPI plasma levels and only partial inhibition of TFPI by the aptamer. This rise in hfl-TFPI was mainly caused by a number of interrelated events including BAX 499 effects on receptor mediated clearance of TFPI. We developed a fusion peptide of a linear and a cyclic peptide, which was shown to be a full inhibitor of TFPI even at highly elevated TFPI levels.

The goal was to show the absence of any impact of the fusion peptide on TFPI clearance in *in vitro* test systems as well as *in vivo*.

The interference of the fusion peptide with low density lipoprotein receptor-related protein 1 (LRP1) and asialoglycoprotein receptor (ASGPR), two TFPI clearance receptors, was assessed using Biacore. Biotinylated recombinant human LRP-1 cluster II Fc chimera protein was bound to the surface via biotin after neutravidin immobilization to a series S sensor chip C1. Then hfl-TFPI or recombinant TFPI1-160 were injected in a single concentration of 10 nM. ASGPR was immobilized to a series S sensor chip CM5 using standard amine coupling chemistry. Hfl-TFPI or TFPI1-160 was injected in the single cycle analysis mode at concentrations ranging from 3.84 to 150 nM. Subsequently, hfl-TFPI was dissociated by changing the flow to running buffer conditions. When the interaction of hfl-TFPI with clearance receptors was assessed in the presence of the fusion peptide, 1 μM final concentration of peptide was added to the hfl-TFPI.

A possible impact of the fusion peptide on the pharmacokinetics of hfl-TFPI *in vivo* was studied in mice. C57Bl/6 mice were treated with hfl-TFPI (775 nM, 5 mL/kg i.v.) or hfl-TFPI complexed to a 10-fold molar excess of peptide. Human TFPI was monitored over 35 min using an ELISA.

Hfl-TFPI interacted efficiently with immobilized LRP and ASGPR with fast on- and off-rates via its K3-C-terminus region as was verified with a TFPI construct devoided of C-terminal parts (TFPI 1-160). When bound to the fusion peptide, hfl-TFPI interacted with LRP and ASGPR with a slightly increased response, which might be explained by the increased molecular weight of the complex. Unchanged association and dissociation kinetics of receptor-TFPI interaction with and without the fusion peptide corroborates that binding of hfl-TFPI to the clearance receptors is unaffected. Pharmacokinetic studies of hfl-TFPI in mice further supported the *in vitro* results. TFPI had a short half-life and a poor *in vivo* recovery. Recovery and half-life of hfl-TFPI remained unchanged when hfl-TFPI was administered in complex with the linear peptide-part of the fusion peptide. This experiment indicates that the peptide does not influence the clearance of TFPI.

The data suggest minimal effects of the fusion peptide on the fl-TFPI plasma level after peptide administration. Fusion peptide binding to hfl-TFPI does not affect binding of hfl-TFPI to the studied clearance receptors and the *in vivo* experiment showed an unaffected circulation time of hfl-TFPI.

PA 1.07-5

Deferasirox prevents cartilage destruction following hemarthrosis in hemophilic mice

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Background and Aims: Joint bleedings in hemophilia result in iron-mediated synovitis and cartilage destruction. It was evaluated whether deferasirox, an iron chelator, was able to prevent the development of hemophilic synovitis and cartilage destruction.

Methods: Hemophilic mice were randomly assigned to oral treatment with deferasirox (30 mg/kg) or its vehicle (control) (30 mg/kg). After 2 months of pretreatment, the right knees of hemophilic mice were punctured to induce hemarthrosis. The mice were sacrificed after another 5 weeks of treatment. Post-mortem, knee joints were isolated and sectioned for histology. Hemophilic synovitis and cartilage destruction were determined by two blinded observers according to Valentino and Glasson respectively. For hemophilic synovitis, sections of tissue were stained with hematoxylin-eosin and scored for evidence of synovial hyperplasia, vascular hyperplasia, hemosiderin depositions, intra-articular erythrocytes, and synovial villi. For cartilage destruction, sections of tissue were stained with safranin O and the intensity of safranin O staining was scored according to Glasson, which is directly proportional to the proteoglycan content in the cartilage, which is a measure of cartilage destruction. The maximum score of the Valentino score is 10, and the maximum score of the Glasson score is 6. An increase in Valentino and Glasson score, represents an increase in hemophilic synovitis and cartilage destruction, respectively. Treatment with deferasirox was compared with control. Categorical data data were analyzed by loglinear analysis and Pearson Chi-Square.

Results: Treatment with deferasirox (823 ± 56 ng/mL) resulted in a statistically significant ($P < 0.01$) decrease in plasma ferritin levels as compared to the control group (1220 ± 114 ng/mL). The presence of hemosiderin was statistically lower ($P = 0.04$) in the deferasirox group compared to the control group. Signs of hemophilic synovitis, as assessed by the Valentino score, were not different ($P = 0.52$) when comparing the control group to the deferasirox group: 1 (12.4% vs. 7.7%), 2 (16.7% vs. 11.5%), 3 (12.5% vs. 38.5%), 4 (29.2% vs. 19.2%), 5 (16.7% vs. 11.5%), 6 (4.2% vs. 7.7%), and 8 (8.3% vs. 3.8%). Deferasirox treatment resulted in a statistically significant ($P < 0.01$) reduction in cartilage destruction, as assessed by the Glas-

son score, when comparing the control group to the deferasirox group: 2 (4.2% vs. 65.4%), 3 (4.2% vs. 26.9%), 4 (20.8% vs. 7.7%), 5 (54.2% vs. 0%), 6 (12.5% vs. 0%), and 7 (4.2% vs. 0%).
Conclusion: Treatment with deferasirox prevented cartilage destruction following the induction of hemarthrosis in hemophilic mice. The data presented herein support the need for further investigation of the potential role for iron chelation in the treatment of joint bleedings in hemophilia. This is in particular of interest in the event of a hemarthrosis despite prophylactic treatment with factor replacement.

PA 1.07-6

In vivo blood loss in a FVIII-inhibited rabbit model and its correlation with global hemostatic assays *ex vivo*

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Background: Hemophilia A is a bleeding disorder caused by mutations in the factor VIII (FVIII) gene. Treatment consists of intravenous infusions with recombinant or plasma-derived FVIII concentrates. In case of inhibitory antibody development, bleeds are treated with bypass therapy. For development of new therapeutic options, hemophilia animal models are crucial. Unlike in human clinical studies, global hemostasis *ex vivo* assays are not commonly included in preclinical studies.
Aim: We used an antibody-induced hemophilia A rabbit bleeding model to correlate *in vivo* blood loss with *ex vivo* parameters generated in two global hemostasis assays. We also present an example for efficacy assessment in this model.
Methods: Forty-two rabbits were injected with high titer FVIII-inhibitor plasma. After 45 min incubation, nail clipping was performed, and blood loss by weight determined over 30 min. Normal and FVIII-inhibited rabbit blood was measured by thromboelastography (TEG) triggered with very low concentrations of tissue factor. Thrombin generation was assessed by calibrated automated thrombography (CAT) under conditions optimized for rabbit plasma. We correlated blood loss (mg), CAT and TEG parameters of FVIII-inhibited blood/plasma using Spearman rank order analysis. We also evaluated the FVIII-sensitivity of the *ex vivo* parameters. For further model validation, the acute efficacy of 75 U/kg activated prothrombin complex concentrate (APCC), compared to a vehicle treated group, was demonstrated in six rabbits by monitoring the blood loss from the same wound for another 30 min.
Results: Peak thrombin, ETP (CAT), R-time, K-time and angle (TEG) were identified as the most FVIII-sensitive parameters in rabbit plasma or blood. For example, peak thrombin and ETP in normal plasma was consistently 2- to 3-fold higher than in FVIII-inhibited plasma. Strong correlations were also seen between CAT and TEG values, e.g. peak thrombin/ETP vs. R-time ($R = -0.6$, $P = 2\times10^{-7}$). Absolute blood loss in FVIII-inhibited rabbits was a highly variable parameter, ranging from 121 to 5043 mg (average 1066 mg). Blood loss did not correlate with CAT or TEG parameters. This demonstrates that absolute blood loss does not merely depend on the hemostatic status of the animal plasma/blood, but likely is influenced by other factors, such as the inflicted cut. Therefore, normalization of the bleeding parameter is indispensable. In our model, this was achieved by calculating relative blood loss (mg blood after treatment/mg blood before × 100%) for the APCC-treated group (average 2%) and vehicle group (average 173%). The difference between these groups was statistically significant. The high procoagulant activity of 75 U/kg APCC was also reflected by global hemostasis assay parameters.
Conclusions: Our results support the wider use of global hemostatic assays in hemophilia preclinical research and drug development. *Ex vivo* assays such as CAT and TEG can add value to the naturally more variable *in vivo* data. We also show the importance of well-controlled bleeding models to obtain meaningful data as accomplished by normalization. Continuous optimization of hemophilia animal models can help to improve data quality, possibly reduce the number of required experimental animals and support the realization of new hemophilia therapies.

PA1.08 – Rare Bleeding Disorders – I

PA 1.08-1

A recurrent Gly43Asp substitution of coagulation Factor X rigidifies its catalytic pocket and impairs catalytic activity and intracellular trafficking

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Background: Coagulation Factor X (FX) deficiency is one of the most severe among inherited bleeding disorders. The homozygous Gly43Asp mutation (chymotrypsinogen numbering) was found in 15 patients, from the border area between Turkey and Iran, with severe FX deficiency (FX:C < 1%). Previous *in vitro* expression studies showed that the introduction of a charged hydrophilic Asp residue instead of a neutral Gly into the protein core caused a secretion defect.
Aim: To thoroughly study the pathogenic mechanisms of the Gly43Asp substitution, by extensively characterizing both the wild-type (WT) and mutant (FX43Asp) FX on a functional and structural basis.
Methods: Kinetic studies were performed on both WT and FX43Asp recombinant FX expressed in HEK293 cells. Molecular dynamics (MD) simulations were performed on the wild-type and mutant FXa with GROMACS ver. 4.5.4 and the AMBER99SB force-field. Four different 60 ns long NPT MD simulations for each system were performed assigning different initial velocities. Continuum Poisson-Boltzmann electrostatic potentials were calculated using the APBS program.
Results: FX43Asp showed a 2–3 folds reduced activation rate by RVV-X and FVIIa/TF, while the amidolytic activity showed a 11-fold reduction of the apparent k_{cat}/K_m, compared to WT, being k_{cat} severely (1.1 vs. 14.6/s) and K_m modestly (26 vs. 40 mM) reduced. Catalytic activity of the FXa43Asp, measured by the prothrombin hydrolysis, was markedly impaired, showing a 30-fold reduction of k_{cat}/K_m, being k_{cat} unchanged and K_m increased (V_{max} 2.69×10^{-5}, K_m 509 ± 86.7 vs. 145.4 ± 22.35 nM) compared to WT. From the MD analysis, it is apparent that the Gly43Asp mutation does not dramatically affect the FXa structure, neither globally nor locally. Analysis of the protein H-bonds during the simulations, however, showed that the mutation modifies the H-bonds network around the catalytic residues, by thickening it. As a possible consequence, the backbone-backbone H-bond between residue 43 and the catalytic Ser195(verystable in the wild type protein: occupancy 98.7%), becomes weaker in the mutant (66.2%). The continuum Poisson-Boltzmann electrostatic potentials calculated for the two systems resulted to be predominantly negative for both along the crevice. However, in the mutant, the electrostatic potential of the binding site, around the oxyanion hole, is reversed from the positive values of the wild type to negative ones.
Conclusion: MD simulations indicate that the Gly43Asp mutation neither disrupts nor destabilizes the FXa native structure, but makes it more rigid. Therefore, by compromising the FXa flexibility, the mutation may also compromise the catalytic efficiency of the enzyme. The increased rigidity of the mutant FX might also affect the molecular recognition by chaperones inside the cell, causing the retention and defective secretion. Altogether, the defects of FX43Asp explain the severe bleeding symptoms in patients with the mutation and outline

the relevance of the plasticity in the FXa catalytic pocket for maintenance of its catalytic competence.

PA 1.08-2

Mapping of inhibitory antibodies directed to the carboxy-terminus of FVIIa in severe FVII deficiency with elongated C-terminal variant (p.A354V-p.P464Hfs†)

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Background: The relationship between the development of inhibitory antibodies and gene defects, extensively investigated in Hemophilia patients, is still an open issue. We studied inhibitory antibodies in severe factor VII (FVII) deficiency, a rare bleeding disorder characterized by a low incidence of this complication.
Aim: To correlate the *F7* gene mutation to major epitopes of anti-FVII inhibitory antibodies developed after replacement therapy.
Patient and Methods: The proposita (female, 59 years old) has been treated with plasma derived FVII (pdFVII) and recombinant activated FVII (rFVIIa) and developed high titers of anti-FVII inhibitory antibodies. The patient displayed very low circulating FVII protein levels (FVII:Ag 1.2%) and undetectable FVII activity (FVII:C < 1%), in accordance with the homozygous condition for the *F7* p.A354V-p.P464Hfs† mutation.
Functional assays were performed in plasma samples and in FVII-deficient plasma upon addition of rFVIIa or engineered FVII recombinant variants. The affinity of antibodies in patient's plasma for rFVIIa, pdFVII and recombinant variants was evaluated by binding and competition studies, using ELISA-based assays and Bio-Plex technology.
Results: Isotypic analysis showed a large prevalence of IgG subtype 1 in patient's plasma and functional assays indicated a type II kinetics. Activated factor X (FXa) and thrombin generation activity assays showed a major impact of inhibitory antibodies on the coagulation initiation phase, leading to prolonged lag times. Binding assays revealed a 1.5 fold higher affinity of the antibody for rFVIIa than for pdFVII. Noticeably, once activated pdFVII was recognized similarly to rFVIIa in competition assays.
The p.A354V-p.P464Hfs† mutation causes frameshift from residue 464 and predicts an elongated carboxy-terminal region (491 vs. 466 residues in FVII-wt). The inferred structural differences between the elongated FVII molecules circulating in patient and the infused factors led us to hypothesize the carboxy-terminal region as bearing a candidate epitope for the antibodies. Binding assays demonstrated that denatured and reduced FVII was not recognized by antibodies, thus supporting the presence of a tridimensional epitope.
To assess whether the carboxy-terminal region contributed to shape the major antibody epitopes we performed functional assays (i) in plasma from a patient homozygous for the p.R462X nonsense mutation, which produces low circulating levels of a truncated FVII with increased specific activity (Branchini et al, 2011) and (ii) by deletion scanning leading to the truncated recombinant FVII-466X, FVII-465X and FVII-464X variants, displaying normal specific activity. Strikingly, functional assays showed that the natural FVII-462X was less inhibited than FVII in pooled normal plasma. Moreover, studies with the truncated recombinant variants demonstrated that the carboxy-terminus length was related to the inhibition rate, being the shortest rFVII-464X the least inhibited variant, as indicated by a significant difference ($P < 0.0001$) in lag time prolongation in FXa generation assays. Accordingly, binding assays on the Bio-plex platform indicated that the rFVII-464X was the least recognized variant by the inhibitory antibodies.
Conclusions: Taken together our findings, which represent the first characterization of an anti-FVII inhibitor, indicated that antibodies were directed toward the carboxy-terminal region of infused FVII molecules. Data may suggest a relation between the *F7* mutation and major inhibitor epitopes.

PA 1.08-3

Association between thrombin generation and bleeding severity in 41 patients with coagulation factor VII deficiency

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Background: Coagulation factor VII (FVII) deficiency is a rare bleeding disorder with autosomal recessive inheritance. The association between levels of FVII (FVII:C) and bleeding severity in FVII deficiency is weak, although patients with levels below 25% have been reported to generally diplay more severe symptoms (J Thromb Haemost. 2012;10:615–21). Measuring FVII:C may therefore provide little information on the bleeding tendency of patients with this condition, hampering the development of optimal management strategies. Measurement of thrombin generation may provide a better method for quantifying the combined effect of procoagulant and anticoagulant factors in determining the bleeding predisposition of a patient.
Aims: To study the association of thrombin generation with bleeding severity in patients with FVII deficiency.
Methods: Between 2009 and 2011, patients with FVII deficiency (i.e. FVII:C below 62%) who had been visited at the Rare Bleeding Disorders Outpatient Clinic of the Angelo Bianchi Bonomi Hemophilia and Thrombosis Center (Milan, Italy) were recalled to the center for a follow-up clinical visit. Bleeding tendency was measured by a tailored bleeding severity score (BSS), compiled by the same physician (S.M.S.) on all participants. Thrombin generation was measured within 2 h of collection on freshly-prepared platelet-rich plasma, with the addition of 1 pM recombinant tromboplastin (IL, Orangeburg, NY, USA). Assay parameters that were used to quantify thrombin generation and their range of normal values (i.e. the 5th and 95th percentiles of the distribution in 43 normal subjects) were: lag-time (5–8 min), endogenous thrombin potential (1699–2937 nM/min), peak level of thrombin generation (137–252 nM), time-to-peak (10–15 min). The association of thrombin generation assay parameters with BSS was assessed by linear regression analysis adjusting for age and sex.
Results: A total of 41 patients with FVII deficiency were included in the study. Patients had a meadian age at enrollment of 40 years (interquartile range [IQR]: 21–49 years), were equally distributed between sexes (21 females, 20 males) and displayed mild to moderate bleeding symptoms (median BSS: 5; IQR: 2–9). Median FVII:C was 23% (IQR: 5–34%). Patients with FVII:C below 25% had higher BSS than patients with activity above 25% (median 8.5 vs. 3.0; difference 5.5, 95% confidence interval [CI]: 0–12). Thrombin generation results were as follows: lag-time, median: 8, IQR: 7–11 min; endogenous thrombin potential, median: 1848, IQR: 1632–2100 nM/min; peak, median: 182, IQR: 145–202 nM; time-to-peak, median 13, IQR: 12–15 min. Thrombin generation was inversely associated with bleeding tendency. Longer lag-time and time-to-peak were associated with higher BSS (lag-time, beta: 0.8; 95% CI: 0.0–1.6; $P = 0.046$; time-to-peak, beta: 0.7; 95%CI: 0.0–1.4; $P = 0.040$) and higher endogenous thrombin potential was associated with lower BSS (per 100 nM/min increase, beta: 0.6; 95% CI: 1.2–0.0; $P = 0.050$). Peak levels were not associated with BSS (beta: 0.0; 95% CI: −0.1 to 0.0; $P = 0.542$).
Summary/Conclusions: In patients with FVII deficiency, increased thrombin generation is associated with milder bleeding tendency.

PA 1.08-4

Characterization of a primary hemostasis abnormality in patients with OCRL1 gene mutations (Lowe syndrome)

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Background: The Lowe oculo-cerebro-renal syndrome (OCRL) is a rare X-linked disorder characterized by congenital cataracts, cognitive disability, and proximal tubular dysfunction. This syndrome results from loss-of-function mutations in the *OCRL1* gene, which encodes OCRL protein. OCRL is a type II phosphatidylinositol bisphosphate 5-phosphatase that contains a Rho-GAP and clathrin domains. Patients with Lowe syndrome often require surgical procedures and an abnormal rate of hemorrhagic events occurring per-or post-surgery have been reported, suggesting clot instability. In a preliminary study, we found that all patients ($n = 6$) had normal coagulation tests excepted for elevated levels of plasma fibrinogen and von Willebrand factor (VWF), but a prolonged closure time in the PFA-100® system.

Aim: The aim of this study was to characterize platelet functions of Lowe syndrome patients compared to controls.

Methods: Fourteen patients and 14 controls were enrolled in a 1-year prospective study. For each patient and control, a bleeding score (BS), derived from VW disease bleeding score, was calculated. Platelet function evaluation was performed using (i) light-transmission aggregation in the presence of various agonists (ii) flow-cytometry assays to quantify platelet membrane glycoprotein level (CD41, CD42, CD62 and CD63) at rest and after activation (iii) lysosome secretion by colorimetric quantification of *N*-acetyl-β-D-glucosaminidase, and (iv) platelet adhesion on collagen under flow. Finally, we studied clot retraction in platelet rich plasma (PRP) and in washed platelets (WP) in the presence of fibrinogen. Clots were formed by adding thrombin and calcium then, at various time points, clots were photographed and clot size was quantified. Clots were solubilized for western blotting analyses. The intensity of Myosin Light Chain (MLC) phosphorylation during clot retraction was evaluated using imageJ. Data presented are median [range] and statistical analysis used paired Wilcoxon tests.

Results: Median patient bleeding score was 3[0–11] (nine patients had positive BS ≥ 3) compared to 0 [(-3)-1] for controls ($P = 0.004$). We confirmed the prolonged PFA-epinephrin closure time in nine out of patients (162 s [126–300] vs. 120 s [97–174], $P < 0.0003$), with no abnormality in platelet aggregation, glycoprotein level or secretion assays. Although no significant difference was denoted in platelet adhesion level on collagen under flow, the size of platelet aggregates was significantly smaller in patients compared to controls 1874 μm^2 [114–5139] vs. 4169 μm^2 [1012–7188] ($P < 0.001$). We also found an abnormal kinetics of clot retraction in Lowe's patients; the PRP-clot retraction was 40% [11.3–71] in patients vs. 60% [30–89] in controls after 20 min ($P < 0.001$). This difference was also observed in WP (63% [27.5–92.5] vs. 70.5% [48–96.2], $P = 0.007$). Moreover, we evidenced a deregulation of MLC phosphorylation during clot retraction in patients compared to controls.

Summary/Conclusions: This study confirms a hemostasis defect in Lowe's patients, especially regarding abnormal clot retraction observed both in PRP and washed platelets. This defect is associated with a decrease of MLC activity, pointing to a deregulation of 'outside-in' signaling pathway. In conclusion, we provide for the first time that loss of OCRL protein is associated with an abnormal MLC signaling pathway during clot retraction. Abnormal clot retraction is in line with the observed re-bleeding events reported in patients.

PA 1.08-5

Measurement of the 5- and 7-carbon aglycone vitamin K metabolites in term and preterm neonatal faecal matter using HPLC with electrochemical detection

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Background: Vitamin K deficiency bleeding (VKDB) is a rare, but potentially life-threatening disease of the newborn. Prophylactic administration of vitamin K_1 at birth is protective, though further dose optimization remains desirable. Urinary excretion of vitamin K metabolites reflects vitamin K status in newborns, but the relationship between vitamin K exposure and vitamin K metabolite excretion in neonatal faecal matter is not yet understood.

Aim: Our aim was to develop a non-invasive method for measurement of the 5C- and 7C-aglycone metabolites of vitamin K in neonatal faecal matter using HPLC with electrochemical detection.

Methods: Faecal samples collected from term and preterm neonates were dried for 48 h at 37 °C and weighed to determine dry mass. Samples were then deconjugated overnight with methanolic HCl. The suspended faecal matter was pelleted by centrifugation and the methanolic HCl decanted. The metabolites were then extracted twice by Bligh and Dyer liquid-liquid extraction and dried under nitrogen. Stabilisation of the metabolites was achieved by conversion to the methyl ester derivatives using MNNG. Further purification was carried out by normal phase, solid phase extraction prior to a final drying stage under nitrogen. The extracts were then reconstituted in methanol and injected to the HPLC-ECD system. A Thermo Betasil C_{18} 100 × 4.6 mm column (3 μm) was used with a mobile phase of 37% polished, deionized water with a sodium acetate buffer (50 mM, pH = 3) and EDTA (0.1%) in methanol. The metabolites were first reduced using an ESA 5011A cell (−1.2 V) controlled by an ESA Coulochem II and subsequently detected using a HyRef VT-03 Electrochemical Flow Cell (+0.3 V, oxidative) controlled by a Decade II. Quantification was achieved using standards made from the pure methyl ester derivatives of the metabolites. LC-MS/MS was used to confirm the identities of the metabolites in the faecal extracts.

Results: Repeat extraction of the faecal matter indicated efficient extraction of the metabolites. The on-column lower limits of qualification (LLOQ) were 3.2 and 10.4 μg/g for the 5C- and 7C-metabolites respectively. The estimated total 5C- and 7C-metabolite excretory half-life values were 12.3 days in a 27-week gestation pre-term infant and 7.3 days in a 39-week gestation term infant, broadly consistent with previously published values for vitamin K_1 clearance in term neonates of 1.1–8.0 days (median 3.2 days). The relative quantity of 7C-metabolite in the preterm neonate declined from 60% of total metabolite excretion to 30% in 25 days (adults ≈ 25%), whereas this ratio was maintained at around 30% in the term neonate. Based on the mass of the parent and daughter ions, LC-MS/MS confirmed the identities of the methyl-ester metabolite derivatives.

Summary/Conclusion: These data provide proof of principle that the 5C- and 7C-metabolites of vitamin K can be detected and quantified in the faecal matter of term and preterm neonates. This method may help provide insight into post-prophylactic vitamin K metabolism in neonates. Further validation experiments are required to determine assay precision, accuracy, and recovery.

PA 1.08-6

Novel homozygous mutation Met362Thr identified as a cause of cross-reacticting material reduced factor X deficiency in Japanese brother patients

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Introduction: Factor X (FX) deficiency is a rare bleeding disorder inherited as an autosomal recessive trait. In this study, we diagnosed a new Japanese patient with congenital FX deficiency and identified a novel missense mutation in the FX gene (*F10*). We performed a recombinant mutant protein expression study to investigate the molecular basis of the genetic defects of the mutant.

Methods: FX activity was determined using a one-stage clotting assay based upon prothrombin time (PT), and FX antigen level was measured with a sandwich enzyme-linked immunoadsorbent assay (ELISA). After receiving informed consent, we obtained blood samples from the patient and his brother, and genomic DNA was extracted. Coding regions and exon/intron boundaries in *F10* were amplified by polymerase chain reaction (PCR). The PCR products were purified and direct sequenced with a 3730 DNA Analyzer (Applied Biosystems). We constructed an FX wild-type plasmid using the *F10* cDNA and the pcDNAtm 3.2 expression vector (Invitrogen). The identified mutation was introduced into the wild-type/pcDNAtm 3.2 plasmid with a QuikChange Site-Directed Mutagenesis Kit (Stratagene). Vectors expressing wild-type and mutant FX proteins were transfected into HEK293 cells with Lipofectamine reagent. Culture media and cells were harvested 60 h later, and culture media were concentrated using Amicon Ultra-15 centrifugal filter units (Millipore).

Patient and Results: The patient, a Japanese man in his early 40s, presented with gingival and nasal hemorrhage. His PT was 51.9 s (control, 11.7 s) and activated partial thromboplastin time (aPTT) was 74.7 s (control, 28.1 s). His younger brother had a past history of postoperative hemorrhage without prior spontaneous bleeding. Other family members had no significant bleeding symptoms. FX activities of the patient and his brother were both < 1%, and their FX antigens were 11% and 12%, respectively. A homozygous missense mutation, Met362Thr (HGVS names: c.1205 t>c, p.Met402Thr), was identified in *F10* from the patient and brother using direct sequencing. A Met362Thr mutation was not detected in the *F10* gene of 82 unrelated normal Japanese individuals. Study of recombinant protein expression revealed that the quantity of secreted FX antigen of the mutant and the wild-type were 1.4 and 4.5 μg/mL in concentrated conditioned media, respectively. It was suggesting that the secreted antigen level of the mutant was about 30% of the wild type. Therefore FX specific activity of the mutant was 19% of the wild-type.

Discussion: The patients were identified as having cross-reacting material reduced (CRM^{RED}) FX deficiency because of the existing of a certain amount of FX antigen with very low activity. Results of the recombinant protein expression study suggested that the mutant would cause a secretion defect and a molecular abnormality of FX.

Conclusion: We identified a novel homozygous Met362Thr mutation located in the catalytic domain of the FX protein as a causative mutation of CRM^{RED} FX deficiency. We will use this FX-Met362Thr mutant protein in our future studies of functional analysis.

PA1.09 – Von Willebrand Factor: Basic

PA 1.09-1

Cysteine 584 is required for correct von Willebrand factor multimerization

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Background: Cysteines have a key role in the dimerization and multimerization of von Willebrand factor (VWF), and their loss may have a great impact on the structure and function of VWF. There are 64 cysteines in the VWF propeptide (VWFpp), some of them involved in favoring interchain disulfide bonds at the N-terminus of VWF.

Aims: The present work describes a new VWF mutation (C584F) located in the VWFpp, reporting the effects of this mutation and using our findings to help explain the physiological process underlying the multimerization of VWF.

Methods: Three type 3 VWD patients were investigated, establishing their phenotype by analyzing their VWF antigen (VWF:Ag), VWF collagen binding (VWF:CB) activity, VWF ristocetin cofactor (VWF:RCo) activity, factor VIII (FVIII), platelet VWF content and multimer pattern. VWF gene mutations were identified by amplifying and sequencing all 52 exons of the VWF gene. Recombinant normal and mutated VWF was produced by transient transfection of HEK293T cells, using pSVvWF vectors. C584F-VWF trafficking was explored in BHK cells by co-expressing VWF and a Green Fluorescent Protein localized in the endoplasmic reticulum (ER) or Golgi apparatus.

Results: Plasma and platelet VWF:Ag were found at almost undetectable levels, as were VWF:CB and VWF:RCo, while FVIII levels were below 10 U/dL. Multimer analysis of plasma VWF revealed no multimers apart from the first oligomer, which was slightly represented. On high-resolution gel electrophoresis (2.2%) the protomer was resolved as a dimer running as the slow- and fast-running satellite of the triplet occurring in the normal counterpart. Genetic analysis showed that the patients were carrying two mutations: a deletion of seven nucleotides in exon 7 (c.729_735del) and the new C584F missense mutation, located in the propeptide of VWF. The C584F mutation was transiently expressed in the HEK293T cells, showing that C584F-VWF was virtually absent from the conditioned medium at homozygous levels. Multimer analysis demonstrated the absence of any multimers in the conditioned medium, except for the slightly represented first oligomer. Conversely, the intracellular VWF content was higher than normal, both when C584F was co-expressed with wild-type VWF, and when C584F-VWF alone was expressed. VWF:CB and its ratio (VWF:CB/VWF:Ag), which are parameters expecially sensitive to large VWF multimer representation, were found much reduced, suggesting that C584F-VWF is synthesized, but not multimerized.

The trafficking of rC584F-VWF showed a normal ER content of C584F-VWF, suggestive of a normal synthesis and dimerization of C584F-VWF, but a reduced localization in the Golgi apparatus, probably due to a defective transport of C584F-VWF from ER and/or its retention in the ER.

Conclusions: We conclude that the C584F mutation abolishes multimerization, but not dimerization, thus interfering with the storage and secretion of VWF. These findings suggest that C584F might be involved in the formation of the intermediate propeptide-dependent species during the VWF multimerization process.

PA 1.09-2

Von Willebrand factor activity determination using new assay principle ristocetin-free for reliable von Willebrand disease diagnosis

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Background: VWF:RCo assay is the reference method for VWD diagnosis despite its poor precision and sensitivity to low VWF activity. Last years, alternative platelets and/or ristocetin free methods have emerged to improve or replace the VWF:RCo assay. The Innovance® VWF Ac Assay (Siemens) is an automated immunoturbidimetric assay using polystyrene particles coated with rGPIb with two gain-of-function mutations avoiding requirement of ristocetin.

Aim and Methods: The aim of the study was to evaluate the performance of this new assay in screening and identification of the various type and subtypes VWD. Results of VWF:Ac, performed in two laboratories on Siemens analysers (Sysmex® CA-1500 and BCS) were compared to VWF:RCo performed using BC von Willebrand reagent on agregometer (SD Medical) or BCS.

Frozen plasma from 43 controls (21 blood group O and 22 non O) and from 172 VWD patients (67 type 1, 101 type 2 and four type 3) phenotypically and genotypically characterized (n = 108) were evaluated.

The two laboratories used the same bath of Standard Human Plasma (Siemens) for the calibration, and same batches of VWF:Ac and VWF:RCo reagents. The sensitivity of the method was evaluated on type 3 VWD samples using a low calibration curve and the imprecision using a normal and pathological control plasma (Siemens).

Type 1, type 3 carriers and type 2N have been grouped in Group A and type 2A, 2B and 2M grouped in group B for results analysis.

Statistical analysis was performed using t test comparison in paired series, Pearson linear regression and agreement estimation according Bland & Altman graphic representation plotting difference vs. mean.

Results: Sensitivity of VWF:Ac was lower than 4%, and lower compared to VWF:RCo methods (5% on agregometry and 10% on BCS). The imprecision was lower than 5% for VWF:Ac while VWF:RCo imprecision was respectively 14% (agregometry) and 15% (BCS).

In controls, a significant correlation between the two methods (r = 0.837, $P < 0.0001$) was observed with an estimated bias +3.5%. In O blood group, VWF:Ac and VWF:RCo means were 65.6 and 65.3 IU/dL respectively. In non O blood group, means were 98.8 and 92.1 IU/dL.

In VWD patients, a very good correlation (r = 0.965, slope = 1.04, $P < 0.0001$) was observed.

VWF:Ac and VWF:RCo means were 33.6 and 33.3 IU/dL respectively in group A, 19.8 and 16.4 IU/dL in group B (mean bias of +2.5% in group A and – 2% in group B).

Discrepant results were observed in only 7/168 samples (type 3 excluded).

In group A, VWF:Ac < VWF:RCo was found in two samples (2N, 1) and VWF:Ac>VWF:RCo in one (2N): [VWF:Ac – VWF:RCo] out of bias ± 2SD, i.e. [+17%; −12%].

In group B, VWF:Ac < VWF:RCo was found in three samples (2A, 2B, 2M/2Alike) and VWF:Ac>VWF:RCo in one (2M/2Alike): bias ± 2SD, i.e. [+7%; −11%].

Conclusion: Despite rare discrepant results (4%), Innovance® VWF Ac assay provides a simple sensitive and reliable test for diagnosing of VWD which seems as good as the reference method.

PA 1.09-3

Identification of VWF gene deletions in nine VWD families using multiplex ligation-dependent probe amplification (MLPA)

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The current classification of von Willebrand Disease (VWD) is based on quantitative (type 1 and 3) or qualitative (type 2) deficiencies of von Willebrand factor (VWF). VWD has a heterogenous mutational basis, but about 10% of severe VWD are caused by either single or multiple exon deletions in heterozygous or homozygous form. Exon deletions in homozygous patients with VWD can be detected by an absence of PCR products of the deleted fragment. However, the detection of deletions in heterozygous form is more difficult because of the presence of the normal allele. Multiplex ligation-dependent probe amplification (MLPA) is a method for the relative quantitation of exonic sequences of a gene in a single reaction. MLPA is a rapid, sensitive and reproducible method based on the ligation and amplification of sequence-specific probes. We have used MLPA to investigate nine families with VWD in whom mutations were either not detected by DNA sequencing, or in whom a second mutation was suspected because of the VWD phenotype. A number of novel abnormalities have been detected using this technique.

In family 1, we identified a novel deletion of exons 4–52 in a male with type 3 disease, in compound heterozygous form with a previously reported single base duplication in exon 14. In family 2, we identified a novel deletion of exons 19–52 in heterozygous form in a male with type 3 disease, in compound heterozygous form with a novel point mutation in exon 14. In family 3, we identified a novel deletion of exon 22 in heterozygous form in a male with type 1 disease. In family 4, we identified a previously reported deletion of exons 33–34 in an individual with type 2 unclassified VWD and abnormal VWF:Ag multimer pattern. Deletion of *VWF* exons 4–5, previously reported to be recurrent among type 3 VWD and to underlie a proportion of dominant type 1 VWD was identifed in three individuals with type 1 VWD. In the remaining two cases, MLPA did not produce a signal for exon 7 in an individual with type 3 VWD, or for exon 45 in an individual with severe type 1 VWD. However, subsequent PCR and DNA sequencing revealed these results to be false postives arising from a novel 2 bp deletion and a novel nonsense mutation respectively in the respective MLPA probe binding sites.

MLPA is a useful addition to DNA sequencing and other screening methods for the identification of deletions, and potentially insertions, in VWF where the genotype determined by standard methods does not fully explain the VWD phenotype. This method facilitates genetic counselling to family members, but care should exercised in data interpretation, particularly with single exon deletions where a false positive may be generated by sequence changes in the probe binding site.

PA 1.09-4

Characterization of von Willebrand factor and ADAMTS13 in plasma derived factor concentrates

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Background: Prevention or treatment of bleeding in patients with von Willebrand disease (VWD) may require infusion of virally-inactivated plasma-derived von Willebrand factor (VWF)-containing concentrates. Three products are FDA approved in the US for treatment of patients with VWD; all contain VWF and factor (F) VIII. Koate®-DVI is a FVIII/VWF containing concentrate FDA licensed in the US for treatment of hemophilia A.

Aims: The aims of the study were to evaluate VWF and ADAMTS13 laboratory parameters in Koate®-DVI and compare to findings in the three FDA-licensed concentrates for treatment of VWD.
Methods: Koate®-DVI, provided by Kedrion Biopharma Inc. (nine lots), and three lots each of the other concentrates (noted as A, B and C), were purchased, and all reconstituted per the manufacturers' recommendations. For VWF and FVIII assays aliquots were diluted in VWF deficient plasma to a concentration of approximately 1 U/mL of FVIII for Koate®-DVI and approximately 1 U/mL of VWF for products A,B, and C. FVIII:C activity was measured by one stage assay (Siemens Actin FSL and deficient plasma), VWF antigen (VWF:Ag) by STA Liatest (Stago) and VWF ristocetin cofactor activity (VWF:RCof) by Siemens BC reagent on the BCS®XP coagulometer. VWF collagen binding (VWF:CB) and FVIII antigen (FVIII:Ag) were determined by ELISA (Technoclone and Affinity Biochemicals, respectively). VWF multimers were analyzed on 1% agarose gels. ADAMTS13 activity was measured in reconstituted concentrate using a FRET-based assay (GTI). Assays were performed in triplicate.
Results: Mean VWF parameters (given as ratios with ranges) for Koate®-DVI were VWF:RCof/VWF:Ag, 0.93 (0.82–1.02); VWF:CB/VWF:Ag, 1.05 (0.95–1.08); FVIII:C/VWF:Ag, 0.50 (0.43–0.55); and, FVIII:Ag/VWF:Ag, 0.45 (0.41–0.48). For concentrates: VWF:RCof/VWF:Ag: A = 0.66 (0.57–0.80), B = 0.53 (0.48–0.67), C = 0.65 (0.62–0.71); VWF:CB/VWF:Ag: A = 0.92 (0.91–0.93), B = 0.98 (0.96–1.0), C = 1.02 (0.92–1.05); FVIII:C/VWF:Ag: A = 0.42 (0.40–0.44), B = 0.67 (0.63–0.69), C = 0.94 (0.89–0.97); and, FVIII:Ag/VWF:Ag: A = 0.28 (0.26–0.32), B = 0.49 (0.47–0.52), C = 0.75 (0.70–0.83). All concentrates contained VWF intermediate-sized molecular weight multimers, defined as > 6–9 bands on the 1% agarose gel. Of the nine Koate®-DVI lots 7 (78%) contained high molecular weight multimers, defined as > 10 bands. This compared with 50% for concentrate B and 100% for concentrates A and C. Koate®-DVI contained more ADAMTS13 activity than the other concentrates, with two concentrates containing virtually no activity (Koate®-DVI: 577.1 ± 96.1; Concentrate A: 23.0 ± 10.4; Concentrate B: 140.1 ± 21.0; Concentrate C: 18.0 ± 4.4). ADAMTS13 activity per labeled FVIII activity for Koate®-DVI was 0.057 and per labeled VWF activity for the other concentrates was: A: 0.002, B: 0.013, C: 0.002.
Summary: Koate®-DVI contains approximately twice the VWF:Ag and activity as FVIII and has parallel VWF protein and functional measurements. VWF:CB was more consistent with the VWF:Ag measurement than was VWF:RCof in the other concentrates. Koate®-DVI contains more ADAMTS13 activity than found in the other concentrates; however, this was not associated with loss of high molecular weight multimers, and all concentrates have considerably less ADAMTS13 activity than labeled FVIII or VWF activity. These data should be of help to physicians treating patients with bleeding disorders.

PA 1.09-5
Development of the self-PBQ (Self-administered pediatric bleeding questionnaire): pre-testing and optimization

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Background: Expert-administered bleeding assessment tools have been developed and prospectively validated for the diagnosis of von Willebrand disease (VWD).
Aim: To generate, pre-test and optimize a self-/parent-administered pediatric bleeding questionnaire.
Methods: The validated expert-administered Pediatric Bleeding Questionnaire (PBQ) (Bowman et al, JTH, 2009; 7:1418) was modified into the Self-PBQ Version #1 by simplifying the language to a Grade 4 reading level. Two groups of research subjects (< 18 years) were recruited: (i) children with known Type 1 VWD; and (ii) healthy control children. Parents completed the Self-PBQ for children < 12 years while children ≥ 12 years completed the Self-PBQ themselves if able to read at a Grade 4 reading level. The expert-administered PBQ was completed at a separate visit by face-to-face interview. All subjects completed the Self-PBQ and the expert-administered PBQ in random order at least 2 weeks apart, and the bleeding scores (BS) obtained from each were compared using the Interclass Correlation Coefficient (ICC). Focus groups were held following the second visit, and revisions made to the Self-PBQ based on feedback before subsequent recruitment.
Results: To date, a total of 36 subjects have been recruited; nine with Type 1 VWD and 27 controls; the Self-PBQ has undergone two revisions. The median age and gender distribution is similar between groups; 9.4 years in the affected group (range of 3–17) and 5.9 years in the control group (range of 9 months–17; $P = 0.078$), $n = 4$ female (44%) in the affected group and $n = 13$ female (48%) in the controls ($P = 1.00$). The mean BS in the affected group was 3 (range 0–7) from the Self-PBQ and 3.8 (range 1–9) from the expert-administered PBQ. The mean BS in the controls was 0.5 (range −1 to 2) from the Self-PBQ and 0.2 (range 0–1) from the expert-administered PBQ. For both the Self-PBQ and expert-administered PBQ the BS were significantly higher in the affected children compared with controls ($P = 0.015$ and $P = 0.003$ respectively). Four affected and 15 unaffected parents/children completed version #1 of the Self-PBQ. The ICC = 0.676, and the main concern during the focus group was the use of the word 'problem' in the question stems (ie: 'Has the research participant ever had a problem with nosebleeds?'), so this word was removed for Version #2. Four affected and eight unaffected parents/children completed Version #2; the ICC = 0.973. The focus group feedback resulted in the addition of the instruction at the beginning of the questionnaire 'If you are not sure whether the answer is yes or no (to each question about bleeding), answer yes' to Version #3. One affected and four unaffected children have been enrolled and are in the process of completing both study visits with Self-PBQ Version #3.
Conclusions: A self-/parent-administered pediatric bleeding questionnaire has been optimized and the BS obtained from its administration show a high degree of correlation with expert-administered BS. Future directions include prospective validation as a screening tool for children referred to Hematology for investigation of a bleeding disorder.

PA 1.09-6
Genetic defect of von Willebrand disease in 30 Taiwanese patients

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Background: Von Willebrand disease (VWD) is the most common inherited bleeding disorder that results from quantitative and/or qualitative defects of the von Willebrand factor (VWF). Genetic study of VWF may provide information useful for VWD diagnosis, subtype classification and management.
Aim: To analyze the genetic defects in our cohort of VWD patients.
Methods: There were 30 patients (13 male and 17 female) from 25 unrelated families with VWD diagnosis enrolled from January 2008 to January 2013. The median age was 31 years with a rage of 14–56 years. Exon 28 of VWF gene was amplified using polymerase chain reaction followed by direct sequencing. Mutations in the other 51 exons were detected by Denaturing High-Performance Liquid Chromatography analysis.

Results: Of the 30 patients in the cohort, 21 had type 1 VWD, seven had type 2 VWD including six type2A and one 2N. Two patients had type 3 VWD. Eight of 30 (27%) patients were identified to have a VWF gene mutation. Four patients from two unrelated families with type2A VWD were found to have c3814T>G, C1272G and c4883T>C, I1628T, respectively. Three patients with type 1 VWD were revealed to have missense mutations including c.4499C>T, A1500V in two and c.7848T>A, S2516R in exon44 in one patient. One patient with type 3 VWD was identified to have IVS27+2T>C transition. Another 17 (57%) patients (16 type 1 and one type 2 VWD) of our 30 patients from 14 unrelated families were found to have polymorphisms of the VWF gene.
Conclusions: Our study demonstrated that around one third of VWD patients had a detectable genetic defect and there was also a high percentage of polymorphisms in our cohort.

PA1.10 – Anticoagulant Agents – I

PA 1.10-1
Observed practice of bridging anticoagulation; guideline adherence and risk factors for perioperative bleeding

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Background: Perioperative interruption of chronic anticoagulation harbors the risk of thromboembolism (TE). To minimize the risk for TE during the anticoagulant free interval, bridging therapy with low molecular weight heparins (LMWH) is applied. This introduces the risk of bleeding. Guidelines characterize patients at risk and strategies to be followed.
Aims: We conducted a search of two electronic patient data systems. Our primary goal was to delineate the features of bridging strategies applied in the region aroundMaastricht, theNetherlands. We intended to assess guideline-adherance and to document the incidence of bridging-related bleeding and TE. The secondary goal was to identify possible risk factors for bleeding during bridging therapy, both patient related risk factors and risk factors associated with the bridging strategy itself.
Methods: We searched from September 2010 until June 2012 the electronic patient data system of theMaastrichtanticoagulation service, which contains data of approximately 4200 patients in relation to anticoagulation therapy and treatment complications. Additional medical information for these patients was retrieved from the patient database of the Maastricht University Medical Centre (MUMC+). We identified 222 patients on chronic anticoagulation who received bridging therapy. We determined guideline-adherence and risk factors for major and overall bleeding. Guideline adherence was defined as low TE risk patients receiving prophylactic doses LMWH and intermediate to high TE risk patients receiving prophylactic or therapeutic doses. Patients without prior surgical bleedings undergoing low risk cataract operations, dental procedures, or dermatological procedures should not be bridged, and the duration of postoperative administration of LMWH should not exceed 7 days. Data were analyzed with SPSS version 19.0.0.
Results: In 27.9% of all cases bridging was installed for a procedure for which bridging was not indicated. The proportion of patients at low TE risk (n = 102) treated with therapeutic doses of LMWH was 84.3% (n = 86). The median duration of postoperative bridging with LMWH was 8 days (mean: 10.0, standard deviation: 6.7). The 30-day incidence of overall bleeding in the entire group (n = 222) was 19.8% (n = 44), the incidence of major bleeding 11.3% (n = 25). Two patients (0.90%) experienced a deep venous thrombosis. In univariable logistic regression analysis high TE risk (odds ratio (OR), 2.59; 95% confidence interval (CI), 1.11–6.04) and dental procedures (OR, 2.99; 95% CI, 1.45–6.13) were found to be risk factors for overall bleeding. Vitamin K administration (OR, 6.55; 95% CI, 1.01–42.52) and dental procedures (OR, 3.77; 95% CI, 1.60–8.86) were identified as independent predictors of overall bleeding, after adjusting for age, total duration of LMWH treatment, TE risk, creatinine clearance, and mitral valve replacement.
Conclusions: Guideline-adherence for bridging therapy was low, leading to prolonged bridging procedures, excess treatment of patients and overall high bleeding rates. The majority of patients had a low thromboembolic risk profile or underwent low risk procedures. The independent risk factor Vitamin K administration may be an indicator for perioperative elevated INR.

PA 1.10-2
The coagulation factor XIIa inhibitor rHA-Infestin-4 potently improves outcome after cerebral ischemia/reperfusion injury in rats

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Background: Ischemic stroke provokes initial brain damage and is still one of the most predominant diseases in industrialized countries with a high rate of mortality or severe disability, respectively. To reduce primary insults based on the main pathophysiological processes of vessel occlusion in the brain, restoration of blood flow is essential. Paradoxically, reperfusion of brain tissue triggers further pathophysiological mechanisms and results in a process termed ischemia/reperfusion injury (I/R injury). The coagulation factor XII (FXII)-driven contact activation system is widely discussed to play an important role within the processes of I/R injury.
Aims: The aim of the current studies was to assess the efficacy of the FXIIa-inhibitor rHA-Infestin-4 in a rat model of I/R injury in a prophylactic and a therapeutic medication.
Methods: Rats were randomly assigned to the treatment groups and all studies were performed in a blinded manner. Within prophylactic treatment, animals were treated intravenously with 100 mg/kg rHA-Infestin-4 or an equal volume of saline 15 min prior to transient middle cerebral artery occlusion (tMCAO). For therapeutic treatment, 100 mg/kg rHA' -Infestin-4 or an equal volume of saline was administered directly after start of reperfusion. tMCAO was induced using the filament technique. Occlusion held up for 90 min with subsequent reperfusion. Twenty-four hours after tMCAO, rats were tested for neurological deficits (Zea Longa score, a composite neurological score, rotarod assay and grip strength test). Thereafter, brains were removed and analyzed for infarct area and edema formation. Furthermore, blood was terminally withdrawn within the prophylactic treatment approach for additional coagulation assays.
Results: Compared to the control group, rHA-Infestin-4 significantly improved survival in both, the prophylactic as well as therapeutic treatment. Moreover, neurological deficits by means of Zea Longa score and a inhibition of FXIIa and aPTT prolongation were still prominent 24 h after tMCAO whilst PT was not affected.
Summary/Conclusions: Although beneficial effects were more prominent within the prophylactic treatment, rHA-Infestin-4 competently ameliorated outcome after acute ischemic stroke in rats. With regard to the central role of FXII-driven contact activation system in stroke, inhibition of FXIIa via rHA-Infestin' – 4 may represent a new and promising treatment approach for brain I/R injury.

PA 1.10-3

Development of a clinical prediction model for an INR = 4.5 in hospitalized patients treated with vitamin K antagonists

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Background: Bleeding is a serious and frequently occurring complication in patients treated with vitamin K antagonists (VKAs). In the Netherlands, over 2.5% of the population is treated with VKAs. Treatment is usually monitored by measuring the clotting time, expressed as the international normalized ratio (INR). Optimal treatment depends on the indication but is normally in the range of 2.0–4.0. The risk of bleeding increases considerably at an INR above 4.5. Many other factors than dosage alone influence the INR. A few prediction models, based on these other factors for a high INR and/or bleeding complications can be found in literature. However, not all risk factors included in these models are readily available in daily practice, thereby limiting the usability of these models.

Aim: To develop a model designed to predict the risk of an INR $\geq$ 4.5 for hospitalized patients treated with VKAs, based on risk factors that are collected during routine care.

Methods: This is a retrospective cohort study conducted in a large university hospital in theNetherlands. Data on medication, laboratory results, admission and discharge dates are embedded in an electronic patient database (EPD).

We included admissions of adult patients in 2006–2009 during which a VKA was prescribed. Patients were followed from start of VKA treatment until their first INR $\geq$ 4.5, the end of treatment with a VKA, discharge, or until the end of the study, whichever came first.

Predictors included in the analysis were: age, sex, blood group, type of patient (surgical or medical), an INR $\geq$ 4.5 during an earlier admission (previous event), and at start of VKA therapy: the number of concomitantly used medicines, concomitant use of low molecular weight heparins (LWMHs) and/or amiodarone, and the laboratory values of liver enzymes: ALAT, ASAT, y-GT, LDH, albumin, haemoglobin, haematocrit, renal function, c-reactive protein (CRP), the number of thrombocytes and leucocytes. Obvious insignificant predictors (P-value > 0.2) were stepwise removed from the model.

Results: In 2006–2009 we included 4762 admissions of 3798 individual patients. We found 1128 admissions (23.7%) during which an INR $\geq$ 4.5 was found for 920 individual patients.

Strongly significant positive predictors were: age (odds ratio (OR) 1.01 per year, 95% confidence interval (CI) 1.004–1.014), being a medical patient (OR 1.56, 95% CI 1.29–1.89), concomitant use of amiodarone (OR 1.52, 95% CI 1.22–1.91), a previous event (OR 1.28, 95% CI 1.03–1.57) and the number of concomitantly use medicines (OR 1.15, 95% CI 1.06–1.25).

Predictors associated with a lower risk of an INR $\geq$ 4.5 were: male sex (OR 0.80; 95% CI 0.69–0.94) and the concomitant use of LMWHs (OR 0.625, 95% CI 0.503–0.776).

Summary/Conclusions: Ten predictors that positively contribute to the increased risk of an INR $\geq$ 4.5 were identified. The strongest predictors were: being a medical patient, concomitant use of amiodarone and an INR $\geq$ 4.5 during a previous hospital admission. The prediction model with routinely collected data can be used to identify high risk patients in every day practice, enabling targeted care by physicians and pharmacists

PA 1.10-4

Inhibiting coagulation factor XIIa potently prevents thrombosis in a rabbit arteriovenous shunt model

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Background: The intrinsic pathway of coagulation is initiated by contact activation of coagulation factor XII (FXII) at negatively charged surfaces, such as glass *in vitro* or platelet polyphosphates and collagen *in vivo*. Contact activation resulting from the exposure of blood to foreign surfaces from implanted prosthetics (e.g. artificial heart valves) or during invasive medical procedures such as cardiopulmonary bypass surgery can trigger life-threatening thrombotic processes and requires robust anticoagulation. However, anticoagulants are associated with an increased bleeding risk and need to be used cautiously. Interestingly, recent studies have demonstrated that FXII-deficient mice as well as rodents treated with FXIIa inhibitors display a robust antithrombotic efficacy without impairment of physiological hemostasis. These findings suggest that inhibiting FXIIa may serve as an ideal antithrombotic approach, especially in medical scenarios involving contact activation at foreign surfaces.

Aims: In the present study an arteriovenous (A/V) shunt model containing a procoagulant glass element was employed to assess the antithrombotic efficacy of two selective and potent inhibitors of FXIIa, i.e. rHA-Infestin-4, a recombinant protein derived from the hematophagus insect *Triatoma infestans*, and a fully human monoclonal antibody (anti-FXIIa MAb).

Methods: Female rabbits were randomly assigned to study groups and treated intravenously with either a single dose of 100 mg/kg rHA-Infestin-4 or 7 mg/kg anti-FXIIa MAb 10 min prior to the start of blood flow through the A/V shunt. Control groups received isotonic saline or 300 IU/kg heparin, respectively. Blood flow was monitored for 30 min in the rHA-Infestin-4 study whilst in the second study (anti-FXIIa MAb) the observation time was extended to 60 min. At the end of the observation period, thrombus weights and bleeding parameters (ear and kidney bleeding) were evaluated.

Results: In saline treated controls profound occlusion of the glass element in the A/V shunt was observed, whereas heparin treatment prevented thrombus formation as expected. Both rHA-Infestin-4 and anti-FXIIa MAb treatment potently inhibited thrombotic occlusion of the A/V shunt. Most notably and in sharp contrast to the increased bleeding observed with heparin treatment, neither ear bleeding time, nor time to hemostasis or blood loss after kidney incision were impaired by rHA-Infestin-4 or anti-FXIIa MAb.

Summary/Conclusion: The results of this study clearly demonstrate a potent antithrombotic efficacy of FXIIa inhibition by rHA-Infestin-4 and anti-FXIIa MAb in a rabbit arteriovenous shunt model where occlusive thrombus formation is initiated by contact activation on a glass surface. Importantly, inhibiting FXIIa had no effect on physiological hemostasis. Therefore, targeting FXIIa may represent a new approach for safe anticoagulation therapy in medical scenarios involving contact activation at foreign surfaces.

PA 1.10-5

Treatment of acute VTE with rivaroxaban. updated results of the prospective Dresden NOAC registry (NCT01588119)

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Background: In the EINSTEIN program rivaroxaban has been found to be at least as effective and safe as warfarin in treatment of acute

venous thromboembolism (VTE), which lead to approval of rivaroxaban in many countries. However, patients in RCT's present a selected population treated under a strict protocol and followed for a short period of time. Consequently, efficacy and safety of new oral anticoagulants (NOAC) need to be confirmed in cohorts of unselected patients in daily care.

Aims: To evaluate the efficacy and safety of acute VTE treatment with rivaroxaban in daily care.

Patients and Methods: The Dresden NOAC registryis a prospective, non-interventional registry. A network of more than 230 physicians from private practice and hospitals enrol eligible patients, who are centrally followed by the registry office. Inclusion criteria are: 1) indication for therapeutic NOAC anticoagulation > 3 month; 2) age > 18 years; 3) written informed consent; 4) availability for follow-up. No Exclusion criteria apply. In the registry, up to 2000 patients will receive prospective follow up (FU) by phone visits at day 30 day and quarterly thereafter to collect efficacy and safety data. All events are centrally adjudicated based on copies of reports, patient charts, autopsy reports and death certificates and using standard definitions.

Results: Until December 31th 2012, 1665 patients were registered. Of these, 233 patients received rivaroxaban for acute VTE treatment (54.9% female). In our registry, the population receiving acute VTE treatment is older than the EINSTEIN population (63.5 vs. 55.8 years). Most patients were treated for DVT vs. PE (74.7% vs. 25.3%) and 28.3% had recurrent VTE.

During follow-up, no recurrent VTE event occurred, but one patient (0.48 per 100 patient years) had a major cardiovascular event (acute limb ischaemia). Furthermore, 71 patients (34 per 100 patient years) had bleeding complications, in eight cases (3.8 per 100 patient years) major bleeding according to ISTH definition, one of which was a fatal intracranial bleeding (0.48 per 100 patient years). Eight patients died (3.8 per 100 patient years) but none of acute thromboembolic or cardiovascular events (three terminal malignant disease, two chronic heart failure, two sepsis, one fatal bleeding).

At 6 month, 61.5% of patients were still taking rivaroxaban. The remaining patients had planned end of treatment (34.2%), were switched to other anticoagulants (4.3%, mostly for side effects or cost issues) or prematurely discontinued (4.3%). Therefore, unplanned discontinuation rate at 6 month was 8.6% (mostly side effects, cost issues, incompliance, malignant disease, unplanned surgery).

Conclusions: In unselected patients in daily care, acute VTE treatment with rivaroxaban is effective and safe with low rates of cardiovascular or bleeding events in the first 180 days of treatment. Overall, adherence to rivaroxaban therapy is over 90% and unplanned discontinuations occur in < 10% and equally because of side effects, cost issues or concomitant diseases.

For presentation at the ISTH meeting, updated results on demographic and clinical characteristics at baseline, cardiovascular and bleeding event rates will be provided.

PA 1.10-6

The FXIIa inhibitor rHA-Infestin-4 safely protects from arterial and venous thrombosis in rodent and non-rodent species

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Background: Ischemic stroke provokes initial brain damage and is still one of the most predominant diseases in industrialized countries with a high rate of mortality or severe disability, respectively. To reduce primary insults based on the main pathophysiological processes of vessel occlusion in the brain, restoration of blood flow is essential. Paradoxically, reperfusion of brain tissue triggers further pathophysiological mechanisms and results in a process termed ischemia/reperfusion injury (I/R injury). The coagulation factor XII (FXII)-driven contact activation system is widely discussed to play an important role within the processes of I/R injury.

Aims: The aim of the current studies was to assess the efficacy of the FXIIa-inhibitor rHA-Infestin-4 in a rat model of I/R injury in a prophylactic and a therapeutic medication.

Methods: Rats were randomly assigned to the treatment groups and all studies were performed in a blinded manner. Within prophylactic treatment, animals were treated intravenously with 100 mg/kg rHA-Infestin-4 or an equal volume of saline 15 min prior to transient middle cerebral artery occlusion (tMCAO). For therapeutic treatment, 100 mg/kg rHA' -Infestin-4 or an equal volume of saline was administered directly after start of reperfusion. tMCAO was induced using the filament technique. Occlusion held up for 90 min with subsequent reperfusion. Twenty-four hours after tMCAO, rats were tested for neurological deficits (Zea Longa score, a composite neurological score, rotarod assay and grip strength test). Thereafter, brains were removed and analyzed for infarct area and edema formation. Furthermore, blood was terminally withdrawn within the prophylactic treatment approach for additional coagulation assays.

Results: Compared to the control group, rHA-Infestin-4 significantly improved survival in both, the prophylactic as well as therapeutic treatment. Moreover, neurological deficits by means of Zea Longa score and a composite neuroscore were significantly reduced after application ofrHA-Infestin-4 in both treatment approaches. In addition, prophylactic application of rHA-Infestin-4 significantly reduced infarct area and edema formation, and furthermore significantly improved performance in the rotarod assay and grip strength test. Using the therapeutic setting, a trend towards improvement in infarct area and edema formation was found whilst performance in the rotarod assay and grip strength test was unchanged between the groups. Following application ofrHA-Infestin-4, inhibition of FXIIa and aPTT prolongation were still prominent 24 h after tMCAO whilst PT was not affected.

Summary/Conclusions: Although beneficial effects were more prominent within the prophylactic treatment, rHA-Infestin-4 competently ameliorated outcome after acute ischemic stroke in rats. With regard to the central role of FXII-driven contact activation system in stroke, inhibition of FXIIa via rHA-Infestin' -4 may represent a new and promising treatment approach for brain I/R injury.

PA1.11 – Blood Coagulation Tests – I

PA 1.11-1

Activated Factor VII. Antithrombin Complex plasma concentration in subjects with or without angiographically demonstrated coronary artery disease and myocardial infarction

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Background: Plasma concentration of activated factor VII (FVIIa)-antithrombin (AT) complex has been recently proposed as a marker of intravascular exposure of tissue factor and, thus, of activation of the extrinsic pathway of coagulation cascade. However, the results of studies investigating the potential relationship between FVIIa-AT complex and cardiovascular disease are only preliminary so far.

Aims: The aim of this study was to investigate the relationship of FVIIa-AT complex plasma concentration with either coronary artery disease (CAD) or myocardial infarction (MI) in a case-control setting.

Methods: Within the framework of the Verona Heart Study (VHS), a regional survey aimed to search for new risk factors for CAD/MI in subjects with angiographic documentation of their coronary vessels, we selected a total of 686 subjects (546 CAD and 140 CAD-free), who

were not taking anticoagulant drugs and for whom plasma citrate samples for FVIIa-AT complex assay were available. Among CAD patients, 312 CAD had a history of previous MI. Plasma concentration of FVIIa-AT complex was determined by ELISA.

Results: There was no significant difference in plasma concentration of FVIIa-AT complex between CAD and CAD-free subjects (84.8 (80.6–88.2) vs. 83.9 (76.7–92.8) pM, respectively – $P = 0.949$). On the other hand, among CAD patients those with history of previous MI had lower FVIIa-AT complex levels than those without history of previous MI (79.8 (75.9–83.9) vs. 90.9 (85.6–97.5) pM, respectively – $P = 0.008$) and this association remained significant in a regression model also after adjustment for all the traditional cardiovascular risk factors.

Evaluating the potential relationships of FVIIa-AT complex with other clinical and laboratory variables in the whole study population, we found significant correlations with gender (higher in females), diabetes (higher in diabetic patients), body mass index (BMI), estimated glomerular filtration rate, total cholesterol, HDL-cholesterol, and triglycerides. In 305 subjects, for whom data about FVIIa levels and FVII genotype were available, a very strong direct correlation with FVIIa was found (beta coefficient = 0.533, $P < 0.001$). Moreover, FVII genotypes known to influence FVIIa levels (e.g. −323 A1/A2 and R353Q polymorphisms) were associated – in a concordant manner with FVIIa levels – also with significantly different concentrations of FVIIa-AT complex.

In a multiple adjusted linear regression model explaining the 34% of FVIIa-AT complex variability, FVIIa (beta coefficient = 0.464, $P < 0.001$), estimated glomerular filtration rate (beta coefficient = −0.131, $P = 0.044$), HDL-cholesterol (beta coefficient = 0.147, $P = 0.039$), and triglyceride (beta coefficient = 0.201, $P = 0.002$) remained significant predictors of FVIIa-AT plasma concentration. On the other hand, the statistical association of FVII genotypes with FVIIa-AT concentration appeared to be mediated by the influence on FVIIa levels.

Conclusions: In this case-control study FVIIa-AT complex plasma concentration was associated with some cardiovascular risk factors, like plasma lipids and renal function, as well as with FVIIa levels. There was no significant difference between subjects with or without CAD. On the other hand, the association of low levels with MI history among CAD subjects should be further investigated and could derive from some potential biases (e.g. survival-related bias). Additional studies are needed to clarify the role of FVIIa-AT complex in CAD and MI.

PA 1.11-2

The appearance of the second peak in platelet rich plasma thrombin generation curve can be provided by antiplatelet compounds: mechanism and possible applications

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Background: Measurement of thrombin generation in clotting blood plasma is now convenient and informative test to analyze a status of coagulation system. Usually the thrombin generation curve obtained in platelet poor plasma (PPP) has one peak. Here we show that this curve obtained in platelet rich plasma (PRP) can have two peaks in the presence of low doses of platelet inhibitors. Also we present the new modification of thrombin generation assay (TGA) based on this phenomenon which can correspond with the tendency of bleeding in severe hemophilia A (HA).

Aims: We explored the mechanism of the second peak appearance and investigated the ability of the new modification of TGA in PRP to determine the clinical phenotypes of severe HA.

Methods: Blood was collected from healthy donors and patients suffering from severe HA (FVIII < 1 IU/dL) by venipuncture. Eleven patients with mild phenotype and 10 with severe one took part in the study. All the donors signed the written inform consent which was approved by ethics committee. Tissue factor induced thrombin generation in PRP and PPP was monitored using continual measurement of hydrolysis rate for thrombin specific fluorogenic substrate Z-Gly-Gly-Arg-AMC. Phosphatidylserine (PS) and CD62P expression on the activated platelets in purified suspension were measured by flow cytometry using annexin' – V-RPE and antiCD62P-FITC staining.

Results: Addition of P_2Y_{12} receptor antagonist 2'-MeS-5'-AMP (160 μM), 83 nM prostaglandin E_1 (PGE_1) or 1.6% DMSO to healthy PRP caused the appearance of two peaks in the thrombin generation curve. All these compounds in used concentrations did not affect significantly endogenous thrombin potential. Supplementation of these agents to purified platelet suspension decreased PS exposure rate after activation with 35 nM of thrombin. However, these compounds did not affect CD62P expression. Decreasing the thrombin concentration to 0.545 nM led to the inhibition of CD62P expression by 830 nM of PGE_1. Supplementation of PRP containing 1.6% DMSO with 830 nM PGE_1 mediated the second peak disappearance and decreased the first peak amplitude. Increasing the platelet concentration in PRP promoted the arrangement of two peaks into one. To investigate the severe HA phenotypic heterogeneity we used TGA in PRP with 1.6% DMSO and the concentration of platelets was equal to 100,000 platelets/μL. HA patients with mild phenotype demonstrated significantly higher second peak amplitude in PRP than ones with severe bleeding tendency.

Conclusions: The second peak in the PRP thrombin generation curve can be mediated by reduced PS expression rate provided by platelet inhibitors. However, α-granules do not take part in the second peak appearance. The modified TGA can determine the bleeding tendency in HA. The possible mechanism of phenotypic heterogeneity is linked with thrombin generation mediated by PS-expressing platelets.

PA 1.11-3

Assessment of thrombin formation in patients with ulcerative colitis without a history of thrombotic events

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Background: It is known that there is a strong relation between inflammation and hemostasis and inflammation causes tendency to the development of thrombosis. Ulcerative colitis (UC) is an inflammatory disease of the colon that follows a course of relapse and remission with a predisposition to thrombosis. The association between thrombin generation and inflammation in certain local and/or general inflammatory disorders with tendency to thrombosis such as ulcerative colitis remains unclear.

Aims: The aim of this study is to evaluate the pattern of thrombin generation tests along with the inflammatory markers in patients with active and inactive UC without a history of thrombotic event.

Methods: Sixty-one patients (age between 18 and 65 years) with UC diagnosed with clinical, endoscopic and histopathological findings and 36 age and gender matched healthy controls have been enrolled in the study. Thirty-three patients had active disease while 28 were in remission. Disease activity was scored using Mayo score of UC. Approval of the local ethical committee and informed consent of all the participants were obtained. Prothrombin time, activated partial thromboplastin time, fibrinogen, D-dimer (Diagnostica Stago), Thrombin Generation Assay (TGA; Thermo Electron Corporation, Flouroscan Ascent; 5 pM TF), Thrombin anti-thrombin complex (TAT; Assay-Max Human TAT), high sensitive C-reactive protein (hs-CRP; DRG CRP-HS, ELISA) tests were performed. The parameters of thrombin generation assay (lag time, thrombin peak, Endogen Thrombin

Potential-ETP, time thrombin peak, start tail) were compared with inflammatory markers. *P*-values < 0.05 were regarded as significant. Sensitivity and specificity values were determined by receiver operating characteristic (ROC) curve analysis for each parameter.
Results: Levels of fibrinogen (452.33 ± 126.97 mg/dL; $P = 0.000$), D-dimer (1.75 ± 3.46 mg/dL; $P = 0.000$) and hs-CRP (9.81 ± 3.23 mg/L; $P = 0.001$) were higher in patients with active disease compared to patients in remission and controls, while TAT values (1.23 ± 0.65 ng/mL; $P = 0.697$) were similar in all groups. Within the TGA, lag time was longer (3.93 ± 0.73 min; $P = 0.000$), thrombin peak higher (391.57 ± 62.94 nm; $P = 0.007$) and start tail was observed to be longer (24.18 ± 3.54 min; $P = 0.001$) in patients with active disease. Thus ETP (2191.42 ± 526.36 vs. 1974.89 ± 408.68 nm; $P = 0.296$) and time thrombin peak (6.21 ± 0.95 vs. 5.80 ± 0.91 min; $P = 0.112$) values were observed to be unchanged. As with the sensitivity and specificity values to distinguish active disease, values regarding fibrinogen (78.8%;70.1%), D-dimer (75.7%;74.1%), hs-CRP (75.8%;58.1%), lag time (75.6%;65.7%), thrombin peak (54.5%;73.4%), ETP (39.9%;82.8%), peak (54.5%;73.4%), tt peak (78.8%;42.1%) and start tail (75.6%;53.1%) were determined respectively.
Summary/Conclusions: Activity index was observed to be well correlated with the increased inflammatory markers (fibrinogen and hs-CRP). D-dimer and thrombin peak were observed to be useful as markers of thrombin generation since levels of D-dimer and TGA-thrombin peak was higher when TAT levels were unchanged. D-dimer and fibrinogen showed the highest sensitivity and specificity while thrombin peak and ETP showed low sensitivity thus highest specificity. According to the results of the study, D-dimer and thrombin peak are the best parameters to demonstrate dynamic nature thrombin generation in patients with ulcerative colitis.

PA 1.11-4

Simultaneous measurement of thrombin generation and fibrin formation in plasma and whole blood applying continuous flow

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Background: Thrombin is the enzyme converting fibrinogen into fibrin. Assays measuring the thrombin generation and fibrin formation are often used separately in research and clinical settings. Recently it was shown that thrombin generation and fibrin formation do not always go hand in hand as factor XII independently of thrombin influences the crosslinking of fibrin and stability of the clot.
Aims: The development of an assay that is able to simultaneously measure thrombin generation and fibrin formation in plasma/whole blood.
Methods: We have redesigned an air-bearing rheometer, based on the plate and cone viscositor, rendering it sensitive enough to measure fibrin formation based on changes in viscosity. Placing a confocal fluorescent camera into the rheometer allows the measurement of thrombin generation based on the conversion of a thrombin-sensitive substrate, as in the Calibrated Automated Thrombogram (CAT) assay. Using this method, the endogenous thrombin potential (ETP), peak height and lag-time can be calculated. The measurement of fibrin formation results in the variables: lag-time, maximum clot strength and time to maximum clot strength. The rheometer further allows the application of different laminar flow conditions within the range of 50/s until 1200/s.
Results: For all variables related to fibrin formation and thrombin generation, the intra- and inter-assay variation were below 10% in platelet-poor and -rich plasma and whole blood. Increasing flow rates (100–1200/s) in platelet-poor plasma triggered with 5 pM TF resulted in reduced ETP and peak values in the thrombin generation (ETP 1113 nM/min [100/s]; 776 nM/min [1200/s] and peak 264 nM [100/s]; 209 nM [1200/s]). Accordingly, maximum fibrin clot strength was inversely related to increased flow rates (7.7 mPa/s [100/s]; 1.9 mPa/s [1200/s]). As the amount of fibrinogen influences both thrombin generation and clot strength we tested the effect of different fibrinogen concentrations (0–5 mg/mL) in our system, applying a shear rate of 300/s in the presence of 5 pM TF. As expected, an increase in fibrinogen was accompanied with dose-dependent elevated ETP (734 nM/min [0 mg/mL]; 1073 nM/min [5 mg/mL]), peak (199 nM [0 mg/mL]; 323 nM [5 mg/mL]) and maximum clot strength (0 mPa/s [0 mg/mL]; 4.7 mPa/s [5 mg/mL]). By simultaneously measuring the thrombin generation and fibrin formation, we calculated that only 1 nM of thrombin is sufficient to polymerize fibrin and cause a change in viscosity. Additionally, we confirmed that an increase in factor XII enhances the clot strength without influencing thrombin generation, when coagulation is initiated via the extrinsic pathway.
Conclusions: We have developed a new method capable of simultaneously measuring thrombin generation and fibrin formation in plasma and whole blood under continuous flow. Our method proved to be sensitive enough to detect differences in fibrinogen content and established that only a small amount of thrombin is necessary to initiate clot formation. Additionally, this technique allows to study the influence of venous vs. arterial flow rates on both thrombin generation and clot strength, bringing coagulation experiments one step closer to physiology.

PA 1.11-5

Preoperative thrombin generation is predictive for the risk of blood loss after cardiac surgery

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Background: Bleeding after cardiopulmonary bypass is a major cause of morbidity and mortality. The contact of blood with the artificial surfaces of the extracorporeal circulation during cardiac surgery results in activation of coagulation, fibrinolysis and platelets that is recognized as potential reasons for increased bleeding tendency.
Aim: To investigate whether the calibrated automated thrombin generation (CAT) can be used as a predictive tool for blood loss after cardiac surgery with cardiopulmonary bypass (CPB) compared to the routine coagulation tests.
Methods: Thirty male patients undergoing first-time coronary artery bypass grafting were enrolled. Blood samples were taken pre-bypass before heparinization (T1) and 5 min after protamine administration (T2). TG was measured both in platelet-rich plasma (PRP) and in platelet-poor plasma (PPP) and the parameters of the CAT (ETP, peak, lag time and time to peak) were evaluated. Activated clotting time (ACT), hematocrit, hemoglobin, platelet number, fibrinogen, antithrombin, D-dimers, prothrombin time (PT) and activated partial thromboplastin time (aPTT) were also determined. Blood loss was measured and the amount of transfusion products was recorded postoperatively until 20 h after surgery. Patients were divided in two groups based on the median volume of postoperative blood loss (group 1: patients with median blood loss < 930 mL; group 2: patients with median blood loss ≥ 930 mL).
Results: Patients with blood loss > 930 mL had a significantly lower ETP and peak thrombin pre-bypass than patients with blood loss lower than 930 mL ($P = 0.000$ and $P = 0.004$ resp.) in PRP, as well as in PPP ($P = 0.004$ and $P = 0.014$). After bypass, ETP and peak thrombin remain significantly lower ($P = 0.011$ and $P = 0.010$) in patients of group 2, measured in PRP but not in PPP. Fibrinogen was higher both

pre- and postoperatively in group 1, but only statistically significant postoperatively ($P = 0.045$).
Platelet level was also significantly elevated in group 1 compared to group 2 ($P = 0.002$), pre- and after bypass. However, no significant difference between the two patient-groups was observed when blood loss was associated with the D-dimers levels or with the clotting time-dependent tests (ACT, aPTT and PT), pre and post-CPB.
Conclusions: This study clearly demonstrates that the thrombin generation assay when performed preoperatively in the absence and presence of platelets is a predictive tool for blood loss after cardiac surgery. The clotting time-dependent tests on the other hand do not show any significant association with bleeding after surgery.

PA 1.11-6
Fibrin monomers improves CHA2DS2-VASc risk prediction in chronically anticoagulated atrial fibrillation patients

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Atrial fibrillation (AF) is associated with abnormalities of haemostasis, endothelium and platelets, which is independent of structural heart disease and AF aetiology – suggesting that AF confers a prothrombotic or hypercoagulable state. To aid decisions for thromboprophylaxis, several risk stratification schemes have been developed using clinical characteristics, the most popular being the $CHADS_2$ score and more recently, the CHA_2DS_2-VASc score. There are limited data on the prognostic role of biomarkers patients taking oral anticoagulants (OAC). Soluble fibrin monomers, as markers of fibrin formation, have been studied as a risk marker in various situations related to a hypercoagulable state as deep vein thrombosis or disseminated intravascular coagulation.
The aim of our study was to analyze the prognostic role of FM in steady anticoagulated AF patients to set if it could refine clinical risk stratification
Methods: We studied 904 patients (50% male; median age 76) with paroxysmal/permanent AF who were stabilised (for at least 6 months) on oral anticoagulation (INRs 2.0–3.0). Plasma FM was measured by immunoturbidimetry using STA-LIATEST FM (Diagnostica Stago, France) at baseline. Patients were followed-up for up to 2 years, and adverse events (thrombotic and vascular events and mortality) were recorded. FM cut-off was assessed by ROC curves.
Results: Median (IQR) values of FM were 8.42 (3.53–12.18) pg/mL. Median follow-up was 955 (785–1096) days, and during this period, 95 (4.04%/year) died whilst 111 patients had an adverse cardiovascular event (4.70%/year), from them 40 were strokes (1.7%/year). On multivariate analysis, high FM (> 4.79 pg/mL) remained significantly associated with both stroke and vascular events even after adjusting for CHA_2DS_2-VASc score, with adjusted hazard ratios (HRs) of 2.66 (1.26–5.58, $P < 0.001$) for stroke and 2.64 (1.69–4.13, $P < 0.001$) for adverse vascular events. For all cause mortality, the adjusted HR was 1.61 (1.04–2.48, $P = 0.031$). Based on c-statistics, FM improved the prediction for stroke/systemic embolism using the CHA_2DS_2-VASc score (0.63 ± 0.04, $P = 0.005$–0.68 ± 0.04, $P < 0.001$).
Conclusion: In a large 'real world' cohort of anticoagulated AF patients, FM levels could provide complementary prognostic information to an established clinical risk score (CHA_2DS_2-VASc) for prediction of long-term stroke/systemic embolism, suggesting that this biomarker may potentially be used to refine clinical risk stratification in AF.

PA1.12 – Coagulation Factor IX – I

PA 1.12-1
Quantitative whole body autoradiography (QWBA) study on the effect of albumin fusion on the biodistribution of recombinant factor rIX

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Background: The recombinant fusion protein linking the human coagulation factor IX (FIX) to recombinant human albumin (rIX-FP) is currently investigated in clinical phase II/III trials (PROLONG-9FP) for prophylaxis and on-demand treatment of bleeding in haemophilia B patients. However, to date, there is only limited information available on the tissue distribution of FIX products following intravenous administration.
Aim: The present study was designed to explore the biodistribution of recombinant Factor IX (rIX) and how albumin fusion may affect it.
Methods: [^{3}H]-rIX-FP, [^{3}H]-rFIX or [^{3}H]-albumin, labeled using the N-Succinimidyl [2,3,-^{3}H] propionate (NSP) method, were administered intravenously to male rats at a single radioactive dose of approximately 400 μCi/kg. Using quantititave whole-body autoradiography (QWBA), tissue radioactivity was determined over 24 ([^{3}H]-rFIX) or 240 ([^{3}H]-rIX-FP, [^{3}H]-albumin) h. In addition to full body sections, the hind limbs were separately subjected to QWBA to obtain more detailed information on the products' distribution within the bone marrow and knee joint region. In parallel, plasma, urine and feces were collected at several time points throughout the observation period to calculate excretion balance and assess physiological elimination pathways. The radioactivity associated with the [^{3}H]-labelled proteins was determined by quantitative radiochemical analysis (QRA) and high performance liquid chromatography (HPLC). The radioactivity associated with plasma, urine and feces samples was also determined using QRA. Biological activity of rFIX and rIX-FP after [^{3}H]-labeling was confirmed by a chromogenic assay *in vitro* followed by *in vivo* measurements of hemostasis in hemophilia B mice treated with 'cold'-labeled rIX-FP material.
Results: Elimination of [^{3}H]-rFIX and [^{3}H]-rIX-FP occurred primarily via the urine. Overall, 73% of radioactivity was recovered in urine (associated with only low molecular weight components), ≤ 5% eliminated in faeces and about 20% remained in tissues after 240 h. The tissue distribution of [^{3}H]-rIX-FP and [^{3}H]-rFIX was comparable, both penetrating predominantly into well vascularized tissues and/or excretion organs including the adrenal gland, spleen, lung, liver, kidney, myocardium and gastrointestinal mucosa. Both proteins were also rapidly present within bone marrow and synovial or mineralized regions of knee joint sections where they seemed to mostly localize to the zone of calcified cartilage within the growth plate regions of long bones. The longest retention times were observed for bone marrow and the endosteum of long bones. Overall, these results suggest similar tissue distribution profile of [^{3}H]-rIX-FP and [^{3}H]-rFIX independent of albumin fusion. In contrast, the biodistribution of [^{3}H]-albumin appeared to be different with only very low initial penetration of bone marrow and liver but rapid, homogeneous distribution throughout the whole body including muscle, skin and connective tissue. Intriguingly, both [^{3}H]-rIX-FP and [^{3}H]-albumin derived radioactivity was well detectable over 72 h, whereas comparable [^{3}H]-rFIX associated radioactivity was only detectable over 24 h, further supporting the notion of extended tissue half-life of [^{3}H]-rIX-FP due to albumin fusion.
Summary/Conclusion: The study indicates that rIX-FP exhibits equal biodistribution compared to other marketed rFIX products, but clearly distinguishes itself by an extended plasma half-life and tissue retention pointing towards a reduction in dosing frequency leading to increased therapeutic convenience and compliance.

PA 1.12-2

Expression and characterization of a novel recombinant factor IX molecule with enhanced clotting activity

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Background: Hemophilia B is an inherited coagulation defect characterized by a deficiency in human factor IX (FIX). Patients with hemophilia B are commonly treated with FIX concentrates. Production of a recombinant human coagulation factor IX (rhFIX) with increased clotting activity is one of the current challenges for hemophilia B treatment. In addition, increasing potency of gene transfer vectors may improve their therapeutic index, as lower plasma concentrations may achieve therapeutic benefit following *in vivo* administration.
Aim: The purpose of our study was to obtain a rhFIX molecule with an increase of specific activity.
Methods: In order to improve the activity of FIX, we focused on an important amino acid sequence known to be potentially involved in the interface region between activated FIX (FIXa) and its cofactor, the activated factor VIII (FVIIIa).
Our group recently showed that the human hepatoma cell line HuH-7 represents an effective cellular system for production of rhFIX with improved posttranslational modifications and therefore significantly better specific activity. Using site-directed mutagenesis, we have developed and produced four mutated rhFIX produced by the HuH-7 cell line. These mutations correspond to the following substitutions on glutamate 410 (E410): rhFIX-E410H, rhFIX-E410A, rhFIX-E410L and rhFIX-E410N.
Results: The four recombinant FIX molecules were produced following stable transfection. Similar secretion rates were observed for both rhFIX-E410 and wild type rhFIX. Western blot analysis showed that the mutated rhFIX molecules presented the same molecular weight than wild type FIX. The activation time course of equimolar amounts of mutated rhFIX-E410 and wild type rhFIX cleaved by the activated factor XI or the activated factor VII – tissue factor complex was also very similar giving FIXa heavy and light chains which appeared within 2 min.
After purification using an ion exchange column, the concentration of rhFIX molecules was determined by ELISA assay. We assessed *in vitro* clotting activity of the rhFIX molecules and evaluated their procoagulant effect using thrombin generation test.
Our results showed that mutated rhFIX-E410 had 3–5 times increased procoagulant activity compared to wild type rhFIX produced in our laboratory using the same cell line. We also observed a significantly higher thrombin generating capacity specifically with rhFIX-E410H compared to wild type rhFIX. Thrombin peak induced by rhFIX-E410H was 4.5 times greater than wild type rhFIX.
Conclusion: Using HuH-7 cell line and mutations on amino acid 410, we produced a rhFIX molecule with significantly improved specific activity as demonstrated by aPTT (activated partial thromboplastine time) based FIX clotting assay and thrombin generation test. We hypothesized that the increased specific activity induced by E410 mutations might be explained by an enhanced affinity between of activated rhFIX towards FVIIIa. Further tests are currently carried in our laboratory to verify our hypothesis.

PA 1.12-3

Pharmacokinetics of buccally and intravenously delivered transgenic recombinant and plasma derived Factor IX

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Introduction: Our previous studies showed that transgenic pig milk containing human Factor IX (tg-FIX) fed to hemophilia B (FIXKO) mice can correct the hemostatic defect in a tail transection model while achieving only low plasma tg-FIX levels. In the present studies we have examined the pharmacokinetics of non- and intravenously administered tg-FIX relative to plasma derived (pd-) FIX. Here, calcium phosphate (CaP:) microparticles of FIX were chosen as a buccal delivery vehicle for both tg-FIX purified from transgenic FIX pig milk and pd-FIX.
Buccal Administration in Mice: Due to the highly keratinized nature of the mouse mouth, relatively large amounts of FIX were buccally administered (10 mg FIX/kg body weight) to obtain changes in plasma FIX antigen levels. The plasma levels of human FIX antigen began to rise within 2 h after buccal delivery of CaP:pd-FIX in three of five normal mice (3/5). The peak values of FIX antigen ranged between 147 and 197 ng/mL and was undetected after about 48–72 h. We also examined the buccal administration of CaP:tg-FIX to hemophilic B mice expressing an inactive human FIX mutant (R333QhFIX). These R333QhFIX mice exhibited baseline levels of human FIX antigen of 100–600 ng/mL. Similarly to that of buccal delivery of CaP:pd-FIX in normal mice, five of seven mice (5/7), antigen levels rose > 200 ng/mL above baseline within 2 h after buccal delivery of CaP:tgFIX. One stage FIX activity values were at the limit of detectability. The low levels of coagulation activity relative to the rise in antigen level could be explained by displacement of inactive endogenous R333QhFIX from intravascular reservoirs by the buccally delivered tg-FIX. Therefore, we next examined the relative partitioning behavior of tg-FIX vs. that of pd-FIX by crossover intravenous infusion in hemophilic B dogs.
Vascular partitioning behavior of intravenously infused tg-FIX vs. pd-FIX in hemophilic B dog. We examined pharmacokinetic behavior of intravenously infused transgenic tg-FIX vs. pd-FIX in crossover studies done over a 7 day period in hemophilic B dogs at 50 IU/kg body weight. Large differences between the partitioning of tg-FIX and pd-FIX into extravascular reservoirs were observed. When the pd-FIX was infused first and then followed 2 days later by an infusion of tg-FIX, the mean residence times were 31.1 h for pd-FIX and 39.0 h for tg-FIX. When the crossover infusion was reversed with tg-FIX followed by pd-FIX, the mean residence time was 5.5 h for tg-FIX and 40.2 h for pd-FIX. Hence, the prior intravenous loading of either species resulted in the lengthening of the mean residence time of the latter infused species. This suggests a significant amount of extravascular reservoir occupation by the agent infused first.
Discussion and Conclusions: These studies of intravenous and buccal delivery of pd- and tg-FIX help to define the general bioavailability of the tg-FIX based upon its rapid extravascular partitioning. Due to the low bioavailability of buccally delivered FIX, transgenic livestock will likely be the only reasonable source of FIX.

PA 1.12-4

Detection of galactose-alpha-1,3-galactose (a-Gal) and *N*-glycolylneuraminic acid (NGNA) in recombinant and plasma derived FIX products

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Background: Recombinant factor IXFc fusion protein (rFIXFc) is a recombinant fusion protein composed of a single molecule of FIX covalently linked to the human IgG1 Fc domain. It is a long lasting clotting factor that has been investigated in a clinical study in Hemophilia B patients. The commercially available FIX products are manufactured in either CHO cell lines or derived from human plasma. The rFIXFc is manufactured using the human cell line HEK293. Non-human glycosylation pattern has been observed in some recombinant proteins. The terminal galactose-alpha-1,3-galactose (α-Gal) antigen is produced by all mammals except humans, old world monkeys, and apes. Antibodies against this antigen constitute approximately 1% of all circulating antibodies in humans. It was reported that CHO cell lines possess the enzyme catalyzing α-Gal synthesis and α-Gal was found in CHO produced therapeutic proteins including FVIII. Another example is the sialic acid *N*-glycolylneuraminic acid (NGNA). Healthy humans are genetically unable to produce NGNA, and circulating anti-NGNA antibodies are present in most normal humans. Therefore, α-Gal and NGNA can be potentially immunogenic.
Aims: The goal of this study is to determine the presence or absence of α-Gal and NGNA in 3 FIX concentrates: Mononine (ZLB Behring), a plasma-derived concentrate; BeneFIX (Pfizer), a recombinant product produced in a CHO cell line, and rFIXFc, recombinant FIXFc fusion protein produced in HEK293 cells.
Methods: a-Gal measurement: the α-Gal moieties were released from FVIII by treating protein samples with α-(1-3,4,6)-galactosidase at 37 °C for 16 h. The α-Gal was labeled by 2-aminobenzoic acid (2-AA) and quantified by reversed-phase chromatography (RP-UPLC) coupled with fluorescence detector. Purified galactose was used to generate a calibration curve. The percentage mole α-Gal per mole of protein was calculated for all samples.
NGNA measurements: all protein samples were incubated with 50 mM sulfuric acid at 80 °C for an hour, and the released NGNA was then derivatized with DMB at 50 °C for 3 h. The DMB derivatized sample was analyzed by RP-UPLC with fluorescence detector. The NGNA standard was used to obtain the calibration curve. The percentage mole NGNA per mole of protein was calculated for all samples.
Results: α-Gal measurements: The limit of detection (LOD) and limit of quantitation (LOQ) were determined to be 0.3 pmol (0.9% for BeneFIX) and 0.1 pmol (0.3% for BeneFIX) respectively, and α-Gal was detected in BeneFIX (1.6%). As expected, no detectable α-Gal was found in either rFIXFc or Mononine (less than LOD of 0.3%).
NGNA measurements: The LOD and LOQ for NGNA were determined to be 2.5 fmol (0.1% for BeneFIX) and 4.5 fmol (0.25% for BeneFIX), respectively. No detectable NGNA was found in rFIXFc and Mononine, whereas NGNA was detected in BeneFIX ($0.27 \pm 0.02\%$).
Summary/Conclusions: The non-human glycosylation α-Gal and NGNA were detected in BeneFIX but not in rFIXFc and Mononine. Both α-Gal and NGNA have been reported to be responsible for generating immune responses in human, and therefore human glycosylation could result in lower immunogenicity, however, the impact of the presence of these antigens on the immunogenicity profile of FIX is not currently known.

PA 1.12-5

Impact of factor IXa content on function, safety and efficacy of recombinant factor IX products

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Background: Baxter has developed BAX 326, a new recombinant factor IX (rFIX) product for the treatment of patients with hemophilia B.
Aims: The presented studies were designed to evaluate the function, safety and efficacy of BAX 326 and a commercially available rFIX with regard to differences in activated FIX (FIXa) content.
Methods: The FIXa concentration of BAX 326 was 0.009 IU/mL and that for the commercially available rFIX was 0.106 IU/mL. As control, BAX 326 samples with an increased FIXa content were prepared. Samples were analyzed *in vitro* by one-stage clotting assay, non-activated partial thromboplastin time assay and thrombin generation assay. The thrombogenic potential of BAX 326 was assessed *in vivo* using a modified Wessler Test in rabbits at 750 IU/kg (10-fold human clinical dose). Efficacy of the rFIX products was studied in hemophilia B (FIX ko) mice that received prophylactic treatment with 75 IU/kg of BAX 326 or the commercially available rFIX. Finally, FIX-deficient mice were treated with BAX 326 or commercial rFIX and analyzed in a carotid occlusion model and one using thrombelastography to study primary pharmacodynamics of the two products.
Results: In all three functional *in vitro* assays, spiking of FIX with FIXa caused an increase in the measured FIX activity, with thrombin generation assay being affected most. No thrombogenic potential was observed with BAX 326 (individual scores of 0), whereas the mean score for the commercially available rFIX was 0.5. After increasing the FIXa concentration of BAX 326 to equalize it with the commercially available rFIX, a mean score of 0.42 (individual scores 0–0.5) was determined. Efficacy was comparable for the two recombinant FIX products in both pharmacodynamic models.
Summary/Conclusions: The *in vitro* results revealed that FIXa interferes with potency assignment of FIX. The data in the thrombogenicity model strongly suggested that the differences in preclinical thrombogenicity were caused by the higher FIXa content of the commercially available rFIX, thereby confirming earlier findings. The efficacy studies demonstrated that despite its lower FIXa content, BAX 326 was as efficacious as a commercially available rFIX at 75 IU/kg ($P < 0.0076$), indicating that FIXa does not contribute to the efficacy of a FIX product. The gathered data thus indicate that rFIX products should preferentially contain a low FIXa content.

PA 1.12-6

Two phosphatidylserine-positive platelet subpopulations are major in binding of coagulation factor IXa

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Background: The importance of membrane phase of proteolitic reactions on the activated platelets in the process of blood coagulation is known. Rate of these reactions if pass on the membrane increases by severalorders of magnitude. To achieve this the enzymatic complexes of protrombinase and intrinsic tenase areassembled on the membrane of activated platelets. Activated platelets include one PS-negative (PS-) non-procoagulant subopulation and two procoagulant phosphatidylserine-positive (PS+) subpopulations which strongly differ in the intracellular calcium concentration. The PS+ subpopulation with low intracellular calcium concentration became known only recently.

Aims: The aim of the study was to describe the properties of binding of the FIXa, a maincomponent of intrinsic tenase, to membranes of each of three known subpopulations of platelets.
Methods: FIXa was covalently labeled with fluorescein. Washed gel-filtered platelets were activatedat 2 × 108/mL with 100 nM thrombin in the presence of 2.5 mM CaCl2 for 15 min. Theywere incubated with different concentrations of fluorescein-labeled FIXa and analyzed witha FACS Calibur cytometer. The surface distribution of FIXa on the activated plateles wasalso imaged using confocal microscopy.
Results: The addition of different concentrations of labeled FIXa to the threesubpopulations demonstrated two types of binding with regard to the amount of bind factorper platelet. Two PS+ subpopulations showed insignificant difference (1.3-fold) in theirFIXa binding. Binding to the PS-negative subpopulation was approximately 12-fold smaller incomparison with the PS+ platelets. Generally, for both PS+ subpopulations thedependence of the bound factor quantity binding on the added FIXa concentration waslinear without any saturation up to very high concentrations of FIXa (2000 nM, which is byseveral orders of magnitude higher than the physiological value). Confocal microscopyshowed that FIXa localizes on the surface of the PS+ platelets to a some 'hat'-shapedformation. This localization could be important for additional acceleration of coagulationreactions.
Conclusions: Two PS+ subpopulations of platelets is better than the PS- in binding of FIXa by one orderof magnitude, their dependence of binding on the concentration of free FIXa is linear andwithout saturation. This suggests their major role in binding of FIXa during the clotting. Non-uniform, localized distribution of the FIXa on the surface of PS+ platelets, it's 'hat'-shaped formation, suggests that such a colocalization with other factors could workfor acceleration of coagulation reactions.

PA1.13 – Coagulation Factor VIII – I

PA 1.13-1

Residues within the BO2C11 epitope may participate in FVIII endocytosis by antigen presenting cells

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Background: Twenty to 30% of severe hemophiliacs develop antibodies to therapeutic factor VIII (FVIII). Immune responses to FVIII require T-cell help and evoke class-switched antibodies that necessitate active antigen uptake and presentation. Currently, our understanding of immune recognition and cellular mechanisms involved in FVIII uptake is limited. Recently, membrane-binding residues within the C1 domain of FVIII (R2090, F2092 and L2093) have been implicated in the interaction with *low-density lipoprotein receptor*-related protein (LRP/CD91) receptor. Alanine substitutions of these residues inhibit LRP-dependent FVIII endocytosis by U87 cells. Conversely, endocytosis of FVIII by monocyte-derived human dendritic cells (MODC) or mouse bone marrow-derived dendritic cells is independent of LRP receptor and may involve other yet unidentified receptor(s). Interestingly, FVIII containing substitutions in the membrane binding residues of C1 domain exhibits diminished immunogenicity *in vivo*, thus possibly implicating the relevance of these residues in antigen uptake.
Aim: Similar to C1, the C2 domain of FVIII interacts with membrane surfaces and involves several basic residues. We targeted these residues with monoclonal antibody to investigate the possible inhibition of FVIII endocytosis by MODCs.
Methods: BO2C11 is a well-characterized human monoclonal antibody that targets C2 domain. It competes for FVIII binding with both phospholipids and VWF. As control, we used the commercially available C2 antibody ESH-8 that targets an epitope that does not compete for membrane binding or VWF interaction. MODCs were generated using standard differentiation protocols for 6 days. MODCs were verified for their immature phenotype by flow cytometry (FACS) and contained greater than 90% differentiated cells. For endocytosis, MODCs were incubated with 20 nM FVIII or FVIII pre-incubated with BO2C11 or ESH8 (20 nM) for 30 min at 37 °C, or at 4 °C as controls. In addition, endocytosis assays were performed using Fab fragments for BO2C11 and ESH8. Subsequently, cells were fixed using 3.7% formaldehyde, permeabilized and stained using FITC conjugated 77IP52 (LFB, Les Ulis, France), a murine anti-FVIII A2 antibody, and uptake was analyzed by FACS. The experiments were performed on 3–5 different donors and as duplicates per donor.
Results: BO2C11 completely inhibited FVIII endocytosis. Pre-incubation of FVIII containing two molar excess of BO2C11 Fab fragment (compensated for bivalency of antibody) also abrogated endocytosis. Surprisingly, ESH-8 interfered with endocytosis of FVIII albeit at diminished capacity as compared to BO2C11 (50% vs. no uptake). Fab fragments for ESH-8 only marginally reduced FVIII endocytosis. FVIII endocytosis was significantly decreased in the presence of both C2 antibodies, and was differentially reduced by the two antibodies, as assessed by one-way ANOVA followed by Newlan-keuls multiple comparisons.
Conclusions: The BO2C11 epitope includes residues involved in endocytosis of FVIII by MODCs. Hence, membrane-binding residues within C2 domain encompass an immunodominant epitope that overlaps with residues involved in its uptake. Currently, we are investigating the *in vivo* significance of this epitope. Identification of the specific C2 residues along with the C1 residues involved in FVIII uptake may help develop FVIII that potentially exhibits a lower immunogenic profile.

PA 1.13-2

Impact on healthcare costs and quality of life of secondary prophylaxis in adolescent and adult patients with severe haemophilia A: the POTTER study

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Background: Progressive arthropathy of large joints of the limbs causes morbidity and quality-of-life impairment in hemophiliac patients. While prophylaxis (primary or early secondary) started early in life has shown to prevent joint bleeding and deterioration and is now gold standard treatment in children, the benefits of late secondary prophylaxis (i.e. in adolescents or adults) are still controversial.
Aims: The POTTER study (Prophylaxis vs. On-demand Therapy Through Economic Report) evaluated clinical effects, economic burden and Health-Related Quality-of-Life (HRQoL) of long-term secondary prophylaxis, compared with on-demand treatment in Italian adolescents and adults (12–55 years) with severe haemophilia-A (factor VIII [FVIII] < 1%). Here we will focus on pharmaco-economic and HRQoL evaluations.
Methods: Patients receiving sucrose-formulated recombinant FVIII on secondary prophylaxis (20–30 IU/Kg three times weekly, $n = 27$) or on-demand ($n = 26$) were prospectively compared over a median

5.4 year-follow-up, being stratified in two age subgroups (12–25 and 26–55 years). Study outcomes included: joint bleedings/year (primary end-point); total bleedings/year, joint status (orthopaedic and radiologic scores), adverse events (AEs), economic assessment and HRQoL. The economic analysis estimated direct costs of treatment (FVIII consumption) and of other healthcare resource consumption (planned and emergency visits, physiotherapy, laboratory/instrumental tests, invasive procedures, hospitalizations) which were quantified with National tariffs (Euros) for the year 2011. Productivity loss assessment counted days lost from work/daily activities by patients/caregivers due to the disease. HRQoL was measured using generic (EQ-5D, SF-36) and disease-specific questionnaires (Haemo-A-Qol). Statistical differences from baseline were assessed by Mann-Whitney U test or paired Student t-test. HRQoL and patients' characteristics association was evaluated by multivariate linear regression analyses.

Results: Patients on prophylaxis showed high adherence to the prescribed regimen (median 93.6%) and achieved significantly better clinical outcomes in terms of joint and total bleedings (an approximately 8-fold lower annual rate) and joint status (reduction of orthopaedic scores and lower progression of radiologic scores) compared to those treated on-demand. Mean direct costs for prophylactic regimen vs. on-demand were respectively: €190,696.84 vs. €60,740.85 in the 12–25 year group and €205,387.35 vs. €103,460.80 in the 26–55 year group ($P < 0.0001$). The difference in costs was mainly due to higher use of FVIII in the prophylaxis arm. Patients treated on-demand showed higher FVIII consumption and costs due to surgery or reasons other than ordinary treatment. Mean days lost from work/daily life activities in the two regimens were: 10.62 vs. 42.99 for patients aged 12–25 years; 13.77 vs. 35.56 for 26–55 year patients ($P < 0.0001$). Patients on prophylaxis always showed statistically better scores in all HRQoL questionnaires and both in physical and mental dimensions ($P < 0.05$) throughout the study. At multivariate analysis, worse HRQoL was associated with higher frequency of bleedings whereas prophylaxis duration directly correlated with better HRQoL.

Summary/Conclusions: The POTTER study provides prospective evidence over the long-term that secondary prophylaxis can significantly improve joint status, HRQoL and social participation of adolescents/adults with severe haemophilia-A. These advantages may counterbalance considerable high treatment costs. Studies with even longer follow-up are needed to assess the full impact of such investment on overall costs of comprehensive care for adult and ageing hemophiliacs.

PA 1.13-3

MD simulation studies of the membrane binding process of the human blood coagulation factor VIII C domains

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Background: Blood coagulation factor VIII (FVIII) is the non-enzymatic cofactor of the FX-activating tenase complex. Human FVIII consists of 2332 amino acids and can be divided into five domains: A1-A2-B-A3-C1-C2 (from N to C terminus). The C1 and C2 domains of FVIII are the main membrane-binding region of the protein.

Aims: We aimed to study the membrane binding mechanism of the FVIII-C domains in near-atomistic detail. Via *in silico* simulation our goal was to identify residues that are of major importance to the membrane binding process and quantitate the contributions of individual residues. The potential cooperativity between the C1 and C2 domain with respect membrane binding is studied.

Methods: Coarse grained Molecular Dynamics (MD) simulations, employing the MARTINI force field, were performed of the C1 domain, the C2 domain and of the C1/C2 domain dimer of human FVIII in the presence of a 20/80 mol/mol PS/PC membrane. Protein-membrane interactions were analysed in terms of energies and individual residues were selected that play major roles during the membrane binding process.

Results: We succeeded in reproducibly performing *in silico* binding experiments for the spontaneous binding of C1, C2 and C1/C2 to PS/PC phospholipid membranes. A consistent mode of binding was observed for each of the experimental systems tested and we obtained novel information about the identity of residues buried in the membrane, of their mode of interaction and of the overall binding mode of individual domains. Overall, membrane binding is steered by hydrophobic spikes of the C domains and discrete phases can be distinguished in the binding process itself.

We observed a deeper burying of residues for the C2 domain as compared to the C1 domain and calculated a net highest binding energy for the C1/C2 dimer as compared to the two individual domains. Cooperativity in binding between C1 and C2 domains was quantitated and the binding mode of the dimer differs from that of the single domains, with the isolated C1 domain binding being most different.

Our simulation results are consistent with data in literature for individual residues such as R2090, H2155, R2159, R2163, V2223 which have previously been recognized as involved in membrane binding and/or causative mutations in hemophilia A. We identified additional residues that appear to be important to membrane binding. Moreover, *in silico* mutation of essential residues results in altered membrane binding or ultimately loss of membrane binding.

Conclusions: We have been able to reproducibly mimic membrane binding *in silico* and provide a method that is in agreement with data in literature. We observed strong interactions between the FVIII C domains and phosphatidylserine containing membranes during MD simulations. The binding of the C domain to the membrane appears to follow several discrete stages: a) approach by electrostatic attraction b) contact by interaction of hydrophobic spikes with the membrane in an upright position along the longitudinal axis of the C-domain, c) tilting of the C-domains to maximize the number of contacts between the domain and the membrane.

PA 1.13-4

Light chain of coagulation factor VIII contains a site for low-density lipoprotein receptor

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Background: The lifetime of blood coagulation factor FVIII (FVIII) in circulation depends on its clearance via hepatic receptors, and one of them was shown to be the low-density lipoprotein receptor (LDLR) (Bovenschen N., et al, 2005). The ligand-binding portion of LDLR is represented by a cluster of seven complement-type repeats (CRs), among which specific adjacent CRs were previously shown to form sites for such ligands as plasma lipoproteins ApoE and ApoB. In regard to FVIII, it was previously shown that its site for another clearance receptor, LDLR-related protein (LRP), is located on the light chain (LCh) (Lenting P., et al, 1998).

Aims: Considering similarity in structure of both receptors, in the present work, we investigated whether the binding site of FVIII for LDLR is also located on the LCh. On the receptor side, we aimed to map the interactive site.

Methods: Expression of recombinant proteins and their mutant forms in baculovirus system, purification of the proteins and performing binding assays in a purified system using surface plasmon resonance.

Results: In the binding assay, we found that FVIII interacted in similar fashion with the LDLR exodomain expressed in mammalian cells, and with the recombinant LDLR cluster of CRs expressed in insect cells. These data indicated that the LDLR site for FVIII is restricted within the CR cluster and not extended to a region beyond it. Next, FVIII was tested vs. a panel of CR doublets expressed in insect cells, overlapping the LDLR cluster. Three such doublets overlapping a four-CR region of the cluster were found positive for the binding. Specificity of these interactions was verified by site-directed mutagenesis of all positive binding LDLR fragments and by using an anti-FVIII antibody

fragment as competitor; both approaches resulted in strong inhibitory effects on FVIII binding. Finally, we tested the FVIII LCh, plasma- and insect cell-derived, for the binding with CR doublets and found that both preparations of the LCh bound to essentially the same receptor fragments as FVIII.
Summary/Conclusion: Thus, the LCh of FVIII contains a site for LDLR, and within the receptor, the LCh interacts with the second through fifth complement-type repeats. In turn, the fact that FVIII interacts with both LDLR and LRP via the LCh, indicates the similarity of these interactions.

PA 1.13-5
Human liver sinusoidal endothelial cells but not hepatocytes contain FVIII

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Background: Although the liver is the major site of Factor VIII (FVIII) synthesis, the FVIII producing cell type within the liver is still unclear.
Aims: We therefore wanted to measure FVIII in extracts of primary liver sinusoidal endothelial cells (LSECs) and hepatocytes, thereby avoiding potential bias due to the modification of the cell phenotype during *in vitro* culture.
Methods: LSECs were purified by flow cytometry cell sorting based on the coexpression of Tie2 and CD32b and the strategy of purification controlled by RNA-Seq.
FVIII in extracts of purified cells was measured with a sensitive FVIII chromogenic assay. The specificity of each test was controlled by the neutralisation of FVIII activity with specific inhibitor antibodies.
Results: FVIII:C concentration in LSECs purified by cell sorting based on the coexpression of Tie2 and CD32b ranged from 0.3 to 2.8 nU/cell. On the contrary, FVIII:C remained undetectable in hepatocytes. The intracellular FVIII:C concentrations are therefore at least 10- to 100-fold higher in LSECs than in hepatocytes.
Summary/Conclusion: Our data demonstrate that LSECs but not hepatocytes contain measurable amounts of FVIII:C and suggest that the former is the main FVIII producing cell type in the human liver.

PA 1.13-6
Myeloid-derived cells a novel target for cell therapy in hemophilia A

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Background: Identification of cells capable of synthesizing and releasing factor VIII (FVIII) is critical for developing therapeutic approaches in hemophilia A (HA). Hepatocytes and endothelial cells (EC), particularly liver sinusoidal endothelial cells (LSEC), express FVIII most in the body. However, recent studies of bone marrow (BM) transplantation suggested additional cell types could synthesize and release FVIII, and also correct bleeding in HA mice.
Aims: To establish the ability of myeloid blood-derived cells in expressing FVIII, we analyzed several murine and human hematopoietic cell types.
Methods: First, we generated polyclonal and monoclonal antibodies against recombinant human FVIII. The specificity of these anti-FVIII antibodies was established by western blotting and staining of lentivirally transduced fibroblasts from HA mice expressing human and mouse B domain-deleted FVIII. Human monocytes were obtained by adhesion from peripheral blood mononuclear cells (PBMC) and differentiated *in vitro* adding M-CSF to the medium for 7 days. Human hematopoietic stem cells (HSC) were isolated from cord blood mononuclear cells (CBMC) by immunomagnetic selection with anti-CD34 antibody. HSC were differentiated in macrophages and megakaryocytes through 2 weeks stimulation with IL-3, M-CSF, Flt-3 ligand, SCF and IL-6, IL-11, TPO respectively. In silico analysis to predict transcriptional factor consensus sequences on FVIII promoter was performed on the website: http://alggen.lsi.upc.edu/recerca/menu_recerca.html. Monocytes were isolated by CD11b immunopositive selection from CBMC and transplanted in NOD-SCID HA mice. Eleven mice were tail vein injected with 15 million cells. FVIII activity was analyzed by chromogenic assay 3 and 7 days after injection. As controls untreated NOD-SCID HA mice were used.
Results: FVIII was expressed in hematopoietic cells isolated from peripheral blood, BM and human cord blood (hCB). The identity of these cell types was verified by costaining for FVIII and cell type-specific markers for monocytes, dendritic cells and megakaryocytes. Moreover, FVIII expression in these cell types was verified by RT-PCR and western blot analysis. Antibody staining confirmed FVIII expression in normal human liver, including LSEC, Kupffer cells (KC), and hepatocytes. We detected FVIII expression in myeloid cells or EC in other organs, e.g., spleen, lymph nodes, lungs, BM and kidneys. Despite molecular progresses, little is reported about the transcriptional regulation of the factor VIII gene. In silico analysis of transcriptional factors (TF) consensus sequences of FVIII promoter predicted in addition of well-known hepatocytes specific TF, the presence of several myeloid-specific TF such as C/EBP and GR family, GATA1, IRF-2 and STAT1 and 4. In order to evaluate the ability of monocytes to release FVIII we performed injections of human CD11b+monocytes hCB-derived in NOD-SCID HA mice. Tissue analysis showed engraftment of CD11b+ monocytes in the mouse liver and spleen up to 1 week. Tail clip challenge of these mice showed bleeding correction in nine of 11 transplanted mice (82%) and plasma FVIII activity was detected in treated mice.
Conclusions: Besides hepatocytes and EC, FVIII is expressed in myeloid cells, offering further opportunities to understand mechanisms in FVIII synthesis and activity for cell therapy approaches.

PA1.14 – Fibrinogen/Fibrin – I

PA 1.14-1
Kinetics and thermodynamics of knob-hole interactions in fibrin from dynamic force measurements in silico

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Background: The physico-chemical properties of fibrin, the primary structural protein of blood clots and thrombi, are essential for hemostasis and wound healing. The polymerization of fibrin occurs primarily via intermolecular interaction between knobs 'A' and 'B' in the central nodule and holes 'a' and 'b' in the γ- and β-nodules.
Aims: To explore the mechanisms of A:a and B:b knob-hole interactions and compare the kinetics and thermodynamics of the forced dissociation of A:a and B:b knob-hole bonds under various solution conditions (pH and temperature).
Methods: Molecular Dynamics simulations of atomic structural models of the complexes of fragment D with peptides GPRP (mimicking knob 'A') and GHRP (mimicking knob 'B').
Results: We found that the main binding determinants in hole 'a' and 'b' are localized to residues in loop I (residues γ315–330 in hole 'a' and β383–398 in hole 'b'), interior region II (residues γ335–365 in hole 'a' and β404–434 in hole 'b'), and moveable flap III (residues γ295–305 in hole 'a' and β359–369 in hole 'b'). The A:a and B:b knob-hole bonds are roughly equally strong when tested mechanically. The average A:a and B:b bond lifetime decreases with increasing pulling force applied

to dissociate the bond, yet the dissociation kinetics are sensitive to pH and temperature variation. Both A:a and B:b interactions are not 'all-or-none' transitions – they occur through distinct pathways via populating intermediate states. This leads to formation of A:a and B:b knob-hole bonds of varying strength. We analyzed the time-dependent maps of residues in hole 'a' and hole 'b' forming binding contacts with their ligands and identified the most important residues critical for binding. The single-step unbinding transitions from the bound state *B* to the unbound state *U*, *B* -> *U*, correspond to longer interaction times, whereas the two-step sequential transitions, *B* -> *I* - > *U* (*I* is the partially bound intermediate state), which occur more frequently in an acidic environment (pH = 5), favor shorter bond lifetimes. There are similar structural changes in the holes 'a' and 'b', which accompany dissociation of the A:a and B:b knob-hole complexes: short 2–4 Å elongation of loop I, limited stretching of the interior region II by 2–4 Å, and large-amplitude 2–6 Å translocation of the moveable flap III. We profiled the binding free-energy as a function of the interaction range underlying the A:a and B:b non-covalent coupling. For pH = 7, the binding energy ΔE decreases from approximately 20 to 17 kcal/mol for the A:a bond, and from approximately 16 to 13 kcal/mol for the B:b bond when the temperature decreases from 35 to 25 °C. For pH = 5, ΔE increases from approximately 2 to 7 kcal/mol for the A:a bond, but decreases from approximately 10 to 8 kcal/mol for the B:b bond upon lowering temperature from 35 to 25 °C.

Summary/Conclusion: These results from equilibrium Molecular Dynamics simulations confirm and clarify the binding determinants from the static X-ray crystallography picture. The pulling simulations provide kinetic and thermodynamic information for the strongest and most significant non-covalent interactions for polymerization of fibrin.

PA 1.14-2

Fibrinogen Birmingham II – a novel variant associated with hypodysfibrinogenemia, due to co-inheritance of Trp334Cys and Asn335Tyr in the fibrinogen Aa chain

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Background: A patient presented with a strong history of thrombosis with paradoxical bleeding diathesis. There was a family history of hypodysfibrinogenemia associated with a range of clinical symptoms, which included thrombosis and/or bleeding, and asymptomatic individuals.

Aims: The aim of this work was to establish whether patient symptoms are due to abnormality in the fibrinogen molecule resulting in altered clot structure and/or lysis.

Methods: Genetic analysis was carried out by PCR and nucleotide sequencing of the entire coding regions/splice junctions of fibrinogen FGA, FGB and FGG genes. Fibrinogen was purified from plasma samples using affinity chromatography. Double mutation in the α-chain of fibrinogen was found in our patient, which was further studied in a recombinant system by site-directed mutagenesis and stable expression of fibrinogen variant in Chinese Hamster Ovary cells.

Clot maximum absorbance (MA) and lysis time (LT) were measured by a turbidimetric assay. Confocal and scanning electron microscopy were used to assess clot structure.

Results: The patient was found to have a novel fibrinogen variant (Birmingham II), consisting of co-inheritance of two previously unreported changes in the fibrinogen FGA gene; c.1002 G>T (p.Trp334Cys) and c.1003 A>T (p.Asn335Trp). Aα Trp334 and Asn335 are both highly conserved residues in the fibrinogen aC domain; there was no evidence that either change was polymorphic. SIFT analysis suggested both substitutions were deleterious (both score 0.03), Polyphen analysis predicted that these changes were 'possibly damaging' and benign' (score 0.85 and 0.41 respectively).

Clots made from plasma purified fibrinogen of our patient showed significantly lower MA compared with control (0.0065 ± 0.0005 and 0.065 ± 0.001 au, respectively; $P < 0.01$). Similar results were obtained in clots made from recombinant fibrinogen with and without the mutations (0.014 and 0.059 au, respectively; $P < 0.01$). These results suggest thinner fibrin fibres in the presence of the mutation, which was confirmed by scanning electron microscopy (32.47 ± 0.91 and 100.23 ± 2.75 nm, respectively for plasma; $P < 0.01$ and 45.40 ± 1.12 and 118.65 ± 3.53 nm, respectively for recombinant protein; $P < 0.01$).

In a purified system, LT was shorter in clots made from the patient compared with control (144 ± 15.49 and 308 ± 5.06 s, $P < 0.01$) with similar results for recombinant fibrinogen with and without the mutations (459 ± 38.69 and 882 ± 22.18 s, respectively; $P < 0.01$). Conversely, LT was longer with variant recombinant fibrinogen when added to fibrinogen depleted plasma (2356 ± 164.72 and 1590 ± 27.93 s, respectively; $P < 0.01$), indicating interactions between clot structure and plasma proteins.

Discussion: We report a novel fibrinogen variant due to co-inheritance of two missense mutations in αC domain (Aa p.Trp334Cys and Aa p.Asn335Trp). This variant is associated with thin fibrin fibres and resistance to fibrinolysis in a plasma environment. Our data using plasma-purified and recombinant fibrinogen confirm the mutations are directly responsible for the changes in clot structure and lysis. Moreover, these findings provide mechanistic explanations for the clinical presentation of our patient. The excessively low clot turbidity and thin fibres are consistent with less robust clots predisposing to bleeding, whereas impaired fibrinolysis may explain the increased thrombotic tendency.

PA 1.14-3

Fibrin structure in subjects with diabetes and aortic aneurysm

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Background: European men over 60 years have a high incidence of abdominal aortic aneurysm (AAA) (5%) and diabetes (12%). Cardiovascular morbidity and mortality is increased in both AAA (excessive and equivalent to that associated with coronary heart disease) and diabetes. Despite similar associations of diabetes and AAA with inflammation and arteriosclerosis, AAA is uncommon in patients with diabetes due to a thicker abdominal aortic wall causing reduction in wall stress and therefore appears protective against AAA. Patients with AAA form denser, smaller pored plasma clots that are more resistant to fibrinolysis and these characteristics correlate with aneurysm size. Clots formed by fibrinogen purified from diabetes subjects have a denser, less porous structure than those from control subjects.

Aims: To investigate fibrin structure, propensity to clot and resistance to clot breakdown in diabetes patients with AAA compared to those without.

Methods: Informed consent was obtained and the study was approved by a local medical ethics committee. Detailed medical history and blood samples were obtained only from patients diagnosed as diabetes as part of LEADS (a large population based study of AAA patients and matched controls recruited from the West Yorkshire) sub-study. Sixty patients had both diabetes and AAA as defined by aortic diameter ≥ 3.0 cm determined by ultrasound scan and 34 patients with diabetes had a normal aortic diameter < 3.0 cm. Turbidity or clot formation was measured using thrombin (5 U/mL) addition to plasma. Absorbency was read every 12 s at 350 nm for 1 h using Bio-Tek ELx 808 reader. Lysis or clot breakdown was measured by using tissue-plasminogen activator (85 ng/mL) to the turbidity assay. Absorbency was read every 120 s at 350 nm for 9 h.

Results: Although groups were sex-matched, diabetes subjects with AAA were older (78 vs. 73 years, $P < 0.001$) than those without. The

diabetes subjects with AAA had a lower prevalence of cardiovascular disease (75 vs. 97%, $P = 0.004$) and use of oral anti-glycaemic medications (3.3 vs. 29%, $P = 0.001$). There were no differences in low density lipoprotein, body mass index, hypertension, insulin or cardiovascular drugs usage. The diabetes subjects with AAA had shortened lag time (386 vs. 472 s, $P < 0.001$), increased clot rate (5.2×10^{-4} vs. 3.6×10^{-4} nm/s, $P < 0.001$) and decreased lysis rate (-9.6×10^{-5} vs. -150×10^{-5} nm/s, $P < 0.001$) than without AAA. Fibre thickness was similar in the two groups. Lag time ($P = 0.006$), clot rate ($P = 0.05$) and lysis rates ($P = 0.007$) remained significantly different (all $P \leq 0.05$) even after adjustment for age, oral anti-glycaemic medication and cardiovascular disease.

Conclusions: Patients with diabetes and AAA form clots, which are more pro-coagulant and resistant to fibrinolysis hence increasing the risk of cardiovascular disease. There are differences in medial wall pathology in diabetes (obliterating arteriosclerosis which causes more occlusion) and AAA (dilating arteriosclerosis which might be protective) hence possibly the cardiovascular disease is decreased in our study in subjects with both diabetes and AAA. Further prospective studies on fibrin clot structure and function in apparently healthy subjects with measure of medial wall changes and follow up for development of diabetes or aneurysm and cardiovascular disease are required.

PA 1.14-4

Prothrombotic plasma fibrin clot phenotype is predictive of recurrent venous thromboembolism following discontinuation of anticoagulant therapy

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Background: Prothrombotic fibrin clot phenotype, including reduced clot permeability and susceptibility to lysis, has been shown to be associated with idiopathic venous thromboembolism (VTE).

Aims: We evaluated the relation of plasma fibrin clot characteristics to risk of recurrent VTE.

Methods: In a prospective cohort study of 162 consecutive VTE patients aged 18–65 years, plasma fibrin clot characteristics were measured following discontinuation of anticoagulation. Recurrent VTE events were assessed during a median follow-up of 30 months.

Results: There were 41 VTE recurrences after 8.2 ± 4.5 months since discontinuation of anticoagulation. Idiopathic index event was associated with increased risk of VTE recurrence (hazard ratio [HR] 3.53, 95% confidence interval [CI] 1.61–7.76). Patients with recurrent VTE had lower K_s, longer $t_{50\%}$, shorter *lag phase* indicating faster fibrin formation, and greater ΔAb_{max}, indicating thicker fibrin fibers. Relative to the middle and upper tertile of clot permeability (K_s) and the rate of D-dimer release from clots ($D\text{-}D_{rate}$), the HRs of recurrent VTE for those from the lowest tertile were 3.68 (95%CI 1.72–7.84, $P = 0.001$) and 2.17 (95%CI 1.07–4.43, $P < 0.001$), $P = 0.003$), respectively. Similar associations were noted for the upper tertile of clot lysis time (HR 3.17, 95%CI 1.46–6.86, $P = 0.032$) and plasma D-dimer following anticoagulation cessation (HR 5.04, 95%CI 2.19–11.61, $P = 0.003$). In ROC analysis ΔAb_{max} has the highest area under the curve (AUC) of 0.784. Its cut-off point of 0.805 had sensitivity of 0.854 and specificity of 0.661. The presence of factor V Leiden mutation and/or prothrombin 20210A mutation were not associated with altered fibrin clot phenotype or recurrent VTE.

Conclusion: Unfavorably altered fibrin clot characteristics measured following discontinuation of anticoagulant therapy can help identify patients at risk for recurrent VTE.

PA 1.14-5

The impact of diabetes duration on plasma fibrin clot properties in type 2 diabetic patients

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Background: It is well known that hyperglycemia is associated with abnormal fibrin clot phenotype. It has been shown that fibrin clots made from fibrinogen obtained from type 2 diabetic patients have more compact fiber networks associated with reduced permeability and impaired fibrinolysis compared with healthy controls. Mechanisms underlying abnormal fibrin clot properties in diabetic patients remain unclear. It has been demonstrated that the level of glycated hemoglobin (HbA1c) is associated with worse fibrin clot phenotype and fibrinogen glycation is a major mechanisms of those alterations.

Aims: We investigate the impact of diabetes duration on plasma fibrin clot characteristics in a cohort of type 2 diabetic patients.

Methods: We studied 156 consecutive patients with type 2 diabetes (87 men, 69 women, aged 66.0 ± 8.5 years, mean disease duration 7.3 years, HbA1c levels from 5.2% to 12.6%). The patients were at high cardiovascular risk, i.e. 149 (95.5%) had hypertension, 126 (80.8%) had dyslipidemia, 103 (66%) were obese, 14 (9.0%) were current smokers, 41 (26.3%) had family history of cardiovascular disease (CVD). *Ex vivo* plasma fibrin clot permeation, turbidity, and efficiency of fibrinolysis using three different assays with various final recombinant tissue plasminogen activator concentrations, along with peak thrombin generation and fibrinolytic proteins were investigated.

Results: There were more patients with obesity, with documented CVD, diagnosed neuropathy and treated with insulin in the group with diabetes duration above 5 years ($n = 68$). There were no differences in laboratory parameters including fibrinogen between the groups with longer and shorter diabetes duration. Five percent lower clot permeability (*Ks*; $P < 0.0001$), 8.2% prolonged clot lysis time (CLT; $P = 0.017$) and 7.8% prolonged half-lysis time ($t_{50\%}$; $P < 0.0001$) were observed in patients with longer diabetes duration. Maximum D-dimer levels released from clots were (+4.5%, $P = 0.011$) higher and D-D rate was (2.8%, $P = 0.002$) lower compared to patients with shorter diabetes duration. Maximum absorbance of a fibrin gel, was (+3.6%, $P = 0.004$) higher, while lag phase was (4%, $P = 0.01$) shorter in patients with longer diabetes duration. Those patients had higher peak thrombin generation (+17.5%), plasminogen activator inhibitor-1 (PAI-1) antigen (+13%), tissue plasminogen activator (tPA) antigen (+17.9%, all $P < 0.001$) and antiplasmin (+7.2%, $P = 0.04$). Thrombin activatable fibrinolysis inhibitor (TAFI) and soluble thrombomodulin tended to be higher in patients with longer diabetes duration ($P < 0.1$). Comparison of patients with worse and better glycemic control (cut-off, HbA1c 6.5%), who did not differ with regard to fibrinogen, showed no differences in thrombin generation and fibrinolysis parameters. Fibrin clots obtained from plasma of patients with HbA1c>6.5% had 5% lower *Ks* ($P = 0.002$), 6.5% prolonged CLT ($P = 0.02$) and $t_{50\%}$ ($P = 0.0002$). Maximum D-dimer levels released from clot in the HbA1c>6.5% group were higher (+4.5%, $P = 0.026$) and Lag phase was (4.5%, $P = 0.027$) shorter in this group. Cardiovascular events and macrovascular diabetic complications as well as cardiovascular risk factors were not associated with less favorable fibrin clot variables.

Conclusions: Prolonged duration of type 2 diabetes is associated with more prothrombotic fibrin clot phenotype, including impaired fibrinolysis and those alterations are largely driven by increased thrombin generation and PAI-1 levels.

PA 1.14-6

Fluid and solute transport in thrombi as function of platelet and fibrin density

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Background: There is growing evidence that fluid and solute transport within the interstitial spaces of thrombi plays a role in thrombus growth, arrest, and dissolution [Stalker et al., *Blood*, in press, 2013]. However, there is a paucity of data on the physical properties, such as the permeability of fluid and diffusivity of solutes, that dictate these phenomena. The data that does exist has been collected at fibrin and platelet concentrations that are orders of magnitude less than what has been reported in thrombi formed *in vivo*. [Wysokinski et al., *J Thromb Haemost,* 2, 2004; Silvain et al., *J Am Coll Cardiol*, 57, 2011]

Aims: The objective of this work was to measure the hydraulic permeability and solute diffusivity in model thrombi as a function of platelet and fibrin density over the range of fibrinogen and platelets concentrations found in thrombi formed *in vivo*.

Methods: A series of thrombi were formed by adding 10 nM α-thrombin to solutions containing 3–156 mg/mL of fibrinogen and 0–5×10^7 platelets/μL. To measure permeability, both fibrin gels and platelet rich clots were formed in 3 mL syringes, which served as a permeation chamber. The flow rate of Tris buffer saline (TBS) through these clots at a constant pressure was used to calculate the permeability using Darcy's law. Fluorescence recovery after photobleaching (FRAP) was used to measure diffusion coefficients of FITC-labeled dextrans of 12, 29, and 54 nm in diameter in fibrin gels and platelet rich clots.

Results: Fibrin formed with fibrinogen concentrations from 3 to 156 mg/mL resulted in gels with a fiber volume fraction of 0.02–0.54 and permeabilities of 1.2×10^{-1}–1.5×10^{-4} μm^2. Platelet rich clots with a platelet volume fraction of 0.01–0.61 and a fibrin volume fraction of 0.03 had permeabilities over a range of 1.1×10^{-2}–1.5×10^{-5} μm^2. Preliminary results from FRAP studies show that diffusion was hindered in fibrin gels compared to free solution. We observed 60%, 66% and 85% reductions of diffusion coefficients for 12, 29, 54 nm probes, respectively, in fibrin gels (100 mg/mL fibrinogen).

Conclusions: The primary outcome of these studies is a catalog of physical properties that determine fluid and solute transport in thrombi as a function of their composition. These data provide bounds on how far zymogens and fibrinolytic agents can be expected to penetrate into clots with different composition. For example, at pressures typical of large human arteries one would expect plasmin travel no further than approximately 100 μm over 1 h into the interior platelet rich clot. In addition, the hindrance of diffusion of coagulation proteins and platelet agonists out of thrombi is a potential mechanism for the arrest of thrombi growth. Finally, these data will aid in the development of the growing number of computational models of thrombi formation, which, to date, have relied on semi-empirical correlations that are not valid for the wide range of solid fractions found in real thrombi.

PA1.15 – Natural Anticoagulants – I

PA 1.15-1

Effect of coagulation factors and heparin on FXa inhibition by TFPI

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Background: TFPI is protease inhibitor which contains three Kunitz domains (KD1, KD2 and KD3) and which down-regulates the extrinsic coagulation pathway by inhibiting FXa and FVIIa. All three Kunitz domains of TFPI contribute to the inhibition of FXa, indicating that inhibition is the result of multiple interactions between FXa and TFPI. FXa as well as TFPI have been reported to interact with several coagulation factors (e.g. protein S, FV, FVa, prothrombin), and with phospholipids and heparin.

Aim: To investigate the effect of proteins and other ligands that bind to FXa and TFPI on the efficacy of FXa inhibition by TFPI.

Methods: Recombinant full length TFPI ($TFPI_{fl}$) and TFPI constructs (KD2, $TFPI_{1–150}$) were purified from bacterial expression systems. Inhibition of FXa by TFPI and TFPI constructs was quantified by measuring progress curves of conversion of the FXa-specific chromogenic substrate CS11-(65) by FXa. The effects of prothrombin, FV, FVa and protein S on FXa inhibition were determined in the presence of negatively charged phospholipid vesicles and Ca^{2+} ions. Heparin and FXa antibody effects were determined in the presence of Ca^{2+} ions.

Results: A monoclonal FXa antibody (aBFX-2b), which is an exosite-directed inhibitor of prothrombinase that does not inhibit the active site of FXa and that has no effect on its amidolytic activity, impairs FXa inhibition by both $TFPI_{fl}$ and KD2. Unfractionated heparin, which binds both FXa and $TFPI_{fl}$, stimulated FXa inhibition by $TFPI_{fl}$ approximately 10-fold at low heparin concentrations (0.2–1 U/mL), but impaired inhibition at higher concentrations (> 1 U/mL). Unfractionated heparin had virtually no effect on FXa inhibition by $TFPI_{1–150}$ and fondaparinux, a synthetic pentasaccharide related to heparin that specifically enhances antithrombin-mediated inhibition of FXa, had also no effect on FXa inhibition by $TFPI_{fl}$ at concentrations up to 800 mg/mL. Protein S, which binds to KD3 of TFPI, and thrombin-cleaved protein S enhanced FXa inhibition by TFPI ($t_{1/2}$ approximately 3 min), but not by $TFPI_{1–150}$. Physiological concentrations FV (20 nM), which like protein S binds $TFPI_{fl}$, enhanced FXa inhibition by $TFPI_{fl}$ approximately 2–3 fold and had virtually no effect on FXa inhibition by $TFPI_{1–150}$. Thrombin-activated FV (FVa) did not enhance the anticoagulant activity of $TFPI_{fl}$, but in contrast impaired the ability of $TFPI_{fl}$ to inhibit FXa, particularly at low TFPI concentrations. $TFPI_{fl}$ appeared to be a potent inhibitor of FXa-catalyzed prothrombin activation in the absence of FVa, but hardly inhibited prothrombin activation by FXa in the presence of FVa.

Summary and Conclusions: $TFPI_{fl}$ is a potent inhibitor of FXa which at physiological concentrations (0.25–0.5 nM TFPI) inhibits FXa with a $t_{1/2}$ = 3–15 min. Direct inhibition of FXa by $TFPI_{fl}$ is modulated by other coagulation factors (prothrombin, FV and FVa), protein S and heparin. These modulating effects indicate that interactions of the various Kunitz domains of TFPI with epitopes on FXa distinct from the active site contribute to FXa inhibition. Modulation of TFPI activity was observed at physiological concentrations indicating that they are important for the *in vivo* expression of anticoagulant activity of TFPI.

PA 1.15-2

Implication of the protein S/Growth arrest-specific gene 6 pathway in the pathophysiology of purpura fulminans

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Background: Purpura fulminans (PF) is an acute skin disorder that involves a complete blood flow blockade in dermal and subcutaneous vasculature. Common causes of PF are severe infection and deficiency of the natural anticoagulants in the blood. Current knowledge on its pathologic mechanisms is still limited.

ProS is an anticoagulant protein, its deficiency is associated to the development of PF as documented by newborns homozygous for ProS

mutations. Beside its role in blood coagulation, ProS exerts cellular functions by binding to and activating cell surface Tyro3, Axl, and Mer tyrosine kinase receptors (TAM). Recently, the characterization of murine model of partial and complete ProS deficiency has shown defects in vessel development and function for those mice.
The product of Growth arrest-specific gene 6 (Gas6) is a protein showing an high degree of similarity compared to ProS both in module organization and at the amino acid level. Gas6 has been shown to be a ligand for the TAM receptors as ProS. Unlike ProS, Gas6 displays a procoagulant effect by amplifying platelet activation and upregulating the expression of vascular tissue factor. Interestingly, both Gas6 and ProS belongs to the family of vitamin K-dependent proteins.
Aims: To investigate the role of ProS, Gas6 and TAM receptors in PF pathophysiology.
Methods: We generated a model mimicking PF by exposing mice to warfarin, a vitamin K antagonist. 0.8 mg warfarin was administered *per os* for 5 days to wild-type (WT), *ProS*, *Gas6*, *Tyro3*, *Axl* and *Mer* deficient mice.
Results: Adult mice with complete *Gas6* or partial *ProS* deficiency did not develop spontaneous PF. To investigate the pathologic mechanisms leading to PF, we administer warfarin. We observed a difference in mortality for *Gas6*−/− (100%, $n = 20$) and *ProS*+/−/*Gas6*−/− (88%, $n = 8$) mice compared to the WT (6%, $n = 17$) mice ($P < 0.05$). In addition, mice deficient in TAM receptors where significantly affected by warfarin challenge (mortality rate: 43% in *Tyro3*−/− ($n = 7$), 38% in *Axl*−/− ($n = 8$) and 14% in *Mer*−/− compared to 6% in WT mice, $P < 0.05$). Notably, most of the mice with ProS/Gas6 pathway deficiency developed skin lesions involving ears, extremities and genital areas, compatible with PF. Such lesions were never observed in WT. Early lesions were erythematous with highly visible ear skin vessels, necrosis appearing in advanced lesions. Ear skin histology of mice with PF showed vascular engorgement, intradermal edema and only rare thrombosis in early lesions, whereas massive red blood cell extravasation, intra-epidermal hemorrhagic blisters and necrosis were found in advanced lesions.
Summary/Conclusions: These data suggest a prominent vascular involvement in PF lesions when the ProS/Gas6 pathway is deficient. Lack of Gas6 or TAM receptors being antithrombotic, the thrombotic process might be less central in PF pathophysiology than currently admitted. Thus, the vasculature might constitute the main target during PF development and the ProS/Gas6 pathway through the TAM receptors in the endothelium appeared to be involved in the process.
Interestingly, our warfarin-induced PF in $Pros^{+/-}/Gas6^{-/-}$could also be used as a mouse model of PF, which to our knowledge is still lacking.

PA 1.15-3
In vivo characterization of double deficiency in protein S and growth arrest-specific gene 6

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Background: Protein S (ProS) and growth arrest-specific gene 6 (Gas6) are vitamin K-dependent proteins. ProS is a natural anticoagulant whose physiological importance is exemplified by life-threatening thrombotic disorders (e.g. purpura fulminans) occurring in newborns with homozygous ProS deficiency. Unlike ProS, Gas6 displays a procoagulant effect by amplifying platelet activation and upregulating the expression of vascular tissue factor. In tissues, ProS and Gas6 exert cellular functions by binding to and activating the same cell-surface tyrosine kinase receptors of the Tyro3 family (TAM).
Aims: To investigate the synergic role of ProS and Gas6 *in vivo* by using murine models.
Methods: We first crossed $ProS^{+/-}/Gas6^{+/+}$ with $ProS^{+/+}/Gas6^{-/-}$ mice. $ProS^{+/-}/Gas6^{-/-}$ strain was then used to obtain mice with a complete double deficiency in ProS and Gas6. In addition, $ProS^{+/-}/Gas6^{+/+}$ were mated in order to generate mice with single ProS deficiency. Genotypic and phenotypic analysis on embryos between E14 and E19, newborns and adults were performed to characterize the double deficiency of ProS and Gas6 in mice.
Results ProS+/−/*Gas6*−/− mice were viable and showed an apparently normal phenotype and development. During adult life, they were crossed to generate $ProS^{-/-}/Gas6^{-/-}$ mice, however among the newborns analyzed ($n = 30$), no double newborn deficient for ProS and Gas6 was observed. Similar results were obtained for the $ProS^{-/-}/Gas6^{+/+}$ genotype.
Macroscopic and histological analysis of $ProS^{-/-}/Gas6^{-/-}$ embryos at different developmental stages (E14–E19) was performed. Interestingly, 24% (5/21) embryos were found dead, showed brownish skin coloration, reduced size and presented developmental defects. Furthermore, 24% (5/21) of them showed relevant pallor with no macroscopically visible vascularization throughout the body, but normal size and development. These abnormalities were never observed in $ProS^{-/-}/Gas6^{+/+}$ embryos. The remaining 52% (11/21) of $ProS^{-/-}/Gas6^{-/-}$ embryos presented massive thrombosis associated with hemorrhages throughout the body, and the absence of the macroscopically visible principal blood vessels; a similar phenotype was previously observed in $Pros^{-/-}/Gas6^{+/+}$embryos. Moreover, we noticed the presence of red blood cells in the extra-vascular compartments, particularly in the head and back, pointing to a severe vascular defect in all $Pros^{-/-}/Gas6^{-/-}$ embryos. We analyzed the vascular network of whole-mounted dorsal skin E15 embryos, by using anti-Ter119 (specific for red blood cells) and anti-VE-cadherin (expressed by endothelial cells) antibodies. The dorsal skin of $Pros^{-/-}/Gas6^{-/-}$ embryos displayed areas with incomplete or collapsed vessels, and widespread red blood cells extravasation. A similar pattern was observed in $Gas6^{+/+}Pros^{-/-}$ embryos (preliminary data).
Summary/Conclusions: Double deficiency in ProS and Gas6 is lethal before birth. The phenotype of $Pros^{-/-}/Gas6^{-/-}$ embryos, characterized by a high percentage of pale embryos and about one fourth of necrotic embryos, appears more severe as compared to the phenotype of $Pros^{-/-}/Gas6^{+/+}$ embryos. Abnormalities in the vascular network were present in both genotypes. Further studies are ongoing to better understand the mechanisms responsible for the observed vascular defects.

PA 1.15-4
Autodegradation of murine activated protein C due to cleavage at Lys43

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Background: Multiple protective effects of pharmacologic activated protein C (APC) are reported in several pathologies, mostly using mouse models. Murine APC mutants are valuable for mechanistic studies and proof-of-concept *in vivo* studies. Murine APC production requires multiple challenging steps due to autodegradation of APC. This is especially important when comparing *in vitro* and *in vivo* potencies of different APC variants.
Aims: To develop a new methodology that will decrease degradation of murine APC during activation and that will enable active site titration of APC.
Methods: Recombinant murine protein C (PC) was produced using conventional methods. Before activation, PC was dialyzed into different saline buffers (citrate pH 6.0, 2-(N-morpholino)ethanesulfonic acid (MES) pH 6.0 or Tris pH 8.0) containing EDTA or various different metal ions (calcium, zinc, manganese, magnesium). PC activation was performed using human thrombin (100 U/mL) at 37 °C for 4 h. SDS-PAGE was used and proteins visualized by Coomassie staining. Amidolytic activity assays were performed at pH 8.0 and 0.4 mM Pefac-

hrome PCa colorimetric substrate. Active site was titrated by incubating mouse APC with biotinylated FPR-chloromethylketone and quantified by Western blotting using infrared dye-conjugated-streptavidin. Human APC was used as a standard. For N-terminal amino acid sequence analysis, APC was run on SDS-PAGE and Western blotted on PVDF membrane. Protein bands were revealed by Coomassie and cleaved APC product bands removed for amino acid sequence analysis.

Results: A major problem during activation involved APC autodegradation of the light chain due the cleavage at the Lys43-Tyr44 peptide bond, as revealed by Western blotting using specific antibodies for the light chain and N-terminal amino acid sequencing. We compared the amount of this cleavage after PC activation in different buffers. Citrate or MES buffers (pH 6.0) showed reduced APC autodegradation in comparison to Tris buffer (pH 8.0). Activation of PC in presence of zinc (0.25–1 mM) at pH 6.0 showed a significant reduction of APC autodegradation. We did not observe differences in the activation rates in the presence of EDTA or zinc. After thrombin inhibition with hirudin, APC was purified by FPLC. We compared amidolytic activity of APC that was activated in EDTA or zinc buffer in a buffer containing 5 mM EDTA or 1 mM Zn2+, since zinc ions inhibit amidolytic activity of human APC. Direct comparison showed that APC generated in presence of Zn^{2+} had a higher activity than APC made in the presence of EDTA.

Conclusions: Murine APC autodegradation involves cleavage at Lys43 that can be avoided by simple buffer modifications. Mutation of Lys43 in murine PC should simplify the production of mouse quality murine APC for mechanistic and *in vivo* studies.

PA 1.15-5

The search for functionally important residues in protein S required for its enhancement of TFPI

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Background: Tissue factor pathway inhibitor (TFPI) is a natural anticoagulant protein that specifically inhibits the initiation of coagulation through direct binding and inhibition of FXa and the TF/FVIIa complex. Protein S acts as a cofactor for TFPI that critically reduces the inhibition constant for FXa to below the plasma concentration of TFPI – thus enabling efficient down-regulation of thrombin generation at physiological concentrations of TFPI. We recently showed that TFPI Kunitz domain three residue Glu226 constitute an essential residue for the interaction of TFPI with protein S during its inhibition of FXa. However, it is not known which domains or residues in protein S are required for its TFPI cofactor activity.

Aims: To fully understand the mechanism underlying the TFPI cofactor function of protein S, the present study aimed to determine the importance of solvent-exposed amino acid residues of protein S in its direct interaction and enhancement of TFPI.

Methods: A panel of 46 protein S variants, spanning the full length of the protein S molecule, were constructed, expressed and quantified in concentrated culture media. Residues were substituted either in composite or single point variants for the protein S Gla-TSR-EGF1-EGF2-EGF3-EGF4 domains. To study the importance of the SHBG-domain we used a previously described protein S chimera in which the SHBG domain was replaced by the corresponding domain of the homologous Gas6. The TFPI cofactor function of the protein S variants was examined using thrombin generation assays and the TFPI-protein S interaction by co-immunoprecipitation.

Results: In thrombin generation assays using protein S deficient plasma, TFPI was efficiently enhanced by recombinant wild-type (WT) protein S in a dose-dependent manner. When protein S variants were screened using this assay, a large proportion exhibited appreciable enhancement of TFPI. However, none of the protein S variants completely lacked TFPI cofactor function. To investigate the interaction between TFPI and the protein S variants a co-immunoprecipitation assay was developed. TFPI was immunoprecipitated using a monoclonal antibody, binding to the TFPI Kunitz domain 1. Co-immunoprecipitation of protein S was investigated by Western blotting. WT protein S co-immunoprecipitated with WT TFPI, whereas no WT protein S was detected after immunoprecipitation of TFPI E226A, suggesting that this method was suitable for screening our protein S variants for variants unable to interact with TFPI. However, similarly to the results from the thrombin generation assay, the protein S variants were co-immunoprecipitated with TFPI in either similar or only moderately decreased levels compared to WT protein S.

Summary: TFPI Glu226 has previously been shown to be essential for the interaction with protein S. The large number of protein S variants showing only modestly decreased enhancement of TFPI in the functional assays suggests that several protein S residues are cooperatively important for its enhancement of TFPI. Further functional assays and direct binding studies using purified components are required to identify these residues.

PA 1.15-6

Stabilization of N-glycosylation in Asn135 of antithrombin by an aromatic sequon

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Background: *N*-glycosylation is a crucial post-translational modification. Two recent studies have demonstrated that an aromatic sequon (Phe-Y-Asn-X-Thr) in reverse β-turns increases the *N*-glycosylation efficiency and the global stability of three different proteins. However, the potential efficiency of this sequon in other contexts has not been evaluated. Antithrombin is a key anticoagulant serpin. Two main glycoforms of antithrombin are present in plasma: a and b, with four and three *N*-glycans, respectively. Although b-antithrombin is the less abundant glycoform in plasma (10%) the rate of secretion of both glycoforms is similar. The reduced efficiency of glycosylation of antithrombin at position Asn135 is not fully understood but has been suggested to be caused by an inefficient consensus sequence, Asn-X-Ser, while in the rest of glycosylation sites (Asn92, 155, 196) the consensus sequence is Asn-X-Thr. Interestingly, Asn135 lays in a loop between helix D and strand 2B.

Aim: To evaluate the effect of an aromatic sequon at Asn135 on the efficiency of glycosylation and the stability of the native conformation of antithrombin.

Methods: Single and multiple mutants affecting the Asn135 sequon (Lys133Phe, Ser137Thr, Ser137Ala and Lys133Phe/Ser137Thr) were produced in HEK-EBNA cells and purified by heparin affinity and anion-exchange chromatography. Biochemical and functional studies included mass spectrometry, differential scanning calorimetry, intrinsic fluorescence, stopped flow and functional chromogenic assays.

Results: The wild type sequence generated similar amounts of both glycoforms (α and β), while the incorporation of a Phe two residues before Asn135 (Lys133Phe), the generation of the common N-glycosylation sequence on antithrombin (Ser137Thr) or both mutations together (Lys133Phe/Ser137Thr) full improved glycosylation efficiency, leading to the secretion of only a-antithrombin glycoform (4 *N*-glycans) according to mass spectrometry and electrophoretic analysis. Differential scanning calorimetry revealed that the presence of the aromatic sequon did neither increase nor reduce the stability of

this conformationally sensitive serpin. However, stopped flow data suggested that the presence of a Phe two residues before Asn135 hindered the conformacional change induced by heparin. Accordingly, the Lys133Phe and the double (Lys133Phe/Ser137Thr) mutations impaired 2- and 4-fold, the heparin affinity, respectively. As expected, the anticoagulant activity of these variants in presence of heparin was paralleled affected in these mutants (80% and 50% of wild type activity, respectively).

Conclusion: Our data support that an aromatic sequon improves the efficiency of *N*-glycosylation in other contexts than in a reverse turn. These results open new possibilities for glycoengineery in other proteins. In antithrombin, this aromatic sequon allows a full glycosylation of Asn135, located in a loop between the helix D and the strand 2B. However, this sequon did not increase the global stability of this serpin, although it preserves the native conformation of the heparin binding domain, impairing the conformational change induced by the binding of heparin.

PA 1.16-1

Predictors of factor Xa generation in breast cancer

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Background: Venous thromboembolism (VTE) is a serious complication commonly associated with hormone therapy for breast cancer. VTE can have a devastating effect on quality of life (e.g. post-thrombotic syndrome; chronic anticoagulation; bleeding complications) as well as result in premature death. Patients with breast cancer may be exposed to multiple VTE risk factors in addition to hormonal therapy over the course of their disease; however, an understanding of prior and concurrent VTE risks among breast cancer patients is not fully elucidated.

Aims: We determined associations with factor (f) Xa generation, a key predictor of thrombosis and therapeutic target for VTE among breast cancer patients.

Methods: In this cross-sectional study, we studied 181 overweight and obese non-smoking breast cancer patients who had completed primary adjuvant therapy. We measured coagulation factors II, V, VII, VIII, IX, X, antithrombin, and tissue factor pathway inhibitor to assess tissue factor-initiated fXa generation. We estimated Kendall's Tau-b and Pearson's correlation coefficients as well as ANOVA to identify predictors of fXa generation. Variables considered included age, anthropometric variables (BMI, waist-hip ratio, fat weight, percent body fat), adipocytokines (adiponectin, leptin), cardio-metabolic risk markers (LDL cholesterol, triglycerides, C-reactive protein, insulin, homeostasis model of assessment- insulin resistance [HOMA-IR]), tumor characteristics (tumor size, stage, lymph node status), prior adjuvant therapies (chemotherapy, radiation), hormonal therapy (tamoxifen, anastrozole) and statin use.

Results: The mean $\pm$ SD time from adjuvant therapy was 5 ± 5 years. Women were on average 55 ± 9 years old with BMI ranging from 25 to 35 kg/m². Of all variables considered, only leptin, LDL cholesterol, and tamoxifen were associated with peak fXa generation; leptin ($r = 0.19$, $P = 0.01$) and tamoxifen ($r = 0.29$, $P < 0.001$) were positively correlated while LDL cholesterol was negatively correlated (-0.21, $P = 0.003$). Tamoxifen users had significantly higher peak fXa generation (5.4 ± 1.7 nM) compared to breast cancer patients on aromatase inhibitor, anastrazole (4.1 ± 1.3 nM), or patients on neither therapy (3.9 ± 1.3 nM; $P < 0.001$).

Conclusion: Among a group of overweight and obese breast cancer patients, tamoxifen use was the strongest driver of peak fXa generation. In contrast, anastrazole therapy was not correlated with fXa. Measures of adiposity were not related to fXa generation, except leptin, a metabolically active adipokine important in the regulation of body weight. To our knowledge, these are the first data elucidating exposures and risk factors in breast cancer patients impacting peak fXa generation.

PA 1.16-2

Clinical course of cerebral venous thrombosis in adults with acute lymphoblastic leukemia

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Background: Venous thromboembolism (VTE) occurs frequently in patients with acute lymphoblastic leukemia (ALL). Reported incidence varies between 2% and 36%. Remarkably, cerebral venous thromboses (CVT) form a relatively large proportion of VTE and represent up to 50% of events.

Aims: To explore the clinical course of a large number of CVT episodes that occurred in a well-defined cohort of adult patients treated for ALL. We analyzed clinical characteristics, prodromal symptoms, radiological characteristics, temporal relationships with treatment components of ALL, clinical outcome of CVT and impact of CVT on ALL treatment.

Methods: CVT incidence was assessed in 240 adults (16–59 years) treated for newly diagnosed ALL in the Dutch-Belgian HOVON-37 multicenter study (1999–2005; NTR228 on www.trialregister.nl). Patients generally received three cycles of combined chemotherapy before stem cell transplantation assessment. CVT was defined as an intraluminal filling defect or presence of a thrombus in one of the cerebral veins or sinuses, detected with magnetic resonance venography or computed tomographic venography. For patients with CVT, we systematically extracted clinical data from patient records and re-evaluated imaging results. We conducted a nested case-control study to explore relevant prodromal symptoms. Associations were expressed as odds ratios (OR) with corresponding 95% confidence intervals (CI).

Results: Nine of 240 patients experienced CVT (4%; median age 33 years (range 17–49), 56% female, five with B-ALL and four with T-ALL). CVT was preceded by headache in eight of nine patients, while only five of 18 matched controls without CVT reported headache (OR 20.8; 95% CI 2–212). Seizures occurred in eight of nine patients with CVT and in none of 18 controls (OR 138.7 (10–6422); six patients with CVT presented with focal neurological deficits vs. two of 18 controls (OR 13.8; 95% CI 2–142). Median time between symptom onset and CVT diagnosis was 1 day (range 0–6). CVT was located in the superior sagittal sinus in eight of nine patients. Seven of nine patients had brain parenchymal lesions (five hemorrhagic infarcts, two non-hemorrhagic infarctions). All CVT events occurred during Cycle I of remission induction treatment; in eight of nine patients during or shortly after L-asparaginase therapy (E.coli) and in all patients after at least one dosage of intrathecal methotrexate. CVT formed 38% of all VTE during Cycle I. Eight of nine patients were treated with therapeutic dose (low-molecular-weight) heparin. Two patients also underwent endovascular thrombolysis; both died in the acute phase due to transtentorial herniation. Two of nine patients with CVT had recurrent VTE, none a recurrent CVT. CVT occurrence was adversely associated with obtainment of complete remission on protocol (adjusted OR 0.12; 95% CI 0.03–0.51).

Conclusions: Although CVT is a rare manifestation of VTE, our study indicates it is much more common among adult ALL patients. CVT often occurred during L-asparaginase and methotrexate therapy in Cycle I and had an adverse impact on ALL treatment outcomes. Presence of headache and seizures were strongly associated with CVT. Close monitoring of headache during ALL treatment may contribute to earlier detection of CVT and could improve its outcome.

PA 1.16-3

Plasma fibrin clot abnormalities in patients with multiple myeloma: association with thromboembolic events during induction therapy

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Introduction: Multiple myeloma (MM) is associated with increased risk of venous and arterial thromboembolism. Formation of denser and poorly lysable fibrin clots is observed in patients with arterial and venous thromboembolism.

Aims: We investigated fibrin clot properties and their treatment-induced alterations in MM patients.

Patients and Methods: *Ex vivo* plasma fibrin clot permeability, turbidity and susceptibility to lysis induced by recombinant tissue-type plasminogen activator (tPA) were evaluated in 106 MM patients (55 men and 51 women aged 34–85 years), vs. 100 age- and sex-matched controls. Patients on thromboprophylaxis with aspirin or heparins were eligible. The patients were evaluated prior to and following 3 months of induction therapy, mainly using thalidomide-based regimens.

Results: MM patients had lower clot permeability (−34%), compaction (+34%), indicating denser fibrin clots, impaired fibrin polymerization with longer lag phase (+20%) and lower final turbidity (−25%), combined with hypofibrinolysis reflected by longer clot lysis time ($t_{50\%}$, −24%) and slower rate of D-dimer release from fibrin clots (D−D_{rate},-10%) compared with controls (all $P < 0.001$). Maximum D-dimer levels released from clots (D−D_{max}) were elevated by 24.3% in the patients compared to controls ($P < 0.001$). Patients with IgG MM had lower clot permeability compared with IgA MM (5.9 [5.1–6.4] vs. 6.3 [5.9–7.2] 10^{-9} cm^2; $P = 0.007$). The MM patients had higher peak thrombin concentration (+63.5%), thrombin-activatable fibrinolysis inhibitor (TAFI) activity (+32.9%), plasminogen activator inhibitor-1 (PAI-1) activity (+56.6%) and FVIII (+72.3%, all $P < 0.01$). Clot permeability in MM patients was associated with peak thrombin generation ($r = -0.63$, $P < 0.001$), PAI-1 activity ($r = -0.57$, $P < 0.001$) and TAFI activity ($r = -0.58$, $P < 0.001$), but not with fibrinogen. Similar but positive associations were observed for clot lysis time. Scanning electron microscopy confirmed abnormal clot morphology. A 3-month therapy resulted in improved clot properties observed in 48 MM (45.3%) patients in whom blood samples were available (all $P < 0.01$). Clot permeability and compaction increased (+10% and +16.7%, respectively). Fibrinolysis was accelerated ($t_{50\%}$ by 7.8% and D−D_{rate} by 7.5%). Posttreatment absorbancy of a fibrin gel increased (+19.7%) and lag phase became shorter (−5.9%). Posttreatment clot permeability and lysis time remained significantly associated with peak thrombin generation, PAI-1 activity and TAFI activity (all $P < 0.01$). Thromboembolism ($n = 12$, venous thromboembolism, $n = 10$) observed in 12 patients during induction treatment despite thromboprophylaxis (nine patients received low-dose ASA, three were on prophylactic heparin) were associated with reduced clot permeability (−8.4%), higher D−D_{max} (+13.3%), lower D−D_{rate} (−9%) and 20% longer lag phase at baseline (all $P < 0.05$). Thromboembolism in MM patients was associated with higher baseline peak thrombin concentration (+18.9%), TAFI activity (+20.8%) and PAI-1 activity (+37.5%) together with slightly lower plasminogen (−7.8%; all $P < 0.05$).

Conclusion: Substantial prothrombotic alterations of fibrin clot properties occur in patients with MM. More compact fibrin clots with reduced lysability characterize subjects who experienced thrombotic complications during induction treatment. Our findings show a new prothrombotic mechanism in MM and its determinants.

PA 1.16-4

Incidental venous thromboembolism in kidney cancer patients: a case–control study

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Background: The incidence of venous thromboembolism (VTE) in kidney cancer patients has been previously reported to be between 1.6% and 3.4%. VTE seems to be associated with an increased mortality among kidney cancer patients (hazard ratio [HR] = 1.3–3.2). However, these studies did not report the localization (e.g. lower vs. upper limb) or extent (e.g. distal vs. proximal) of the included VTE. Furthermore, it is unclear if all events were suspected or incidental.

Computed Tomography (CT) is routinely used for staging of the underlying malignancy. As the resolution of CTs increases, more incidental VTEs are being detected. Previous reports showed that incidental VTE might be associated with lower overall survival in cancer patients. However, a wide variety of tumor sites were included and a standardized definition of thrombotic events was not used. The clinical significance of symptomatic and incidental VTE in kidney cancer patients is unknown.

Aims: To determine the effect of suspected and incidental VTE on overall survival in kidney cancer patients.

Methods: A case-control study of all kidney cancer patients treated at our institution between January 1, 2005 and July 1, 2012 was conducted. Cases with VTE (suspected and incidental) were matched in a ratio of 1:2 to controls by gender, age (± 3 years) and stage at cancer diagnosis (TNM stage 1–4). Imaging studies were reviewed to identify the location of the VTE and whether it was symptomatic or incidental. VTE was defined as: (i) proximal DVT of the lower and upper extremities; (ii) pulmonary embolism (PE; segmental or more proximal); and (iii) any intra-abdominal VTE. The primary outcome was overall survival 2 years following the diagnosis of cancer for all symptomatic and asymptomatic VTE.

Results: A total of 1140 primary kidney cancer patients were reviewed for inclusion into the study. Sixty-eight patients (6.0%) had a VTE event. Men represented 69% of cases, mean age 62 years old (range 35–79 years), 53% were early stage (stage 1–3). Twenty-four (35%) cases of VTE were incidentally detected.

Compared to matched controls, all patients with VTE did not have worsened overall survival (HR = 0.97; 95% CI: 0.60–1.56). Treated symptomatic or incidentally discovered VTE were not associated with an increased risk of death compared to controls (HR = 0.82; 95% CI: 0.46–1.46 and HR = 1.31; 95% CI: 0.68–2.52 respectively). Similarly, symptomatic or incidentally discovered PE were not associated with lower overall survival (HR = 1.48; 95% CI: 0.70–3.13 and HR = 0.92; 95% CI: 0.37–2.30 respectively).

Conclusions: Kidney cancer patients are at high risk of VTE (6.0%), of which 35% were found incidentally on staging CT. Treated symptomatic and incidental VTE are not associated with worse overall survival among kidney cancer patients. Future studies assessing the clinical impact of VTE (incidental or symptomatic) among cancer patients need to adjust for tumor type, stage and other important confounders.

PA 1.16-5

Association of interleukins with venous thromboembolism and mortality in cancer patients

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Background: Elevated rates of interleukins (ILs) were reported to be related to the risk of cancer development and cancer progression. The

survival of cancer patients seems to correlate with various IL-levels and IL-levels differ depending upon the tumor type. Studies showed an association between elevated IL-levels and venous thromboembolism (VTE) in non-cancer patients. There is also some evidence that such an association is present in cancer patients.

Aims: The present study aims to evaluate an association between different ILs and the occurrence of VTE as well as the patients' overall survival.

Methods: Patients with solid tumors, either newly diagnosed or progressing after partial or complete remission, were included. Recruitment took place within the framework of the Vienna Cancer and Thrombosis Study (CATS), which is an on-going prospective and observational study. At inclusion, the patients are questioned regarding their medical history and a blood sample is drawn. All participants have been observed for a maximum period of 2 years. Study endpoints are the occurrence of objectively confirmed VTE, death or completion of the study period. IL-levels (ie. IL-3, -4, -6, -8, -10, -11 and -1ß) were determined by using the xMAP technology of Luminex.

Results: We included 726 patients with solid tumors (344 female/382 male, mean age 60 years). Thereof, 337 patients died within the observation period. During the median follow-up time of 705 (quartiles: 262–731) days, 52 patients developed a VTE. The investigated ILs did not show a statistically significant association with the occurrence of VTE. In univariate analyses, levels of IL-1β, IL-3, IL-4 and IL-10 were not associated with survival of patients, whereas an increase of IL-6 [hazard ratio (HR) comparing values > 0–0 of levels: 1.60, 95% confidence interval (CI) 1.29–1.99, $P < 0.001$] and IL-8-levels [HR 1.57, 95% CI 1.04–2.39, $P = 0.032$], respectively, correlated with an increased risk of death. We have also noticed a higher mortality in patients with IL-11-levels above 0 (HR 1.373 [95% CI, 1.103–1.709], $P = 0.005$); however, this difference was not affected by the extent of increase (HR 0.971 [95% CI, 0.908–1.037], $P = 0.376$). In multivariate analyses after adjustment for age and gender, the associations between IL-6-, IL-8- and IL-11-levels and survival were statistically significant. Regarding different tumor entities, higher IL-6-levels correlated with worse prognosis in patients with colon carcinoma (HR 2.405, [95% CI 1.252–4.618], $P = 0.008$) and elevated IL-10-levels with an increased risk of death in patients with lung cancer (HR 1.824, [95% CI 1.098–3.031], $P = 0.020$).

Summary/Conclusions: We did not find an association between the investigated IL-levels and the risk of developing VTE in cancer patients. However, there was a significant higher risk of mortality in patients with elevated IL-6- and IL-8-levels. Furthermore, worse prognosis was observed for patients with lung cancer and increased IL-10-levels, and for patients with colorectal cancer and increased IL-6-levels.

PA 1.16-6

Prolonged thromboprophylaxis with low molecular weight heparin to prevent venous thromboembolism after abdominal cancer surgery: a systematic review

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Background: Patients undergoing major abdominal surgery are at high risk of postoperative venous thromboembolism (VTE). The efficacy and safety of thromboprophylaxis (TP) with low molecular weight heparin (LMWH) administered during the in-hospital period has been proven in several clinical studies. Accordingly, LMWHs are recommended during the in-hospital period. However, clinical studies have shown that patients remain at risk of VTE complications even after hospital discharge.

Aims: In order to clarify the role of prolonged TP with LMWH in patients undergoing abdominal surgery for malignant diseases, we performed a systematic review of all randomized clinical trials, assessing the efficacy and safety of prolonged TP with LMWH after abdominal surgery.

Methods: Electronic searches were performed in the Medline, Embase, Lilacs and the Cochrane Library databases. The most recent search was performed in November 2012. We assessed both randomized and non-randomized controlled clinical trials comparing prolonged LMWH prophylaxis with TP during the in-hospital period only. The patient population in the trials included patients undergoing major abdominal or pelvic surgery for malignant diseases. The outcome measures included symptomatic and asymptomatic cases of VTE, as assessed by objective tests. Safety outcomes were defined as bleeding complications and mortality within 3 months after surgery.

Results: Our search revealed five studies which met the inclusion criteria and were included in the meta-analysis. The total number of patients undergoing curative or palliative surgery for malignant disease was 1210. All studies used bilateral ascending venography to evaluate the VTE end-point. All patients received LMWH during the in-hospital period usually for 1 week, and were then randomized to receive an additional 3-week treatment with LMWH or placebo/control. The venography was performed 30 ± 5 days after surgery. The administration of prolonged TP significantly reduced the incidence of overall VTE compared to in-hospital administration of TP by 50% (14.0% vs. 7.0%), leading to an OR of 0.46 (95% CI, 0.31–0.68; $P < 0.0001$). Also the incidence of proximal DVT was significantly reduced from 4.4% to 0.8% (OR 0.20; 95% CI 0.08–0.50; $P = 0.0006$). There was no significant difference in the incidence of major bleeding complications (OR 1.3; 95% CI, 0.69–2.50). The number needed to treat in order to prevent one episode of the end points was 14 patients for all VTE and 28 for proximal DVT. The overall mortality was comparable in the two groups (4.7% in the treatment group and 4.1% in the control group), with OR 1.21 (95% CI, 0.73–1.99; $P = 0.46$).

Summary/Conclusions: This meta-analysis provides further compelling evidence in support of extending the administration of LMWH for up to 4 weeks after operation in patients undergoing major abdominal or pelvic surgery for malignant diseases. The strong reduction in both asymptomatic and clinically relevant major VTE events, which can be achieved by prolonging LMWH in low doses for a few additional weeks beyond the hospital stay, is not offset by bleeding complications.

PA1.17 – Angiogenesis

PA 1.17-1

Role of alpha6 integrin subunit in tumor growth and tumor angiogenesis

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Background: We have previously shown that alpha6 integrin subunit (α6) plays a major role in post-ischemic angiogenesis. α6 has been implicated in cancer cell migration and in the progression of several malignancies, but its role in tumor angiogenesis is still a matter of debate.

Aims: The aim of our study was to assess the role of α6 in tumor angiogenesis and thus in tumor growth.

Methods: Since α6 KO mice die at birth, we used the cre-lox system to generate a mouse line, α6 fl/fl-Tie2Cre+, with α6 gene deletion specifically in Tie2-expressing cells. Tie2 is a receptor of angiopoietin-2, expressed by endothelial cells, a subset of hematopoietic stem cells, and Tie2-expressing monocytes/macrophages (TEMs), known for their proangiogenic properties. To study the effect of this endothelial deletion of α6 on tumor growth and angiogenesis, one million B16F10 melanoma cells were injected subcutaneously in the right flank of

α6 fl/fl Tie2Cre+ and α6 fl/fl Tie2Cre- (control) male mice and the tumor growth was quantified. Twelve days later the mice were sacrified and tumors were harvested to study the vascular density and the macrophages infiltration in α6 fl/fl'-Tie2Cre+ compared to α6 fl/fl'-Tie2Cre- mice.

Results: Tie-2 dependent deletion of α6 gene reduced tumor growth in a murine B16F10 melanoma model by 56% ($P < 0.001$). Immunohistological analysis of the tumors showed that Tie2-dependent α6 gene deletion was associated with a decrease by 34% of tumor vascularization ($P < 0.05$). We also observed a decrease of 55% of tumor infiltration by proangiogenic Tie2-expressing macrophages ($P < 0.05$), whereas the total number of macrophages in tumors was not statistically significant between α6 fl/fl-Tie2Cre+ and α6 fl/fl-Tie2Cre- mice.

Conclusion: These findings demonstrate that α6 integrin subunit plays a major role in tumor angiogenesis and TEM infiltration. Targeting α6 could be used as a strategy to reduce tumor growth.

PA 1.17-2

Intramyocardial release of engineered chemokines from biodegradable hydrogels prevents injury extension after myocardial infarction

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Background: Myocardial infarction (MI) induces an inflammatory immune response, which causes a progressive expansion of the infarcted area and dilation of the ventricles with replacement of the adjacent myocardium by connective tissue, the so-called 'remodeling' of the heart muscle. The regeneration of the blood vessel network system by recruitment of hematopoietic stem cells for recovering the supply of the infarcted area with oxygen, nutrients, and the prevention of apoptosis of myocytes can be beneficial for heart function.

Methods and Results: We designed a novel therapeutic strategy with Met-CCL5, a chemokine receptor antagonist to suppress initial neutrophil infiltration, and a proteaseresistant CXCL12 (S4V) variant, which is inert to matrix metalloproteinase-2 and exopeptidase cleavage, but retains its chemotactic bioactivity for recruitment of circulating hematopoietic stem cells, promoting neovascularization after MI. To control the proper timing and local release of Met-CCL5 and CXCL12 (S4V), two different biodegradable hydrogels were implemented, a fast degradable hydrogel (FDH) for delivering Met-CCL5 over 24 h and a slow degradable hydrogel (SDH) for a gradual release of CXCL12 (S4V) over 4 weeks.

Summary/Conclusions: We demonstrate that time-controlled release using Met-CCL5-FDH and CXCL12 (S4V)-SDH blocks neutrophil recruitment to the inflamed myocardium, recruits stem cells from circulating blood, and improves cardiac function after MI. Therefore, this study describes a novel and promising therapeutic strategy to sustain the endogenous reparatory mechanisms, and may represent a viable alternative to cell-based therapies, with notable clinical significance.

PA 1.17-3

Characterization of a reduced form of plasma plasminogen as the precursor for angiostatin formation

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Plasma plasminogen is the precursor of the tumor angiogenesis inhibitor, angiostatin. Generation of angiostatin in blood involves activation of plasminogen to the serine protease plasmin and facilitated cleavage of two disulfide bonds and up to three peptide bonds in the kringle five domain of the protein. The mechanism of reduction of the two allosteric disulfides has been explored in this study. Using thiol alkylating agents, mass spectrometry and an assay for angiostatin formation, we show that the Cys462–Cys541 disulfide bond is already cleaved in a fraction of plasma plasminogen and that this reduced plasminogen is the precursor for angiostatin formation. From the crystal structure of plasminogen, we propose that plasmin ligands such as phosphoglycerate kinase induce a conformational change in reduced kringle 5 that leads to attack by the Cys541 thiolate anion on the Cys536 sulfur atom of the Cys512–Cys536 disulfide bond, resulting in reduction of the bond by thiol/disulfide exchange. Cleavage of the Cys512–Cys536 allosteric disulfide allows further conformational change and exposure of the peptide backbone to proteolysis and angiostatin release. The Cys462–Cys541 and Cys512–Cys536 disulfides have ‴/+RHHook and ‴LHHook configurations, respectively, which are measures of the geometry of the disulfide bond. Analysis of the structures of all known allosteric disulfide bonds identified six other bonds that have these configurations. This suggests that the ‴/+RHHook and ‴LHHook disulfides, along with the ‴RHStaple bond, are potential allosteric configurations.

PA 1.17-4

Targeting VEGFR1 on endothelial progenitors modulates their differentiation potential

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We studied whether plasma levels of angiogenic factors and endothelial markers in coronary artery disease patients or undergoing cardiac surgery are modified, and whether those factors modulate endothelial progenitor's angiogenic potential.

One hundred and forty-three patients' plasmas from two different studies were analyzed (30 coronary artery disease patients, 30 patients with stable angina, coupled with 30 age and sex-matched controls; 53 patients underwent cardiac surgery). Among factors screened, only placental growth factor (PlGF) was found significantly increased in these populations. PlGF has been described acting in pathological angiogenesis through binding to VEGF receptor-1 (VEGFR1)/Flt-1, which also links VEGF-A and -B isoforms. PlGF-1 and -2 were then tested on human endothelial colony forming cells (ECFC). We found that PlGF-1 and -2 induce VEGFR1 phosphorylation and potentiate ECFC tubulogenesis *in vitro*. ECFC VEGFR1 gene expression was further inhibited using a specific small interfering RNA (siRNA, sc-29319, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and the chemical compound 4321 (firstly described in Gautier et al, Chem Biol. 2011). We then observed that the VEGFR1-siRNA and the compound 4321 decrease ECFC tubulogenesis potential *in vitro*. Finally we tested the compound 4321 in the preclinical Matrigel®-plug model with C57Bl/6J mice as well as in the hindlimb ischemia model with Swiss Nude mice. We found that 4321 inhibited the plug vascularization, attested by the haemoglobin content and the VE-Cadherin expression level, and that 4321 inhibited the post-ischemic revascularization in the hind limb ischemia model.

PlGF plasma levels were found increased in cardiovascular patients. Disrupting PlGF/VEGFR1 pathway could modulate ECFC induced tubulogenesis, the cell type responsible for newly formed vessels *in vivo*.

PA 1.17-5

Imbalances in angiopoietin 1 and 2 levels in steady state sickle cell disease

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Background: Angiopoetins (Ang) 1 and 2 are two important factors in the regulation of embryonic and post-natal angiogenesis. Ang-1 is important in vessel maturation, whereas Ang-2 is involved in vascular disruption and destabilization. Hence, imbalances in the relative concentrations of these antagonistic modulators of angiogenesis have been associated with the pathogenesis of several conditions associated with hypoxia and inflammation such as sepsis and tumour growth. In sickle cell disease (SCD), tissue hypoxia may also serve as a potent stimulus for this angiogenic switch, which could be related to the pathogenesis of complications such as proliferative retinopathy.
Aims: We herein aimed to evaluate serum levels of Ang-1 and Ang-2 in steady state SCD, and correlate our findings with clinical and laboratory parameters.
Methods: This was a cross-sectional study conducted on a cohort of adult SCD patients, with no history of painful crisis or transfusions in the previous 3 months. Biomarkers were measured by commercially available ELISA kits. Comparisons between patients and controls were performed by Mann-Whitney test. Correlations were evaluated by Spearman Rank correlation coefficient.
Results: A total of 79 adult SCD patients were included in the study, 28 HbSS and 51 HbSC, and 20 healthy age-matched controls. Ang-2 levels were significantly elevated in SCD patients in comparison to controls (3081 vs. 1944 pg/mL; $P = 0.003$), whereas an inverse finding was observed with Ang-1, which was lower in SCD patients than in controls (47.835 vs. 56.292 pg/mL; $P = 0.03$). Since the imbalances between Ang-2 and Ang-1 ratio have been considered more informative than the isolated values of these biomarkers, the Ang-2/Ang-1 ratio was analyzed. As expected, the Ang-2/Ang-1 ratio was much higher in SCD patients than in controls (0.07 vs. 0.03; $P < 0.0001$). When HbSS and HbSC patients were compared, significantly higher Ang-2 levels were observed in HbSS patients compared to HbSC (4877 vs. 2338 pg/mL; $P < 0.0001$). Although no difference was observed in Ang-1 levels between these two patient groups (43.799 vs. 48.566 pg/mL; $P = 0.80$), the Ang-2/Ang-1 ratio was significantly higher in HbSS than in HbSC patients (0.12 vs. 0.05; $P < 0.0001$). When analyzing the whole SCD cohort, Ang-2 levels presented significant correlations with hemolysis markers (hemoglobin, hematocrit, reticulocyte count, lactate dehydrogenase levels, total and indirect bilirrubin) and leukocyte and platelet counts (all P values < 0.001). Ang-1 was significantly correlated with lactate dehydrogenase, total bilirrubin, leukocyte and platelet counts (all P values < 0.05). No significant associations were found between the presence of retinopathy and Ang-2, Ang-1 levels or Ang-2/Ang-1 ratio.
Conclusions: SCD patients present higher serum levels of Ang-2 and a high Ang-2/Ang-1 ratio, both suggestive of the activation of pro-angiogenic programs in these conditions. These findings are present in both HbSS and HbSC patients, but are much more intense in HbSS patients. In other conditions associated with hypoxia and inflammation, a high Ang-2/Ang-1 ratio represents a poor prognosis marker and an important factor in the pathogenesis of these conditions. Further studies are warranted to elucidate the relevance of these findings in the pathogenesis of SCD complications.

PA 1.17-6

Thrombin induces pro- angiogenic signals and disruption of the retinal blood barrier- an *in vitro* model

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Purpose: Thrombin is a multifunctional protease. Apart from its central role in coagulation, thrombin was found to exert some cellular effects and to be involved in inflammatory processes. The retinal pigment epithelium (RPE) forms the outer blood retina barrier (BRB) and its integrity is essential for normal function of the retina. Retinal pathologies such as pathological choroidal neovascularization (CNV), age related macular degeneration, inflammation and vascular occlusions may be accompanied by local thrombin elevation. Moreover, fibrin matrixis found in the areas of pathological newly CNV and acts as a scaffold upon which new blood vessels are grown.
Aims: In the present work, we aimed to explore the impact of thrombin on the integrity and function of the RPE blood barrier. We induced a short-term exposure of RPE to pathological levels of thrombin *in-vitro* and studied the immediate and long lasting changes in the RPE permeability and angiogenic balance.
Methods: ARPE-19 cells were grown for a month to achieve definite polarity properties. Following a short (10 min) exposure to thrombin (1–10 units/mL), the cells were washed and covered with new medium until measurements were performed. Permeability was evaluated based on spectrophotometric monitoring of the leakage of labeled dextran molecules. The expression of pro- and anti- angiogenic genes was evaluated using real time PCR. Protein levels were measured by ELISA or by FlowCytomix[TRADEMARK]. Matrix metalloproteinases (MMPs) activity was examined by zymography.
Results: Short-term exposure to thrombin induced an increase in RPE permeability to 10, 40 and 70 kD dextran that persisted for several hours. Sixty percent decrease in pigment derived growth factor (PEDF) mRNA and twofold increase in vascular endothelial growth factor (VEGF) mRNA expression and protein levels were detected even 24 h after the 10 min exposure to thrombin. MMPs 2 and 9 activities were also increased for several hours after the short-term exposure to thrombin.
Conclusions: Short-term exposure to thrombin induced proangiogenic signals in RPE and caused disruption of barrier properties. The data indicate that short-term exposure to thrombin induces changes in permeability, gene expression, protein levels and activity that persisted for hours following the exposure to thrombin. Based on our *in vitro* findings, we suggest that accumulation of short-term thrombin exposures over years may contribute to the pathological processes of retinal barrier breakage and CNV development in the elderly.

PA1.18 – Antiphospholipid – I

PA 1.18-2

A systematic review and meta-analysis of pathogenic mechanisms of the antiphospholipid syndrome

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Background: The antiphospholipid syndrome (APS) is diagnosed by the combination of vascular thrombosis and/or pregnancy morbidity and the detection of antiphospholipid antibodies (aPLs) in plasma. A

vast amount of mechanisms have been proposed to explain why aPLs can cause thrombosis and/or pregnancy morbidity. The focus of the mechanism studies seems to be largely diffused, given the heterogeneity in the proposed mechanisms. This heterogeneity hinders the effectiveness in pursuing the ultimate goal of benefiting the patient care. Attention and efforts are required to find the underlying reasons and solutions for this problem.

Aims: (i) to provide a systematic review of the studies investigating the pathogenic mechanisms of aPLs, (ii) to identify differences in methodology in these studies that may contribute to the heterogeneity of the proposed mechanisms.

Methods: One hundred and twenty-four studies focusing on the mechanistical actions of aPLs, published between February 2006 and March 2012, were analyzed. Besides the proposed mechanisms, we investigated the following aspects in the selected studies: (i) origin of the aPLs used (mouse/human), (ii) character of the antibodies (monoclonal or polyclonal), (ii) the criteria used to diagnose the APS patients from whom the antibodies were derived, and (iv), confirmation of the involvement of β_2GPI.

Results: The main focus of studies from the last 6 years have been the effects of aPLs on monocytes, platelets, and endothelial cells (37%). aPLs have been demonstrated to cause dysfunctions of these cells via various receptors, signaling pathways and mediators. Other studies focused on the effect of aPLs on the coagulation system (13%), fetal loss (15%), immunopathology (9%), arthrosclerosis (4%) and central nervous system (6%), resulting in five subcategories. Our analysis revealed a great heterogeneity in the methodology part in these studies: (i) heterogeneity in types of aPLs used (patient-derived total IgG antibodies 54%, human/mouse monoclonal antibodies 36%, and others 19%); (ii) heterogeneity in application of APS classification criteria (criteria not mentioned 54.4%, old Sapporo criteria 22.3%, new Sydney criteria 23.3%); and (iii) heterogeneity in the involvement of β_2GPI (anti-β_2GPI together with β_2GPI in the *in vitro* experiments 10%; β_2GPI as the primary reagent 10%, purified human aPLs alone without β_2GPI 55%). Interestingly, the subcategories of aPLs-mediated mechanisms demonstrated different profiles regarding the type of antibody that was used, the applied selection criteria and the involvement of β_2GPI, illustrating that the methodology can influence the eventual demonstrated mechanism.

Conclusions: The great diversity in methodology studying the pathogenicity of aPLs renders the mechanism studies less effective in making noticeable progress in either true knowledge seeking or patient care. This diversity may be minimized by finding a consensus on the optimal approach, more specifically regarding the type of antibody to use, the application of selection criteria if patient-related materials are used, and the eventual necessity to include β_2GPI as a control. By following such consensus methodology, the main target of these aPLs can hopefully be identified, ultimately leading to a strategy tackling the actual cause instead of the peripheral mechanisms.

PA 1.18-3

Increased levels of thrombin activatable fibrinolysis inhibitor – TAFI in patients with the antiphospholipid syndrome

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The Antiphospholipid syndrome (APS) is diagnosed when arterial/venous/small vessel thrombosis or obstetric morbidity occur together with positive laboratory tests for antiphospholipid antibodies (aPL, including anticardiolipin (aCL) and/or anti-$\beta_2$glycoprotein-I (a β_2GPI) and/or the functional lupus anticoagulant (LA) test). The pathophysiology behind aPL associated thrombosis is still unclear. Thrombin activatable fibrinolysis inhibitor (TAFI) represents a link between coagulation and fibrinolysis. Active form of the enzyme removes carboxyl-terminal lysine residues from partially degraded fibrin, thus maintaining fibrin clots and having a prothrombotic function. TAFI has been considered a potential acute phase protein while it may mediate anti-inflammatory effects by regulating complement anaphylatoxins.

Objective: To investigate underlying mechanisms behind the development of thrombotic complications in patients with APS with the focus on TAFI levels, the tightness of the fibrin network and fibrinolytic function.

Methods: Twenty-four patients with APS and 14 controls were included. The levels of pro-TAFI (proenzyme) and TAFI/TAFIi (complex of active and inactive form which corresponds to the concentration of the active form that cannot be measured because of instability) were analysed with a chromogenic assay for TAFI and a specific ELISA for TAFIa/ai (both from Diagnostica Stago, Asnieres, France). Permeability of the fibrin network was assessed by flow measurement technique and clot density together with fibrinolytic function, analysed by a turbidimetric clotting and lysis assay.

Results: The levels of proenzyme – Pro-TAFI (%) as well as the levels of active enzyme TAFIa/TAFIai (ng/mL) were significantly increased in patients with APS compared to controls (117 ± 4.9 vs. 88.7 ± 16.5; $P < 0.001$ and 18.7 ± 2.3 vs. 12.2 ± 0.7; $P < 0.001$, respectively). Fibrin network permeability expressed as permeability coefficient Ks ($cm^2 \times 10^{-9}$) was lower in samples from APS-patients compared to controls (6.4 ± 0.6 vs. 9.8 ± 0.8; $P < 0.001$) indicating a tighter fibrin structure. This finding was corroborated by the turbidimetric clotting assay which demonstrated higher maximum absorbance and longer clot lysis time – CLT ($P < 0.01$) in APS-samples compared to controls. TAFI levels did not correlate with CLT, neither with fibrin clot permeability in the investigated samples.

Conclusions: Increased levels both of TAFI-proenzyme and active form of TAFI were found in patients with APS. This is to the best of our knowledge a novel observation. Thus, TAFI activation may contribute to the thrombotic tendency in the APS by a mechanism which is independent of the antifibrinolytic action of this enzyme. However further studies are required to elucidate TAFIs role in the pathophysiology of APS.

PA 1.18-4

A novel method for the diluted Russell's viper venom time (dRVVT) test that abrogates the effects of vitamin K antagonist (VKA) treatment

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Background: Lupus anticoagulants (LA) are immunoglobulins that affect phospholipid-dependent coagulation reactions *in vitro*. Patients with LA have increased risk of thrombus formation and are treated with anticoagulants such as vitamin K antagonists (VKA). The diluted Russell's viper venom time (dRVVT) test and activated partial thromboplastin time (aPTT) test are clinical assays used for LA detection. Detecting LA in patients on VKA is difficult because basal clotting time is prolonged. To avoid false positives and negatives, ISTH guidelines recommend discontinuation of VKA or 1:1 mixing with pooled normal plasma. However, mixing studies are not always effective, because LA titer is diluted 2-fold and pooled normal plasma quality varies between laboratories.

Aims: In this study, we developed and assessed a new diluent for dRVVT that abrogates the effect of VKA treatment.

Methods: This study was performed comparing plasma samples obtained from healthy volunteers, LA-positive patients without VKA treatment, non-LA patients with VKA treatment and LA-positive patients with VKA treatment. The assays were performed using an in-

house dRVVT reagent, of low (screening) and high (confirming) phospholipids concentrations, with samples: (A) diluted with a new diluent including coagulation factors, (B) diluted 1:1 with normal plasma, and (C) undiluted. The assays were performed on the COAPRESTA [TRADEMARK] 2000 (Sekisui Medical), which has an automatic dilution system. We compared the performance of the assay to the Gradipore (GRA) dRVVT test performed on the Diagnostica Stago STA-R analyzer. Normalized lupus ratio (NLR) was determined, which is calculated according to the following formula:
NLR = (screening test – patient result/confirming test – patient result)/(screening test – mean normal time/confirming test – mean normal time).
Results: Based upon the analyses of samples of patients treated with VKA, we developed a diluent which include a high concentration of coagulation factors the lack of which affects clotting time. A cutoff based on the 99th percentile was determined by evaluating the diluent on samples from 52 healthy volunteers. Subsequently, commercially available samples from 50 LA patients not undergoing VKA treatment were assessed. Two of the patient samples were classified as normal when diluted 1:1 with normal plasma (B), whereas the sensitivity using the new diluent (A) was the same as undiluted (C) samples. We also assayed samples from 56 VKA-treated patients with LA or other diseases. Four of these patients, positive by method (A), (B), and GRA, were classified as negative using undiluted method (C). Twenty-one of these samples, negative by other methods, were classified as positive by GRA. The rate of agreement between the new method (A) and the mixing method (B) was 96%, with the former being more sensitive than the latter.
Summary/Conclusion: We developed a diluent for dRVVT that abrogates the effect of VKA treatment. This diluent can be used on Coapresta[TRADEMARK] 2000 which has an automatic dilution system. Using the new diluent enables sensitive detection of LA, regardless of the presence of VKA.

PA 1.18-5

Antibodies against domain I of beta2-glycoprotein I are a better predictor for the antiphospholipid syndrome than antibodies to the total protein

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Background: Antiphospholipid syndrome (APS) is difficult to diagnose, because currently used laboratory tests have limited specificity. One important assay is the detection of anti-beta2-glycoprotein I (anti-beta2GPI) antibodies. Recently, domain I of beta2-glycoprotein I (beta2GPI-DI) has been suggested to be an important target for antibodies in patients suffering from APS.
Aim: To evaluate whether beta2GPI-DI antibodies are more specifically associated with APS compared to antibodies to total beta2GPI using the same detection technique.
Methods: From May 2010 until November 2012 blood samples were collected from patients referred to our hospital for suspicion of APS. Final diagnosis of APS was based on results of local routinely used tests (lupus anticoagulant and anti-cardiolipin antibodies) in combination with clinical criteria following the International Consensus Statement classification criteria. Antibodies (IgG) against beta2GPI-DI and the complete protein were determined in all samples using the chemiluminescence immunoassay technique based on the BIO-FLASH system (INOVA Diagnostics). Results were correlated with diagnosis of APS. Ethical approval was obtained for conducting the study.
Results: Seventy-nine patients were included and screened, containing 24 (30%) patients with APS and 55 (70%) patients without APS. Domain I antibodies were demonstrated in 12 patients where of 67% were diagnosed with APS. On the other hand, 22 patients were found positive for IgG antibodies against the complete beta2GPI protein of which 41% were APS patients. This resulted in an odds ratio for anti-domain I of 6.4 (1.7–24.0), 95% confidence interval) for APS. In contrast to antibodies to total beta2GPI which were not significantly correlated with APS in our study (odds ratio: 1.9, 0.7–5.5, 95% confidence interval).
Conclusion: In this patient population, the detection of IgG antibodies directed against domain I of betaGPI proved to be more strongly associated with APS than the detection of antibodies against the total beta2GPI protein. These preliminary results must be confirmed in larger clinical studies.

PA 1.18-6

Investigation of resistance to exogenous activated protein C and activation of endogenous protein C in thrombotic patients with or without antiphospholipid syndrome

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Background: It has been suggested that activated protein C resistance (APCr), in the absence of the factor V Leiden genotype, may indicate an increased risk of thrombosis in patients with antiphospholipid syndrome (APS).
Aim: The aim of this study was to investigate APCr with either exogenous recombinant human APC (rhAPC) or by activation of endogenous protein C, in thrombotic patients with and without APS receiving warfarin anticoagulation.
Methods: Plasma from 70 individuals was analysed: 60 patients with a history of venous thromboembolism on warfarin (30 with APS diagnosed in accordance with the international [revised Sapporo] consensus APS criteria, 29 of whom had lupus anticoagulant (LA); 30 non-APS patients; and 10 [not anticoagulated] normal controls). Patients with protein C/S deficiency, factor V Leiden, on hormone replacement therapy or oral contraceptive preparations, were not included in the study.
APCr was expressed as the percentage inhibition of the endogenous thrombin potential (ETP) using the calibrated automated thrombography CAT system in the presence and/or absence of either rhAPC (Eli Lilly) or Protac® (Pentapharm), to activate endogenous protein C. All results were normalised using pooled normal plasma (PNP) as a reference and all patient samples were mixed 1:1 with PNP to correct the reduced coagulation factor activity due to warfarin. rhAPC and Protac concentrations producing approximately 50% inhibition of ETP in the reference plasma were selected. APCr was expressed as normalised ETP, and as normalised percent inhibition of ETP by dividing the percent inhibition of ETP in test plasma, caused by either rhAPC or Protac, by the percent inhibition of ETP determined in PNP alone where decreased values indicate APCr.
Results: Using rhAPC, no significant differences were observed in APCr with exogenous rhAPC between APS patients, non-APS patients and normal controls, median values (observed ranges) for APCr: 123.9 (21.9–221.5), 120.0 (59.2–241.6) and 145.5 (68.2–319.2) respectively. However using protac to activate endogenous protein C, significant differences were observed between the three groups ($P = 0.0015$), with APS patients showing more resistance to activation endogenous protein C than non-APS patients ($P = 0.023$), median values (observed ranges): 77.0 (5.6–96.3), 91.4 (41.6–347.2) and 168.9 (60.2–213.5) in APS patients, non-APS patients and normal controls, respectively. There was a significantly greater degree of resistance to activation of endogenous protein C than to exogenous rhAPC in both APS ($P < 0.0001$) and non-APS thrombotic patients ($P = 0.0021$). Five patients in the APS group demonstrated marked APCr with both method and these patients have experienced clinically more severe disease, i.e. both arterial and venous thrombosis and/or multiple events whilst on therapeutic anticoagulation.

Conclusion: These results suggest that irrespective of the presence of APS/LA, thrombotic patients have a greater degree of resistance to activation of endogenous protein C. Whether this is due to the presence of autoantibodies that interfere with protein C activation, and/or changes in the activity of other haemostasis modulators, remains to be elucidated. APCr appears to be a marker of thrombosis risk not just in APS populations and may indicate a more severe clinical phenotype.

PA1.19 – Innate and Acquired Immunity

PA 1.19-1

Plasma endothelial protein C receptor influences innate immune response in ovarian cancer by decreasing the population of natural killer and TH17 helper cells

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Background: In spite of the growing importance of endothelial protein C receptor/active protein C (EPCR/aPC) in tumor biology, their impact on immunological homeostasis remains largely unexplored.
Objective: To assess whether soluble plasma EPCR (s-EPCR), which is a regulator of circulating aPC, can be involved in innate immune response in cancer patients.
Patients/Methods: (i) In Ovcar-3 ovarian cancer line, the role of aPC in secretion of cytokines was analyzed by proteinarray using a set of 174 membrane coupled anti-cytokines. (ii) In 33 patients, with a diagnosis of ovarian epithelial cancer (asymptomatic disease in progression detected by increase in CA 125 level), s-EPCR was measured using Asserachrom s-EPCR immunoassay, blood immune cell phenotypes were determined by flow cytometry, using a set of 30 antibodies against factors of the immune system and plasma cytokines were evaluated using an array of 100 cytokine antibodies. Spearman's rank correlation coefficients (r) and Coefficient significance was determined by a statistical hypothesis test ($\alpha = 0.05$).
Results: (i) aPC induced the secretion of several cytokines in Ovcar-3 cells. (ii) 61% of patients exhibited a concentration of plasma s-EPCR well above the base-line (normal plasma level, 100 ± 28 ng/mL). Comparing immune cell phenotypes in patients having a normal level of s-EPCR with those having a high level of s-EPCR, it was found that s-EPCR level was correlated with high intensity of cells expressing CD45ra, CD3, CD8, CD25 and low intensity of cells expressing CD56 (NK cells), IL2, IL10, IL17a (TH17 cells), IL21 (TH 21 cells) and CD29 markers ($r \geq 0.60$). In addition, when level of s-EPCR was high, a decrease in IL17a-expressing cells was associated with decrease of cells expressing CD161 and ROR-γ involved in the secretion of IL17. (iii) High level of s-EPCR correlate with high levels of plasma bio active proteins such as insulin-like growth factor-2 (IGFII), IL13rα, macrophage inflammatory protein (MIP1α) and matrix metalloproteinase-7 (MMP7) that have already been proposed as biomarkers for ovarian cancer and particularly those with poor prognosis.
Conclusions: s-EPCR produced by ovarian cancer cells, by modulating circulating aPC, influences secretary behavior of tumor cells (cytokines and interleukins). Consequently, s-EPCR in turn acts on the innate immune response by decreasing the effectors cells such as Natural killer and T helper cells. (TH2, TH17, TH21).

PA 1.19-2

Acute hypoxia induced adhesion of leukocyte are mediated through toll-like receptor3-interferon? Signal transducers and activators of transcription1 pathway

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Background: Leukocyte adhesion associated with local or systemic hypoxia can lead to prothrombotic diatheses. Cell adhesion molecules (CAMs) like Intra Cellular Adhesion Molecule-1 (ICAM) and Platelet Endothelial Cell Adhesion Molecule (PECAM) are critical for the recruitment, and transendothelial migration of leukocyte at the site of vascular injury. Inflammatory cytokines play an important role in leukocyte adhesion and trans-endothelial migration. But the molecular mechanism of acute hypoxia (AH) induced vascular inflammation and leucocytes adhesion remains unknown. Ligands released from dying cells activate pattern recognition receptors including toll-like receptor3 (TLR3). The TLRs are innate immune receptors which play a key role in immune activation and inflammation. Therefore, we hypothesized that AH might up-regulate TLR3 which subsequently facilitates leukocyte adhesion to endothelium through interferon (IFN α/γ)-STAT1 pathway.
Aim: Aim of the present study is to delineate the role of TLR3 in AH induced adhesion of leukocyte and underlying signalling mechanism.
Methods: Mice (Swiss albino, 25–30 g, male, each group contains 4–6 mice) were subjected to AH (in an environmental chamber) equivalent to the prevailing atmospheric conditions at an altitude of 7628 m (282 mm Hg barometric pressure) having O_2 content approximately 8.5% for 0–24 h. The temperature and humidity were constantly maintained to 25 ± 2 °C and 55 ± 5% respectively throughout the whole experiment. Age and sex matched control mice (normoxia, not exposed to AH) were maintained in 21% O_2 with same environmental conditions. Leukocyte was isolated from freshly drawn blood though density gradient centrifugation. Lung immunohistochemistry were performed as described. The expressions of TLR3, IFNs and CAMs were analyzed by immunoblotting, flowcytometry, enzyme-linked immunosorbant assay (ELISA) and Immunohistochemistry. The interaction between human umbilical vein endothelial cells (HUVEC) and leukocyte were evaluated by *in-vitro* adhesion assay. *In vivo* inhibition of TLR3 and IFNs were performed by tail vein injection of anti-TLR3 and anti-IFN neutralizing antibody 2 h before AH exposure respectively. A similar injection of poly I:C for indicated time was made to observe the TLR3 activation. Selective inhibition of STAT-1 phosphorylation was performed through tail vein injection of AG 490 (10 mg/kg) 2 h before AH or poly I:C treatment.
Results: The expression of TLR3, IFNs and CAMs were increased significantly following AH exposure. Pretreatment of TLR3 immunoneutralising antibody significantly decreased AH induced IFNs and CAMs expression as well as adhesion of leukocyte to HUVEC. We also showed that pretreatment of IFNγ neutralizing antibody significantly inhibited CAMs expression and leukocyte adhesion to HUVEC, but pre-treatment of IFNα neutralizing antibody fails to do so. We also found increased STAT-1 phosphorylation both in AH and poly I:C treatment. But the pre-treatment of TLR3 neutralizing antibody or STAT-1 inhibitor (AG490) 2 h prior to AH exposure or poly I:C treatment completely abrogated STAT-1 phosphorylation, CAMs expression and leukocyte adhesion to endothelium.
Conclusions: Collectively, these data show that AH induced adhesion is mediated through TLR3-Interferon γ- STAT1 axis.

PA 1.19-3

Protease-activated receptor (PAR) 1 impairs host defense during severe Gram-negative sepsis (melioidosis)

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Background: Melioidosis, an endemic disease in Southeast Asia caused by the gram-negative bacterium *Burkholderia* (*B.*) *pseudomallei*, is associated with pneumonia often leading to severe sepsis. Protease-activated receptor-1 (PAR-1) is a G-coupled transmembrane receptor expressed by multiple cell types present in the lungs that can be activated by various proteases generated during acute inflammation.
Aims: In this study we aimed to investigate the role of PAR1 during melioidosis.
Methods: Wildtype (WT) and PAR1 knockout (KO) mice were infected intranasally with *B. pseudomallei* to induce melioidosis. Mice were sacrificed after 24, 48 or 72 h and survival studies were done. Lungs, liver and blood were harvested to measure bacterial loads, cytokines, clinical chemistry, pathology scores and coagulation parameters. Additionally, bronchoalveolar lavages (BAL) were done and the BAL-fluid (BALF) and cells were analyzed.
Results: PAR1 KO mice showed decreased bacterial loads in lungs, BALF and liver (all $P < 0.01$ vs. WT). In addition, 72 h after infection, PAR1 mice displayed a decreased cell influx in the lungs ($P < 0.05$), which was due to a lower number of neutrophils. No differences in levels of pro-inflammatory cytokines (TNF-α, IL-6, IL-10, IFN-γ and MCP-1) and lung histopathology were seen between WT and PAR1 KO mice. Mortality was similar in WT (65%) and PAR1 KO mice (82%) ($P = 0.2$).
Conclusion: PAR1 impairs host defense during severe Gram-negative sepsis caused by *B. pseudomallei* by facilitating bacterial growth.

PA 1.19-4

Thrombin receptor activation impairs TLR mediated whole blood TNF production by thrombin induced MKP-1 expression and p38 deactivation

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Blood coagulation may play a decisive role during infections and the sepsis syndrome. However how coagulation influences host defense and cytokine responses in whole blood and infection models is poorly understood. In a new *in vitro* human recalcified whole blood coagulation model we found that the early TNF response to viable *E. coli* is specifically down regulated by thrombin generation. Prevention of whole blood clotting by either APC, heparin, LMWH, TFPI or the specific thrombin inhibitor Lepirudin all increased TNF production by *E. coli*. Prevention of blood clot formation by fibrin polymerization inhibitor did not influence TNF production, and it could be concluded that thrombin itself downregulates TNF production. Accordingly, thrombin receptor activation by the PAR-1 activation peptide TRAP decreased TNF production induced by TLR4 or TLR2 stimulation in heparin blood. This indicates that PAR-1 stimulation actively inhibits TLR mediated TNF production. Phospho-P38 is pivotal for TNF expression and we found that MKP-1, the most important p-P38 inhibitor, is upregulated at the mRNA and protein level through PAR-1 activation by TRAP in combination with LPS. In line we found in our whole blood coagulation model that thrombin generation synergizes with *E. coli* in the induction of MKP-1. Accordingly, phospho-P38 was consistently increased in leukocytes from *E. coli* stimulated whole blood when thrombin generation was inhibited compared to clotted controls or control with fibrin polymerization inhibitor. Moreover, thrombin inhibited TNF production by LPS stimulated isolated PBMC's. During sepsis, mice with impaired thrombin generation (lowTF) displayed decreased MKP-1 expression and increased TNF production compared to TF+ mice in affected organs. This indicates that thrombin may suppress local host defense during sepsis when TNF is limited and crucial for resistance to microbes. Indeed during *E. coli* peritonitis lower bacterial burden is observed in FVIII-deficient and TF-deficient mice in line with increased host defense to virulent *E. coli* when thrombin is inhibited. Our data indicates that thrombin receptor activation hampers TNF production during infection and sepsis.

PA 1.19-5

Thrombelastographic studies reveal a new mechanism in MASP-1-induced fibrin clotting

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Background: The complement system is an integral part of the innate immune system and consists of plasma proteins which monitor the blood for foreign particles and participate in their clearance. It is not only phylogenetically related to the coagulation system, in addition a number of interactions between the two systems have been described. Mannan-binding lectin-associated serine protease-1 (MASP-1) is a protein of the complement lectin pathway. Due to structural features closely resembling thrombin, MASP-1 was assumed to interact with the coagulation cascade; recent findings have supported this hypothesis.
Aim: The aim of this study was to examine the effects of MASP-1 on kinetics of clot formation and clot structure along with comparing its effects on coagulation between whole blood (WB), platelet-poor plasma (PPP) and a purified system.
Methods: Thrombelastography using a ROTEM® device was the method of choice for this study because it allows to test coagulation in a global environment and study clot formation kinetics and viscoelastic properties of the clot. Freshly drawn citrated WB and directly derived PPP were incubated with three different concentrations of either MASP-1, thrombin or a combination of both flanking their physiological concentrations (10 μg/mL and 20 nM respectively, whereby the chosen thrombin concentration corresponds to the observed concentration during the initiation phase of plasmatic coagulation). The experiment was repeated in prothrombin-depleted plasma. In a second approach the same concentrations of thrombin and MASP-1 were tested in a purified system containing fibrinogen only or fibrinogen supplemented with $CaCl_2$ and factor XIII.
Results: In PPP, addition of the physiological concentration of MASP-1 reduced the clotting time (CT; the time until onset of clotting) by up to 15%, while thrombin decreased the CT by up to 89%. When PPP was supplied with both enzymes, the CT was not further reduced as it was with thrombin only. Similar results were obtained in WB.
The clot formation time (CFT; representing the duration from onset of clotting until reaching a clot of a certain firmness), was also shortened by MASP-1 in PPP and WB (29% and 41% respectively). The same was observed with thrombin, where a CFT-reduction of 38% (PPP) and 47% (WB) was reached. When both enzymes were added in their physiological concentration their effects on CFT added up to a 44% (PPP) and 67% (WB) reduction.
In the purified system as well as in prothrombin-depleted plasma, thrombin was able to generate a clot in both environments, while MASP-1 failed to induce clotting.
Conclusions: For the first time we used thrombelastography to investigate the influence of MASP-1 on whole blood and plasma clotting. MASP-1 showed strong and synergistic effects with thrombin on clot formation. The observed effects were more distinct in WB than in PPP suggesting that MASP-1 may interact with platelets. In the isolated system and in prothrombin-depleted plasma MASP-1 failed to induce clotting, indicating that it triggers the coagulation in an indirect way

via prothrombin activation. Further work is ongoing to unveil the underlying mechanisms and potential physiological relevance.

PA 1.19-6

The role of autoantibodies to heat shock proteins (HSP-70) in immune reactions in old-aged patients with chronic generalized periodontitis and coronary heart disease

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Introduction: Autoimunne process is accompanied by any chronic inflammation and chronic generalized periodontitis is no exception. However, the role of autoimmune reactions in chronic periodontal inflammation in modern dentistry have not been evaluated.

We believe that one of the reasons supporting the chronic inflammation of the periodontium are autoimmune processes associated with the expression of HSP in the destruction.

Aim: To investigate the intensity of formation of autoantibodies to heat shock proteins (HSP-70), and some immunological parameters in peripheral blood and saliva of the elderly before and after treatment of chronic generalized periodontitis and coronary heart disease.

Materials and Methods: Ninety-six patients with chronic generalized periodontitis of moderate severity (age 64–75 years) and 25 controls aged 64–75 years with no signs of periodontal inflammation. Patients with chronic generalized periodontitis and controls enrolled in Veterans Hospital of war with clinical manifestations of coronary heart disease, and received on this occasion cardiovascular therapy. Patients with inflammatory periodontal received additional local anti-inflammatory therapy. The control and experimental groups did not include patients with lesions of the gastrointestinal tract, respiratory tract, allergic manifestations and with oncopathology.

Blood samples and saliva was performed before and after treatment. In the blood and saliva studied concentration of autoantibodies to HSP-70 content of interleukin and immunoglobulins (s IgA, IgA, IgM, IgG) by ELISA.

Results and Discussion: Our studies have shown that autoantibodies to HSP-70 in older people not suffering from chronic periodontitis, in saliva exist, but not at high concentrations (66.6 $\pm$ 7.1 ng/mL) in human blood of the control group required significantly more autoantibodies (164 $\pm$ 13.6 ng/mL).

When inflammation of the periodontal maintenance of autoantibodies to HSP-70 increased (212.2 $\pm$ 16.3 ng/mL) in saliva and blood. HSP act on antigen-presenting cells, that cause release of proinflammatory cytokines and chemokines. Our studies have shown that in chronic periodontitis concentration of Il-8, Il-a, IgG, IgA, Ig M, s/IgA were increased. In the blood of patients noted a significant increase in the level of Il-1a, Il-8 and IL-4. At the same time there is polyclonal activation of adaptive immunity to the increase in blood concentrations of IgG, IgM, IgA, and autoantibodies to HSP-70.

Following the local anti-inflammatory therapy and concomitant coronary artery disease symptoms, correction of immune parameters occurs in part. The level of IgG in saliva and blood is not changed, indicating a preserved because of their synthesis. The concentration of autoantibodies to HSP is somewhat reduced in the saliva and blood, but does not reach the reference values.

Conclusions: High levels of autoantibodies to HSP-70 in saliva and blood suggests about pathogenetic role of protein-chaperones in the development of periodontal inflammation and chronicity of the process.

PA1.20 – Recurrent Venous Thrombosis – I

PA 1.20-1

Aspirin for the prevention of recurrent venous thromboembolism (VTE): the INSPIRE collaboration

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Background: In patients with a first unprovoked venous thromboembolism (VTE) who have completed initial and long-term oral anticoagulation, the risk of recurrent VTE is substantial after anticoagulant treatment is discontinued. The WARFASA and ASPIRE studies have recently shown that aspirin reduces the risk for recurrent VTE in these patients (NEJM 2012). An individual patient data meta-analysis of these two trials was planned in the INSPIRE project (ACTRN12611000684921) before the results of either study were known.

Aims: To assess effect of aspirin vs. placebo on recurrent VTE, major vascular events and bleeding.

Methods: WARFASA (n = 402) and ASPIRE (n = 822) were both randomized, double-blind, placebo-controlled studies with harmonized study protocols designed to evaluate the effect of aspirin on VTE recurrence, major vascular events and bleeding. Primary analysis of treatment was intention-to-treat using individual time to event data stratified by study and within 4 years of randomization on the primary outcome of VTE.

Results: Baseline characteristics were well balanced between the two treatment groups pooled over the two studies (1224 patients, 30.4 months median follow-up). WARFASA patients were older than ASPIRE (61 $\pm$ 15 vs. 54 $\pm$ 16 years, $P < 0.001$), had higher smoking rates (16% vs. 9%, $P < 0.001$) and were more often males (64% vs. 54%, $P < 0.01$). Aspirin reduced the risk of recurrent VTE (7.5% per annum (pa) vs. 5.1% pa; Hazard Ratio (HR) 0.68, 95% CI 0.51–0.90, $P = 0.008$) with a similar relative reduction of deep vein thrombosis (HR 0.66, 95% CI 0.47–0.92, $P = 0.01$) and pulmonary embolism (HR 0.66, 95% CI 0.41–1.06, $P = 0.08$). A similar reduction was also observed on the rate of major vascular events (recurrent VTE, MI, stroke and CVD death): 8.7% pa vs. 5.7% pa; HR 0.66, 95% CI 0.50–0.86, $P = 0.002$. The rate of major bleeding was low and similar in the two study groups (0.4% and 0.5% pa for placebo and aspirin, respectively). Similar relative reductions in risk were seen in pre-specified subgroups (each interaction $P > 0.1$), but larger absolute benefits were seen in men and those of older age. Larger treatment benefits were seen in the first year of treatment (11.0% pa vs. 5.4% pa; HR 0.49, 95% CI 0.32–0.76, $P = 0.001$) than in years 2–4: (3.6% v 3.2% pa HR 0.89, 95% CI 0.60–1.31, $P = 0.56$).

Conclusions: Aspirin given after anticoagulant treatment reduces the overall risk for recurrence by about one third in patients with a first unprovoked VTE without significantly increasing the risk of bleeding. Higher rates of VTE recurrence and larger absolute treatment benefits are seen during the first year.

PA 1.20-2

Low level of residual thrombotic obstruction following 6 months of anticoagulant treatment for acute pulmonary embolism

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Background: In patients with acute pulmonary embolism (PE), systematic assessment of residual thrombotic obstruction after long-term anticoagulation has been understudied. This information may be of clinical importance for diagnostic baseline imaging, in case of clinically suspected recurrent PE or as a tool for risk stratification for recurrent venous thromboembolism (VTE). On the basis of retrospective, selective studies, nowadays physicians often order repeat CT scans after 6 months of anticoagulant treatment without solid evidence for clinical relevance.
Methods: In this prospective, multi-center cohort study, consecutive patients with acute PE underwent baseline CT pulmonary angiography (CTPA) following 6 months of anticoagulant treatment. Two independent, expert thoracic radiologists systematically and independently assessed all CTPAs for the presence of residual thrombosis. The degree of pulmonary obstruction was calculated using the obstruction index of Qanadli. A two-year follow-up was performed to assess the correlation between residual thrombotic obstruction and recurrent VTE. Ethical approval and informed consent was obtained.
Results: A total of 141 patients were included. At time of diagnosis, the mean obstruction index was 30% (standard deviation (SD) 20). After 6 months of treatment, 85% of the patients had complete clot resolution. Residual thrombotic obstruction was identified in 21 patients (15%; 95% confidence interval [CI]: 10–22%). Of these, seven (5%; 95% CI: 2–10%) had residual thrombosis in at least a segmental pulmonary artery, accounting for a mean obstruction index of 8% (SD: 5). In the other 14 patients (10%; 95% CI: 6–16%), non-occlusive post-thrombotic webs or strictures were found. During follow-up, 11 (7.8%) patients experienced recurrent VTE. Residual thrombosis did not correlate with recurrent VTE; hazard ratio: 0.6 (95% CI 0.08–4.7).
Conclusion: This study reveals that the incidence of residual thrombotic obstruction following treatment for acute PE is considerably lower than currently assumed. These findings, combined with the absence of a correlation between residual thrombotic obstruction and the occurrence of recurrent VTE, do not support the use of baseline CTPA imaging in patients treated for acute PE.

PA 1.20-3

The prognostic significance of residual vein obstruction in patients with treated deep vein thrombosis: a patient-level meta-analysis

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Background: Risk stratification for recurrence after unprovoked deep vein thrombosis (DVT) could improve clinical decisions on the optimal duration of anticoagulant therapy. Residual venous obstruction (RVO) may be pivotal in clinicians' decision-making but results from clinical studies and study-level meta-analyses are conflicting
Aims: We aimed to determine if RVO is a valid predictor of recurrent venous thromboembolism (VTE) in patients with a first symptomatic unprovoked proximal DVT who had received at least 3 months of anticoagulant therapy.
Methods: Systematic search of electronic databases (Medline, Embase, Cochrane Library) until September 2012, supplemented by manual reviewing of the reference lists and contacting content experts. Prospective studies that investigated the association between RVO and recurrent VTE in patients with a first unprovoked proximal DVT were selected. Individual patient-level data were obtained from the datasets of selected studies and merged into a single database. A multivariate, shared-frailty Cox model was used to calculate hazard ratios (HRs) for recurrent VTE which included the following covariates: RVO; age; sex; anticoagulation duration before RVO assessment; and anticoagulation continuation after RVO assessment.
Results: There were 2527 patients studied from 10 prospective studies. RVO was found in 1380 patients (55.1%) after a median of 6 months (inter-quartile range [IQR]: 3–7.6) from a first unprovoked DVT. Recurrent VTE occurred in 399 patients (15.8%) during a median follow-up of 23.3 months (IQR: 12.8–30) from RVO assessment. After multivariate Cox analysis, RVO was independently associated with recurrent VTE (HR = 1.32, 95% CI: 1.06–1.65). RVO was a stronger predictor of recurrent VTE if detected early, i.e. at 3 months (HR = 2.17; 95% CI: 1.11–4.25), but no longer predictive if detected later, i.e. > 6 months (HR = 1.19; 95% CI: 0.87–1.61) after DVT is diagnosed.
Conclusion: In patients with unprovoked DVT who have received at least 3 months of anticoagulant therapy, RVO is a weak overall predictor of recurrent VTE and has predictive utility if detected at an earlier time (3 months) but not later time (> 6 months) after a diagnosis of DVT.

PA 1.20-4

Complication rates among patients treated for upper extremity thrombosis: a meta-analysis and systematic review

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Background: Upper extremity deep vein thrombosis (UEDVT) accounts for 4–11% of all thromboses in the deep veins. Current guidelines advise to treat patients with an UEDVT similarly to patients with a lower extremity thrombosis, i.e. a minimum duration of treatment of 3 months. The risk of recurrent venous thromboembolism (VTE), recurrent pulmonary embolism (PE) and recurrent fatal VTE after a first lower extremity thrombosis in the first 3 months of anticoagulation have been reported to be 3.2% (95% CI, 2.4–4.1%), 1.3% (95% CI, 1.0–1.7%) and 0.3% (95% CI, 0.2–0.5%) respectively. The rate of major and fatal bleeding episodes have also been reported to be 1.6% (95% CI, 1.2–2.1%) and 0.2% (95% CI, 0.1–0.3%) respectively. However, the rates of recurrent VTE and major (fatal) bleeding episodes in patients with UEDVT receiving anticoagulant therapy are unknown. These point estimates are important to assess the risks and benefits of anticoagulation and to help counsel patients with UEDVT.
Aim: To summarize the risk of recurrent VTE (UEDVT, PE and fatal VTE), major, fatal bleeding rates and overall mortality in patients with UEDVT undergoing the first 3 months of anticoagulation therapy.
Methods: A systematic literature search was conducted to identify potential studies on MEDLINE, Embase, and Cochrane Central Register of Controlled Trials using an OVID interface to 20 December 2011. We included randomized controlled trials and prospective cohort studies. We calculated the pooled proportions for the different outcomes at 3 months of follow-up. Ninety five percent confidence intervals (95% CI) were calculated for each rate.

Results: Eleven studies met all inclusion criteria for our meta-analysis and 1162 patients with confirmed UEDVT were included in the analysis (all prospective cohort studies). The majority of patients were treated with unfractionated or low-molecular-weight heparin in combination with vitamin K antagonists. During the initial 3 months of anticoagulation, the rates of recurrent UEDVT was 1.7% (95% CI, 0.9–2.6%; I^2 = 7.6%), 1.6% (95% CI, 1.0–2.4%; I^2 = 0%) for PE, and 2.3% (95% CI, 1.2–3.8%; I^2 = 27.9%) for major bleeding. The rate of fatal PE and fatal bleeding was 0.5% (95% CI, 0.2–1.0%; I^2 = 0%) and 0.87% (95% CI, 0.39–1.54% I^2 = 0%) respectively. The overall mortality was 13.1% (8.3–19.0%; I^2 = 76.2%). All studies had adequate representativeness, assessment of outcome measures and follow-up.
Conclusion: The rate of recurrent VTE on anticoagulant therapy for patients with UEDVT is similar to patients with a lower extremity proximal deep vein thrombosis, however the rates of major and fatal bleeding episodes appear higher for patients with UEDVT. These results might suggest that a less aggressive treatment strategy for patients with UEDVT is warranted. There is an urgent need for randomized therapeutic management studies in UEDVT patients.
Acknowledgement: M. Tan was funded by the Dutch Heart Foundation (grant no. 2011T026).

PA 1.20-5

The risk of venous thromboembolism in renal cell carcinoma patients with residual and non-resected tumor thrombus

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Background: Renal cell carcinoma (RCC) has a unique ability to invade the venous system in 4–10% of the cases. Radical nephrectomy with thrombectomy is the most effective treatment in patients with kidney cancer and tumor thrombus (TT), but the surgical approach depends on the tumor thrombus level, presence of metastases, comorbidities and patient performance status. Complete tumor thrombus extraction is not always possible and residual tumor fragments may remain within the venous system post-surgical thrombectomy. The long-term risk of venous thromboembolism (VTE) in RCC patients with TT and residual thrombus post extraction or not surgically removed is unknown.
Aims: Our objective was to determine the long-term risk of VTE in patients with persistent (residual and non-resected) tumor thrombus.
Methods: A cohort study of patients with stage 3–4 RCC was undertaken. All diagnostic imaging studies were reviewed to identify patients with tumor thrombus. TT was defined as the presence of an intra-luminal filling defect in the renal veins, hepatic veins, or inferior vena cava (below and above the diaphragm) directly extending from a renal mass detected on computed tomography (CT) or magnetic resonance imaging (MRI). Residual tumor thrombus was defined as the presence of TT fragments post thrombectomy seen on imaging. Patients with tumor thrombus in whom VTE occurred prior to surgical removal of the TT were excluded. The primary endpoint was the rate of VTE during a 2 year follow-up period. VTE was defined as proximal lower limb (popliteal vein or more proximal) deep vein thrombosis or pulmonary embolism. Rates between groups were compared using Pearson's Chi-square statistic.
Results: A total of 304 RCC patients with stage 3–4 kidney cancer were included in this study, of them 126 (41.4%) had tumor thrombus. 65.8% were males, median age was 65.0 years (range 22–91) and 63.9% were stage 4. Post-operatively, 61 (20.1%) patients had persistent tumor thrombus (37 residual tumor thrombus detected on CT or MRI post thrombectomy and 24 not surgically removed) whereas 243 did not (65 complete thrombectomy and 178 no tumor thrombus). The rate of VTE within 2 years of cancer diagnosis was 23.0% (95% CI: 14.2–34.9%) for patients with persistent TT compared to 6.2% (95% CI: 3.5–10.7%) for patients without TT ($P < 0.0001$), with a relative risk of 3.7 (95% CI: 1.8–7.7). The 2-year rate of VTE in patients with residual TT and in patients who could not have TT resection was 24.3% (95% CI: 13.4–40.1%) and 20.8% (95% CI: 9.2–40.5%), respectively. RCC patients who had complete resection of their TT had a 2-year VTE rate of 4.6% (95 CI: 1.6–12.7%). The 2-year relative risk of VTE in patients with residual TT compared to those with completely resected TT was 2.9 (95% CI: 1.2–7.4%).
Conclusions: The presence of persistent tumor thrombus is an important risk factor for VTE among RCC patients. Future trials assessing the clinical impact of thromboprophylaxis among RCC patients with residual tumor thrombus are needed.

PA 1.20-6

Predictors of recurrent venous thromboembolism in cancer patients: findings from the worldwide RIETE registry

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Background: Patients with malignancy have a significant increased risk for venous thromboembolism (VTE), compared with non-cancer patients. A better understanding of the characteristics that may influence the risk of VTE recurrence in cancer patients is needed, with the aim to offer a better tailored treatment approach for cancer-associated VTE.
Aims: Aim of the present analysis was to evaluate potential predictors of recurrent VTE within 3 months from an index event, in patients with cancer.
Methods: RIETE is an ongoing, international, prospective cohort of consecutive patients presenting with symptomatic VTE confirmed by objective tests, and followed-up for at least 3 months. Each episode of recurrent deep vein thrombosis (DVT) or pulmonary embolism (PE) has to be documented by objective tests as well. Death is considered VTE-related if occurred within the first 10 days after a PE episode, or in case of sudden, unexpected death in the absence of alternative diagnosis. The association between occurrence of recurrent VTE (fatal or non-fatal) and potential predictors was evaluated by means of a multivariable logistic regression analysis. Odds ratios (OR) and corresponding 95% confidence intervals were calculated and a P-value < 0.05 was considered statistically significant.
Results: The overall population of cancer patients included in RIETE at January 2013 (n = 9280) was considered. Three-month recurrent VTE occurred in 3.8% of patients (fatal PE: 2.3%). Advanced disease was the strongest predictor of recurrent VTE (OR 2.09, 95% confidence interval 1.73–2.52). Lung and pancreatic cancer were significantly associated with the risk of recurrent VTE (OR 1.49, 1.18–1.88, and 1.52, 1.04–2.22, respectively), while no significant increase was shown in case of stomach and ovarian cancer, and patients with breast or colorectal cancer had lower risk of recurrent VTE (OR 0.50, 0.35–0.72, and 0.68, 0.50–0.91, respectively). Patients with reduced mobility had more frequent VTE recurrence (OR 1.50, 1.22–1.85), as it was in case of PE as VTE presentation (vs. isolated DVT, OR 1.72, 1.45–2.08), increased creatinine levels (OR 1.48, 1.20–1.83) and previous recent major bleeding (OR 1.75, 1.16–2.85). No significant correlation with risk of recurrent VTE was pertinent to other variables, namely

age, concomitant chronic pulmonary disease, history of VTE, presence of anemia, and body weight.

Summary/Conclusions: Recurrent VTE is a not negligible complication in cancer patients, and to evaluate characteristics of patients which lead to increased or decreased risk is of clinical interest. Some findings from our study may have been influenced by the observational approach of data collection. However, to our knowledge the present analysis is that with the highest statistical power ever conducted to examine predictors of recurrent VTE in cancer patients. In this perspective, some items we assessed as strongly related to increased risk (tumor stage, some types of cancer, PE at presentation, reduced mobility) could be considered for risk stratification for secondary prophylaxis in daily practice. The identification of patients at increased risk of recurrent VTE may be important for optimally adapting treatment and patients' management, a hypothesis to be addressed in future clinical trials.

PA2.01 – Antiplatelet Agents: Aspirin – I

PA 2.01-1

The response to enteric-coated aspirin is weight-dependent in multiple assays

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Background: The response to aspirin as measured by serum thromboxane levels has been shown to be affected by weight and the use of enteric coating. However, it is not known if this applies to aspirin non-response as determined by other assays especially as they do not agree with non-response determined by serum thromboxane levels.

Aims: To determine if non-response to aspirin, as determined by VerifyNow® and platelet aggregation are affected by weight and enteric coating.

Methods: Patients (138) who had been prescribed aspirin for secondary prevention of cardiovascular disease were recruited after providing informed consent. They were designated as non-responsive if serum thromboxane levels were > 10 ng/mL, VerifyNow exceeded 550 aru or arachidonic acid-induced platelet aggregation was > 20%. This study was approved by Beaumont hospital, Dublin, REC.

Results: Thirty-five patients were considered to be non-responsive by at least one of the three assays. Only three of these patients were non-responsive by all three assays. After a reminder to take their medication the patients were re-tested and 14 patients were non-responsive. After being observed taking aspirin only seven were non-responsive. Weight was a key predictor of non-responsiveness (non-responders: 102.6 ± 20.6 kg, responders: 78.5 ± 14.0 (SD); $P = 0.0016$). After switching to plain aspirin only three were non-responders. After increasing their dose of aspirin to 150 mg all were subsequently responsive.

Conclusion: Approximately 25% of patients were identified as non-responsive to aspirin by at least one assay. However, 80% of these patients were found to be non-compliant. While there is little agreement between assays weight was an important factor in determining the response to aspirin in all three assays. Thus, it would be difficult to select one assay over the others, as all assays would miss some patients. However, a strategy of restricting 75 mg enteric-coated aspirin to patients under 90 kg and using plain aspirin in patients over 90 kg is a cheap and effective strategy to ensure full response to aspirin. One hundred and fifty milligram aspirin may be required in patients over 120 kg. However, as non-compliance was the biggest cause of non-response patient education is also important to ensure that patients achieve the full benefits of aspirin.

PA 2.01-2

Lack of aspirin resistance in patients with coronary artery disease

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Background: Due to its antiplatelet effect, aspirin (acetylsalicylic acid) is widely used in the prevention of atherothrombotic diseases. By acetylating Ser529 residue in platelet cyclooxygenase-1 (COX-1) it prevents the binding of arachidonic acid (AA) to the catalytic site and inhibits the production of thromboxane (TX) A_2. As aspirin prevents acute vascular events only in part of the patients, the controversial term aspirin resistance was introduced. We define aspirin resistance as the lack of sufficient acetylation of platelet COX-1 at Ser529 by aspirin, while insufficient protection against acute vascular events is considered as ineffectiveness. Most recently we developed two novel methods that directly and indirectly detects the acetylation of COX-1 by aspirin and demonstrated the lack of aspirin resistance in healthy volunteers taking aspirin for a week (Kovács et al. Thromb Res. 2013; accepted).

Aims: Using these new methods, we investigated the occurrence of aspirin resistance among patients with coronary artery disease (CAD) and evaluated several methods routinely used to detect the effect of aspirin.

Patients and Methods: One hundred and fifty-nine patients with CAD being on long-term aspirin monotherapy (100 mg aspirin/day) were enrolled in the study. Based on a priori exclusion criteria 11 subjects were excluded. The study was approved by the Regional Medical Ethics Committee; all patients gave informed consent.

Specific monoclonal antibodies, developed in our laboratory, were used to detect acetylated and non-acetylated COX-1 (acCOX-1, nacCOX-1) by Western blotting. Determination of AA-induced TXB_2 production in platelet rich plasma was used as an indirect measure of COX-1 inhibition. Both methods were sensitive enough to detect the presence of 2.5% of nacCOX-1 content of platelets from non-treated individuals. Methods used for the detection of aspirin effect in the clinical routine were also tested. Platelet COX-2 mRNA was determined by RT-PCR.

Results: With the exception of five patients only the band representing acCOX-1 appeared on the Western blot and TXB_2 production decreased into the very low reference interval (4–35 pg $TXB_2/10^6$ platelets) established for healthy volunteers on controlled aspirin intake. In a telephone interview the attention of the five patients demonstrating nacCOX-1 band and higher TXB_2 production was drawn to the danger of non-compliance and after 2 weeks they were recalled for repeated testing. Only in a single case of recalled patients was TXB_2 production slightly above the reference interval (42 pg TXB2/10^6 platelets) or appeared a faint nacCOX-1 band on the Western blot. In those platelets that expressed COX-2 mRNA the effect of aspirin was not diminished.

Results of AA-induced aggregation and ATP release were in complete accordance with those obtained by the above two methods, while three patients did not show definite aspirin effect by VerifyNow Aspirin assay. Platelet aggregation induced by ADP, epinephrine and collagen and PFA-100 assay with collagen/epinephrine cartridge revealed the effect of aspirin only in part of the patients.

Conclusions: If non-compliance was eliminated no aspirin resistance was observed, i.e. full acetylation of platelet COX-1 occurred in all patients with CAD. Only methods using AA as inducer detect the effect of aspirin on COX-1 reliably.

PA 2.01-3

The relationship between antiplatelet drug therapy and platelet microparticle formation and procoagulant activity in normals and patients with cardiovascular disease

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Background: Dual antiplatelet therapy with aspirin and clopidogrel is commonly used in order to prevent recurrent ischaemic events in patients with cardiovascular disease. It is unclear however whether antiplatelet drug therapy inhibits the formation of platelet-derived microparticles or their procoagulant activity. Platelet-derived microparticles are increasingly being recognised as pathogenic mediators in vascular disease.
Aims: The aim of this study was to investigate the relationship between antiplatelet therapy and its effect on platelet microparticle formation and procoagulant activity.
Methods: In *ex vivo* studies, blood samples from normal individuals ($n = 7$) who were not receiving antiplatelet therapy were incubated with buffer, aspirin or a P2Y12 inhibitor (MeSAMP). *In vitro* studies were performed on blood samples obtained from patients with known cardiovascular disease receiving long-term dual antiplatelet therapy ($n = 19$). Multiplate impedance aggregometry was used to assess the degree of platelet inhibition. Microparticle formation and procoagulant activity in response to adenosine diphosphate (ADP), arachidonic acid (AA) and thrombin receptor activating peptide (TRAP) stimulation was assessed by flow cytometric and Procoag-PL assays respectively. Multiplate reference ranges were established using 20 normal individuals not receiving antiplatelet therapy.
Results: In *ex vivo* studies, P2Y12 inhibition of normal platelets resulted in a significant inhibition of platelet aggregation to ADP and AA and a non-significant inhibition in response to TRAP. Aspirin significantly inhibited AA induced aggregation as expected. P2Y12 inhibition resulted in impaired microparticle formation in response to AA, ADP and TRAP, however increased procoagulant activity was detected in the presence of TRAP stimulation. Aspirin only inhibited microparticle formation and procoagulant activity following AA incubation.
In patients receiving dual antiplatelet therapy, there was a significant decrease in platelet aggregation in response to ADP, ADP-HS and AA. Very few patients remained in the reference range. Aggregation to TRAP was also significantly decreased, but the majority of patients still remained in the reference range. Microparticle-associated procoagulant activity in patients receiving dual anti-platelet therapy was inhibited in response to AA only and not to ADP or TRAP.
Summary/Conclusion: This study shows that the inhibition of P2Y12 is more effective at preventing platelet microparticle release and procoagulant activity when compared to aspirin. It also demonstrates that there are different effects of anti-platelet agents in *ex vivo* compared to *in vitro* studies. From these results, dual antiplatelet therapy appears to be effective at decreasing platelet aggregation, however it fails to completely inhibit microparticle release, particularly in response to ADP and TRAP. This could explain why some patients administered dual antiplatelet therapy experience ischaemic events.

PA 2.01-4

Safety and efficacy of dual antiplatelet therapy in transcatheter aortic valve implantation

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Background: Dual antiplatelet therapy (DAPT) is widely used in patients undergoing transcatheter aortic valve implantation (TAVI). Clinicians and valve producing manufacturers recommend DAPT for 6 months after TAVI. However, this recommendation is not evidence-based and rather empirical.
Aims: The objective of our study was to examine the efficacy and safety in association to drug responsiveness in patients with DAPT after TAVI.
Methods: We investigated a retrospective cohort of 71 patients (age of 66–91, mean 81 years, 34 males, 37 females) undergoing TAVI procedure.
Prior to TAVI, 100 mg ASA and a loading dose of 300 mg clopidogrel were administered. DAPT was continued with 100 mg ASA and 75 mg clopidogrel daily for 6 months. Responsiveness to clopidogrel was measured postinterventionally by maximal intensity of ADP-induced platelet aggregation (5 μM) and phosphorylation of vasodilator-stimulated protein (VASP assay).
Results: Mean values ± SD for ADP-induced platelet aggregation were 50 ± 20% and 53 ± 26% for platelet reactivity index (PRI), respectively (correlation $r = 0.647$, $P < 0.01$). Low response to clopidogrel, defined as PRI > 61% and ADP-induced platelet aggregation > 46%, was seen in $n = 30$ patients (42%). No thrombotic adverse events were reported, but bleeds occurred in 20/71 (28%) patients. In 14 of 71 patients (19%) minor bleeds and in six of 71 (8%) patients major hemorrhagic complications were recorded according to the ISTH criteria. However, there were no significant differences for ADP-induced platelet aggregation and PRI comparing patients without documented bleeds (50 ± 21% and 53 ± 27%) to patients with minor (49 ± 20% and 56 ± 22%) and major bleeding (59 ± 20% and 42 ± 26).
Conclusion: Despite the high proportion of low clopidogrel responsiveness, the absence of thrombotic adverse events, i.e. valve thrombosis and embolization, indicate that DAPT appears to be effective in patients who underwent TAVI. However, the high incidence of reported bleeding events suggests re-evaluation of the need for DAPT after TAVI in future. The present results, obtained in a limited number of patients, do not support a critical influence of clopidogrel on post-procedural bleeding. Larger randomized prospective trials with more robust statistical power are required to determine optimal antiplatelet intensity and duration.

PA 2.01-5

Twice-daily administration of low-dose aspirin to patients with ischemic cerebrovascular disease

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Background: Aspirin is widely used for the secondary prevention of ischemic cerebrovascular disease (ICD). Patients may have increased platelet turnover due to atherosclerosis. We previously reported a reduction of platelet reactivity by the administration of twice-daily low-doses of aspirin to patients with vascular diseases [1] and that residual TXA_2 synthesis in patients was a determinant of aspirin resistance [2]. Down-regulation of platelet function by twice-daily administration of aspirin in patients with diabetes or coronary diseases and the kinetics of recovery of platelet cyclooxigenase-1 activity in patients with and without diabetes have been recently reported [3]. However, no data is presently available on the effect of twice-daily aspirin administration in patients chronically treated after ICD.
Aim: To compare the effect of a daily-dose of aspirin with a twice-daily regimen on platelet reactivity in patients with ICD.
Methods: Patients ($n = 59$) with a history of ICD treated with 200 mg/day aspirin for more than 3 months were consecutively included in the study after informed consent. The study was approved by the Institutional Review Board of the University Hospital la Fe. After an initial evaluation of platelet function, the aspirin regimen was changed in a

cross-over design to receive twice-daily 100 mg aspirin ($n = 35$ patients) or 50 mg twice-daily ($n = 24$ patients) for at least 2 months, and platelet function was evaluated again. Platelet function testing included collagen (1 μg/mL)-induced TXA_2 synthesis, dense granule release (^{14}C5HT), platelet recruitment (proaggregatory activity of cell-free releasates and light transmission aggregometry induced by arachidonic acid (AA, 1 mM), collagen (1 μg/mL) and ADP (3 μM) [2]. Compliance with the treatment was assessed by a personal interview and platelet TXA_2 inhibition > 80% [2].

Results: As compared with the administration of 200 mg/daily, the twice-daily 100 mg regimen significantly reduced the residual TXA_2 synthesis ($P = 0.002$), dense granule secretion ($P = 0.000$), recruitment ($P = 0.000$) and AA-induced aggregation ($P = 0.045$), while no differences were found when collagen or ADP were used as agonists for platelet aggregation. No significant differences in platelet reactivity were detected between the twice-daily 50 mg aspirin and 200 mg/day regimens.

Conclusions: A twice-daily dose of 100 mg aspirin administered to patients with ICD under chronic treatment improves the anti-platelet effect of the same dose of aspirin (200 mg) administered daily. Reducing the dose to 50 mg twice-daily is as effective as a double dose of aspirin administered daily. The most effective strategy for managing patients with high on-treatment platelet reactivity is not known, but the administration of a twice-daily low-dose aspirin may be a reasonable approach, particularly in patients at high risk of recurrence. However, more studies are needed.

Grants: PI07/0463; Retics06/0026.

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PA 2.01-6

Microfluidic assay of platelet deposition on collagen using perfusion of whole bllod from healthy subjects taking aspirin

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Background: Microfluidic devices can create hemodynamic conditions for platelet assays. An 8-channel device was validated in a study of inter-donor reponse to acetylsalicylic acid (ASA, aspirin) using whole blood from 28 healthy subjects)

Aims: To recreate the hemodynamic conditions for platelet assays while testing inter-donor whole blood response to aspirin and to domonstrate a novel and rapid assay for measing the effects of aspirin on platelet function under physiologically relevant conditions.

Methods: Platelet deposition was assessed prior to treament or 24-h after 325 mg ASA ingestion. Whole blood (plus 100 μM H-D-Phe-Pro-Arg-chloromethylketone to inhibit thrombin) was further treated *ex vivo* with ASA (0–500 μM) and perfused over fibrillar collagen for 300 s at a venous wall shear rate (200/s).

Results: *Ex vivo* ASA addition to blood drawn prior to aspirin ingestion caused a reduction in platelet deposition ($IC_{50} \approx 10$–20 μM), especially between 150 and 300 s of perfusion when secondary aggregation mediated by thromboxane was expected. Twenty-seven of 28 subjects displayed smaller deposits (45% average reduction; 10–90% range; $P < 0.001$) from blood obtained 24-h after ASA ingestion (no ASA added *ex vivo*). In replicate tests, an *R*-value to score secondary aggregation [deposition rate from 150 to 300 s normalized by rate from 60 to 150 s] showed $R < 1$ in only two of 28 subjects without ASA ingestion, with $R > 1$ in only three of 28 subjecst following 500 μM *ex vivo* ASA addtion. At 24-h after ASA ingestion, 21 of 28 subjects displayed poor secondary aggregation ($R < 1$) without *ex vivo* ASA addition, while the seven subjects with residual secondary aggregation ($R > 1$) displayed insensitivity to *ex vivo* ASA addition. Platelet deposition was uncorrelated with platelet count, *Ex vivo* ASA addition caused similar inhibition at venous and arterial wall shear rates.

Conclusions: Microfluidic devices quantified platelet depostion following ingestion or *ex vivo* addition of aspirin.

PA2.02 – Platelet Activation: Novel Proteins – II

PA 2.02-1

Mitochondrial permeability transition pore dependent intracellular pH elevation is associated with enhanced calpain activity and integrin aIIbβ3 inactivation

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Background: Thrombin (Thr) plus convulxin (Cvx) stimulation of platelets results in irreversible integrin $\alpha_{IIb}\beta_3$ inactivation, which is mediated by opening of the mitochondrial permeability transition pore (mPTP). Our lab previously showed that deletion of cyclophilin D (CypD), a regulator of mPTP formation, markedly blunts mPTP opening and abrogates integrin inactivation. However, the mechanism by which mPTP opening mediates integrin $\alpha_{IIb}\beta_3$ inactivation remains uncertain.

Aim: Investigate the mechanisms, subsequent to mPTP formation, that mediate integrin $\alpha_{IIb}\beta_3$ inactivation.

Methods: Platelets from wild type and $CypD^{-/-}$ mice were isolated. Cleavage of platelet integrin β_3 intracellular domain (C-terminus) and cytoskeleton protein talin were assessed simultaneously by Western blot analysis using their respective antibodies. Platelet function was evaluated in static conditions by platelet aggregation assay and in flow conditions by microfluidic assay. Intracellular pH was measured by flow cytometry assay using Snarf-1 AM as pH indicator.

Results: In strongly-stimulated (Thr/Cvx) platelets, 50% of both the integrin β_3 intracellular domain and talin was cleaved within 5 min. In platelets stimulated with either thrombin or convulxin alone, only minimal (10%) cleavage was observed. Cleavage of β_3 was temporally correlated with integrin $\alpha_{IIb}\beta_3$ inactivation. Calpain inhibition (MDL 28170) blocked the Thr/Cvx induced cleavage of β_3 and talin and integrin $\alpha_{IIb}\beta_3$ inactivation. Consistent with this inhibition of integrin inactivation, calpain inhibition resulted in increased platelet aggregation in static and flow conditions. Cleavage of integrin β_3 and talin and integrin inactivation were abrogated in Thr/Cvx treated-$CypD^{-/-}$ platelets. Calpain inhibition did not affect loss of mitochondrial membrane potential indicating that enhanced calpain activation occurred subsequent to mPTP formation.

A major regulator of calpain activity is an increased intracellular Ca^{2+} concentration. Surprisingly, an unaltered Ca^{2+} response was observed in $CypD^{-/-}$ platelets, despite the marked impairment of calpain-mediated integrin cleavage. This result indicates that sustained Ca^{2+} elevation alone is not sufficient for the enhanced calpain activity observed in strongly-stimulated platelets. Calpain activity can also be regulated by intracellular pH. Evaluation of intracellular pH by flow cytometry revealed that Thr/Cvx stimulation caused a marked increase in intracellular pH from a baseline pH of approximately 7.3–7.8–8.0. This increase in pH was primarily limited to the platelet subpopulation in which integrin inactivation had occurred. In contrast, in platelets treated with thrombin or convulxin alone and in CypD null platelets, only a slight increase of pH (approximately 0.1–0.2) was observed.

Conclusions: In strongly-stimulated platelets, mPTP formation results in enhanced calpain activation, enhanced integrin β_3 and talin cleavage, and irreversible integrin inactivation. An mPTP-mediated increase in intracellular pH may be the mechanism driving this enhancement of calpain activity in strongly-stimulated platelets.

PA 2.02-2

Redundant functions of TRPC6 and Orai1 in murine platelets

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Background: Elevation of the intracellular Ca^{2+} concentration is an essential step during platelet activation. Store-operated Ca^{2+} entry (SOCE) through Orai1 is well described as the major route of Ca^{2+} influx in platelets. Recently, we found that diacylglycerol (DAG) activated Ca^{2+} influx was completely abolished in transient receptor potential cation channel 6 knockout (*Trpc6−/−*) platelets establishing TRPC6 as the only DAG induced receptor operated Ca^{2+} (ROC) channel in murine platelets. Surprisingly, this abolished ROCE did not affect agonist induced Ca^{2+} responses and SOCE. Furthermore, platelet activation and aggregation *in vitro* and thrombus formation *in vivo* were normal in *Trpc6−/−* mice. All together, these results indicated that TRPC6 mediated Ca^{2+} influx in platelet physiology may be dispensable or fully compensated by other Ca^{2+} channels.
Aims: In order to understand the functional redundancy between TRPC6 and Orai1 in Ca^{2+} homeostasis, platelets lacking both Ca^{2+} channels were analyzed.
Methods: Chimeric mice were generated by transplanting 13 days old fetal liver cells from *WT, Orai1−/− and Orai1−/−/Trpc6−/−* embryosinto lethally irradiated 6 weeks old C57Bl6 mice. Mutant platelets were analyzed using a wide range of *in vitro* and *in vivo* platelet functional assays.
Results: Decreased Ca^{2+} store content and severely altered agonist induced Ca^{2+} store release were observed in *Orai1−/−/Trpc6−/−* platelets whereas these responses were normal in *WT* and *s*ingle knockout platelets. Agonist and thapsigargin induced Ca^{2+} influx were further reduced in *Orai1−/−/Trpc6−/−* platelets in comparsion to *Orai1−/−* platelets. These insufficient Ca^{2+} responses inhibited GPVI mediated integrin activation, P-selectin and phosphatidyl-serine (PS) exposure in *Orai1−/−/Trpc6−/−* platelets, but did not affect G-protein coupled receptor (GPCR) – mediated platelet activation. Surprisingly and in sharp contrast to *Orai1−/−* platelets, *Orai1−/−/Trpc6−/−* platelets could form stable thrombi under flow resulting in comparable surface coverage and thrombus volume as *WT* platelets. Moreover, in the presence of high concentration of apyrase, aggregation response to platelet agonists were enhanced in *Orai1−/−/Trpc6−/−* platelets when compared to *Orai1−/−* platelets.
Conclusions: TRPC6 and Orai1 regulate Ca^{2+} store content in platelets. Orai1 induced SOCE dominates the process of platelet activation, aggregation and thrombus formation, whereas TRPC6 plays a minor role in these processes. Severe Ca^{2+} deficiency due to the blockade of SOCE and DAG mediated ROCE in platelets could enhance so far unknown signalling pathways which trigger platelet aggregation and thrombus growth.

PA 2.02-3

The chaperone protein, heat shock protein 47 (Hsp47): a novel platelet collagen receptor that contributes to thrombosis and haemostasis

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Background: Heat Shock Protein 47 (Hsp47) is a collagen binding protein that is normally localised within the secretory system of collagen producing cells, functioning as a chaperone for procollagen. However, some studies have shown they can be present on the surface of cells. We previously showed that HSP47 is present on the surface of platelets and that levels increased upon platelet activation. Since platelet function is dependent on their tethering to collagen exposed in the arterial wall upon injury, we investigated whether Hsp47 functions as a receptor for collagen.
Aim: To determine the role of Hsp47 in normal platelet function and thrombosis.
Methods: Immunofluorescence was performed on resting human and mouse platelets using specific Hsp47 antibodies. The role of Hsp47 on platelet function *in vitro* was established using platelet aggregation and thrombus formation assays in the presence and absence of Hsp47 inhibitors. The significance of platelet Hsp47 was studied *in vivo* using tail bleeding assay of haemostasis and intravital microscopy to measure thrombosis using laser injury model on mouse cremaster arterioles.
Results: The presence of Hsp47 on platelet surface and their progenitor cells, megakaryocytes was established by immunofluorescence. An inhibitory polyclonal antibody against Hsp47 (Rb anti-Hsp47) and a small molecule inhibitor of Hsp47(SMIH) were found to attenuate platelet aggregation induced with collagen fibrils (Horm, equine tendon) at 1 μg/mL, but not with CRP-XL (a GPVI selective agonist) and thrombin. Both SMIH and inhibitory antibodies reduced the size of thrombi formed when whole human blood was perfused over collagen *in vitro*. Further to findings from the *in vitro* assays, the role of Hsp47 in haemostasis and thrombosis was confirmed using the *in vivo* assays. Inhibition of Hsp47 using SMIH in mice increased tail bleeding times and the volumes of blood lost. Following the inhibition of Hsp47 a reduction of 66% in thrombus size was observed using a laser injury model of thrombosis on cremaster muscle arterioles.
Conclusions: Hsp47 on the platelet surface functions as a receptor for extracellular collagens that are exposed at sites of blood vessel injury. This facilitates the adhesion of platelets to the site of vessel damage, and thus inhibition of Hsp47 may be useful for the prevention of thrombosis.

PA 2.02-4

Inhibition of the Gas6/Mer pathway with novel compounds recapitulates the antithrombotic phenotype of Gas6−/− or Mer−/− mice in arterial and venous thrombosis models

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Background: Growth Arrest Specific gene 6 (Gas6) signals through platelet-surface Mer receptors, leading to platelet activation and thrombus stabilization. We previously used two novel compounds to inhibit this pathway, decreasing platelet activation in functional assays. iMer is an alternatively spliced form of the Mer receptor tyrosine kinase's extracellular domain that likely acts as a Gas6 trap. A selective UNC Mer small molecule inhibitor (UNC Mer TKI) inhibits Mer tyrosine phosphorylation.
Aims: We hypothesized that inhibiting the Gas6/Mer pathway with either compound would protect wild type (WT) mice against arterial and venous thrombosis to a similar degree as that seen in Gas6- or Mer-null mice.
Methods: We used a 6% ferric chloride ($FeCl_3$)-induced carotid artery injury model and a collagen/epinephrine-induced pulmonary embolism (PE) model to compare thrombosis protection between WT C57BL/6 mice treated with inhibitors or vehicle, and also Gas6- and Mer-null animals. Experiments ran for 30 min and reported values are

means ± standard error. Paired t-tests were used to compare times to initial and stable occlusions in the $FeCl_3$ model and survival time in the PE model. Bleeding time was assessed by tail-clip with stump insertion into 37° saline.

Results: In the $FeCl_3$ model, vehicle-treated control mice ($n = 6$) had faster times to initial (6.08 ± 0.12 min) and stable occlusion (7.36 ± 1.3 min) compared to 30 mg/kg iMer-treated mice ($n = 6$, 7.97 ± 0.64 min initial [$P = 0.03$] and 20.49 ± 4.61 min stable [$P = 0.02$]). Also, vehicle-treated control mice ($n = 10$) had faster time to initial (6.93 ± 0.25) and stable occlusion (15.59 compared to 3 mg/kg UNC Mer TKI-treated mice ($n = 9$, 7.97 ± 0.48 initial [$P = 0.06$] and 46.64 ± 7.72 min stable [$P = 0.002$]). Mer-null mice ($n = 3$) had prolonged time to both initial (5.9 ± 0.42 min, $P = 0.06$) and stable occlusion (15.87 ± 6.47 min, $P = 0.15$) compared to WTs ($n = 3$, 4.10 ± 0.59 min initial occlusion, all of which were stable). These experiments, including Gas6-null mice, are currently underway.

In the PE model, 30 mg/kg iMer-treated mice ($n = 9$) survived longer (15.7 ± 4 min) than controls ($n = 10$, 5.5 ± 2.7 min, $P = 0.05$), with three experimental mice (33%) and only one control (11%) surviving for 30 min. Similarly, 3 mg/kg UNC Mer TKI-treated mice ($n = 9$) survived longer (21.25 ± 4.7 min, $P < 0.01$, 6 [66%] surviving 30 min) than controls ($n = 7$, 3.09 ± 0.41 min, no survivors). Both Gas6-null mice ($n = 6$, 17.58 ± 5.11 min, $P = 0.04$, 3 [50%] survivors), and Mer-null mice ($n = 6$, 21.03 ± 5.26 min, $P = 0.02$, 4 [66%] survivors) survived longer than WT controls ($n = 5$, 2.70 ± 0.19 min, no survivors). Tail clip bleeding times did not vary significantly between WT controls ($n = 8$, 6.41 ± 0.89 min) and Gas6-null mice ($n = 5$, 6.5 ± 1.25 min, $P = 0.6$) or Mer-null mice ($n = 7$, 4.8 ± 0.8 min, $P = 0.2$).

Conclusions: Compared to genetic absence of Gas6 or Mer, pharmacological inhibition of the Gas6/Mer pathway protects mice from arterial and venous thrombosis with comparable effects. iMer and UNC Mer TKI may have translational applications as novel anti-platelet agents.

PA 2.02-5

VacA, the vacuolating cytotoxin of *Helicobacter pylori*, binds to multimerin 1 associated with platelet membranes

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Background: Immune thrombocytopenia is a frequent complication with *H. pylori* infection in Japan. While the mechanism by which *H. pylori* induces thrombocytopenia remains largely undetermined, there are several lines of evidence to suggest that its infection activates platelets. In this study, we investigated the role of vacuolating toxin A (VacA) in inducing platelet activation, and attempted to identify the target protein with which VacA interacts.

Results: The CD62P expression in platelets was 1.5 ± 3.9% in ITP patients free from *H. pylori* infection ($n = 18$). However, its expression was significantly increased to 9.5 ± 8.1% with *H. pylori* infection ($n = 17$, $P < 0.001$), and with those who underwent eradication successfully ($n = 5$), the CD62P expression was significantly lowered (1.9 ± 0.8%, $P < 0.02$).

Acid-activated VacA did not induce platelet aggregation, however, it increased the expression of CD62P upon interaction with platelets. Previously reported that VacA reacted with its receptors (EGFR, RPTPa, RPTPb, CD18) present on platelet membranes. However, we were not able to detect the binding between VacA and each of these receptors with the use of either anti-VacA antibody or the antibody against each of these receptors. We therefore analyzed VacA associated proteins obtained through VacA affinity chromatography, using MALDI-TOF-MS. As a result, a set of the peptides matched from the database suggested that multimerin 1 (MMRN1) was detected in two consecutive experiments, as the binding protein for VacA. A GST-fusion protein of MMRN1 corresponding to the 291–391 a.a peptide sequence reacted with VacA assessed by a Biacore system. We synthesized the 20-aa-length peptides, which partly overlap with one another, and checked the binding of VacA with the dot blot method. As a result, the peptide sequence corresponding to 321–340 a.a. showed the highest reactivity to VacA, suggesting that VacA binds to MMRN1 by interacting with this region.

Conclusion: We found that VacA binds MMRN1, and that its binding site for VacA appears to reside within the 321–340 aa sequence. However, how this translates into VacA-induced platelet activation remains an issue.

PA 2.02-6

Rational targeting of Rac-NOX2 pathway prevents ROS generation and platelet activation involving MLC, Akt and Ca++-PKC signaling induced by diverse agonists

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Reactive oxygen species (ROS) play a critical role in platelet activation induced by diverse agonists. ROS generation by NOX2 depends on binding of activated Rac to $p67^{phox}$. We have shown that rationally designed small molecule inhibitors of Rac- $p67^{phox}$ interaction (Phox-I) inhibit ROS generation (Chem. Biol. 19, 228–242, 2012). In this study we investigated the role of Rac-NOX2 axis in platelet activation by determining the effects of a second generation $p67^{phox}$ inhibitor Phox-I3 on ROS generation and platelet activation induced by collagen, collagen related peptide (CRP) or thrombin. ROS generation in washed human platelets was quantified by flow cytometry using CM-H2-DCFDA. Thrombin, collagen or CRP stimulation dynamically increased ROS levels in platelets. Addition of NSC23766 (1–10 μM), a specific inhibitor of Rac GTPase activity, or Phox-I3 (1–10 μM), to platelets 2 min before stimulation with collagen, CRP or thrombin inhibited ROS generation in a concentration dependent manner. DPI, a non-selective inhibitor of NOX2, also inhibited ROS generation. Pre-incubation of washed human platelets with Phox-I3 inhibited: (i) thrombin or U46619, a TXA_2 analog, induced expression of P-selectin on platelet membranes; (ii) collagen or thrombin induced secretion of ATP from platelet dense granules; (iii) collagen, CRP or thrombin induced platelet aggregation. These data, taken together with our earlier findings that NSC23766 inhibits platelet secretion and aggregation by blocking activation of Rac GTPase (J Thromb Haemost 5, 1747–55, 2007), suggest that inhibition of the Rac-NOX2 pathway suppresses ROS generation and consequently platelet activation. Downstream from the Rac-NOX2-ROS axis, cytosolic calcium response, as well as phosphorylation of myosin light chain (MLC) and Akt, were inhibited in platelets treated with Phox-I3 prior to stimulation with collagen or thrombin, suggesting that the NOX2-ROS pathway regulates signaling through cytosolic calcium and phosphorylation of MLC and Akt. Further, Phox-I3 inhibited collagen, but not the phorbol dibutyrate (PDBu), induced phosphorylation of Pleckstrin, a critical substrate of Ca^{++}-Protein kinase C (PKC) signaling in platelets. All together, our data show that Rac-NOX2 pathway regulates ROS generation and subsequent MLC, Akt and Ca^{++}-PKC responses that are essential for platelet activation induced by diverse agonists.

PA2.03 – Platelet Signal Transduction – I

PA 2.03-1

Antiphospholipid antibodies-mediated platelet activation: a key role of PI3Ks beta and alpha

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Background: The antiphospholipid syndrome (SAPL) is a systemic autoimmune disorder characterized by the persistent presence of antiphospholipid antibodies (aPL) that cause the clinical features of vascular thrombosis. There is strong evidence that anti-β2GPI antibodies have thrombogenic properties. However, the mechanisms by which aPL induce platelet activation are still poorly characterized. Anti-β2GPI antibodies, through the formation of immune complexes with the plasmatic ß2-glycoprotein 1, induce a dimerization of the protein and the recruitment of platelet membrane receptors such as GPIbα, ApoER2 and possibly TLR4.
Aim of the Study: The goal of this study was to identify the cell surface receptors and the intracellular pathways involved in platelet activation by anti-β2GPI autoantibodies.
Methods: Antiphospholipid antibodies (aPL) were either commercial or isolated from plasma of thrombotic SAPL patients. Control IgG antibodies were isolates from plasma of healthy donnor in same conditions. Platelet activation was studied in presence of aPL (50 μg/mL) ± pharmacologic inhibitors. Anti-CD42b, anti-TLR2 and anti-TLR4 antibodies were used to block the signaling pathways initiated by these receptors. Platelet adhesion and aggregation was analyzed *ex-vivo* using either human or mice blood.
Results: We show that anti-β2GPI antibodies increase platelet adhesion to a collagen or von Willebrand matrix under flow at a physiological shear rate compared to control IgG with an implication of both GPIbα and TLR4. Using washed platelets, we found that aPL antibodies significantly sensitize platelet aggregation in the presence of suboptimal doses of thrombin (2.5 fold increase, $P > 0.001$ vs. control igG). This effect was totally prevented by blocking of GP1ba, TLR2 and TLR4 receptors. Moreover, inhibitors of PI3K totally blocked the aPL effect on human platelet aggregation as well as AKT activation. The implication of Class IA PI3K alpha and beta was confirmed using platelets from mouse deficient in these isoforms of PI3K selectively in megakaryocytes and platelets.
Conclusion: Overall our results suggest that inhibition of class IA platelet PI3K downstream of GP1ba and TLR could be an interesting therapeutic strategy in order to prevent thrombotic events in SAPL patients without increasing the hemorrhagic risk.

PA 2.03-2

Partial inhibition of serine/threonine phosphatase PP2A blocks clot retraction without reducing alphaIIbbeta3 receptor activation

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Background: Platelet aggregation depends on changes in the integrin $\alpha_{IIb}\beta_3$ that allow fibrinogen binding. However, to retract the clot and stabilize the growing thrombus a new set of signal transduction mechanisms with origin at $\alpha_{IIb}\beta_3$ must take place. The $\alpha_{IIb}\beta_3$ receptor is known to be regulated by protein phosphorylation-dephosphorylation processes. Knowledge about the role of PP2A serine/threonine phosphatase in $\alpha_{IIb}\beta_3$ signalling is scarce, and no data is available on its participation in clot retraction.
Aim: To study the role of serine/threonine phosphatase PP2A in the mechanisms regulating clot retraction: (i) inside-out process that trigger $\alpha_{IIb}\beta_3$ activation and platelet aggregation, and (ii) in the subsequent outside-in signaling that reinforces aggregation and leads to clot retraction in thrombin activated platelets.
Methods: We used washed human platelets from healthy controls without medication. As inhibitor of PP2A we used okadaic acid (OA). Platelet agonist thrombin (1 U/mL) was employed. Platelet aggregation, cytoskeleton isolation, flow cytometry and inmunodetection of proteins were performed as previously described (1). Clot retraction was started by adding 1 U/mL thrombin to washed platelets in the presence of fibrinogen, and the evolution of the clot was photographed at intervals up to an hour.
Results: Inhibition of PP2A with OA strongly inhibited clot retraction of platelets at 100 or 500 nM. In contrast, OA 100 nM did not inhibit $\alpha_{IIb}\beta_3$ activation (PAC-1 binding) or platelet aggregation, while 500 nM strongly inhibited both processes. The down-regulation of the integrin activation with OA 500 nM could be related to the inhibition of tyrosine phosphorylation of proteins (1), since we observed that treatment with OA 500 nM but not with OA 100 nM strongly inhibited tyrosine phosphorylation of proteins in whole platelets lysates. OA 500 nM inhibited several signal transduction mechanisms associated with outside-in signaling such as cytoskeletal reorganization and incorporation of tyrosine phosphorylated proteins, β_3 and tyrosine kinases to the cytoskeleton; in addition, β_3 and FAK phosphorylation were also strongly reduced. In contrast, OA 100 nM only affected FAK incorporation to the cytoskeleton and FAK phosphorylation.
Conclusion: The level of inhibition of PP2A by low or high OA concentration differentially regulates platelet aggregation and integrin signalling, but have a common effect in blocking clot retraction. The latter may be associated with the presence of phosphorylated FAK in the cytoskeleton. This study reveals PP2A as a novel target for anti-platelet treatment to block clot retraction without affecting the platelet haemostatic function if a partial inhibition of PP2A could be achieved.
Reference: 1. Santos MT Circulation 2000;102:1924.

PA 2.03-3

Autocrine amplification of integrin aIIbβ3 activation and platelet adhesive responses by deoxyribose-1-phosphate

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Background: Platelet activation is a complex series of events originating from the stimulation of surface receptors by biochemical signals linked to vascular damage, such as the exposure of the subendothelial matrix and the generation of thrombin in the coagulation cascade. Besides responding to their microenvironment and to the biochemical messages released by other circulating or vascular cells, platelets also release their own rich array of extracellular signals. Amongst the small molecules released by platelets, thromboxane A_2 (TXA_2), adenosine diphosphate (ADP), epinephrine, and serotonin (5-HT) are key autocrine regulators of platelet responses. Recently, we utilised gas-chromatography mass spectrometry (GC-MS) to identify new molecules released by platelets. The pro-angiogenic metabolite deoxyribose-1-phosphate (dRP) was detected in micromolar concentrations in stimulated platelet supernatants.
Aims: In light of the ability of dRP to stimulate the generation of reactive oxygen species (ROS) in different cell types and the pro-aggregatory role of ROS in platelets, we investigated whether platelet-derived dRP plays any autocrine role in the regulation of the redox balance, the intracellular signalling, or the functional responses of platelets.

Methods: The experiments were performed on human platelets from healthy donors or mouse platelets, either wild type or transgenic characterised by genetic deletion of thymidine phosphorylase and uridine phosphorylase ($TP^{-/-}$ $UP^{-/-}$), which results in impaired dRP release upon platelet activation. The effects of deprivation of endogenous dRP (transgenic platelets) or addition of exogenous dRP were tested on washed platelet aggregation (by turbidimetry), integrin αIIbβ3 activation (by flow cytometry), washed platelet static adhesion (by phase contrast imaging), whole blood thrombus formation (by microfluidics and time-lapsed epifluorescence microscopy), ROS accumulation (by live platelet confocal imaging), and signal transduction pathway (by immunoblot).
Results: The addition of exogenous dRP to human platelets significantly increased platelet aggregation and integrin αIIbβ3 activation in response to thrombin. In parallel, genetically modified platelets with double genetic deletion of thymidine phosphorylase and uridine phosphorylase were characterised by reduced release of dRP, impaired aggregation and decreased integrin αIIbβ3 activation in response to thrombin. *In vitro* platelet adhesion onto fibrinogen and collagen under physiological flow conditions was potentiated by treatment of human platelets with exogenous dRP and impaired in transgenic platelets with reduced dRP release. Human and mouse platelets responded to dRP treatment with a sizeable increase in reactive oxygen species (ROS) generation and the pre-treament with the antioxidant apocynin abolished the effect of dRP on aggregation and integrin activation. Experiments directly assessing the activation of the small G protein Rap1b and protein kinase C suggested that dRP increases the basal levels of activity of these two pivotal platelet-activating pathways in a redox-dependent manner.
Summary/Conclusions: Taken together, we present evidence that dRP is a novel autocrine amplifier of platelet activation, which acts on platelet redox levels and modulates integrin αIIbβ3 inside-out signalling.

PA 2.03-4
Canonical and non-canonical cleavage patterns for protease-activated receptor (PAR) 1 and 3 by factor Xa reveal novel insights into the allosteric modulation of cytoprotective PAR1 signaling by PAR3

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Endothelial barrier protective effects by APC require binding to the endothelial protein C receptor (EPCR) and protease activated receptor 1 (PAR1). In contrast, activation of PAR1 by thrombin results in endothelial barrier disruptive signaling, thus creating a paradox how PAR1 can mediate both of these opposite effects. Recently, we provided a partial explanation by demonstrating that APC cleaved PAR1 at Arg46 and that tethered-ligand peptides beginning at Asn47 mediated APC-like barrier protective effects *in vitro* and *in vivo*. Thus, PAR1 is a biased receptor that can initiate endothelial barrier protective or disruptive effects depending on the cleavage site of its tethered-ligand at either Arg46 or Arg41. We found another piece of the puzzle analyzing PAR3 cleavage by APC. Requiring EPCR, APC cleaved PAR3 at non-canonical Arg41, whereas thrombin cleaved at canonical Lys38. PAR3 non-canonical tethered-ligand peptides beginning at Gly42 mediated APC-like barrier protective effects *in vitro* and *in vivo* that required PAR1, whereas canonical PAR3 tethered-ligand peptides beginning at residue Thr39 had no effect. Similar to APC, factor Xa (FXa) blunts thrombin-induced barrier disruptive effects *in vitro*. To determine whether non-canonical PAR1 and PAR3 cleavages are generally applicable mechanisms for endothelial barrier protection, the PAR1 and PAR3 cleavage profiles for FXa were determined, using SEAP-PAR1 and PAR3 cleavage constructs in HEK293 cells. Cleavage of PAR1 by FXa was EPCR-dependent and required the FXa GLA-domain. Mutation of the canonical Arg41 to Gln abolished PAR1 cleavage, whereas FXa cleaved Arg46Gln PAR1 similar to wt-PAR1. Thus, FXa cleaved PAR1 at Arg41 like thrombin and not at Arg46 like APC. FXa cleaved PAR3 in the presence of EPCR but not in the absence of EPCR and required the GLA-domain. PAR3 cleavage by FXa was inhibited by blocking antibodies against FXa, PAR3, and the direct FXa inhibitor rivaroxaban. Interestingly, PAR3 could still cleave Lys38Gln-PAR3 but failed to cleave Arg41Gln-PAR3 or Lys38Gln/Arg41Gln-PAR3. Thus, FXa cleaved PAR3 at the non-canonical Arg41 similar to APC but not at the canonical Lys38 like thrombin. Since FXa cleaved PAR1 at Arg41 and PAR1 desensitization can give the false impressions of protection against thrombin-induced permeability, barrier protective effects of FXa were verified using histones to induce PAR1-independent barrier disruption. Both FXa and APC blunted histones-induced permeability, indicating that barrier protective effects were not due to PAR1 desensitization. The PAR3 non-canonical tethered-ligand peptide but not the canonical PAR3 tethered-ligand peptide also blunted histones-induced barrier disruption. In summary, the unique canonical/non-canonical PAR1/3 proteolysis profile of FXa, intermediate between thrombin and APC, provides unique opportunities for novel insights on the role of non-canonical PAR cleavages and of PAR3 in particular. Our data help conceptualize novel functions for non-canonical PAR3 activation at Arg41 as an allosteric modulator of canonical PAR1 activation.

PA 2.03-5
Immobilized heparin enhances aIIbβ3-dependent outside-in signaling in human platelets

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Backround: Heparin is the most commonly used parenteral anticoagulant worldwide. Upon its administration, 10–30% of patients develop a mild, nonimmune thrombocytopenia. The nature of this heparin-induced thrombocytopenia (HIT, type I) is unknown (in contrast to the antibody-mediated HIT, type II). Recently, it has been suggested that heparin can promote platelet responsiveness by potentiating αIIbβ3-mediated outside-in signaling, thereby inducing HIT type I (Gao et al, Blood 2011; Yagi et al, Thromb Res 2012).
Aims: To analyze the role of αIIbβ3 in heparin-platelet interaction, we first studied platelet aggregation in the presence of unfractionated heparin (UFH). Moreover, we assessed platelet spreading and quantitatively investigated the activation of two tyrosine kinases, Src and focal adhesion kinase (FAK), in human platelets on immobilized UFH or its synthetic derivative fondaparinux. To examine the specificity of αIIbβ3 we used the αIIbβ3 antagonist abciximab and αIIbβ3-overexpressing HEK293 cells which lack other platelet receptors. As positive control, we applied immobilized fibrinogen.
Methods: We used human washed platelets or αIIbβ3-transfected HEK293 cells. Aggregation with platelets was performed in the presence of soluble UFH (0.2 or 1.0 U/mL). Adhesion was carried out under static conditions either on immobilized UFH (5, 10 and 50 U/mL) or fondaparinux (3 μg/mL) or fibrinogen (100 μg/m). BSA was used as negative control. Images of FITC-phalloidin-conjugated platelets and YFP-conjugated αIIbβ3-transfected HEK293 cells were taken with an Axiovert 100M microscope (Carl Zeiss). Specific phosphorylations of Src (pY418) and FAK (pY397) were determined by Western blot.
Results: UFH alone did not induce platelet aggregation, but it caused a 2-fold potentiating response upon platelet stimulation with ADP (300 nM). Platelets showed spreading on immobilized UFH to a similar extent as on a fibrinogen matrix. We detected the same effect with immobilized fondaparinux. In the presence of abciximab or on BSA, no platelet spreading was visible. We examined Src and FAK phosphorylation at various concentrations of immobilized UHF and detected maximal activation at 10 U/mL UFH matrix (twofold higher than at 5 U/mL; 1.5-fold higher than at 50 U/mL). Comparing Src and FAK signaling in platelets on immobilized UFH with that on immobilized fibrinogen, signaling to both kinases in platelets on

immobilized UFH were approximately 40% of that in platelets on a fibrinogen matrix. We observed comparable extents of activity in platelets on fondaparinux matrix as on UFH (5-fold increase in Src pY418 and 6-fold increase in FAK pY397 signaling compared to BSA control). Abciximab completely blocked the activation of both Src and FAK. αIIbβ3-transfected HEK293 cells also exhibited enhanced signaling on immobilized UFH. However, in contrast to platelets, we detected maximal kinase activities on UFH matrices with higher concentrations (Src at 100 U/mL and FAK at 50 U/mL UFH concentration, respectively).

Summary: We demonstrated enhanced Src and FAK activations both in platelets and αIIbβ3-transfected HEK293 cells on heparin and fondaparinux matrices. This activation is blocked by abciximab. Our results suggest a significant role of αIIbβ3 in the platelet-heparin interaction and are in accordance with earlier reports referring to enhanced platelet responsiveness by heparin.

PA 2.03-6

Purinergic receptor modulation of platelet function *in vitro* and *ex vivo*

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Background: Platelets express $P2Y_1$ and $P2Y_{12}$ receptors which are involved in ADP-induced activation under both aggregatory and inflammatory conditions. Whilst the relative role of $P2Y_1$ and $P2Y_{12}$ receptors has been extensively documented for platelet aggregation and thrombus formation, their role in inflammatory conditions is less understood.

Aims: Aim of the present study was to assess the role of P2Y receptors in platelet-induced leukocyte chemotaxis, platelet leukocyte conjugation and platelet aggregation in an established mouse model of allergic asthma.

Methods: Studies were carried out in an established model of allergic asthma in mice. Balb/c mice were immunised with ovalbumin (OVA, 30 μg/mouse) on day 1, 5 and 11. On days 15 mice were pre-treated with MRS2500, (3 mg/kg i.v.), AR-C66096 (3 mg/kg i.v.) or Clopidogrel (75 mg/kg) and challenged with 3% nebulised OVA for 30 min. Using flow cytometry and the platelet and leukocyte antibodies CD41 and CD45, platelet-leukocyte aggregation were quantified 6 h post allergen challenge. Platelet aggregation induced by 2–10 μM ADP was also measured at 6 h. For *in vitro* chemotaxis studies, platelets were isolated from OVA sensitised Balb/c mice and pre-treated with either MRS2500 or AR-C66096 (0, 1, 10, 100, 1000 nM). Platelets were then stimulated with 100 nM ADP and mixed with PMNs isolated from Balb/c mice. Using chemotaxis plates (3 μM pores), leukocyte migration towards 100 nM MDC was quantified after 90 min incubation.

Results: Circulating platelet-leukocyte aggregates, expressed as mean fluorescence intensity (MFI) of the platelet antigen CD41/CD61 on the leukocyte surface, were significantly elevated 6 h post allergen challenge compared to sham controls (1.8 ± 0.4% vs. 0.9 ± 0.15%, $P < 0.01$). Circulating platelet-leukocyte conjugates remained significantly elevated in animals pre-treated with AR-C66096 compared to sham controls (1.85 ± 0.3%, $P < 0.05$) but not in animals pre-treated with MRS2500 (1.3 ± 0.2%).

MDC enhanced platelet-induced PMN chemotaxis compared to vehicle (PMN + platelets: chemotaxis index (CI) = 1.00 ± 014; PMN + platelets + MDC: CI = 2.69 ± 0.13). Pre-incubation of platelets with the $P2Y_1$ antagonist MRS2500 inhibited MDC-induced PMN chemotaxis in a concentration dependent manner (1 nM: 2.99 ± 0.23, 10 nM: 2.54 ± 0.16, 100 nM: 2.03 ± 0.21 $P < 0.05$, 1000 nM: 1.68 ± 0.14 $P < 0.001$). In contrast, incubation with ARC60996 did not affect MDC-induced PMN chemotaxis (1000 nM [top dose]: 2.42 ± 0.14).

ADP (2–10 μM) induced platelet aggregation (52.50 ± 7.50%) was signficantly suppressed (by 75%, $P < 0.05$) in mice treated with Clopidogrel (75 mg/kg) and it was reduced by 40% in AR-C66096 (3 mg/kg) pretreated animals ($P < 0.05$). On the contrary, in mice administered MRS2500 (3 mg/kg) ADP-induced platelet aggregation was not inhibited (−12%, P = NS).

Conclusions: Our data show that the $P2Y-_1$ but not the $P2Y_{12}$ receptor plays an important role in the inflammatory function of platelets in allergic asthma in mice (platelet-leukocyte conjugation and platelet-leukocyte chemotaxis), whilst in contrast in $P2Y_{12}$ receptor plays a major role in ADP-induced platelet aggregation.

PA2.04 – Megakaryocytes and Thrombopoiesis – II

PA 2.04-1

Microtubule plus-end tracking protein CLASP2 KO mice phenocopy CAMT: a role for CLASP2 in hematopoiesis and hematopoietic stem cell maintenance

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Background: Haematopoietic cell lineage commitment is determined by signalling mechanisms that require ligands of various origins, as well as the appropriate expression and functioning of the cognate receptors. Receptor activation leads to intracellular signalling cues that trigger different processes, e.g. cell division, attachment, or migration. Mammalian CLASPs (CLASP1 and CLASP2) are microtubule plus-end tracking proteins whose essential function as regulators of microtubule behaviour has been studied mainly in cultured cells. However, the role of these proteins in haematopoiesis is not yet explored.

Aims and Methods: We generated Clasp2 KO mice to analyze its role in haematopoiesis. We used cell culture assays, flow cytometry, immunohistochemistry, confocal microscope imaging, RNAseq, transplant experiments.

Results: We show here that absence of murine CLASP2 *in vivo* results in thrombocytopenia, progressive anaemia, and pancytopenia, due to defects in megakaryopoiesis, in erythropoiesis, and in the maintenance of haematopoietic stem cell activity, similarly to what occurs in Congenital Amegakaryocytic Thrombocytopenia patients (CAMT). Interestingly, CLASP2-deficient haematopoietic cells show accumulation of intracellular cMpl and cKit receptors (personal observation). Furthermore, microtubule stability and organization are affected upon attachment of Clasp2 knockout haematopoietic stem-cell-enriched populations, and these cells do not home efficiently toward their bone marrow niche. Strikingly, CLASP2-deficient haematopoietic stem cells contain severely reduced mRNA levels of c-Mpl, which encodes the thrombopoietin receptor, an essential factor for megakaryopoiesis and haematopoietic stem cell maintenance.

Summary/Conclusion: CLASP2 is a microtubule-stabilizing protein and Clasp2 knockout mice display progressive pancytopenia which phenocopies the human syndrome congenital amegakaryocytic thrombocytopenia (CAMT). Our data suggest that thrombopoietin signal-

ling is impaired in Clasp2 knockout mice. We propose that the CLASP2-mediated stabilization of microtubules is required for proper attachment, homing, and maintenance of haematopoietic stem cells and that this is necessary to sustain c-Mpl transcription. Furthermore, we hypothesize that CLASP2-mediated microtubule stabilization is necessary for the trafficking of cMpl and cKit receptors in haematopoietic cells.

PA 2.04-2

The epigenetic landscape of platelet and red blood cell traits

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Platelet count and volume are independent risk factors for heart attacks and ischaemic stroke. We used meta-analysis of genome-wide association (GWA) studies to identify genes encoding novel regulators of megakaryopoiesis and platelet formation. Over three-quarters of the 68 genetic signals associated with platelet count and volume are located at non-protein coding regions (Nature 2011; 480, 201). We postulated that the common variants at many of the discovered platelet quantitative trait loci (QTLs) exert their effects through cell type-dependent transcriptional regulation. To investigate this, we integrated GWA data of 75 genetic signals associated with red blood cell indices (Nature 2012; 492, 369).

We aimed to assess the role of candidate regulatory variants associated with platelet and red blood cell QTLs in the regulation of lineage-specific gene expression during blood stem cell differentiation.

We used formaldehyde-assisted isolation of regulatory elements followed by next-generation sequencing (FAIRE-seq) to map regions of open chromatin genome-wide in blood stem cell-derived megakaryocytes and erythroblasts, the precursors of platelets and red blood cells, respectively, as well as in peripheral blood monocytes. We applied these epigenomic data sets to define cell type-dependent enrichment patterns of GWA signals associated with platelet and red blood cell QTLs at regions of open chromatin.

In megakaryocytes and erythroblasts, we found that open chromatin patterns reflect the corresponding hematopoietic lineages of the studied cell types, and associate with the cell type-specific gene expression patterns. Open chromatin regions coinciding with platelet trait-associated SNPs were more likely to be restricted to megakaryocytes than expected by chance. We observed the same cell type-dependent effect for red blood cell trait-associated SNPs at erythroblast-restricted open chromatin sites. The majority (63.6%) of candidate regulatory variants at platelet QTLs overlapped with binding sites of transcription factors key to regulating megakaryopoiesis, namely FLI1, GATA1, GATA2, RUNX1 and TAL1 (also known as SCL). We experimentally tested 13 candidate regulatory variants at 10 platelet QTLs in gel shift assays (MLSTD1, PTGES3-BAZ2A, RAD51L1, DNM3, LRRC16, PDIA5, ABCC4, CTSZ-TUBB1, KALRN and SATB1). We found all but 3 candidate SNPs affected nuclear protein binding, suggesting this to be a frequent mechanism by which regulatory variants influence the formation of platelets from megakaryocytes.

GWA studies have placed a plethora of genetic markers at genes encoding novel regulators of platelet and red blood cell formation. Here, we show that epigenetic annotation of precursor cell genomes combined with GWA results aids the identification of candidate regulatory variants, which may exert their effect through disruption or introduction of nuclear protein binding sites. We have recently shown that differential binding of transcription factors at the PIK3CG (PLOS Genetics 2011; 7, e1002139) and DNM3 (Blood 2012; 120, 4859) platelet QTLs affects expression levels of genes critical in megakaryopoiesis and platelet formation.

PA 2.04-3

Biogenesis of the demarcation membrane system in megakaryocytes

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Background: The demarcation membrane system (DMS) in megakaryocytes (MKs) forms the plasma membrane of future platelets. One MK is supposed to produce an average of 4000 platelets and during its maturation the DMS increases its total surface about 26-time. The exact origin of this unique membrane system and the molecular mechanisms driving its explosive growth are unknown. A current view is that the DMS develops from the invagination of the MK plasma membrane (PM).

Aims: Considering the importance of the DMS as the major membrane supply for platelet production, the aim of this study was to investigate the biogenesis of the DMS.

Methods: Using confocal microscopy, dual axis electron tomography, and large volume FIB-SEM analysis, we have determined the sequential developmental steps of DMS formation, both *in situ* in mouse bone marrow MKs, and *in vitro* in cultured MKs generated from progenitors. Early DMS phenotypes have been identified using the platelet lineage specific markers glycoprotein GPIb, known to be expressed at the early stages of MK maturation.

Results: We have identified a pre-DMS precursor form which initiates at the cell periphery through invagination of GPIb-positive membranes, and which gradually develops towards a well-defined perinuclear oriented membrane complex. Confocal microscopy and electron tomography analysis revealed that at all developmental stages the DMS is continuous with the cell surface, suggesting that its origin is determined through invagination of the plasma membrane (PM). Remarkably, the precise location of the pre-DMS is between the nuclear lobes, and the number of PM invaginations coincides well with MK ploidy, suggesting a relation with cleavage furrow formation. Upon DMS expansion Golgi complexes assemble around the pre-DMS and fusion profiles of TGN-derived vesicles with the DMS are observed. Treatment with Brefeldin A, an inhibitor of anterograde membrane transport, reduced the expansion of the DMS, indicating that the exocytic pathway is an essential determinant for DMS biogenesis. In addition, close contacts (< 30 nm) between endoplasmic reticulum (ER) and DMS are frequently detected suggesting a physical interaction between the two membranes. These ER-DMS contact sites were free of ribosomes and contained filamentous electron-dense material reflecting cross-linking/anchoring molecules. FIB-SEM analysis of late stages reveals that the DMS forms a highly intertwined tubular membrane network with numerous connections with the cell surface, resembling the future open canalicular system of platelets.

Conclusion: We propose the following model for the biogenesis of the DMS: (i) DMS formation starts at focal locations at the cell surface, and (ii) is followed by an invagination process that resembles cleavage furrow formation; (iii) formation of the pre-DMS and further expansion require vesicular membrane delivery from multiple targeted Golgi complexes to the DMS, and possibly direct lipid transfer through ER to DMS tethering.

PA 2.04-4
Platelet bioreactor-on-a-chip

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Background: Morbidity and mortality from bleeding due to low platelet count is a major clinical problem encountered across a number of conditions. Despite serious clinical concerns owing to their immunogenicity and associated risk of sepsis, and inventory shortages owing to their high demand and 5-day shelf life, platelet transfusions total more than 14 million units per year worldwide.
Aims: By modeling human physiology and mimicking nature's design principles *in vitro* we will identify components of the vascular bone marrow microenvironment that promote thrombocytopoiesis, and yield novel approaches to accelerate platelet production *in vivo*"circumventing transfusion-related complications. An alternative source of infusible platelets will obviate risks associated with platelet procurement and storage, and help meet growing transfusions needs.
Methods: We have developed a microfluidic platelet bioreactor that recapitulates key aspects of bone marrow physiology, including bone marrow rigidity, extracellular matrix composition, microchannel size, hemodynamic vascular shear, and endothelial cell contacts. Bone marrow biochips are based on custom-built PDMS devices bonded to glass slides, and comprise of an upper and lower microfluidic channel separated by a series of columns spaced 3 μm apart. Primary megakaryocytes infused along the top channel sequentially become trapped between the columns, and extend proplatelets into the lower channel.
Results: Physiological shear rates (500–2500/s) can be tightly regulated and were shown to increase both the rate and extent of proplatelet production. Median proplatelet extension rates under physiological flow velocities were roughly one order of magnitude higher than has been calculated for proplatelet-producing megakaryocytes under static conditions (36 μm/min vs. 0.85 μm/min) and correspond to recent estimates from *in vivo* multiphoton intravital microscopy studies of mouse bone marrow (Zhang et al. J Exp Med. 2012). Interestingly, increasing shear rate from 500 to 2500/s did not further accelerate proplatelet extension, and suggests that elongating microtubule bundles comprising the proplatelet shaft actively resist vascular shear forces to regulate proplatelet telescoping. To more accurately reproduce the bone marrow stiffness (approximately 1000 Pa), the uppermost channel was selectively filled with a low melting point agarose solution comprising primary megakaryocytes, and polymerized inside this space to trap megakaryocytes in a physiologically relevant three-dimensional environment. Extracellular matrix proteins native to the bone marrow vascular niche were included in the low melting point agarose solution individually and in combination to recapitulate the bone marrow vascular niche. The lowermost channel was selectively coated with extracellular matrix proteins and seeded with human endothelial cells to generate a sinusoidal blood vessel adjacent the megakaryocyte microenvironment. Proplatelet production was visualized by high resolution live-cell microscopy. Platelets released into the lower channel by megakaryocytes enter the fluid stream. Bioreactor-derived platelets were collected from the effluent, and are structurally equivalent to human blood platelets.
Summary: Platelet bioreactor-on-a-chip devices recapitulate the bone marrow vascular microenvironment to increase/accelerate proplatelet production *ex vivo* and support physiological platelet release.

PA 2.04-5
Sp1/Sp3 transcription factors are essential for megakaryopoiesis

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Specificity proteins (Sp) are closely related family of zinc finger transcription factors that are important components of the eukaryotic cellular transcription machinery. They have been implicated in the regulation of many physiological processes including cell cycle, growth control, apoptosis, hormonal activation and angiogenesis.
Sp1 and Sp3 are ubiquitously expressed and can either activate or repress the expression of a variety of genes by interacting with the GC- and GT-boxes of the DNA with similar specificity and affinity. They play a critical role during embryogenesis as *Sp1* knockout embryos die around embryonic day 10.5. Sp3 deficient embryos are postnatal lethal. *Sp1ko/wt::Sp3ko/wt* compound heterozygous embryos display a multitude of developmental abnormalities resulting in late prenatal mortality. Thus, functional redundancy exists between the Sp factors. To investigate their functions beyond embryogenesis we generated mice with conditional *Sp1* and *Sp3* knockout alleles. Using a pan-hematopoietic Cre line (Mx1-Cre), we conditionally deleted the *Sp1* and *Sp3* genes from the entire hematopoietic system. The results revealed the importance of these genes in hematopoiesis particularly in megakaryopoiesis.
Pf4-Cre mediated deletion of *Sp1* and *Sp3* (dKO) confirmed the importance of these genes in megakaryopoiesis. These mice exhibited a significant reduction in platelet counts but normal TPO levels in plasma and an increase in platelet volume. The dKO platelets responded normally when stimulated with PMA (fibrinogen receptor) but showed a decreased response to aggretin A (Clec-2), collagen (integrin β1 and GPVI) and did not respond to Botrocetin (vWF receptor or GPIb complex). These platelets were also functionally impeded as they displayed a decreased adhesion to collagen type1. Flow cytometry analysis of the four vWF receptors revealed very low expression levels of GP1bα in the dKO platelets.
Our results suggest that these mice are a phenocopy of patients with Bernard-Soulier syndrome (BSS), a coagulopathy caused by a defect of the vWF receptor due to mutation in either the GP1b or GPIX subunits leading to macrothrombocytopenia characterized by prolonged bleeding time and the absence of Botrocetin/Ristocetin induced platelet agglutination. Our data also suggested that GP1bα is a target for Sp1/Sp3 in megakaryoctes.
We are currently analyzing RNA sequencing data and will perform ChIP sequencing to unravel functional genomic targets of Sp1/Sp3 in megakoryocytes.

PA 2.04-6
Microtubule plus-end tracking APC negatively regulates proplatelet formation

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Background: Platelets are produced from mature megakaryocytes following profound cellular reorganization leading to proplatelet elongation, a highly dynamic process mainly driven by microtubules (MT). Despite some progress, the mechanisms regulating MT dynamics and reorganization remain poorly defined. Adenopolyposis Coli (APC) is a microtubule plus-end tracking protein (+TIPS) involved in the regulation of MT in a number of cell systems (Akhmanova and Steinmetz,

2010) and its inactivation has been reported to alter hematopoiesis (Qian et al., 2008).
Aim: The aim of our study was to investigate a role for APC in megakaryopoiesis.
Methods: We used a loss of function approach using shRNA and a knock-out murine model. Megakaryocytes (MKs) were obtained by culturing hematopoietic stem cells (Lin-) and APC was knocked down using two different APC shRNAs lentiviral constructs. Mice with APC-deficiency in the megakaryocytic lineage were obtained by crossing APCflx/flx (Qian et al., 2008) with Pf4-Cre mice.
Results: APC shRNA induced a 70% (shAPC1) and 54% (shAPC2) decrease in APC transcript levels, respectively, when compared to a control shRNA. Down-regulation of APC promoted endomitosis, as evidenced by a higher percentage of Mks > 8 N. In addition, an increased proportion of mature megakaryocytes was observed that were able to extend proplatelets (68.8% (shAPC1) and 52.5% (shAPC2) vs. 28.1% (control); $n = 3$). Loss of APC also exacerbated proplatelet branching. Similar results, in terms of ploidy and amplification of the proplatelet network were also observed when MKs were differentiated from Lin- cells from mice with megakaryocyte-restricted APC deficiency. In addition these mice exhibited increased platelet counts when compared to control mice (1200 ± 61 vs. 990 ± 36 platelets/μL; $n = 25$, $P = 0.0061$**) with a similar size, ultrastructure and number of microtubules coils. Platelet functions were also preserved. A ggregation in responses to several agonists and von Willebrand factor and fibrinogen-mediated platelet adhesions were normal.
Conclusion: Loss of APC leads to an amplification of proplatelet formation, indicating a negative role of this +TIP stabilizing factor in thrombopoieisis.

PA2.05 – Platelets and Genes

PA 2.05-1

The platelet response is inherited and dependent on the number of shared alleles

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Background: The platelet response to agonist stimulation shows a high degree of inter-individual variability. While this may be the result of external factors, the fact that the individual platelet response is reproducible over time, suggests that the platelet response must somehow also be genetically controlled.
Aim: Since a well-defined platelet phenotype is informative when assessing the risk of an individual to develop e.g. arterial thrombosis, the current study aimed to elucidate the relative contribution of inherited gene variation on platelet count and function.
Methods: Blood was collected from an unselected cohort of 18 year-old monozygotic ($n = 12$ pairs) and dizygotic twin pairs ($n = 19$ pairs). After measurement of the platelet count, the response of platelets to agonist stimulation was assessed in whole blood by flow cytometry by measuring fibrinogen binding and surface expression of P-selectin, each in response to two concentrations of adenosine 5'-diphosphate (ADP), or the glycoprotein (GP) VI-specific collagen peptide mimetic, cross-linked collagen-related peptide (CRP-XL). Major secondary signaling pathways were inhibited to guarantee ADP- and CRP-XL-pathway specificity. Data were recorded as percentage of cells positive for the activation markers. Agreement between the platelet counts or platelet responses of subjects of monozygotic or dizygotic twin pairs, or between non-related subjects (subject from one twin pair randomly paired with a subject from another twin pair) was quantified by the Pearson correlation coefficient with two-tailed P-value after logit transformation of the data to ensure Gaussian distribution of the data populations. The study was approved by the institutional review board of the VU University, Amsterdam. Written informed consent was obtained from all participants.
Results: Platelet counts were not significantly different between monozygotic and dizygotic twins compared to subjects from a singleton population. However, heritability of the platelet count was shown to correlate with the number of shared alleles: within monozygotic twins the correlation between twins was high ($r^2 = 0.73$, $P < 0.001$), whereas correlation was lower within dizygotic twins ($r^2 = 0.196$, $P = 0.066$) and completely absent in a random population of subjects ($r^2 = 0.01$, $P = 0.60$). Similarly, there was a high degree of correlation within the pairs of twins for both fibrinogen binding and surface expression of P-selectin after stimulation with ADP or CRP-XL. The level of correlation was highest within pairs of monozygotic twins and less profound in case of dizygotic twins. No correlation was found for the platelet response in the random population.
Summary/Conclusions: These observations suggest that the platelet response through $P2Y_1/P2Y_{12}$ or GPVI is under genetic control and dependent on the number of shared alleles.

PA 2.05-2

Genome-wide association study of aspirin and clopidogrel response in patients with percutaneous coronary intervention

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Background: Inter-individual variability exists in response to antiplatelet therapy. A significant minority of patients show poor platelet inhibition, which has been associated with adverse clinical events.
Aims: To investigate possible genetic determinants of platelet reactivity in patients receiving antiplatelet therapy.
Methods: Patients who underwent percutaneous coronary intervention (PCI) and were on both aspirin and clopidogrel were enrolled in an Institutional Review Board approved Observational Study (ASCLOGEN). Platelet function was measured using light transmission aggregometry (LTA) with arachidonic acid (AA) and ADP, Verify*Now* with aspirin (ARU) and P2Y12 (PRU) cartridges, *ex vivo* TxB_2, urinary $11dhTxB_2$, and VASP (PRI) assays. A genome-wide association study (GWAS) was performed in 409 Caucasian patients using a cardiovascular gene-centric Illumina 50 K SNP array. TaqMan assay was also utilized for additional SNPs selected from P2RY1, P2RY12, and CD36 genes. SNPs were filtered for MAF, H-W equilibrium, and call rate. Subjects were filtered for excess heterozygosity, genetic relatedness, gender mismatch, call rate and genetic population outliers. Gene-based tests were conducted using a linear kernel machine approach. All Ensembl gene IDs containing at least three typed SNPs were tested (Ensembl 69; 2297 gene IDs) for the association with platelet function measures. Among the tested genes, we also selected a focused set based on literature review. Regression analyses were adjusted for gender, age at PCI procedure and genetic relatedness. For genome-wide associations, a P-value < 2.16E-6 and < 1E-4 was deemed significant for SNP- and gene-based tests, respectively, and P-value < 1E-2 for candidate gene test.
Results: Three SNPs from *CYP2C19* (rs2281891, rs1322179, and rs4244285) showed significant association with high PRI and a SNP from *NR5A2* (rs2821369) was associated with higher urinary $11dhTxB_2$ levels. Gene-based tests indicated a significant association between *P2RY12* and PRI, and also *NLRP1* and $11dhTxB_2$. Candidate-gene approach revealed association of *CYP2C19* with clopidogrel-related assay measures including PRU, LTA-ADP, and PRI. Other candidate genes that have shown association with platelet mea-

sures are: *PTGS2* (LTA-ADP), *HTR2B* (PRU), *ITPR1* (LTA-ADP), *PLAT* (LTA-AA) and *UGT1A6-A10* (11dhTxB_2).
Conclusion: This GWAS revealed an association between *CYP2C19* and clopidogrel response. Other genes identified in this study require further evaluation to establish their relationship with platelet reactivity.

PA 2.05-3
Systematic characterization of human platelets by quantitative proteomics

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Anti-platelet treatment is of fundamental importance in combatting functions/dysfunction of platelets in the pathogenesis of cardiovascular and inflammatory diseases. Dysfunction of anucleate platelets is likely to be completely attributable to alterations in protein expression patterns and post-translational modifications. Combining elaborate protocols for platelet isolation from fresh blood donations in conjunction with quantitative mass spectrometry, we created the first comprehensive and quantitative proteome of highly pure human platelets, comprising almost 4000 unique proteins with copy number estimates for approximately 3700 of those and relatively quantified approximately 1900 proteins between four different healthy donors – with negligible contamination by leukocytes, erythrocytes and plasma, respectively. For the first time, our data allow for a systematic and weighted appraisal of protein networks and pathways in human platelets, and indicate the feasibility of differential and comprehensive proteome analysis from small blood donations. Since 85% of the platelet proteome show no variation between healthy donors, this study represents the starting point for disease-driven platelet proteomics. These findings allow for correlation to genome-wide association studies which identified in a retrospective manner a set of chromosomal regions affecting the risk of cardiovascular diseases. While respective gene products could be identified in platelets, a comprehensive and quantitative comparison of protein patterns between patients and relevant controls such as relatives and spouses to validate risk factors is still missing. In order to improve cardiovascular risk management, genomic and proteomic analyses of respective corresponding gene loci and proteins using next generation sequencing and targeted MS strategies are applied with the final goal to characterize valuable biomarkers for biomedical screenings.

PA 2.05-4
Association between the microarray-based CYP2C19 genotyping assay and the platelet function test in cardiovascular patients receiving clopidogrel

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Background: Clopidogrel is a widely used antiplatelet agent that irreversibly inhibits platelet P2Y12 ADP receptors after transformation into an active metabolite by cytochrome P450 (CYP) in the liver. Especially, CYP2C19 polymorphism has been reported to be associated with altered antiplatelet activity of clopidogrel, and this polymorphism reveals considerable inter-ethnic variation.
Aims: In this study, we would perform the CYP2C19 genotyping in Korean cardiovascular patients receiving clopidogrel to assess the frequency of polymorphism and analyze the association with the efficacy of treatment. And we would investigate the applicability of CYP2C19 genotyping to decide the direction of treatment as well as the prediction of the efficacy of treatment.
Methods: Polymorphisms of CYP2C19*2, *3, *17 and the degree of inhibition of platelet function were determined in 155 patients who were being treated with clopidogrel following cardiovascular interventions. CYP2C19 genotyping was performed by the Verigene® system CLO+ test (Nanosphere, USA) for the first time in Korea, and the results were categorized as ultra-rapid metabolizer (UM, including *17/*17 and *1/*17), extensive metabolizer (EM, including *1/*1), intermediate metabolizer (IM, including *1/*2 and *1/*3), and poor metabolizer (PM, including *2/*2, *3/*3, and *2/*3). The degree of platelet inhibition was assessed by the VerifyNow P2Y12 system (Accumetrics, USA), and the pre-procedure/post-procedure PRU and percent inhibition were measured to check the association with genotype. This study was approved by the Institutional Review Board of Dong-A University Hospital. Informed consent was obtained.
Results: Four of 155 patients were UM (2.6%), 56 EM (36.1%), 69 IM (44.5%), and 26 PM (16.8%). The pre-procedure/post-procedure PRU values of EM patients (268.0 ± 81.0/222.2 ± 91.3) were lower than those of IM and PM patients (294.8 ± 90.5/246.8 ± 105.7 and 336.2 ± 79.9/259.5 ± 96.0, respectively; $P = 0.001/0.168$). The pre-procedure/post-procedure percent inhibition values of EM patients (20.5 ± 23.1/31.1 ± 27.1) were higher than those of IM and PM patients (10.5 ± 16.4/21.9 ± 23.6 and 7.7 ± 18.2/12.7 ± 16.2, respectively; $P \leq 0.001/0.004$).
Conclusion: About 60% of Korean patients with cardiovascular diseases receiving clopidogrel have CYP2C19 loss-of-function genotypes classified as IM or PM, and the frequency is similar with the reported data of Asian. And these genotypes are significantly associated with reduced antiplatelet activity of clopidogrel. There are no consented cutoff values of the platelet function test, the prediction of therapeutic efficacy and the decision of therapeutic direction based on the genotyping could be useful. And the Verigene® system used in this study is fast and simple, so genotyping with this instrument would improve the clinical usefulness of genotyping.

PA 2.05-5
Genes in arterial thrombus formation: a comparison of *in vivo* and *in vitro* studies

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Background: Arterial thrombus formation relies on activation of blood platelets and the coagulation system. Although antiplatelet and anticoagulant agents are successful in the prevention of cardiovascular diseases, they are directed against one of only few molecular targets. On the other hand, *in vivo* studies of arterial thrombosis using genetically modified mice show that multiple platelet- and plasma-derived proteins contribute to thrombus formation. In many of these studies also flow chamber technology for screening of thrombus formation at arterial shear rates *in vitro* is included. Systematic comparisons of the *in vivo* and *in vitro* measurements have not been made up to now.
Aims: (i) Systematic comparative within-study analysis of effects of genetic modification on arterial thrombus formation *in vivo* and whole-blood thrombus formation in parallel-plate flow devices *in vitro*. (ii) Use bioinformatics technology to construct networks of genes of human signaling proteins implicated in arterial thrombus formation to identify novel candidate genes.
Methods: An extensive search in PUBMED was performed, including original studies where effects are reported of genetic modification on murine thrombus formation both *in vivo* and *in vitro* from 1993 to 2013. Reviews were excluded. Information was categorized with respect to the type of genetic modification, the vascular bed, the

thrombosis model, dose and duration of vascular injury and pulmonary thromboembolism *in vivo*; the thrombogenic surface, flow protocol and flow device *in vitro*. Three different scoring scales were employed to quantify antithrombotic or pro-thrombotic effects of mass-, time- or stability-dependent parameters of thrombus formation indicating thrombus size, thrombus build-up in time and degree of embolization respectively. Statistical analysis was performed using the program SPSS version 19. The Reactome database was used to construct extended networks of human proteins corresponding to the analyzed mouse genes.

Results: Information was obtained of > 200 mouse genes with a positive or negative role in arterial thrombus formation. These included genes encoding for platelet proteins (68%), for transcription factors expressed in megakaryocytes (3%), for plasma coagulation and other plasma factors (20%) and for endothelial-expressed proteins (5%). The majority of genes contributed positively to thrombus formation *in vivo* (75%) and *in vitro* (71%).

Time and mass-dependent parameters, reflecting thrombus build-up in time and thrombus size respectively, correlated for all thrombosis models and vascular beds ($P < 0.01$). Thrombus size also correlated with the degree of death of mice due to pulmonary thromboembolism ($P < 0.01$). Mass-dependent parameters *in vitro* correlated with mass-dependent parameters *in vivo* ($P < 0.01$). Over-representation analysis and construction of extended protein networks using Reactome indicated a potential role of multiple novel genes in less well studied intracellular signaling pathways.

Conclusions: This logistic analysis indicates that: (i) The overall set of measurements *in vivo* of thrombus buildup (in time) and thrombus size (mass) produces a coherent database, in spite of the differences in experimental models and conditions used in individual laboratories. (ii) Mass parameters of thrombus formation *in vitro* are predictive for those *in vivo*. (iii) Combined information on mouse genes involved in thrombus formation can be used to obtain logical networks of human proteins, potentially involved in cardiovascular diseases.

PA 2.05-6

A new aIIb platelet antigen p.M841 (HPA-27bw) involved in materno-fetal alloimmunization and HPA-27bw genotyping by PCR-HRM (high resolution melting) technique

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Fetal/Neonatal Alloimmune Thrombocytopenia (FNAIT) diagnosis relies on materno-fetal incompatibility and alloantibody identification. In unsolved FNAIT, genotyping for rare platelet polymorphisms allowed the identification of three families with materno-fetal incompatibility for the αIIb-c.2614C>A mutation (Halle et al, Transfusion 2008). This mutation leads to the L841M substitution. In one case, the alloimmunization was confirmed by detecting an antibody to the M841 form of αIIb.

The mutation was originally detected in 2001 as it introduced an unexpected restriction profile by using the Fok-I enzyme in a PCR-Restriction Length Polymorphism genotyping technique. To quickly explore unsolved FNAIT, a PCR- sequence specific primer amplification (SSP) assay was designed to genotype the αIIb-c.2614C>A mutation. Seven hundred and fifty-two DNA samples collected over 10 years were genotyped and four parents presenting the αIIb-c.2614A allele were detected. Nonetheless the materno-fetal incompatibility was suspected or confirmed in only three cases. The classic Monoclonal Antibody Specific-Immobilization of Platelet antigen Assay (MAIPA) initially used in diagnosis failed to detected alloantibodies to the αIIb-M841 antigen in maternal sera. Thus HEK-293 cells expressing αIIb-leu841 or αIIb-Met841 αIIbβ3 forms were used to probe the reactivity of maternal sera. Tested by flow cytometry, one serum specifically reacted with αIIb-M841 but not with αIIb-L841 αIIbβ3. This specificity revealed the αIIb-L841 polymorphism as a new alloantigen named HPA-27bw in the international nomenclature. New blood samples from the concerned family obtained in the context of a new pregnancy, allowed detecting the alloantibody using heterozygous platelets in flow cytometry. The anti-HPA-27bw alloantibody remains undetectable using either transfected HEK-293 cells or platelets in MAIPA. As observed for HPA-3a (or -3b) and HPA"-9bw alloantibodies, anti-HPA-27bw alloantibodies appeared difficult to detect and required whole platelet assays when classical MAIPA failed. Co-localization of the HPA-27bw, HPA-3a (or -3b) and HPA"-9bw antigens on the same very flexible αIIb loop (amino acids 827–855) may explain the difficulty to detect antibodies upon platelet solubilization. Similarly to alloimmunizations in HPA-3 and -9 systems, the immune response to the HPA-27bw antigen might induce severe thrombocytopenias and life-threatening hemorrhages. The p.L841M substitution has limited effects, if any, on expression and functions of αIIbβ3 in transfected HEK-293 cells. MD simulations done with 3D structural models of the αIIb peptide 827–855 correlated these last results as the L841M substitution had any effect on the model structures.

The HPA-27bw variant, apparently rare in Caucasian, reaches an allelic frequency up to 8.2% in Sub-Saharan African populations, where homozygous people are observed (Halle et al, transfusion 2008).

This case of alloimmunization underlies the necessity to perform genotyping of rare variants in unsolved FNAIT. Furthermore, it also highlights that other serologic techniques must be used to complete FNAIT diagnosis when MAIPA failed. We have developed a PCR-High Resolution Melting (HRM) for quick simultaneous genotyping of the HPA-3, -9 and -27bw variants to provide a technique complementary to the PCR-SSP in population studies. Furthermore PCR-HRM may help to detect new variants. Future studies will concern the HPA-27a allele immunogenicity to further characterize this particular immune response.

PA2.06 – Haemophilia A: Clinical – V

PA 2.06-1

Treatment for life for severe haemophilia A. A cost-utility model for prophylaxis vs. on-demand treatment

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Introduction: Prophylaxis has been established as the treatment of choice in children with haemophilia and its continuation into the adult years has been shown to decrease morbidity throughout life. The cost of factor therapy has made the option questionable in cost-effectiveness studies.

Aim: The role of prophylaxis in pharmacokinetic dosage and tolerisation against inhibitor formation were used to model the cost-utility of prophylaxis vs. on-demand (OD) therapy over a lifetime horizon in severe haemophilia A.

Methods: Commercial software (TreeAge[TRADEMARK]) was used to construct a Markov model with 80 cycles of 1 year each. The model was populated with variables for costs and effectiveness for haemophilia outcomes including joint and soft tissue bleeds, inhibitors and dosage. Key inputs into the model which differed from previous exercises included the use of pharmacokinetic dosage and effect of prophylaxis on the probability of developing inhibitors. The model was applied to a single provider national health system exemplified by the

United Kingdom's National Health Service and a third party provider in the United States. The incremental cost effectiveness ratio was (ICER) was estimated and compared to threshold values used by payer agencies to guide reimbursement decisions. A cost per quality adjusted life year (QALY) was also estimated for Sweden.

Results: Applying a bidiurnal dosage regimen and using the early tolerisation protocol of Kurnik et al (Haemophilia. 2010;16(2):256–62), prophylaxis was shown to be more effective and less costly (dominant) relative to OD treatment in the UK. In the USA, the model resulted in an ICER – $68,000, which is within the range of treatments reimbursed by third party payers in that country. In Sweden, a cost/QALY of SEK 1.1 million was also within the range of reimbursed treatments in that country, and prophylaxis was dominant over OD treatment when daily dosage was applied. Sensitivity analysis showed that dosage and treatment-induced inhibitor incidence were the most important variables in the model.

Conclusion: Subject to continuing clinical evidence of the effectiveness of pharmacokinetic dosage and the role of prophylaxis in decreasing inhibitor incidence, treatment for life with prophylaxis is a cost-effective therapy, using current criteria for the reimbursement of health care technologies in a number of countries.

PA 2.06-2

Severe haemophilia a: different regimens for starting prophylaxis (in PedNet)

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Background: The practice of prophylaxis in children with severe haemophilia A (FVIII < 1%) is changing. Prophylaxis is started at a younger age and at lower frequencies and doses. Different step up regimens are used for patients starting at low frequencies. The general objective is to eventually progress to full prophylaxis (3–3.5×/week) for optimum protection against bleeding.

Aims: To describe regimens of starting prophylaxis and compare their effects on bleeding and use of central venous access devices (CVADs).

Methods: Data for this project were extracted from the 'European Paediatric Network for Haemophilia Management' (PedNet) registry. Patients with severe haemophilia A, born January 2000–January 2010, who started prophylaxis before inhibitor development were included. Detailed data on treatment and bleeding were collected until May 2012 or up to 7 years of age. Data were censored at inhibitor development or loss to follow up. Different regimens of starting prophylaxis were compared. Cumulative incidences and age at start of full prophylaxis (≥ 3× per week) were calculated using Kaplan Meier survival analysis.

Results: 319/425 patients with severe haemophilia A started prophylaxis before the age of 7 years. Median follow up was 2.6 years (range: 0.1–7.0). Three regimens for starting prophylaxis were identified: 138 (43%) patients started 1×/week, 97 (30%) 2×/week and 84 (26%) ≥ 3×/week. Patients started prophylaxis around a median age of 1.4 years and median one joint bleed (range 0–15) for all regimens. Significantly less CVADs were used when starting with lower frequencies: 18% for 1×/week, 36% for 2×/week and 41% for full prophylaxis (*P*-value < 0.01).

Almost all (90%) patients who started prophylaxis 1×/week first progressed to 2×/week before progressing to full prophylaxis. Patients in both regimens reached full prophylaxis around the same age: 5.4 and 5.1 years respectively. Median dose used was 500 IU/infusion for all regimens.

At the end of follow up, 4% of patients infused 1×/week and 24% 2×/week without having progressed to full prophylaxis. Compared to those who started with full prophylaxis (after a median of one joint bleed, 41% without joint bleeds), patients who started with less frequent infusions had suffered more bleeding at reaching full prophylaxis: for 1×/week this was after median four joint bleeds (16% no joint bleeding) and for 2×/week after median three joint bleeds (20% no bleeding, *P*-value < 0.01).

Summary/Conclusion: Overall, prophylaxis was started at a median of 1.4 years of age after one joint bleed. Starting prophylaxis with less frequent infusions but progressing to full prophylaxis in 3.9 years was associated with limited additional joint bleeding, and a lower use of CVADs. More research on the effects of step up regimens on life-threatening bleeds, and long term outcome is needed.

PA 2.06-3

Population pharmacokinetic analysis of long-lasting recombinant factor VIII Fc fusion protein (rFVIIIFc) in patients with severe haemophilia A

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Introduction: In a recently completed Phase 3 clinical study (A-LONG), rFVIIIFc, a recombinant fusion protein composed of a single molecule of human B-domain-deleted recombinant coagulation factor VIII (BDD-rFVIII) attached to the Fc domain of human immunoglobulin G_1 (IgG_1), was well-tolerated and effective in the treatment of bleeding, routine prophylaxis, and perioperative management in patients with haemophilia A. The half-life of rFVIIIFc was prolonged, compared to a marketed recombinant FVIII product, octocog alfa (Advate).

Objectives: To characterize the activity-time profiles of rFVIIIFc in haemophilia A patients by population pharmacokinetic (PK) analysis and to identify intrinsic covariates that may affect the variability of rFVIIIFc PK parameters.

Methods: The modelling dataset included activity-time profiles of 180 subjects (15 from a Phase 1/2a study and 165 from a Phase 3 study [A-LONG], collected over ≤ 52 weeks of treatment). Subjects were 12–65 years old and weighed 41–132 kg. The analysis was done with NONMEM 7 software, and included model building, covariate search, and model qualification steps.

Results: A two-compartmental model adequately described the activity of rFVIIIFc. The population estimate for clearance (CL) was 1.65 dL/h; and for volume of distribution at steady state (Vss) was 44.4 dL. The inter-individual variability (IIV) of CL was moderate (24.3%) and central volume of distribution (V1) was low (13.4%). The inter-occasional variability (IOV) of both CL and V1 was low (20.6% and 12.0% respectively). The additive residual error was very low (0.208 IU/dL), as was the proportional residual error (13.6%), approximating the variability of the one-stage clotting assay for FVIII activity. Von Willebrand Factor (VWF) level was identified as the major covariate for CL; higher levels of VWF yielded lower clearance values, reflecting the protective role that VWF has on FVIII activity.

Body Weight (BW) and haematocrit (HCT) were identified as weak covariates on V1.

Conclusion: This is the first population PK analysis that systematically describes and characterizes the prolonged activity profile of long-lasting rFVIIIFc. The population PK model of rFVIII activity adequately describes the observed activity-time profiles. The clearance of rFVIIIFc activity is lower than the clearance observed for octocog alfa, resulting in longer duration of activity. The relatively low IIV underlines the consistency and homogeneity of the activity profiles. The low IOV indicates that rFVIIIFc maintains stable and predictable activity with long term administration over time. The set of covariates identified is physiologically relevant. Therefore, the population model developed can be used to simulate various dosing scenarios in support of dosing regimen selection and other decision making related to rFVIIIFc therapy. This approach represents an advance over the current approach, wherein the efficacy of a treatment regimen cannot be evaluated until after a patient has a bleeding episode.

PA 2.06-4

High-resolution peripheral quantitative computed tomography (HR-pQCT): a novel imaging technology detects microarchitectural skeletal pathology in hemophilia patients

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Background: Osteoporosis is a pathologic bone disorder characterized by low bone mineral density (BMD) and microarchitectural bone disruption that can result in increased risk of fracture. Recent studies based on dual X-ray absorptiometry (DXA) showed a high prevalence of low BMD in persons with hemophilia (PWH). However BMD measured by DXA is limited by its inability to measure true volumetric BMD and to determining bone microarchitecture that is directly related to bone strength and resistance to fracture. HR-pQCT is a novel technology that provides 3D imaging of human bone microarchitecture by measuring BMD and characteristics of different bone compartments, and can predict resistance to fracture using finite element (FE) method.

Aim: To determine the microarchitectural differences in the bone of moderate and severe PWH compared to age and sex-matched controls using HR-pQCT. (Approved by University of Calgary Research Ethics Board)

Methods: Informed consent was obtained from severe and moderate adult hemophilia patients (males, ≥ 18 years-old, factor VIII or IX level ≤ 5%) recruited from the Southern Alberta Rare Blood and Bleeding Disorders Clinic, Calgary. BMD and bone measurements included DXA (reported as *T*-scores) and HR-pQCT imaging. HR-pQCT results were compared to age-and sex-matched normative data from a healthy patient cohort collected from a previous study (Canadian Multicentre Osteoporosis Study) conducted at our centre. Bone turnover markers (C-telopeptide CTX, N-telopeptide NTX) and bone formation markers (osteocalcin, P1NP) were measured.

Results: Sixteen hemophilia patients (A:B = 14:2, 87.5%(A); 12.5% (B), mean age: 34 years) were included in this pilot study. Vitamin D deficiency was present in 47% of patients. Bone turnover markers were elevated (mean: CTX 385.7 ng/L, NTX 83.3 nmol/mmol Cr) in 44%, and bone formation markers were decreased (mean: P1NP 66.2 μg/L, osteocalcin 4.2 μg/L) in 40%. Preliminary analysis of BMD measurements showed a mean *T*-score of −1.0 (hip) and −1.2 (L-spine) with 53% of patients meeting criteria for osteopenia. HR-pQCT measurements of patients in the hemophilia group (compared to control group) showed (i) lower total volumetric BMD (282 ± 75 vs. 331 ± 44 mg hydroxyapatite (HA)/cm3); (ii) lower trabecular BMD (expressed as Bone/Trabecular volume fraction) (0.142 ± 0.04 vs. 0.178 ± 0.02); but (iii) higher cortical BMD (872 ± 87 vs. 784 ± 52 mg HA/cm^3). At the microarchitectural level, the hemophilia group compared to the controls demonstrated (i) fewer trabecular number (TbN); (ii) thinner trabeculae (TbSp) but (iii) thinner cortex thickness (CtTH) compared to the controls (data not shown). Finite element (FE) analysis estimating bone strength (failure load) showed significantly lower mean failure load, in the hemophilia group compared to controls (6288 ± 1850 vs. 13,720 ± 1561 Newtons, $P < 0.05$).

Conclusion: This preliminary data demonstrates remarkably poor bone health in PWH with detectable skeletal pathology at the microarchitectural level in addition to low BMD. The resulting poor bone quality may result in increased fracture risk in PWH. This study provides some insight into how hemophilia affects bone metabolism and bone structure, and suggests HR-pQCT may serves as a tool for early detection of poor bone quality. However, further studies are need to confirm the significance of our findings and translation into clinical practice.

PA 2.06-5

Association between predicted FVIII levels and risk of bleeding episodes in clinical trials with turoctocog alfa, a new rFVIII product from Novo Nordisk

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Background: Novo Nordisk has conducted phase III clinical trials (guardian[TRADEMARK]) with the new rFVIII product turoctocog alfa in adult (≥ 12 years) and paediatric (< 12 years) patients with severe haemophilia A. In these trials, patients received prophylactic turoctocog alfa doses in the range 20–50 IU/kg (adult patients) or 25–60 IU/kg (paediatric patients), the doses being adjusted according to bleeding patterns of the individual patient during the trial.

Aims: To demonstrate the association between predicted FVIII:C and estimated bleeding rates for spontaneous and traumatic bleeding episodes in adult and paediatric patients.

Methods: All patients ($N = 214$) reported detailed information about dosing and bleeding episodes (spontaneous and traumatic), including the time points of these events, in diaries. Initial results showed that the estimated annualised bleeding rates (ABRs) increased with time since the latest prophylactic dose. Furthermore, a subset of patients ($N = 50$) underwent pharmacokinetic assessments. Based on the observed PK properties and the patient-reported information, a profile of predicted FVIII:C at all time points during the trial was constructed for each patient and correlated to the time pattern of reported bleeding episodes. This amounts to a population pharmacodynamic analysis of the association between predicted FVIII:C and the risk of experiencing either a spontaneous or traumatic bleeding episode. FVIII:C profiles from patients ($N = 67$) who did not experience a bleeding episode during the trial also contributed to the analysis, whereas patients ($N = 3$) with insufficient reporting of dosing were excluded.

Results: In all, the 211 patients were exposed to turoctocog alfa in daytime hours (06.00–24.00) for a total of 76.1 patient years (58.1 patient years for adults and 18.0 patient years for children) and reported a total number of 550 bleeding episodes during these hours (326 spontaneous, 188 traumatic and 36 not categorised). Overall, the results of the analysis demonstrate that the risk of experiencing a bleeding episode decreases with increasing FVIII:C. The protection provided by FVIII:C in the range of 1–5%, corresponding to moderate haemophilia, appears to be better for paediatric than for adult patients, particularly for spontaneous bleeding episodes. Between-patient

variability of bleeding risk at any specified FVIII:C was substantial in both age groups.

Conclusion: The protective effect of increasing FVIII:C appears more pronounced for our paediatric vs. adult patients, and for spontaneous vs. traumatic bleeding episodes. The high variability between patients reflects the global character of the trials, spanning patients with highly different treatment histories reflecting different economic resources in the participating regions. The estimated relations between predicted FVIII:C and bleeding risk could serve as a model to predict mean ABRs not only in trials with multiple dosing regimens of standard FVIII products, but also in trials using next generation FVIII compounds with prolonged PK profiles.

PA 2.06-6

Parameters influencing factor VIII half-life and recovery in patients with haemophilia A

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Background: Factor VIII (FVIII) half-life and recovery vary widely among patients with haemophilia A. Therefore, for tailored prophylactic treatment regimens the knowledge of FVIII half-life and recovery in individual patients is important.

Aim: The aim of the current study was the identification of factors influencing FVIII pharmacokinetics in patients with haemophilia A.

Methods: Peripheral venous blood samples were collected in adult patients (≥ 18 years) with severe and moderate haemophilia A pre- and post-infusion after a washout period of at least 72 h after the last factor VIII injection. Patients were on prophylactic or on-demand treatment and received their usual recombinant or plasma-derived FVIII concentrates. All patients had to give their written informed consent. Exclusion criteria were a current or past FVIII inhibitor, bacterial infection or inflammation at inclusion, active bleeding or other coagulation disorders. Blood samples for the pharmacokinetics were collected before and at ten time points after FVIII infusion (according to the recommendations of the ISTH). If desired by patient, sampling was reduced to at least five time points after FVIII infusion. All FVIII half-lives were evaluated by the Bayesian analysis (Bjoerkman, Blood, 2012, 119:612–618).

Results: Forty adult patients with haemophilia A (35 severe, five moderate, median age 30 years [range: 18–61]) were recruited. Thereof, 20 patients had blood group 0. Fifty percent of all patients (n = 20) had an inversion in the factor FVIII gene as underlying mutation (19: inversion 22, 1: inversion 1), 16 a point mutation, and one each had a deletion, a nonsense mutation, a splice mutation and no identified mutation, respectively. The median BMI was 25.7 (17.9–35.5) and the median FVIII dosage used was 23.7 IU/kg (10.5–50.0). Regarding the treatment regimen, 58% (n = 23) were on prophylaxis and 42% (n = 17) on demand. The majority of patients (n = 29) received recombinant and 11 a plasma-derived FVIII product. The median FVIII recovery was 2.4/dL/U/kg (1.0–3.7). Patients with higher BMI (> 25) showed a significantly higher FVIII recovery (P = 0.013). No correlation was found between FVIII recovery and blood group or age. The FVIII half-life varied from 5.8 to 19.2 h (median 10 h) and the median clearance was 0.27 L/h/kg (0.12–0.65). In patients with blood group 0, FVIII half-life was significantly shorter than in patients with non-0 blood group (median half-life: 9.5 h, range: 6.3–14.8 h vs. median: 11.8 h, range: 5.8–19.2 h, P = 0.046). Patients aged > 30 years had a longer median half-life compared to those 18–29 years of age (median: 12.4 h, range: 6.3–19.2 h vs. median: 9.2 h, range: 5.8–17 h, P = 0.004). Moreover, von Willebrand antigen and activity levels were associated with FVIII half-life, patients with higher levels had a longer FVIII half-life (r = 0.608 and r = 0.402, respectively).The mutation type (inversion vs. other mutations) had no impact on FVIII recovery, half-life or clearance.

Summary/Conclusions: FVIII pharmacokinetics, performed in adult patients in routine clinical practice, using the usual medication and dosage of an individual patient, revealed blood group, von Willebrand factor antigen and age as major determinants for FVIII half-life, whereas the underlying F8 gene mutation had no influence. Results can be used for individualization of the prophylactic treatment regimen.

PA2.07 – Haemophilia B – II

PA 2.07-1

A retrospective observational multicenter cohort study on peri-operative Factor IX consumption in Hemophilia B ('OPTI-CLOT' studies)

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Background: Hemophilia B is an X-linked bleeding disorder, caused by a deficiency of clotting factor FIX (FIX). Treatment of hemophilia with coagulation factors is costly. Previous studies have demonstrated that FIX consumption in prophylactic treatment can be significantly reduced by individualized dosing regimens. However, data on peri-operative FIX consumption, clearance and modifying factors are scarce.

Aim: To evaluate peri-operative FIX consumption and achieved FIX levels in hemophilia B with attention to patient- and surgical characteristics.

Patients and Methods: In this multicenter retrospective observational study, we included patients with severe and moderate hemophilia B (FIX levels < 0.05 IU/mL), without an inhibitor, undergoing elective and low or medium risk surgery from January 2000 until July 2012, from three Dutch Hemophilia Treatment Centers. The study was not subject to the Medical Research Involving Human Subjects Act and was approved by the Medical Ethics Committee. FIX concentrates were administered with the aim to achieve the following target levels advised by the Dutch Hemophilia Consensus: day 1: 0.8–1.0 IU/mL; day 2–5: 0.5–0.8 IU/mL; > day 6: 0.3–0.5 IU/mL. The achieved FIX plasma levels were generally checked daily. Data were collected on clinical and surgical characteristics, peri-operative dose and mode (bolus infusion: BI or continuous infusion: CI) of FIX concentrate administration, achieved FIX levels and bleeding complications.

Results: A total of 43 surgical procedures was performed in 21 patients; 16 adults (36 surgeries; median age 49 years; median weight 80 kg) and five children (seven surgeries; median age 4 years; median weight 18 kg). In adults, mainly orthopedic surgeries were performed (63%); in children insertion or removal of intravenous catheters and abdominal surgery was most frequent (57%). Peri-operative bleeding complications were reported in six procedures (14%); in three patients (7%) reoperation was required for persistent bleeding.

Median amount of infused FIX concentrate during hospitalisation was 44,575 IU (686 IU/kg) per surgical procedure. Forty-seven percent of achieved peri-operative levels was above highest required level (median + 0.17 IU/mL) according to the Consensus; 41% was within target range and 11% of levels was below targeted range (median −0.11 IU/mL). CI was used more frequently (63%) than BI (37%); children received only FIX by BI.

CI and BI did not differ in bleeding complications or achieved FIX levels. For adults the median amount of infused FIX concentrate was

significantly higher when treated by CI (788 IU/kg) compared to BI (408 IU/kg) ($P < 0.001$). After correction for duration of hospitalisation this difference did persist; CI 60 IU/kg/day and BI 44 IU/kg/day ($P = 0.01$). The severity of surgery was not associated with the amount of FIX concentrate administered per day. Further analyses are ongoing to differentiate between indications for surgery.

Conclusion: Forty-seven percent of peri-operative FIX levels was above the predefined target levels. These data support that there is room for improvement in the efficacy of peri-operative dosing. PK-guided dosing with iterative pharmacokinetic modelling in the peri-operative period is therefore promising.

PA 2.07-2

Is hemophilia B less severe than hemophilia A in young children with same level of factor deficiency?

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Background: Several studies, mainly in adults, have suggested that the bleeding phenotype in hemophilia B (HB) is milder compared with hemophilia A (HA) at similar residual clotting factor VIII or IX activities. More knowledge on children's bleeding phenotype in both HA and HB, especially at the start of treatment, is important in order to optimize childhood patient care.

Aims: This study was designed to describe and compare the severity of the bleeding phenotype and the variation in bleeding sites in young patients with severe or moderate HA and HB.

Methods: Consecutive untreated children with severe HA and HB (factor VIII/IX < 0.01 IU/mL) or moderate HA and HB (factor VIII/IX 0.01–0.05 IU/mL) born between January 1st 2000 and January 1st 2010, who were diagnosed in one of the participating hemophilia treatment centers were included. Approval from every centre's review board and written informed consent was obtained from the parents or guardians of all participants. The primary outcome of the study was the clinical phenotype expressed as the severity of bleeding tendency, i.e. the age when bleeding necessitating treatment occurred. The secondary outcome was variation in bleeding sites. The determinant was the type of hemophilia, HA and HB. Patients were censored when they started on prophylactic treatment.

Results: Among 582 patients with severe HA and 76 with severe HB there were no differences in age at diagnosis (median 0.42 vs. 0.43 years [$P = 0.65$]), age at first exposure to clotting factor (0.81 vs. 0.88 years [P 0.20]), age at first bleed (0.82 vs. 0.88 years [$P = 0.36$]), and age at first joint bleed (1.18 vs. 1.20 years [$P = 0.59$]), respectively. As expected, these respective ages were higher in patients with moderate hemophilia. Also in moderate hemophilia there were no clear differences between HA and HB. The age at start of prophylaxis in HA and HB were similar. Target joints occurred with similar frequencies. The proportion of children without bleeding before the age of 2 years was equal.

Summary/Conclusions: The present study demonstrates that the severity and the variation in bleeding phenotype of hemophilia A and hemophilia B are similar during the early stage of the disease in children with severe and moderate hemophilia. Young children with hemophilia B should be observed and treated as intensely as those with hemophilia A.

PA 2.07-3

Identification of 11 new mutations in the factor IX gene in patients with haemophilia B

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Introduction: Haemophilia B (HB) is an X-linked hereditary bleeding disorder caused by factor IX (FIX) deficiency. The FIX gene (*F9*) is located at Xq27.1 with eight exons and seven introns and encodes a 2.8 kb mRNA molecule, resulting in the mature serine protease FIX with 461 amino acids. Mutations that cause haemophilia B are restricted entirely to the *F9* and include point mutations, such as missense, nonsense and splice site mutation, and deletions or insertions. Point mutations are regarded as cause of approximately 85% of the haemophilia B cases.

Objective: The aim of this study was to determine the genotype of patients with the diagnosis of haemophilia B, correlating it with their phenotype, and to evaluate the most frequent mutations in our population.

Patients and Methods: Patients with HB from six Haemophilia Centers located in different regions inBrazil were included in this study. DNA samples extracted from peripheral blood leukocytes were used for direct sequence analysis, using the reference NCBI Ref Seq NM_000133.3 and the King's College London Haemophilia B database.

Results: A total of 154 patients with haemophilia B of 88 unrelated families were included in this study, with 11 cases of sporadic haemophilia B. Sixty-nine patients (47 families) were classified as severe haemophilia B (FIX ≤ 1 IU/dL). Sixty-one *F9* distinct mutations were identified, including 11 mutations not previously described. Novel mutations included two frameshift mutations (c.31044_31046delC and c.31298_31303ins) and the point mutations p.L326R, p.D359H, p.R116P, p.H221N, p.I382T, p.P193L, promoter -21 T>A, p.E17D and p.Q246R. Forty-seven unrelated families had severe haemophilia B, among them we observed 32 (68%) missense mutation, 8 (17%) of nonsense mutation, 3 (6%) splice site mutation and 4 (9%) frameshift mutation. In 41 unrelated families with moderate and mild haemophilia B (34 moderate and seven mild) we observed 1 (2.5%) frameshift mutation, 1 (2.5%) splice site mutation and 39 (95%) missense mutations. Regarding the most frequently observed mutations in this population we can highlight two codons in exon 8 recognized as hotspots in *F9* (p.R248 and p.R333). The mutation p.R248Stop (30863 C>T) was present in three families with severe haemophilia B. Four distinct families with haemophilia B presented the mutation p.R333Q (31119 G>A) and in one family with severe haemophilia B the mutation p.R333Stop (31118 C>T) was identified.

Discussion: The present study identified the causal mutation of 154 Brazilian haemophilia B patients. Sixty-one different *F9* mutations, including 11 novel mutations, were detected. Identification of the mutations related to haemophilia B allows the carrier detection and prenatal diagnosis. This study is of great interest for genetic counseling of HB carriers and to better understand the pathophysiology of the disease.

PA 2.07-4

Long-lasting recombinant factor FIX Fc fusion (rFIXFc) for perioperative management of subjects with haemophilia B in the phase 3 B-LONG study

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Background: A long-lasting recombinant factor IX Fc fusion protein (rFIXFc) was developed to improve current treatment of haemophilia B. A monomeric rFIX molecule was fused directly to the Fc domain of immunoglobulin G_1(IgG_1) to utilise an endogenous pathway that delays lysosomal degradation by cycling these proteins back into circulation, resulting in a longer plasma half-life. The prolonged activity of rFIXFc was confirmed in the recently completed phase 3 B-LONG study to provide prophylactic protection at 1–2 week dosing intervals and effective management of bleeding episodes.
Aim: To evaluate the efficacy of rFIXFc for haemostatic control in the setting of major surgery.
Methods: Male subjects with severe haemophilia B (≤ 2 IU/dL [2%] endogenous FIX), ≥ 12 years old, with no current or previous FIX inhibitors, and a history of ≥ 100 documented prior exposure days (ED) to FIX were enrolled in one of four treatment arms: Arm 1, weekly prophylaxis; Arm 2, individualised interval prophylaxis; Arm 3, episodic (on-demand) treatment; and Arm 4, perioperative management. Subjects requiring major surgery (newly recruited or from Arms 1–3) participated in Arm 4. Treatment for subjects in Arm 4 was chosen by the investigators and surgeons based on considerations of the subject's rFIXFc PK profile, the type of planned surgery, and clinical status of the subject. Endpoints for Arm 4 included investigators'/surgeons' assessments of subjects' response to surgery with rFIXFc; number of injections and dose required to maintain haemostasis during the surgical period; estimated blood loss during and after surgery; and number of transfusions required for surgery.
Results: Overall, 14 major surgeries were performed in 12 subjects, including arthroscopic meniscectomy of knee (n = 1), arthroscopic ankle fusion (n = 1), knee replacements (n = 5), and other (n = 7). Haemostasis was rated as excellent (13/14) or good (1/14) by the investigator/surgeon. The median estimated blood loss was 65.5 mL (range: 0.0–300.0 mL) during surgery and 0.0 mL (range: 0.0–500 mL) post-operatively. No blood transfusions were required during surgery, but two subjects received transfusions in the postoperative period. A single injection of rFIXFc was required in 85.7% of surgeries to maintain haemostasis during surgery, at a median dose of 90.9 IU/kg per injection. Most procedures required 1–2 injections the day preceding and day of surgery and most subjects required 2–3 injections during post-operative Days 1–3. On the day of surgery, post-operative Days 1–3, and post-operative Days 4–14, the median rFIXFc consumption (summarized over all injections during the time period) was 146.1, 164.6, and 277.1 IU/kg, respectively. Overall, one or more adverse events (AE) were reported for 10 (83.3%) of the 12 subjects, and three subjects reported six serious AEs, all of which resolved and were judged as unrelated to rFIXFc treatment.
Summary/Conclusions: In this study, long-lasting rFIXFc maintained perioperative haemostasis in patients with haemophilia B. High perioperative efficacy was reported by investigators/surgeons, suggesting that haemostasis achieved after infusion of rFIXFc was comparable to that for similar surgeries in subjects without haemophilia B.

PA 2.07-5

Clinical implications of population pharmacokinetics of rFIXFc in routine prophylaxis, control of bleeding and perioperative management for haemophilia B patients

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Background: Clinical dosing of factor IX (FIX) in treatment of haemophilia B is well established based on empirical practice and clinical outcomes. Since pharmacokinetics (PK) of FIX activity is a surrogate efficacy marker, we utilised population PK (popPK) modelling and simulation to evaluate dosing regimens of long-lasting recombinant FIX Fc fusion protein (rFIXFc). The PK of rFIXFc, in 12 phase 1/2a and 123 phase 3 study subjects from 12 to 76 years old (body weight (BW): 45–186.7 kg), was best described by a three-compartmental model, which showed modest inter-individual variability (IIV) of 17.7% for clearance (CL) and 21.7% for volume of central compartment (V1). The proportional residue error of 10.6% approximates the variability of the one-stage clotting assay for FIX activity. The only covariate that showed a weak association with rFIXFc PK was BW, which accounted for approximately 3% of IIV for CL and V1, suggesting that BW-independent (i.e. flat) dosing of rFIXFc may be feasible for treating adult haemophilia B patients.
Aims: To simulate the BW-based and flat dosing regimens for routine prophylaxis, control of bleeding and perioperative management in the haemophilia B population.
Method: The validated three-compartmental popPK model, including inter-occasion variability and BW as the covariate on CL and V1, was used for dosing simulations. For each regimen, PK profiles were simulated for 1000 subjects with BW distribution representative of the phase 3 study, and for three populations (n = 1000 each) stratified by BW: low (≤ 10th percentile), typical (10th–90th percentile) and high (≥ 90th percentile). Variability of exposure parameters, percentage of population maintaining target C_{max} and trough, and deviations of median exposure parameters in extreme BW groups were compared between BW-based and flat dosing regimens.
Result: Consistent with the observations from the phase 3 study, popPK simulation of 50 IU/kg once weekly or 100 IU/kg every 10–14 days predicted peak FIX activity within the physiologic range (C_{max} < 150%) and trough ≥ 1% in the majority of the population. Analysis of 12 major surgeries found that the FIX activities measured during the perioperative period were largely consistent with the prediction by popPK based on subjects' pre-surgery PK characteristics, indicating no substantial factor consumption in these surgeries. Furthermore, BW-based and flat dosing resulted in comparable PK profiles, eg, 50 IU/kg and 4000 IU once weekly predicted a median (5th, 95th percentile) C_{max} of 52.6 (32.1, 89.3) IU/dL and 56.1 (36.2, 90.9) IU/dL, respectively. Both dosing regimens predicted that > 95% of the population maintains C_{max} < 150% and trough ≥ 1%. However, BW-based and flat dosing showed differential effects on the exposure parameters in extreme (≤ 10th or ≥ 90th percentile) BW populations.
Conclusion: PopPK provides a robust and effective means to evaluate potential dosing regimens. The predictions by popPK simulation for rFIXFc corroborate the results from the phase 3 study. The simulations of BW-based and flat dosing of rFIXFc achieved similar PK pro-

files. Considering the wide therapeutic range for factor replacement therapy, flat dosing of rFIXFc and rFIX products may be a viable approach in adult haemophilia B patients that warrants further clinical investigation.

PA 2.07-6

Treatment of bleeding episodes in subjects with haemophilia B with the long-lasting recombinant factor IX Fc fusion protein (rFIXFc) in the phase 3 B-LONG study

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Background: Fc fusion technology has been utilised to develop a long-lasting recombinant factor IX (rFIXFc) for the treatment of haemophilia B. Recombinant FIX was covalently linked to the Fc domain of human immunoglobulin G_1 (IgG) to utilise an endogenous pathway that delays lysosomal degradation by cycling Fc-containing proteins back into circulation, resulting in a longer plasma half-life. The improved PK of rFIXFc was demonstrated in the recently completed phase 3 B-LONG study, in which rFIXFc provided sustained protection from bleeding with prophylaxis regimens of injections every 1–2 weeks, and excellent control of bleeding episodes.

Aim: To evaluate the treatment of bleeding episodes with rFIXFc in the B-LONG study.

Methods: Male subjects with severe haemophilia B (≤ 2 IU/dL [2%] endogenous FIX), ≥ 12 years old, with no current or previous FIX inhibitors, and a history of ≥ 100 documented prior exposure days (ED) to FIX products were included in one of four treatment arms: Arm 1, weekly prophylaxis (starting at 50 IU/kg; PK-driven dose adjustments); Arm 2, individualised interval prophylaxis (100 IU/kg starting at every 10 days; PK-driven interval adjustments); Arm 3, episodic (on-demand) treatment (20–100 IU/kg); and Arm 4, perioperative management. The number of bleeding episodes, number of injections, and median dose required to resolve bleeding episodes were evaluated. Subject and physician subjective assessment of efficacy were recorded.

Results: One hundred and twenty-three subjects were enrolled at 50 centres (Arm 1 = 63, Arm 2 = 29, Arm 3 = 27, Arm 4 = 12 [including eight from Arms 1–3]), and 93.5% completed the study. A total of 636 bleeding episodes (Arm 1 = 167, Arm 2 = 67, Arm 3 = 402) in 89 subjects were treated, 90.4% of which required a single dose of rFIXFc for resolution of the bleed and 97.3% required ≤ 2 injections. The median dose per injection was 46.1 IU/kg and the median total dose for the bleeding episodes was 47.0 IU/kg. Overall, subject assessment of response to treatment with rFIXFc was excellent or good for 76.4%, 77.0%, and 85.4% of injections in Arms 1, 2, and 3, respectively. Physicians' global assessment of subject response to their rFIXFc regimen was rated as excellent or effective for 98.9%, 99.2%, and 97.9% of visits in Arms 1, 2, and 3, respectively.

Summary/Conclusions: Results of this analysis indicate that long-lasting rFIXFc offers a robust option for treatment of bleeding episodes in persons with severe haemophilia B. Most bleeding episodes in this study required one injection only to resolve the bleed. Assessments of haemostatic response reflect high subject ratings of treatment response to breakthrough bleeding and high physician ratings of the efficacy of rFIXFc injections.

PA2.08 – Von Willebrand Factor: Clinical – I

PA 2.08-1

Clinical efficacy and safety of DDAVP with or without tranexamic acid in inherited VWD: final results of the prospective and international study on 229 patients

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Background: Desmopressin (DDAVP) is the treatment of choice for most patients with mild-moderate forms of von Willebrand disease (VWD) because it can induce the release of von Willebrand factor (VWF) from cellular compartments. However, despite the large use of DDAVP since 1977, there are only a few prospective clinical trials aimed at determining benefits and limits of this therapeutic approaches.

Aims and Design of the Study: To correlate efficacy and safety with biological response of DDAVP in inherited VWD, 268 patients were enrolled and exposed to 0.3 μg/kg DDAVP intravenous injection and to blood withdrawal for measuring VWF/FVIII activities before and after 0.5, 1, 2 and 4 h (biological response). Then efficacy and safety of DDAVP were evaluated prospectively during 24 months when VWD were exposed to bleedings and minor/major surgeries.

Methods: The following criteria were used for analyses: VWD = VWF:RCo < 55 U/dL; VWD2 = VWF:RCo/Ag ratio ≤ 0.6; VWD1 accelerated clearance: VWF:RCo/FVIII increase after 0.5–1 h with return to baseline after 2–4 h.

Results: 229/268 (85%) patients met inclusion criteria as VWD1 (n = 196), VWD2A (n = 18), VWD2B (n = 1), VWD2M (n = 14), VWD2N (n = 3). Biological response was complete, partial and absent in 89%, 10% and 1% of VWD and correlated with baseline levels of VWF:RCo < 30 U/dL (Fisher's exact = 0.001). VWD1(n = 15) with C1130F and R1205H mutations showed accelerated clearance. During the 24-month follow-up, 62/86 (72%) patients received > 1 injection of DDAVP for bleedings (n = 102), deliveries (n = 13), dental extractions (n = 27), minor/major surgeries (n = 46). Total injections were 652 with median, range/episode during bleedings (2.1–12), deliveries (3.1–3), dental extractions (1.1–10), surgeries (3.61–16). Tranexamic acid (TA) was used together with DDAVP not only in most bleeding episodes [GI bleeds (100%), epistaxis (77%), menorrhagia (71%)] and in most dental extractions (83%) but also in minor (53%) and major (57%) surgeries. Clinical efficacy was excellent/good in bleedings (92.1%), deliveries (84.6%), dental extractions (100%), surgeries (90.6%) being poor in six cases with VWD2A (n = 4) during GI bleedings (n = 3) or abdominal surgery (n = 1) and VWD1(n = 2) during deliveries and surgery. The rate of efficacy were not significantly different in the two groups receiving DDAVP with or without TA. Side effects (16 cases) were mainly minor, such as headache, facial flushing and tachycardia with water retention reported in two cases (one delivery, one surgery) after > 6 injections.

Conclusions: Based on the results of this large prospective study, we confirm that DDAVP is an effective and safe drug at low costs for

managing patients with VWD1, VWD2A, VWD2M and VWD2N once tested for their biological response: therefore it should be always recommended as the treatment of choice in responsive patients with moderate-mild VWD.

PA 2.08-2

The merging project: a machine learning approach to merge and analyze data from four different bleeding questionnaires

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Background: The accurate assessment of hemorrhagic symptoms is a critical component in the diagnosis of mild bleeding disorders including von Willebrand disease (VWD). The value of standardized bleeding histories and quantitative bleeding scores has been recognized and multiple bleeding assessment tools have been developed, validated and published for use in both adult and pediatric populations.

Aim: To create a bioinformatics system for merging and analyzing data derived from different bleeding assessment tools.

Methods: Data obtained from index cases (IC) with VWD and healthy controls (defined as having no known problem with bleeding or bruising) using one of four different bleeding questionnaires [MCMDM1-VWD Bleeding Questionnaire (Tosetto, JTH, 2006;4:766–73), the Condensed MCMDM1-VWD Bleeding Questionnaire (Bowman, JTH, 2008;6:2062–6), the Pediatric Bleeding Questionnaire (PBQ) (Bowman, JTH, 2009;7:1418–21) and the ISTH-BAT (Rodeghiero, JTH, 2010;8:2063–5)] were normalized and cleaned prior to merging. Attribute selection was performed to determine features best able to distinguish individuals affected with VWD from unaffected. Information Gain and Rank search methods were used to determine the score to assign to each attribute with a score of 1.0 having perfect predictive value. The validity of the top 25 attributes was confirmed using classification analysis (Naïve Bayes Decision Tree Classifier) with 5-fold cross validation. Performance of the classifier was assessed by calculating sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV).

Results: Data from 2073 subjects were cleaned and merged (774 MCMDM1-VWD Bleeding Questionnaire, 735 Condensed MCMDM1-VWD Bleeding Questionnaire, 401 PBQ and 163 ISTH-BAT) comprising 679 cases of VWD and 1394 controls. The average age was significantly different between the affected subjects and controls [22.1 (range 1–81) and 34.8 (range 0.4–90), respectively ($P \leq 0.001$)]. The gender distribution did not differ [$n = 432$ females (64%) among cases and $n = 902$ (65%) among controls ($P = 0.785$)]. Of the 679 VWD cases, 612 are Type 1 VWD, 23 are Type 2 VWD and 44 are Type 3 VWD. The overall bleeding score from all four of the bleeding questionnaires included is the best predictor of affected status (score = 0.43) followed by the score for the bruising category (0.23), whether excessive bruising occurs or not (score = 0.23), size of the average bruise (score = 0.19) and location of bruises (exposed vs. unexposed) (score = 0.19). The sensitivity, specificity, PPV, and NPV of the top 25 attributes are 91%, 87%, 0.83 and 0.94 respectively. The analysis did not substantially differ when performed for only cases with Type 1 VWD and controls, with the exception of an increase in PPV to 0.93.

Conclusions: We found that the overall bleeding score is better than any single attribute at predicting the presence of VWD. Thus, an accurate assessment of bleeding symptoms must be comprehensive in order to have the greatest predictive value. Finally, our results highlight the strength in pooling existing datasets to improve our knowledge about diagnostic accuracy in VWD.

PA 2.08-3

Hemophilia arthropathy occurs in a significant percentage in von Willebrands disease

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Background: In von Willebrand disease (VWD), the occurrence of joint bleeds has been well described, but no valuable information on joint status and outcome is currently available. The objective of this study is to learn more about the occurrence and impact of arthropathy in VWD.

Methods: The Willebrand in the Netherlands (WiN) study is a large nation-wide cross-sectional study that collected data on adults and children with moderate and severe VWD (VWF of 30 IU/dL or lower) from all 13 Haemophilia Treatment Centers in the Netherlands between October 2007 and October 2009. Participants were asked to complete an extensive self-administered questionnaire, including questions on bleeding episodes, treatment, concomitant disease, joint damage, joint surgery and Quality of Life. Furthermore, at inclusion, blood was drawn from most patients for measuring von Willebrand parameters in a central laboratory.

Results: Twenty-three percent of the 664 included adult VWD patients suffered from haemarthrosis ($n = 147$). Sixty-one patients (61 out of 664, 9%) self-reported treatment with DDAVP or coagulation factor for joint bleeds. FVIII:C was a strong determinant and joint bleeds were mostly seen in VWD patients with FVIII:C < 5 IU/dL. Joint bleeds had significant impact on their health related quality of life (physical component summary of the SF36 in men, $P = 0.044$, 95% CI [−5.37, −0.07] and in women, $P = 0.007$, 95% CI [−5.20, −0.85]. Mental component summary of the SF36 ns in men, in women $P = 0.004$, 95% CI [−5.71, −1.10]). Joint damage related to joint bleeding was reported by 80 of the 147 patients who reported joint bleeds (54%), which was most often joint damage of the knee (31%). Ninety-four patients had been admitted to the hospital for orthopedic joint surgery (94 out of 664, 14%) and in up to 33% of these patients the orthopedic surgery was probably related to the JB.

Conclusion: This study shows that the problem of arthropathy in VWD clearly exists and is not marginal. Self reported joint damage related to joint bleeds occurred in a significant percentage of moderate and severe VWD patients and a significant percentage of the patients needed orthopedic surgery related to the joint bleeds. Joint bleeds affect health related quality of life in VWD patients and it could be speculated that arthropathy due to joint bleeds partly explains this finding. Further research is needed to learn more about the severity and onset of arthropathy in VWD patients, its impact on daily functioning and on health-related quality of life.

PA 2.08-4

Validation of a micro assay for the diagnosis and characterisation of Von Willebrand disease

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Background: Von Willebrand disease (VWD) is the most common inherited bleeding disorder, defined by a quantitative or qualitative defect in the Von Willebrand Factor (VWF). Conventional diagnosis of VWD is based on quantification of VWF antigen levels and assessment of its activity and composition. These tests are laborious, require at least 15 mL of blood and can only be performed by specialised technicians in equally specialised laboratories. We have developed a simple VWF function micro assay which requires only 40 μL of blood for the diagnosis and characterisation of VWD.

Aims: Our aim was to develop and validate a simple, accurate and fast single step assay to distinguish healthy donors from VWD and characterise VWD subtypes, using a mere 40 μL of blood.

Methods: This study was approved by the Medical Ethics Committee of the University Medical Center Utrecht. The procedures followed were in accordance with the Helsinki Declaration. After oral informed consent, blood samples were collected from 60 healthy adult volunteers and 60 adult patients with established diagnosis of VWD (ten of each subtype 1, 2A, 2B, 2M, 2N, 3). The micro assay consists of an eight serial dilution of ristocetin (0–0.75 mg/mL) containing a fixed concentration of FITC labelled anti-VWF antibody. Five microllitre of citrated blood was added to each well and VWF binding was assessed with fluorescent activated cell sorting. Results were plotted in a dose response graph. Analysis of the control vs. VWD groups and the characterisation of the sub groups was performed using ROC analysis.

Results: VWD can be distinguished from healthy subjects in two steps. First, type 2B patients can be distinguished from other patients and controls by increased binding of VWF to the platelet at a low ristocetin concentration (0.13 mg/mL ristocetine, $P < 0.0001$, sensitivity 100.0%, specificity 99.0%). All other VWD types show a significantly lower area under the dose response curve (AUC) compared to healthy controls ($P < 0.0001$, sens 93.1%, spec 100.0%). We have developed an algorithm to further distinguish between type 1 and type 2A (P 0.0056, sens 88.9%, spec 90.9%), type 2M and type 3 ($P < 0.0001$, sens 100.0%, spec 99.1%). As expected, type 2N patients were not identified, which is in accordance with its assumed unaffected ristocetin induced binding capacity.

Summary/Conclusion: We have developed a simple, accurate and fast new assay to diagnose and characterise VWD in unprocessed blood using flow cytometry. This is a major step forward in the current complex and laborious diagnostic process of VWD.

PA 2.08-5

Comparison of two automated latex-based VWF activity assays of differing principles with a standard VWF:RCo platelet aggregometry technique

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Background: Assessing VWF activity is a crucial diagnostic tool in detection and sub-typing of VWD. The VWF:RCo platelet aggregometry assay has been the mainstay technique for decades yet it is a technically demanding manual method with low sensitivity and high inter-assay and inter-laboratory variability. Newly available, fully automated assays potentially offer improved diagnostic performance.

Aims: To compare two new generation automated VWF activity assays with a VWF:RCo platelet aggregometry technique.

Methods: VWF:RCo was performed using locally-prepared formalin-fixed platelets on a PAP4 aggregometer (Alpha Laboratories). Both automated assays employ latex beads coated with a recombinant fragment of GpIbα through a monoclonal antibody. In the HemosIL VWF:RCo assay (Instrumentation Laboratory), VWF in the sample binds to the GpIbα fragment via ristocetin and the degree of agglutination is directly proportional to VWF activity, determined by decreased light transmittance due to aggregation. The Innovance® VWF activity assay (Siemens) similarly employs latex-bound GpIbα fragment but it contains two gain-of-function mutations and agglutination is ristocetin-independent. The HemosIL assay was performed on an ACL TOP® 500 (Instrumentation Laboratory) and the Innovance® assay on a CS2100i (Sysmex). Fifty nine samples from patients with various VWD sub-types and 14 samples with normal VWF activity levels were tested with all three assays.

Results: Both automated assays had sensitivity limits of approximately 7.0 IU/dL, compared to 12 IU/dL for the aggregometry technique. Intra-assay CVs with a commercial normal and an abnormal control were 4.21% and 3.20% respectively for HemosIL and 4.20% and 3.20% respectively for Innovance®. Forty three of the known VWD samples gave results within the measurable range for the aggregometry technique, which along with the normal samples gave good correlation with the automated techniques. Mean and median values and ranges for aggregometry, HemosIL and Innovance® were 40.7, 38.5, 12.0–114.0; 47.7, 45.8, 4.6–106.7 IU/dL and 46.6, 47.4, 4.0–132.2 IU/dL respectively. Coefficients of determination (R^2) for aggregometry vs. HemosIL, aggregometry vs. Innovance® and HemosIL vs. Innovance® were 0.86, 0.86 and 0.89 respectively. A positive proportional bias was noted with HemosIL and Innovance® when compared to aggregometry, the mean bias for HemosIL was 6.5 IU/dL and for Innovance® was 5.9 IU/dL, which interestingly improved concordance with VWF:Ag values in type 1 patients. Of the 16 samples reported as < 12 IU/dL by aggregometry, 11 gave quantitative results < 12 IU/dL with both automated assays. Mean and median values and ranges for HemosIL and Innovance® respectively were 6.1, 6.3, 3.3–9.6 IU/dL and 6.5, 5.6, 4.0–10.0 IU/dL. The remaining samples gave the following HemosIL/Innovance® paired results (IU/dL): 11.7/12.4, 12.9/16.8, 12.5/14.6, 12.9/12.3, 13.8/13.0. Based on an activity:antigen ratio of < 0.7 indicating a type 2/multimeric abnormality, six samples from type 1 patients would have been misclassified as type 2 (four with aggregometry and two with HemosIL), and two samples from type 2 patients misclassified as type 1, one each with aggregometry and HemosIL.

Conclusions: New automated, latex-based VWF activity assays offer increased precision and diagnostic accuracy compared to standard VWF:RCo platelet aggregometry.

PA 2.08-6

RNA analysis for Von Willebrand disease; it has a role but it's not the answer

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Type 1 and type 3 von Willebrand disease (VWD) are quantitative deficiencies of von Willebrand factor (VWF) in plasma. Diagnosis of VWD requires phenotypic data (VWF:Ag; WVF:RCo, VWF:CBA) to support the clinical symptoms of muco-cutaneous bleeding and bruising. Low levels or absence of VWF and proportionate activity define type 1 or type 3 VWD. Unfortunately, many factors other than the VWF gene affect VWF levels in plasma, including ABO blood group. Molecular analysis of the VWF gene can assist diagnosis but is complex as it is large (approximately 175 kb spread over 52 exons), highly polymorphic and has a highly homologous partial pseudo-gene. Anal-

ysis of VWF cDNA, reverse transcribed from leukocyte RNA, significantly reduces the size (approximately 9 kb) and removes the issue of the pseudo-gene.
We report three cases which illustrate the benefit and limitations of an RNA approach to genetic screening of quantitative VWD and its contribution to our understanding of the disease.
In the first case, analysis confined to the genomic level suggested the lack of a pathogenic mutation, clearly visible at the RNA level. Heterozygosity for a 41 base-pair insertion after exon 5 was detected in a mother and daughter each with type 1 VWD. In silico analysis identified this sequence as originating within intron 5. Analysis of this region in gDNA identified a heterozygous G>A substitution within a region containing a possible cryptic splice site. In this case, RNA analysis provided a definitive result, missed by gDNA analysis.
The second case is a demonstration of additional information provided by RNA analysis. A male with severe type I VWD was shown, at the gDNA level, to have a homozygous c.5455+2T>C substitution. cDNA analysis demonstrated a transcript lacking exon 31, but also some expression of normal transcript. This explains the patient having higher levels (VWF:Ag 14 IU/dL) than would be predicted by the DNA result.
These cases argue the case for a role for cDNA analysis in the investigation of VWD. However a third example raises concerns. The only variant detected in the RNA from a female with 'severe type I' VWD was a heterozygous c.4751A>G mutation. As this was not consistent with the diagnosis, DNA samples from her two children, both with type 3VWD, were analysed. Neither had the c.4751A>G mutation but both were homozygous for a c.7130delC mutation. Further analysis of the maternal gDNA confirmed her to be heterozygous for the c.7130delC mutation. Preferential reverse transcription of the normal RNA species or selective amplification of the wild type cDNA obscured detection of this mutation in the heterozygous state and led to a false negative result.
Non reproducible sequence data from cDNA transcripts have been observed in several other cases. These are suggestive of the presence of aberrant leucocyte VWF transcripts.
In conclusion, genetic analysis of both gDNA and RNA has a role in VWD diagnosis. However, neither represents a totally effective tool, and a combined approach may resolve a maximum number of cases.

PA2.09 – Von Willebrand Factor – II

PA 2.09-1

Von willebrand factor as a surrogate marker for coronary artery calcification: the rotterdam study

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Background: Increased levels of von Willebrand factor (VWF) are associated with increased risk of coronary heart disease (CHD). As VWF mediates platelet adhesion, there might be an association with atherosclerosis and plaque formation. Understanding the association between atherosclerosis and VWF levels will help us further understand the pathogenesis of CHD.
Aims: We studied the association between coronary atherosclerosis and VWF levels in a population-based cohort study.
Methods: This study was part of the Rotterdam study, a population-based cohort study among subjects ≥ 55 years. We included 3485 subjects of whom blood samples and electron-beam computed tomography (EBT) or multidetector computed tomography (MDCT) were available and who were free from CHD at baseline. VWF antigen (VWF:Ag) levels were measured using ELISA and the coronary artery calcification (CAC) score was quantified according to the Agatston score. CAC scores were divided into four categories: 0–10, 11–100, 101–400 and > 400 Hounsfield Units. The association between the CAC score categories and VWF:Ag levels was assessed using a linear regression analysis. Analyses were adjusted for potential confounders including age, sex and cardiovascular risk factors.
Informed consent was obtained and the study was approved by a recognised medical ethics committee.
Results: The CAC score was significantly and positively associated with VWF:Ag levels (*P* for trend 0.007). VWF:Ag level (Mean ± SD) was lowest among individuals in the 0–10 CAC score category (1.10 ± 0.44 IU/mL) and highest among the individuals with the > 400 CAC score category (1.25 ± 0.54 IU/mL).
Summary/Conclusion: Our findings demonstrate an association between the coronary artery calcification score and VWF:Ag levels in the older subjects, without coronary heart disease. This suggests that the well-known association between VWF and risk of coronary heart disease may be determined by atherosclerosis.

PA 2.09-2

Von Willebrand factor propeptide (VWFpp) a marker useful for identifying adverse platelet activation in murine blood phlebotomy samples

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Background: Von Willebrand factor (VWF) and its propeptide, VWFpp, are secreted in an equimolar ratio into plasma. Since the VWFpp has a shorter half-life than VWF (2–3 vs. 8–14 h), the concentration of plasma VWFpp is less than VWF although each is defined as 1.0 U/mL with a VWFpp/VWF ratio of 1.0. After DDAVP administration, the plasma concentration of both proteins is increased due to release from endothelial cell (EC) Weibel-Palade bodies (WPB), but VWFpp/VWF is elevated (range of 4–10), reflecting regulated release on an equimolar, but not 'equi-unit' basis. Platelet activation should result in an increased VWFpp/VWF ratio.
Aims: To determine the most reliable method for blood sampling in mice and to identify the source of exocytosed VWFpp and VWF (platelet or endothelial cell).
Methods: 'Platelet-only' and 'EC-only' mice were generated by bone-marrow transplant between C57BL/6J and VWF−/− mice. As control, murine VWF expression was induced in the livers of VWF−/− mice by hydrodynamic tail vein injection (HDTV) which lack regulated VWF release. Blood sampling methods used include single retro-orbital (single capillary tube), multiple retro-orbital (multiple successive capillary tubes), cardiac puncture, submandibular, and vena cava bleeds. Blood collection after injection of heparin into the mouse was assessed. VWFpp and VWF levels were determined by ELISA.
Results: Assay of VWFpp/VWF demonstrated that single retro-orbital bleed, and multiple retro-orbital or cardiac puncture after heparin injection resulted in a VWFpp/VWF range of 0.6–1.6. Multiple retro-orbital bleeds without heparin injection had an elevated VWFpp/VWF of 4.5, suggesting activation of regulated release. Cardiac puncture and submandibular bleeds demonstrated variable results with VWFpp/VWF ranging from 1.0 to 18.4. Vena cava bleeds consistently showed the least activation with average VWFpp/VWF of 0.5. HDTV mice that lack regulated VWF secretion had VWFpp/VWF of 0.2 regardless of collection method. The low ratio in vena cava bled mice and HDTV mice compared to our VWF standard collected by cardiac puncture indicates the standard itself suffers from a degree of platelet/EC activation. To determine the source of released VWFpp and VWF, we compared vena cava and multiple retro-orbital bleeds of 'platelet-only', 'EC-only', and HDTV mice, measuring VWFpp and VWF released into plasma. In EC-only mice, VWF and VWFpp levels after vena cava collection averaged 0.5 U/mL with a ratio of 1.0. In contrast, after multiple retro-orbital bleeds, VWF and VWFpp were ele-

vated (0.9 and 2.3 U/mL, respectively) with average ratio of 3.1. In platelet-only mice, no VWFpp or VWF could be detected in plasma after vena cava sample collection as expected. In contrast, after multiple retro-orbital bleeds, VWFpp and VWF were elevated (3.5 and 0.12 U/mL) with VWFpp/VWF of 25.8 indicating substantial platelet activation.

Conclusions: Activation of platelets appears to be the more predominant mechanism than EC activation. Vena cava blood collection in mice appears to be the most reliable method, although a single retro-orbital bleed is a non-terminal method with only moderate platelet activation. Assay of VWFpp has a clear utility in determining the acceptability of murine plasma samples.

PA 2.09-3

Isolation of blood outgrowth endothelial cells from early infantile epileptic encephalopathy 4 (EIEE4) and familial hemophagocytic lymphohistiocytosis 5 (FHL5) patients

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Background: Syntaxin binding proteins (STXBPs) are Sec1/Munc18-like (SM) proteins which allow the docking of secretory granules to the plasma membrane and regulate the formation of the SNARE-complex prior to exocytosis. Recently, a number of putative binding partners of STXBP1, such as synaptotagmin-like protein 4-a (Slp4-a) and the small GTPase Rab27A, have been implicated in regulation of secretion of Von Willebrand factor (VWF), which is released from endothelial-specific secretory organelles called Weibel-Palade bodies (WPBs). Mutations or deficiencies in some STXBP proteins are linked to diseases characterized by a secretory defect: Early infantile epileptic encephalopathy type 4 (EIEE4) is a severe neurological disorder caused by de novo mutations in the gene encoding syntaxin binding protein 1 (STXBP1). Familial hemophagocytic lymphohistiocytosis type 5 (FHL5) is severe immunological disorder caused by mutations STXBP2.

Aims: In this study we will address a potential role of STXBP1 and STXBP2 in regulation of VWF secretion and WPB exocytosis using endothelial cells isolated from individuals carrying disease causing mutations in STXBP1 or STXBP2.

Methods: Blood outgrowth endothelial cells (BOECs) were isolated from peripheral blood. Expression of STXBP1 and STXBP2 was determined by Western blotting. VWF antigen plasma levels were determined by ELISA.

Results: We have isolated BOECs from a 16-year old EIEE4 patient carrying a de novo mutation in STXBP1 [c.963+ ?.(1967+?); p.Thr322_Glu603 del], which is predicted to result in a truncated protein. Moreover we isolated BOECs from two siblings homozygous for a splice-site mutation [c.1247-1G>C] in STXBP2, which results in a 17–19 residue substitution and one patient compound heterozygous [c.1247-1G>C; c.1621G>A], the latter resulting in a Gly541Ser substitution. By Western blotting we found that FHL5 BOECs had severely reduced levels of STXBP2, whereas STXBP1 levels in EIEE4 BOECs were relatively unperturbed. Both EIEE4 and FHL5 BOECs contained normal numbers of morphologically normal WPBs. VWF plasma levels in the EIEE4 patient and the FHL5 siblings were reduced, but normal in the compound heterozygote FHL5 patient.

Summary/Conclusions: Our findings suggest a moderate effect of clinically relevant mutations in STXBP proteins on VWF plasma levels, in line with the absence of immediate hemostatic abnormalities in the pathology of EIEE4 and FHL5. Further characterization of the secretory function of EIEE4 and FHL5 BOECs is necessary and ongoing to determine a role for STXBP1 and/or STXBP2 in regulation of WPB exocytosis.

PA 2.09-4

Active VWF predicts 4 week mortality in patients with SIRS

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Background: Systemic Inflammatory Response Syndrome (SIRS) and sepsis are among the leading causes of death in industrialized nations and adequate early diagnosis is a major challenge. Endothelial cell function is a major determinant of thrombosis in patients with SIRS and sepsis and might be used to identify high risk patients or guide the treatment. Von Willebrand factor (VWF) released from Weibel-Palade bodies of endothelial cells is different from the majority of VWF in plasma because it is present in a GPIb binding conformation ('active' von Willebrand factor, aVWF).

Aims: The aim of this study was to determine if the amount of circulating aVWF is able to predict mortality in patients with SIRS.

Methods: A prospective cohort study of 275 consecutive SIRS patients, aged 18 years or older and an anticipated Intensive Care Unit (ICU)-stay of at least 24 h. Blood samples on admission and the following 9 days were collected. aVWF was measured in plasma with an ELISA that recognizes aVWF but not VWF[1]. The end-point of the study was 4-week hospital mortality.

Results: aVWF levels were higher in non-survivors on admission (0.47 vs. 0.69 μg/mL, $P = 0.019$) on day 1 (0.52 vs. 0.78 μg/mL, $P = 0.011$) and on day 2 (0.50 vs. 1.03 μg/mL, $P < 0.01$). On day 3, non-survivors tended to have higher levels of VWF (0.51 vs. 0.76 μg/mL, $P = 0.055$) but subsequently, these levels were comparable to those of survivors. Kaplan Meier Curves showed that patients in the highest tertile of aVWF levels on admission had a lower cumulative survival than patients in the lower two tertiles (75% vs. 86%, $P = 0.017$) and a 2-fold increased risk of mortality [HR = 1.98; 95% CI: 1.1–3.5]. When adjusted for the APACHE IV score, this association remained significant [adjusted HR = 1.82; 95% CI: 1.03–3.3]. Total VWF levels were higher in patients who developed septic shock (53 μg/mL; IQR = 31.5–76.3 μg/mL) and severe sepsis (47.8 μg/mL; IQR = 35.3–63.3 μg/mL) than in patients with only SIRS or sepsis (42.2 μg/mL; IQR = 30.3–61.9 μg/mL), but these differences did not reach significance ($P = 0.071$).

Conclusions: Higher levels of circulating VWF in its GpIb binding conformation predict 4-week mortality in SIRS patients, independently of the APACHE IV Score. Our results show that biomarkers that reflect endothelial activation are of value in SIRS and sepsis patients.

Reference: [1]Blood 2005; 106: 3035–42.

PA 2.09-5

Matrix metalloproteinase-13 influences thrombus formation through the cleavage of VWF

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Background: Following atherosclerotic plaque rupture, the exposure of flowing blood to collagen-rich surfaces triggers platelet activation and aggregation. The engagement of GPIb-IX-V/GPVI by VWF and collagen is pivotal to thrombus formation on the exposed endothelium. MMP-13 is a collagenolytic metalloproteinase that is up-regulated in the vulnerable plaque and released upon rupture.

Aim: Here we aim to determine the effects of MMP-13 on platelet receptor and VWF function.

Methods: Solid phase binding assays, receptor-specific compounds and blocking single-chain variable fragment antibodies (scFvs) were used

to determine the interaction of MMP-13 with platelet receptors. The degradation of recombinant human platelet receptors by MMP-13 was assessed by SDS-PAGE and corresponding assays performed *in vitro*. Aggregation experiments were used to elucidate the effect of MMP-13 and catalytically inactive (E204A)MMP-13 on washed platelet activation in response to CRP-XL, A23187, thrombin and fibrillar Type I collagen. The degradation of VWF by MMP-13 was confirmed via SDS-PAGE and Edman degradation. MMP-13 on coated and plasma-borne VWF proteolysis on platelet adhesion, rolling and thrombus size were analysed under flow conditions in whole blood. The effect of MMP-13 on VWF multimerisation was assessed via agarose gel electrophoresis.

Results: Both latent and active forms of MMP-13 bind to washed platelets via GPVI and αIIbβ3 and can be inhibited by scFvs and RGD peptides respectively. Although MMP-13 is able to cleave rhGPVI, no evidence was found for sheddase activity *in vitro*. These interactions, while resulting in some degree of platelet shape change, do not impede the activation of platelets or a significant inhibition of aggregation in response to any agonist tested. Pre-incubation of active MMP-13 and (E204A)MMP-13 with whole blood however resulted in a differential reduction in surface coverage and volume of the thrombi formed on fibrillar collagen. Pre-treatment of whole blood with MMP-13 resulted in a decrease in thrombus height and platelet rolling on intact coated VWF. In contrast, surface coverage and height of thrombi formed on MMP-treated VWF were increased. SDS-PAGE following incubation of MMP-13 with VWF confirmed cleavage of the glycoprotein between the D3 and A1 domains. This MMP-mediated proteolysis resulted in an impairment of VWF multimer assembly as assessed by agarose gel electrophoresis.

Summary/Conclusions: Under high shear VWF is unfolded and is cleaved by the protease ADAMTS13. Here we report that the collagenase MMP-13 is also able to cleave VWF; albeit at a different site than ADAMTS13. Further studies will determine how pro- and anti-thrombotic flow conditions affect VWF cleavage by MMP-13.

PA 2.09-6
Von Willebrand factor and ADAMTS-13 in hypertensive disorders of pregnancy

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Background: Normal pregnancy is associated with hypercoagulability, endothelial activation and suppression of fibrinolysis. Haemostatic changes are, mostly, exaggerated when pregnancy is complicated with hypertensive diseases of pregnancy (HDP). HDP are a common cause of maternal morbidity and mortality, but much remains to be known about the underlying pathophysiology. Earlier studies have shown that HDP are accompanied, and occasionally preceded, by significant endothelial cell damage, platelet activation and enhanced intravascular thrombin generation. von Willebrand Factor (VWF) is an adhesive glycoprotein that plays a major role in primary haemostasis. Increased levels are commonly reported in normal pregnancy. The main mechanism regulating VWF molecular size and function is its cleaving protease ADAMTS13 (A disintegrin and metalloprotease with thrombospondin) that controls the release of the highly thrombogenic ultra large ULVWF multimers. Factors disturbing this balance may cause pathologic conditions ranging from bleeding to thrombosis.

Aims: We aimed to study the changes in plasma antigen levels of VWF, and ADAMTAS-13 in Saudi patients with HDP in the antenatal and postnatal periods in comparison with normal pregnancy.

Methods: This case-control cross-sectional study included 109 patients with HDP; 49 preeclampsia (PE), 60 gestational hypertension (GH) and 100 women with normal pregnancy as control group. Subjects were recruited in the third trimester and 24 h post-delivery at a tertiary care facility in the capital Riyadh. Commercially available Enzyme immunosorbant assay (ELISA) kits were used to measure plasma antigen concentrations of VWF and ADAMTS-13.

Results: The plasma levels of VWF were significantly higher in PE and GH in both the antenatal and postnatal periods as compared to normal pregnancy ($P < 0.001$). The levels of ADAMTS-13 were significantly lower in PE and GH in both the antenatal and postnatal periods, as compared to normal pregnancy ($P = 0.001$, $P = 0.002$ respectively). VWF levels were elevated, 24 h after delivery, in all three groups and reached statistically significant levels in GH patients ($P = 0.02$). However, ADAMTS-13 did not change significantly in the postnatal period. No significant correlation was found between VWF and ADAMTS-13 plasma levels in all groups. Also, levels were not associated with O and non-O blood groups.

Conclusion: PE and GH and associated with significant increase of VWF and decrease of ADAMTAS-13 antigen levels which reflect an exaggeration of the prothrombotic state that characterizes normal pregnancy. Also, the further elevation of VWF levels after delivery and with no change in of ADAMTS13 levels add to the heightened hypercoagulability which is believed to underlie the pathogenesis of HDP. All level changes seem to be independent of ABO blood groups.

PA2.10 – Anticoagulant Agents – VII

PA 2.10-1
Novel VKORC1 variants are associated with the higher acenocoumarol requirements

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Background: Algorithms combining both clinical and genetic data have been developed to improve oral anticoagulant therapy. All of them have confirmed that three polymorphisms in two genes, *VKORC1* and *CYP2C9*, are the main coumarin dose determinants. Additional genetic elements have to be involved in the response to oral anticoagulant, although no further polymorphisms (SNPs) with a relevant pharmacogenetic importance have been identified, and according to recent GWAS analysis is not expected.

Aim: To identify new pharmacogenetic variations involved in oral anticoagulant therapy in *VKORC1*, the main element of this pathway.

Methods and Results: Our retrospective study recruited 3949 consecutive Caucasian patients taking acenocoumarol. Patients were eligible to take part in the study if aged 18 years or over and were steadily anticoagulated (defined as a period of at least 3 weeks with three or more consecutive INR measurements with < 10% change within the target range in the acenocoumarol dose). Exclusion criteria were a diagnosis of cancer, alcohol or drug abuse, acenocoumarol allergy/intolerance, terminal disease, known or suspected pregnancy and intake of amiodarone, antifungal drugs, anti-platelet drugs and/or statins. Clinical and demographical data were obtained from the medical records and the database: 1947 males (49%); median age, 74 ± 10 years; median body mass index, 29.5 ± 7.4. Most patients (90%) were anticoagulated with an INR target range of 2.0–3.0% and 10% of them had an INR target range of 2.5–3.5. All 3949 patients were genotyped for the *VKORC1* rs9923231 and *CY2C9** polymorphisms. These polymorphisms defined 18 genetic profiles. For each profile, we identified the average dose of carriers. Patients with dose requirements outside the mean ± 2 SD value according to their genetic profile were identified as *"outlier"* patients ($N = 145$). Patients with clinical factors that explained the abnormal dose requirements (i.e., autoimmune diseases, liver failure, severe heart failure, severe chronic obstructive pulmonary disease, acenocoumarol interacting drugs, new thrombotic events, obesity and

associated tumoral diseases) as well as those patients with no available clinical data were excluded ($N = 88$). In the remaining 57 patients (all with higher doses than expected) the promoter and coding regions of *VKORC1* were sequenced. We identified *VKORC1* genetic changes in 14 patients. Four patients carried *VKORC1* variants previously related to high coumarin doses (L128R, $N = 1$ and particularly D36Y, $N = 3$).Three polymorphisms were also detected: rs17878544 ($N = 5$), rs55894764 ($N = 4$) and rs7200749 which was in linkage disequilibrium with rs17878544 ($N = 2$). Finally, 2 patients had lost the rs9923231/rs9934438 linkage. The prevalence of these variations was higher in these patients than in the whole sample. Multivariate linear regression analysis revealed that only D36Y and rs55894764 variants significantly affect the dose, although the improvement in the prediction model is small (from 39% without their inclusion to 40% if the analysis included these mutations).

Conclusion: Our strategy identified *VKORC1* variants associated with higher acenocoumarol doses. Further studies are necessary to test their influence on the *VKORC1* function and the cost/benefit of their inclusion in pharmacogenetic algorithms.

PA 2.10-2

Ensuring an efficient transition from rivaroxaban to warfarin: a pharmacodynamic study in healthy subjects

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Background: Clinical scenarios may arise when it is necessary to transition patients from rivaroxaban to a vitamin K antagonist such as warfarin. Maintaining appropriate anticoagulation, without increasing the risk of thrombosis or adverse bleeding events, is key when transitioning patients between anticoagulants.

Aims: The primary objective of this study was to evaluate the pharmacodynamic changes when transitioning healthy subjects from steady-state rivaroxaban 20 mg once daily (od) to warfarin, dose-adjusted to an international normalized ratio (INR) range of 2.0–3.0.

Methods: This was an open-label study conducted in 46 healthy adults. This study was comprised of a screening phase and two treatment periods (TP1 and TP2) with a ≥ 14 day wash-out in-between. During TP1, subjects received rivaroxaban monotherapy 20 mg od for 5 days followed by rivaroxaban 20 mg od + warfarin 10 mg od for 2–4 days (warfarin dose could be adjusted after 2 days based on the trough INR value on Day 8), co-administered each morning. When a trough (pre-dose) INR ≥ 2.0 was achieved during this transition phase, subjects were switched to warfarin monotherapy (dose range: 0 [no dose] to 15 mg od) for 4 days to achieve/maintain an INR range of 2.0–3.0 by the last day of warfarin monotherapy. TP2 involved just a warfarin monotherapy control arm, in which subjects received the same warfarin regimen administered in TP1. Each TP was followed by a single oral dose of vitamin K.

Results: During the TP1 transition phase, when rivaroxaban was co-administered with warfarin, INR and peak prothrombin time (PT) values were higher than those observed with either agent administered alone. The mean maximum effect (E_{max}) for INR values ranged from 2.79 to 4.15 (mean PT E_{max} values ranged from 41.0 to 62.7 s), with individual INR values reaching as high as 5.90. In comparison, warfarin monotherapy during TP2 produced mean INR E_{max} values ranging from 1.41 to 1.74 (mean PT E_{max} values ranged from 20.1 to 25.2 s). However, rivaroxaban had a minimal effect on INR values at trough drug levels during the TP1 transition phase (mean trough INR values during co-administration were similar to those observed with warfarin monotherapy). Maximum plasma concentrations (C_{max}) for both rivaroxaban and warfarin seemed to be unaffected when the drugs were co-administered: a finding that is consistent with results obtained from a previously conducted drug-drug interaction study. Study drugs were generally well tolerated (alone or combined), although three subjects withdrew owing to adverse events. No significant bleeding events were observed, even in subjects with elevated INRs during the transition phase, and no discontinuations or serious adverse events occurred because of bleeding events.

Summary/Conclusions: This study showed that healthy adult subjects can be safely transitioned from steady-state rivaroxaban to warfarin based on a target INR of 2.0–3.0. Importantly, when transitioning patients, the INR value used to determine the next warfarin dose should always be taken at a trough (24 h after the last rivaroxaban dose) in order to minimize the transient additive INR effect of rivaroxaban.

PA 2.10-3

Postdischarge mortality risk and risk factors for mortality in acutely ill medical patients following hospitalization

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Background: Conflicting evidence exists regarding the benefit of venous thromboembolism (VTE) prophylaxis on mortality in hospitalized acutely ill medical patients, who may also be at-risk after discharge. We identified characteristics associated with receiving thromboprophylaxis after discharge and compared the risk of within-90-day mortality among those patients who did or did not receive thromboprophylaxis.

Aims: To evaluate post-discharge antithrombotic drug use, risk of short-term mortality, and risk factors for mortality.

Methods: We conducted a retrospective healthcare claims analysis of patients aged ≥ 40 years hospitalized ≥ 2 days for nonsurgical reasons between 2005 and 2009 using the HealthCore Integrated Research Database. VTE and rehospitalization were identified by ICD-9-CM codes while use of antithrombotics was captured from pharmacy claims. All-cause mortality was ascertained using the Social Security Death Index and cause of death was identified utilizing the National Death Index. Hazard ratios (HR) for mortality risk factors were estimated using multivariate survival analysis and Cox proportional hazards models.

Results: A total of 327,578 patients were included (median age, 65 years; mean hospital stay, 4.6 days). Of those, 11.6% received at least one post-discharge antithrombotic medication, with 3.9% receiving anticoagulants only. The final post-discharge mortality cohort consisted of 11,922 patients with a median age at death of 80 years compared to 64 years for patients that did not die ($P < 0.001$). Cancer was the most common cause of death ($n = 4695$; 39.4%) followed by overall cardiovascular disease ($n = 3293$; 27.6%). Only 24 and 171 of patients had VTE or major bleeding indicated as a cause of death (0.2% and 1.4%, respectively). Compared with patients that survived within 90 days, significantly fewer patients in the mortality cohort received post-discharge antithrombotics (5.3% vs. 11.9%, $P < 0.001$), and for a significantly shorter mean [SD] duration of time (29 [19] vs. 59 [24] days, $P < 0.001$). In general, mortality rates of patients who did not receive any antithrombotics were significantly higher than those of patients who did. Absence of antithrombotic use (HR 1.6252, 95% Confidence Interval [CI] 1.4999–1.7610), stroke (HR 1.1275, 95% CI 1.0489–1.2120), severe lung disease (HR 1.0693, 95% CI 1.0293–1.1108), age (HR 1.0049, 95% CI 1.0032–1.0065) and index hospitalization length of stay (HR 1.0077, 95% CI 1.0054–1.0100) were significant predictors of post-discharge mortality. Absence of anticoagulants and antiplatelets were also predictors for

mortality (HR 1.5573, 95% CI 1.4003–1.7320 and 1.6774, 95% CI 1.4967–1.8798, respectively). For patients receiving anticoagulants, cancer was a significant risk factor for mortality (HR 1.0620, 95% CI 1.0120–1.1146).

Conclusions: Patients prescribed antithrombotic medications in a heterogeneous medical population had a lower risk of short-term mortality. The described risk factors for mortality may guide future studies assessing potential mortality benefits with thromboprophylaxis in specific subsets of medical patients and are hypothesis generating.

PA 2.10-4

A high risk of falls is associated with an increased risk of medically-relevant non-major bleeding in elderly patients receiving anticoagulants

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Background: Whether a high falls risk significantly increases the risk of bleeding in patients receiving anticoagulants is still a matter of debate.

Aims: We aimed to evaluate whether a high falls risk is associated with an increased risk of bleeding in elderly patients receiving anticoagulants for acute venous thromboembolism (VTE).

Methods: In a multicenter prospective Swiss cohort, we prospectively enrolled consecutive patients aged ≥ 65 years who received anticoagulants (low-molecular-weight heparin, fondaparinux, and/or vitamin K antagonists) for acute VTE between September 2009 and March 2012. We prospectively assessed the falls risk by asking patients two validated questions: (i) 'Did you fall during the last year?', (ii) 'Did you notice any problems with gait, balance or mobility?'. If at least one of these questions was answered positively, the falls risk was considered high. The primary outcome was the occurrence of a first major bleeding, defined as fatal bleeding, symptomatic bleeding in a critical site, or bleeding causing a fall in hemoglobin ≥ 20 g/L or leading to the transfusion ≥ 2 units of red blood cells. The secondary outcome was the occurrence of a first non-major bleeding requiring medical attention. To examine the association between falls risk and bleeding, we used a Cox proportional hazards model, adjusted for age, gender, history of major bleeding, anemia, thrombocytopenia, cardiac disease, diabetes mellitus, arterial hypertension, active cancer, chronic liver disease, chronic renal disease, overt pulmonary embolism, surgery in the last 3 months, polypharmacy, concomitant treatment with antiplatelet agents, and history of stroke or transient ischemic attack.

Results: Of 991 enrolled patients, 458 (46.2%) had a high risk of falls. The mean ± SD follow-up duration was 17 ± 10 months. The overall incidence rate of major and medically-relevant non-major bleeding was 7.9 events per 100 person-years and 12.0 events per 100 person-years, respectively. Patients with a high falls risk had a somewhat higher rate of major bleeding (9.6 vs. 6.6 per 100 patient-years; $P = 0.06$) and a significantly higher rate of medically-relevant non-major bleeding (16.7 vs. 8.3; $P < 0.001$) than patients with a low falls risk. After adjustment, a high falls risk was significantly associated with non-major bleeding (hazard ratio [HR] 1.67, 95% confidence interval [CI] 1.18–2.36; $P = 0.004$) but not with major bleeding (HR 1.18, 95% CI 0.78–1.77; $P = 0.43$).

Discussion: In this prospective cohort of elderly patients with acute VTE, patients at high falls risk had a significantly increased risk of medically relevant non-major bleeding. A high falls risk may be an argument against prolonging anticoagulation duration beyond 3 months in such patients.

PA 2.10-5

Reversal of the effects of new oral anticoagulants by administration of FEIBA

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Rivaroxaban and dabigatran, a direct factor Xa inhibitor and a direct thrombin inhibitor, respectively, are new oral anticoagulants (NOACs) used in a large number of patients to prevent thromboembolic disease following orthopedic surgery and non-valvular atrial fibrillation. In the event of a major bleed, however, there is no specific way to reverse their anticoagulant effect. The presented studies evaluated the potential of FEIBA, a licensed activated prothrombin complex concentrate, to reverse the anticoagulant effect of rivaroxaban or dabigatran.

Six male NZW rabbits per group received an i.v. dose of 25, 50, or 100 IU/kg FEIBA. Saline was used as a negative control item. Efficacy was defined as a correction of the animals' thrombelastogram (*R*-time) compared with saline-treated controls. Other coagulation variables were assessed for additional information.

After assessment of baseline coagulation parameters, animals received 1.2 mg/kg of the respective NOAC. Buffer was used as a negative control item to validate the model. Ten minutes later, coagulation was assessed followed by i.v. administration of test or reference item. Another 5 min and 1 day later, coagulation was assessed again.

Administration of NOACs led to an increased median *R*-time (from 21.5–35.5 to 67.3–120.0 min), aPTT, PT and reduced thrombin generation in all groups. Administration of saline showed no effect on coagulation parameters.

Treatment with 25, 50, or 100 U/kg FEIBA led to a dose-dependent correction of the prolonged *R*-time (28.6, 24.9 and 12.6 min with rivaroxaban; 46.5, 42.0 and 33.8 min with dabigatran). *R*-time on day 1 was similar to baseline values.

This procoagulant effect of FEIBA was also seen in other coagulation variables: Treatment with 25, 50 or 100 U/kg FEIBA led to a correction of the prolonged PT (9.15, 8.3 and 9.1 s with rivaroxaban; 9.90, 7.70 and 6.80 s with dabigatran) and dose-dependent increase in thrombin generation (56.0, 81.1 and 103.6 nM with rivaroxaban; 77.5, 167.5 and 406.7 nM with dabigatran) 5 min after administration. Intravenous administration of FEIBA also corrected aPTT after pretreatment with rivaroxaban (39.55, 38.80 and 40.40 s). After pretreatment with dabigatran, however, aPTT remained above baseline 5 min after treatment with FEIBA (56.40, 62.90 and 62.00 s).

In summary, the results of our studies show that FEIBA effectively and quickly corrects NOACs' anticoagulant effect, and thus may be a suitable antidote for bleeding complications after treatment with NOACs.

PA 2.10-6

Methodological problems in network meta-analysis of studies with the new oral anticoagulants

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Background: The efficacy and safety of the new oral anticoagulants (NOACs) dabigatran, rivaroxaban and apixaban have been demonstrated for prevention of venous thromboembolism following total knee- (TKR) and hip-replacement surgery (THR), and of ischemic stroke and systemic embolism in atrial fibrillation (AF). Direct comparisons of the NOACs on these indications are unlikely to be conducted in the near future. Patients and practioners may wish a scientific rationale for which NOAC to preferentially use on a specific indication. Network meta-analyses (NMA) are statistical tools feasible to compare indirectly the NOACs on their efficacy and safety outcomes. Several network NMAs have recently been presented. The statistical methods of the indi-

rect comparisons differ to some extend and there is a need for standardized procedures to document more precisely endpoints in the studies and to follow rules for conducting NMAs.
Aim: The aim of the present study is to identify the methodological differences of the NMAs performed with NOACs in THR/TKR surgery and in patients with AF and to relate their methods to the published guidelines for Preferred Reporting Items for Systematic reviews and Meta-aAnalyses (PRISMA) and for conducting NMAs published by the International Society for Pharmacoeconomics and Outcome rResearch (ISPOR).
Methods: The NMAs were subjected to the adherence to the PRISMA and ISPOR guidelines. NMAs were checked (i) for presentation of data on risk of bias of each trial regarding the definition and documentation of bleeding and thromboembolic events (PRISMA item 12 and 19, (ii) for handling of potential statistical biases, the on relative-effect estimates (odds ratio, relative risk, hazard ratio, differences in change from baseline), levels of significance and differences across studies that may violate similarities (ISPOR).
Results: PubMed and internet search strategies identified seven NMAs with NOACs for prevention of venous thromboembolism following TKR and THR and 10 NMAs for prevention of stroke and systemic embolism in patients with AF. As major differences between the NMAs were identified: (i) analyzed only efficacy and not safety endpoints, (ii) combined results of NOACs, (iii) combined results of control groups, (iv) analysed subgroups without access to original data, (v) not analysed differences in biographic trial-data, (vi) or determined hazard ratio without access to the original data (time of event relevant), (vii) not reported *P*-values, (viii) not reported the use of ISPOR criteria.
Conclusion: We conclude that further standardization of the definition and reporting of efficacy and safety endpoints in trials on anticoagulants are required as well as how to adopt PRISMA and ISPOR criteria in NMAs before drawing definite conclusions on which NOAC that is preferable in a given situation. Guidelines based on a wider matrix of scientific information than purely trial-data should be recommended until an international standard on all key levels of the anticoagulant drug development process has been agreed. Until such guidelines are available only results of NMAs should be recommended which used the rigorous methods published by PRISMA and ISPOR.

PA2.11 – Blood Coagulation Tests – VI

PA 2.11-1

Thrombin generation monitored in capillary blood with a calibrated automated thrombogram-based assay

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Background: The calibrated automated thrombogram (CAT) assay in plasma is a versatile tool to investigate patients with hypo- or hypercoagulable phenotypes. Recently we have published a thrombin generation (TG) method applicable to whole blood.
Aim: The aim of the current study was to develop a method capable of measuring thrombin generation from capillary blood (via finger-puncture) enabling near-patient testing.
Method: To study TG in capillary blood we have developed a miniaturized bedside device that consists of a microfluidic chip, handheld dual-channel fluorometer, and foil heater. The device is connected to a handheld computer. The device has been technically validated using citrated blood and finger prick blood from healthy volunteers and patients taking oral anti-coagulants. Thrombin generation was initiated with 5 pM tissue factor (TF). The read-out thrombogram parameters were endogenous thrombin potential (ETP), thrombin peak (TP), lagtime and time to peak.
Results: In the newly developed miniaturized device, repeated TG measurements ($n = 8$) with citrated blood from one healthy volunteer gave inter-assay coefficients of variation (CV) for ETP and TP of 7% and 11%, respectively (in whole blood CAT, CV of 12% for ETP and 11% for TP).
Applying this technique to samples from patients on warfarin treatment in the range INR 1–6 we found a good correlation between INR and ETP ($R^2 = -0.97$, $P < 0.01$) as well as between INR and TP ($R^2 = -0.91$, $P < 0.01$).
Validation with blood from a finger puncture (capillary blood) showed an inter-assay CV for ETP and TP of 5% and 14%.
Conclusions: We have developed a small portable device capable to measure with high precision TG in a single drop of (capillary) blood.

PA 2.11-2

Optical tracking of acoustic radiation force induced motion in plasma to estimate viscoelastic measurements of blood coagulation

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Background: Several viscoelastic measurements of blood coagulation have been used in clinical care to diagnose and guide the treatment of bleeding or thrombotic disorders. The current devices have been shown to improve patient outcome while reducing the use of blood products. Sonorheometry is a novel viscoelasticity measurement technique that assesses the relative stiffness of a sample by application of acoustic radiation force from ultrasound pulses. The returning echoes are analyzed to determine a relative measure of displacement of the material in response to the applied force. The algorithm used in sonorheometry depends on time delay estimation of the ultrasound pulse, an indirect measure of particle displacement, in order to make its viscoelastic measurements.
Aims: Our work investigates the viscoelastic measurement capabilities of direct optical.
Methods: of measuring particle displacement by acoustic radiation force in order to verify that sonorheometry measurements are indeed representative of changes in stiffness. We hypothesize that (i) optical tracking of bead movement due to acoustic radiation force can detect the viscoelastic changes that occur in the coagulation of plasma, and (ii) that the optical measure of viscoelasticity will provide comparable displacement measurements to those obtained with sonorheometry.
Methods: We utilized 15 μm polystyrene beads in plasma as a source of acoustic reflectors and targets for motion tracking. The bead motion in response to the applied ultrasound pulses was observed by microscopy and quantified using a particle tracking program implemented in Image J. The end point displacements of the bead after 1 s of ultrasound pulse sequence were plotted over the course of up to 40 min of sample coagulation to give a relative compliance curve.
Results: In this study, we show that plasma in which coagulation was initiated with kaolin developed significantly increased stiffness over the course of minutes. Bead displacement reduced from initial velocities of 30 μm/s to near zero 20 min after initiation of clotting. The relative compliance curves collected had the same shape as the curves produced by thrombelastography or sonorheometry. At 15 min after clot initiation, inhibition of $\alpha_{IIb}\beta_3$ by the Fab fragment abciximab reduced platelet rich plasma clot stiffness by 43% relative to control plasma. Bulk clot stiffness was reduced 48% relative to control for platelet rich plasma treated with cytochalasin D, an inhibitor of platelet cortical actin polymerization.
Conclusions: We observed a time dependent change in stiffness due to clotting based on tracking microparticles under the influence of acoustic radiation force. In addition we observed reduced stiffness of platelet-inhibited plasma as compared to control platelet rich plasma. In summary, motion of microparticles in response to the application of acoustic radiation force by ultrasound can be used to measure changes in viscoelasticity of the blood plasma.

PA 2.11-3

Six novel missense mutations causing factor X deficiency and application of thrombin generation test

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Background: Inherited factor X (FX) deficiency is a rare hemorrhagic condition characterized by a variable clinical presentation poorly correlating with laboratory phenotype and genotype. Thrombin generation test (TGT) is a newly developed global coagulation assay, which offers potential clinical advantages in the evaluation of hypocoagulable states, but its application in FX-deficient patients is rarely reported.
Aims: In the present study, we investigated the role of thrombin generation as a tool for assessing bleeding tendency in four unrelated pedigrees with FX deficiency.
Methods: Five FX assays were performed using clotting, chromogenic and immunological methods. FX gene defects were analyzed by direct sequencing. Thrombin generation was measured using a standard procedure in platelet-poor plasma of patients and normal control using 1 and 5 pM of tissue factor (TF), respectively.
Results: Seven missense mutations were identified in four probands, six of which (Ser425Pro, Ala-29Pro, Phe324Leu, Ala235Thr, Cys111Arg and Met362Thr) were novel and associated with type I FX deficiency. Arg347Cys was previously reported by us and accounted for type III FX deficiency (proband 3). Peak and Rate parameters measured at 1 pM TF were closely correlated with the clinical manifestations of the patients and the results revealed a higher sensitivity for FX deficiency at 1 pM TF compared with 5 pM TF.
Conclusion: TGT may serve as a useful laboratory tool to assess the individual clinical manifestation of patients with FX deficiency. Lower TF concentration (1 pM) is suggested to make the assay more sensitive to discriminate clinical severity of patients.

PA 2.11-4

Fibrinogen activity determined by rotational thromboelastometry is affected by high haematocrit

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Background: Whole blood viscoelastometry may help selection of appropriate coagulopathy treatments in settings such as cardiac surgery. However, viscoelastometry assay end-points are influenced by the red cell concentration in whole blood which may affect interpretation of test results.
Aim: To investigate the effect of high haematocrit (HCT) on whole blood clot formation and clot viscoelastic strength, by comparing ROTEM® thromboelastometry and plasma coagulation test results in patients with cyanotic congenital heart disease (CCHD) and in model high HCT blood.
Methods: Blood samples from adults with CCHD and stable HCT (> 0.55 L/L) were collected into tubes containing HCT-adjusted volumes of 0.105 M tri-sodium citrate during routine clinic phlebotomy.
Model *high* (0.44–0.62 L/L) and *very high* (0.63–0.75 L/L) HCT blood was generated by adding autologous packed red cells to blood samples from healthy controls with *normal* HCT (0.35–0.43 L/L). The HCT, platelet count, prothrombin time (PT), activated partial thromboplastin time (APTT) and Clauss fibrinogen (CF) were determined using standard techniques. The whole blood fibrinogen concentration (WBFC) was calculated using the formula WBFC = (1−HCT) × CF (g/L). The clot time (CT), clot formation time (CFT), α-angle and maximum clot firmness (MCF) were determined by ROTEM viscoelastometry using the EXTEM® and FIBTEM® reagents.
Results: Compared to normal HCT controls, high HCT blood samples from CCHD patients displayed prolonged EXTEM CT and CFT, reduced EXTEM α-angle and MCF, prolonged FIBTEM CT and reduced FIBTEM MCF. The platelet count and WBFC were reduced in the CCHD blood samples compared to controls. The PT, APTT and CF were similar to controls.
The model *high* and *very high* HCT blood samples displayed the same pattern of ROTEM changes as the CCHD patients, which were greatest in the *very high* HCT group (median EXTEM CT *normal* 50 s vs. *very high* 81 s; EXTEM CFT *normal* 75 s vs. *very high* 151 s; EXTEM α angle *normal* 75° vs. *very high* 62°; EXTEM MCF *normal* 64 mm vs. *very high* 52 mm; FIBTEM CT *normal* 47 s vs. *very high* 85 s; FIBTEM MCF *normal* 14 mm vs. *very high* 5 mm, all $P < 0.0001$). The WBFC was reduced in the model high HCT blood compared to controls (median WBFC *normal* 1.59 g/L vs. *very high* 0.85 g/L; $P < 0.0001$). There were no differences in PT, APTT or CF between model groups and controls. Across all model groups, the FIBTEM MCF correlated linearly with WBFC ($r^2 = 0.59$; $P < 0.0001$) but not with CF ($r^2 = 0.03$; $P = 0.18$).
Summary and Conclusions: We show for the first time using blood samples with HCT-adjusted anticoagulant volume, that high HCT in blood samples from CCHD patients and in model blood causes changes in ROTEM test results suggestive of hypocoagulability. Although the FIBTEM MCF predicts plasma fibrinogen concentration (CF) at normal HCT, this relationship is disrupted at high HCT. However, FIBTEM MCF remains correlated with WBFC irrespective of HCT suggesting that high HCT reduces FIBTEM MCF by a simple dilution effect of the increased red cell concentration. The influence of HCT should be considered carefully during interpretation of ROTEM results in clinical settings.

PA 2.11-5

Evaluation of the activated partial thromboplastin time assay for clinical monitoring of PEGylated recombinant factor VIII (BAY 94-9027) for hemophilia A

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Background: BAY 94-9027, a site-specific PEGylated factor VIII (FVIII) with a longer plasma half-life than native FVIII, is currently in clinical development for prophylactic treatment of hemophilia A. Clinical monitoring of FVIII:C activity in patients with hemophilia (PWH) is performed using the activated partial thromboplastin time (aPTT) assay.
Aim: It is highly important for diagnostic and patient care that aPTT results are interpreted correctly. Therefore, we evaluated the aPTT for clinical monitoring of BAY 94-9027.
Methods: BAY 94-9027 and the World Health Organization 8th International Standard for FVIII (WHO-8) were spiked into pooled and individual severe hemophilia A plasma samples at 1.0, 0.25, and 0.05 IU/mL. Five commercial aPTT reagents widely used in clinical laboratories were compared and evaluated for BAY 94-9027 activity in plasma from PWH using the ACL TOP and STAGO STA compact analyzers.
Results: BAY 94-9027 and WHO-8 bestow similar clot times (CTs) and excellent precision in both analyzers when ellagic acid aPTT reagents (SynthAFax, Dade Actin, and Cephascreen) are used. In contrast, BAY 94-9027 shows significantly prolonged CT and poor precision compared with WHO-8 using silica aPTT reagents (APTT-SP and STA-PTT5). Furthermore, free polyethylene glycol (PEG)-60 kD used for the conjugation of FVIII shows dose-dependent prolongation of CT in the APTT-SP assay. There was no effect on the SynthAFax-aPTT, the prothrombin time, or the activated factor XI-initiated

thrombin generation assay, demonstrating that the PEG moiety on FVIII has no general effect on coagulation cascade and most likely interferes with contact activation on the silica surface.
Conclusion: Ellagic acid aPTT reagents (SynthAFax, Dade Actin, Cephascreen) are most suitable for evaluating potency of BAY 94-9027 and should be used in clinical laboratories for monitoring FVIII activity after infusion of BAY 94-9027 to PWH.

PA 2.11-6
Investigation of the use of a proteomic approach to detect intravascular coagulation

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Background: At present, intravascular coagulation is diagnosed by measuring the level of activation markers such as Prothrombin fragments F1 + 2, thrombin-antithrombin complex and D-dimer. However in other areas of medicine (e.g. cancer), the use of proteomic fingerprinting is under development since it can be used to characterise the underlying mechanism driving the disease, to detect disease at the early stages and to determine therapeutic efficacy, as part of the movement towards personalized medicine.
Aim: To compare the proteomic fingerprint of plasma to that of serum to determine the potential of proteomic fingerprinting for the diagnosis of intravascular coagulation.
Method: Forty-five microlitre of citrated NHP at 37 °C was mixed with 5 μL of diluted tissue thromboplastin (IL)/$CaCl_2$ 100 mM mixture. At 0, 4 and 180 min following recalcification, reactions were terminated by the addition of 950 μL of 0.1% trifluoroacetic acid (TFA). After removal of the clot, the sample was centrifuged at 13,000 r.p.m. for 2 min. Peptides were extracted from the sample by the addition of 80 μL of RPC-18 beads (Dynabeads) to 200 μL of the TFA treated sample. After 5 min incubation, the beads were washed and extracted with 10% acetonitrile (AcN). The AcN extract was pipetted onto the surface of a Bio Rad Protein Chip NP-20 array, the chip was allowed to dry and Sinapinic acid according to the manufacturer's instructions. Chips were read using a Ciphergen Protein Biological Systems II Ca ProteinChip reader (Bio-Rad). The detector was optimized in the range of 1–8 kDa and mass spectrometry profiles were generated at three different laser intensities. Peak detection was performed using ProteinChip Biomarker Wizard software version 3.1 and the intensity of each compared between each of the three groups (NHP and 4 and 180 min after initiation of clotting, respectively) using Biomarker Wizard.
Results: Around 70 separate peaks of mass between 1022 (M/Z) to 7154 (M/Z) were detected in the AcN extracts. From the profile of the change in intensity over the time course of clotting, the peaks could be divided into four groups. The majority of the peaks were in Group 1 in which the intensity of the peak remained unchanged from plasma apart from a slight fall at 180 min. Group 2 contained seven peaks which increased abruptly and significantly ($P < 0.02$) at 4 min but then dropped at 180 min (e.g FpA which decays to des Ala FpA). Three peaks were in Group 3 which showed a progressive decrease at 4 and 180 min. Group 4 contained 20 peaks which exhibited an increase from low levels at $t = 0$ and 4 min to high levels at 180 min.
Conclusion: The large number of peptides (in the range of 1189–4403 M/Z) which have been shown to change in level during the clotting of plasma suggests that this approach can be used to develop proteomic fingerprints for the diagnosis of intravascular coagulation using statistical approaches such as decision tree analysis or neural networks.

PA2.12 – Coagulation Factor VIII – III

PA 2.12-1
Enhancing expression of coagulation factor VIII through bioengineering: strategic introduction of Asn-linked glycan improves secretion

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Coagulation factor VIII (FVIII) is an essential cofactor of the intrinsic blood coagulation pathway and its deficiency results in hemophilia A. Commercial production of recombinant FVIII (rFVIII) in heterologous expression systems is 2–3 orders of magnitude lower than similarly sized proteins owing to multiple factors. FVIII is a complex glycoprotein containing upto 25 asparagine (N)-linked glycans and deletion of the entire B domain, that contains the bulk of these oligosaccharide structures, does not affect either expression or its procoagulant activity. Our preliminary results from site-directed mutagenesis of the non-B domain N-linked glycans indicated that N-239, present in the A1 domain, was the most crucial and N239Q reduced secretion by 35–50%. N-239, positioned at the A1–A2 interface, is situated just upstream of the putative BiP-binding site. BiP, a peptide dependent ATPase is known to interact with FVIII and retain unfolded protein within the endoplasmic reticulum. We therefore hypothesized that additional N-linked glycans in the vicinity of this region could improve efficiency of protein folding and improve secretion. Potential N-linked glycosylation sequons (K213N, L216S, A222N, M231N, and Y237S) were introduced into the A1 domain of full length wild type (WT), B domain deleted (BDD) and 226/N6 (bioengineered FVIII variant with enhanced secretion efficiency) FVIII backbones, through site-directed mutagenesis techniques. Transient transfections in COS-1, CHO and HEK-293t cells showed a 2-fold higher expression of K213N and L216S mutants across all three FVIII variant backbones. While the expression of A222N mutant was not significantly different from that of native FVIII, the mutants M231N and Y237S were poorly secreted. A similar increment in expression was observed for the K213N and L216S mutants over BDD (2.4 vs. 1.1 U/mL) and 226/N6 (11.6 vs. 5.4 U/mL) following hydrodynamic tail vein injections into $F8^{-/-}$ hemophilia A mice. An additional twofold enhancement in expression was observed upon incorporation of F309S, an A1 domain mutation which improves secretion possibly by diminishing FVIII association with BiP, into the 226/N6-L216S variant. Confirmation of the presence of an additional N-linked glycan structure in the L216S mutant through mass spectrometric analysis is currently underway. Previous studies have shown that porcine BDD-FVIII is secreted more efficiently than human BDD-FVIII. While the precise mechanism is still unclear, it has been suggested that structural variations between the A1, ap (activation peptide) and A3 domains of human and porcine FVIII might contribute to this secretion differential. Sequence alignment analysis revealed that porcine FVIII contains a potential glycosylation site at N-213 that is not conserved in human FVIII and is analogous to the K213N and L216S mutants engineered in this study. This additional N-linked glycan in the porcine A1 domain could partly account for the enhanced expression levels observed for recombinant porcine BDD-FVIII in cell culture systems. Also this region of the A1 domain was observed to be the most divergent between human and porcine BDD-FVIII with respect to the primary sequence.
Such targeted bioengineering strategies may facilitate more efficient secretion of rFVIII thereby enhancing commercial production for lower cost factor replacement therapy.

PA 2.12-2

Functional consequences and founder effect of a recurrent mutation of F8 (c.6046C>T, p.R2016W) in patients with hemophilia A from Northern Italy

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Background: Hemophilia A, the most common hereditary bleeding disorder, is caused by mutations in coagulation Factor VIII gene (*F8*). Knowledge on recurrent *F8* mutations may facilitate screening and genetic diagnosis in patients from specific geographic areas. By screening more than 300 haemophilia A patients from Northern Italy who referred to the Angelo Bianchi Bonomi Hemophilia and Thrombosis Center (Milan, Italy), we identified a recurrent mutation occurring in exon 19 of *F8*.

Aims: To characterize the functional effect of the mutation and determine the existence of a founder effect for the c.6046C>T (p.R2016W) mutation in exon 19 of *F8*.

Methods: Mutations of *F8* were searched for by PCR and Sanger sequencing in 335 patients with hemophilia A referred to the Angelo Bianchi Bonomi Hemophilia and Thrombosis Center (Milan, Italy). The functional effects of the c.6046C>T (p.R2016W) mutation were assessed by *in silico* annotation on *F8* gene models (URL:http://conseq.bioinfo; URL: http://bioinf.cs.ucl.uk/psipred) as well as on Polyphen 2 and SIFT prediction software (URL: http://genetics.bwh.harvard.edu/pph2/; http://sift.jcvi.org/). Analysis of *F8* mRNA was performed by reverse transcription (RT)-PCR and subsequent Sanger sequencing. Haplotype analysis using three intragenic (STR13, STR22 and STR24) and three extragenic (DXS1108, DXS1073, and DXS7423) polymorphic microsatellites markers was employed to investigate a potential founder effect. DMLE+2.3 software was used to estimate mutation age using linked markers.

Results: A c.6046C>T (p.R2016W) mutation occurring in exon 19 of *F8* was identified in 35 of 335 haemophilia A patients from Northern Italy (prevalence 10.4%; 95% confidence intervals 7.6–14.2%). The mutation was the second most frequent in our cohort, after the intron 22 inversion. Patients carrying the c.6046C>T (p.R2016W) mutation had moderate to severe clinical phenotype. *In silico* annotation predicts that the substitution of an Arginine by a Tryptophan introduces a polar residue within the A3 domain of FVIII, which may alter the conformation of the protein. Polyphen 2 and SIFT software predicted the mutation to be potentially deleterious for FVIII function. RT-PCR and sequencing of the *F8* mRNA region spanning exons 17–22 clearly showed that the C>T substitution also induces an impairment of the splicing process causing the skipping of exon 19. Characterization of splicing patterns of *F8* in patients' leukocytes identified both in- and out-of-frame *F8* transcripts. Haplotype analysis found a peculiar haplotype (H1) in 21/35 (60%) haemophilia A patients with the recurrent mutation. The haplotype was not identified in 52 Italians without hemophilia (Fisher's exact test, $P < 0.0001$). These data strongly suggest a founder effect, supporting the existence of a single mutation event. DMLE+ software estimated that c.6046C>T (p.R2016W) mutation likely arose approximately 300 years ago.

Conclusions: We identified a mutation of *F8* that is highly prevalent among haemophilia A patients from Northern Italy. The variant, which likely arose from a single mutation event occurring approximately three centuries ago, acts by the dual mechanism of affecting the protein sequence of FVIII and inducing abnormal splicing of *F8*.

PA 2.12-3

Characterization of missense mutations in the B domain of coagulation factor VIII reported to be associated with mild/moderate hemophilia A

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Background and Significance: Factor VIII (FVIII) serves an essential role as a co-factor in the intrinsic coagulation pathway and its deficiency leads to hemophilia A. FVIII is a complex glycoprotein with the domain structure A1-a1-A2-a2-B-ap-A3-C1-C2. The B domain is dispensable for procoagulant activity and is not involved in von Willebrand factor affinity or phospholipid binding. However, several missense mutations within the B domain have been reported in patients with hemophilia A of varying clinical severity. We have demonstrated earlier that missense mutations within the B domain are unlikely to cause severe hemophilia A (Ogata, J Thromb Haemost 2011). This study focuses on missense mutations in the B domain reported in patients with mild/moderate hemophilia A. A total of 17 such missense mutations were identified from the HAMSTeRS and CHAMP hemophilia mutation databases and current literature. These include T751S, P851R, D944N, E1038K, M1074I, N1091Y, S1095S, P1134A, F1175V, R1260K, T1295P, R1310H, Q1317K, L1450S, S1511T, E1623G and N1639H. Four of these were unpublished submissions to the HAMSTeRS database. While T1295P and M1074I were reported in female patients, R1260K was found in conjunction with the well known polymorphism D1241E in four patients of the same family. Interestingly, S1095S, a synonymous substitution has also been reported to be associated with mild hemophilia. M1074I and Q1317K were excluded from the study since they were secondary mutations found in addition to causative lesions.

Methods: *In silico* analysis using online tools predicted that while the majority of these mutations were considered benign, P851R and E1623G were considered likely to affect protein function. The B domain contains several Asparagine (Asn)-linked glycans that might influence FVIII expression and intracellular transport, but none of the missense mutations reported so far have been shown or predicted to interfere directly with the Asn-linked glycosylation process. Plasmid vectors containing the B-domain missense mutations were generated using site-directed mutagenesis techniques and were analyzed through transient transfections into COS-1, CHO and HEK-293t cell lines. FVIII activity and antigen levels were assayed by aPTT and ELISA respectively and compared to FVIII-wild type (WT). Previously well-defined mutations, R2150C, R593C and C179G were utilized as controls for mild, moderate and severe clinical phenotypes respectively.

Results: FVIII activity and antigen levels of all the mutants studied were similar to that of FVIII-WT levels in all three cell lines indicating that these B domain missense mutations did not significantly impact FVIII expression or activity.

Conclusion: In accordance with our previous observations for FVIII B domain missense mutations associated with severe hemophilia, our findings in the present study indicate that missense mutations reported to be associated with mild/moderate hemophilia also do not have an impact on expression or activity of FVIII. It is therefore unlikely that any of these mutations could be causative of hemophilia A. These reported amino acid changes are more likely to represent polymorphisms or non-pathological mutations. This will be important to recognize as genotyping gains momentum globally through new research initiatives, particularly as it pertains to genetic counseling and genotype/phenotype correlations.

PA 2.12-4

In vivo and hepatocellular distribution of FVIII in rats is independent of high affinity binding to VWF

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Background: FVIII is predominantly cleared in the liver. In circulation, the majority of FVIII is bound to VWF. Tyrosine sulfation of FVIII-Y1680 is required for high affinity VWF binding[1] and FVIII-Y1680F lacking high affinity VWF binding displays a shorter half-life *in vivo*. Currently, it is not known if high affinity VWF binding influences the clearance pathways of FVIII.

Aims: To determine the *in vivo* anatomical and hepatocellular accumulation of wild type FVIII and FVIII-Y1680F in rats, in order to investigate the contribution of high affinity VWF binding in FVIII clearance.

Methods: Anatomical distribution of a biostable radio-iodinated human recombinant FVIII and FVIII-Y1680F was assessed following i.v. tail injection in rats. At time points up to 24 h for native FVIII and 1 h for FVIII-Y1680F plasma samples were collected, blood removed by systemic perfusion with saline, and organs harvested. Radioactivity in plasma and organs was counted. At time points up to 7 h, cells were harvested from the liver by collagenase perfusion followed by isopycnic density gradient centrifugation in Percoll®. Hepatocellular distribution was assessed by comparing amount of radioactivity per million cells in parenchymal (PCs), Kupffer (KCs) and sinusoidal endothelial cells (LSECs). The uptake per total cell population was calculated according to the ratio between KCs, LSECs and PCs in rat livers of 1:2.5:7.7.[2]

Results: More than 94% of radioactivity remained bound to FVIII and FVIII-Y1680F for the duration of the study and < 1% of radioactivity was found in the thyroid demonstrating sufficient stability of the iodine-labeled FVIII proteins to analyze not only the initial distribution of FVIII, but also accumulation in organs for up to 2–3 half-lives. As expected, the clearance of FVIII-Y1680F was significantly faster than for FVIII but the relative distribution between organs was the same for both FVIII molecules with the liver being the organ primarily responsible for clearance. Radioactivity was observed in the intestines after 1 h, in concordance with FVIII degradation via the liver. In hepatocellular studies, both FVIII molecules were predominantly taken up by PCs with approximately 64 ± 5% of total radioactivity, while 34 ± 4% radioactivity was found in LSECs. Calculations of radioactivity per cell showed that LSEC had slightly higher capacity than PC to take up FVIII or FVIII-Y1680F. Minimal radioactivity was seen in KC for both molecules. The relative pattern of cellular distribution did not change over time (between 1–7 h for FVIII or 0.5–2 h for FVIII-Y1680F).

Conclusion: In rats, the liver is the predominant organ showing accumulation of FVIII and FVIII-Y1680F. Hepatocellular distribution was also similar for both FVIII molecules with PC and LSEC having a major role. Thus, the lack of high affinity VWF binding does not alter the distribution of FVIII.

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PA 2.12-5

A combinatorial library approach to generate rFVIII variants with multiple XTEN insertions and improved pharmacokinetic properties

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Background: XTEN is an unstructured polypeptide that has been shown to increase the circulating half-lives of a number of proteins. The impact of XTEN on the clearance and function of rFVIII molecules can be optimized by varying the location, composition, length and number of XTEN insertions, all of which can be achieved by recombinant technology. With the identification of locations in rFVIII that can accommodate intra-domain insertions of XTEN, a multivariate approach towards XTEN modification of FVIII was explored to develop novel long-lasting rFVIII-XTEN variants.

Aim: To evaluate the effects of multiple XTEN insertions on the activity and pharmacokinetics of rFVIII.

Methods: rFVIII-XTEN combinatorial libraries were constructed comprising over 400 rBDD-FVIII variants with 2–6 XTEN insertions within the A domains, at the B domain junction, and at the C-terminus. Variants were expressed in HEK293 cells by small-scale transient transfection, and FVIII activity in conditioned medium was measured by FVIII chromogenic assay. The pharmacokinetic (PK) properties of variants with ≥ 0.3 IU/mL FVIII activity in culture medium were evaluated in FVIII knockout (HemA) and FVIII/VWF double-knockout (DKO) mice by monitoring plasma FVIII activity over time. DKO mice were used for initial ranking purposes to eliminate the influence of endogenous VWF on half-life. Concentrated conditioned medium or partially purified rFVIII-XTEN preparations were used in PK studies to increase the throughput of PK screening, since the PK profile obtained is comparable to those of the purified proteins.

Results: Based on the FVIII chromogenic activity, the rFVIII variants remained functional with up to five XTEN insertions. In DKO mice, which lack the protective benefit of VWF, the half-lifeimprovement conferred by XTEN was insertion site-dependent, with single XTEN insertions in the A1, A2 and A3 domain extended half-live up to 3.5, 2.5, and 4.5 h, respectively, as compared to 0.25 h for unmodified rBDD-FVIII. The optimal composition and length of XTENs for activity and half-life at each intra-domain insertion site is site-dependent, and the effects on PK of multiple XTEN insertions were generally additive when insertion sites were in different domains. rFVIII with three XTEN insertions achieved a half-lifeof 16 h in DKO mice, representing a 64-fold increase relative to rBDD-FVIII, but the introduction of additional XTENs resulted in only a nominal increase to 18 h, indicating that half-life extension with XTENs is additive but saturable. Selected rFVIII-XTEN variants that had exhibited half-lives of 3–18 h in DKO mice all had similar half-lives in HemA mice (approximately 14 h).

Conclusions: Multiple intra-domain XTEN insertions are tolerated by rFVIII and provide greater protection for rFVIII compared to the endogenous VWF multimers. The normalizing effect of VWF on the half-lives of these variants observed in HemA mice highlights a limit on half-life extension imposed by VWF. Protein engineering of a rFVIII-VWF fusion protein for which the clearance is independent from endogenous VWF will be necessary to exceed this limit on half-life extension by the modification of XTEN.

PA 2.12-6

B domain-deleted FVIII lacking mannose-ending glycans at Asn239 and Asn2118 retains pro-coagulant activity and corrects bleeding in FVIII-deficient mice

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Background: Hemophilia A (HA) is a rare inherited bleeding disorder due to the absence of the coagulation protein factor VIII (FVIII). Administration of therapeutic FVIII to severe hemophilia A patients induces, in up to 30% of the cases, inhibitory anti-FVIII antibodies. Previously, we have shown that blockage of mannose receptor on human monocytes-derived dendritic cells reduces FVIII uptake and presentation to a human FVIII-specific T-cell line. The data suggested that a recombinant FVIII lacking mannose-ending would be less immunogenic.
Aim: The aim of this study was to validate in a mouse model of hemophilia A the production and procoagulant activity of recombinant human FVIII lacking oligomannose carbohydrates at Asn239 and Asn2118.
Methods: FVIII-deficient mice were injected with 100 μg of pLIVE encoding wild-type (WT) human B domain-deleted FVIII (Δ2-FVIII, LFB, Les Ulis), and the corresponding single Asn239Gln mutant, single Asn2118Gln mutant or double Asn239Gln/Asn2118Gln mutant (DM). FVIII:Ag, FVIII:C and hemostatic function were assessed 4 days after hydrodynamic injection using Asserachrom, functional coagulation assay and tail vein clip assay, respectively.
Results: Our results show reduced levels of circulating demannosylated FVIII compared to WT FVIII in hydrodynamic injection-treated mice. Indeed, the expression of Δ2-FVIII mutated at position Asn2118Gln was slightly reduced compared to that of WT (FVIII:Ag 3.61 ± 1.11 IU/mL and 1.60 ± 1.53, respectively), whereas the expression levels of the Asn239Gln and DM FVIII mutants were significantly decreased (FVIII:Ag 0.25 ± 0.05 and 0.63 ± 0.28 IU/mL). Similar observations were made when FVIII:C was measured in plasma. Interestingly, the specific activity of the different mutants did not drastically differ from that of the WT Δ2-FVIII: 1.02 ± 0.1, 2.09 ± 0.54, 1.4 ± 0.4, 1.66 ± 0.44 IU/mL for WT Δ2-FVIII, and the Asn239Gln, Asn2118Gln, and DM, respectively.
In agreement with the latter findings, the amount of blood loss by hemophilia A mice expressing the DM Δ2-FVIII was significantly lower following tail clipping than that loss by untreated hemophilic mice (226 ± 94 vs. 63 ± 56 μL, respectively, $P = 0.013$), and was not statistically different from the blood loss by wild-type mice.
Conclusion: The absence of mannose-ending sugars at Arg239 and/or Arg2118 does not alter the specific pro-coagulant activity of FVIII. It however affects the levels of circulating FVIII, probably by reducing the secretion yield by hepatocytes. Mutation at Arg239 was found to affect FVIII secretion the most. Importantly, the double mutant corrects bleeding in an acute model of hemorrhage.

PA2.13 – Factor II/Prothrombin – I

PA 2.13-1

Structure-function analysis of prothrombin-D519E, a causative mutation in a type 1/2 prothrombin deficient patient with a severe bleeding tendency

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Background: Recently we have described two mutations in the prothrombin gene of a young boy of Caucasian descent from non-consanguineous parents who presented with a serious bleeding disorder as a neonate. Functional analysis showed that the patient had a prothrombin activity below the detection level (< 1%). Genetic analysis revealed the presence of two mutations: one introducing a premature STOP codon at position Arg296, the other coding for a Asp519Glu missense mutation, together giving rise to a compound heterozygous prothrombin deficiency phenotype.
Since complete prothrombin deficiency results in embryonic and neonatal lethality in mice, and furthermore given that amino acid 519 in prothrombin is crucial to enzyme catalysis by thrombin, being located at the base of the catalytic pocket, we hypothesized that the relative conservative mutation of Asp into Glu has prevented embryonic lethality, yet causes a severe bleeding tendency.
Aims: We performed a detailed structure-function analysis of the human prothrombin Asp519Glu mutant molecule.
Methods: Human wild-type prothrombin, PT-Asp519Glu and PT-Asp519Ala were stably expressed in HEK293 cells. Prothrombin was subsequently purified from conditioned serum-free medium to homogeneity. Activation of prothrombin was achieved with the prothrombin activator from *Oxyuranus Scutellatus*, after which thrombin was purified. Wet laboratory experiments were supported by *in silico* structural bioinformatics. Methods involving 3D structure analyses of (pro) thrombin and its *in silico* prepared variants, molecular dynamics simulations and docking studies.
Protein analysis was performed via SDS-PAGE analysis and spectrophotometry. Prothrombin variants were tested for their ability to function as substrates in the prothrombinase complex both in reaction systems composed of purified components and in plasma. Membrane binding was assessed via surface plasmon resonance. Thrombin variants were analysed by quantitation of their amidolytic activities towards the chromogenic substrate S2238, and for their ability to activate physiological thrombin substrates such as FV, fibrinogen and platelets.
Results: *In silico* analysis predicted no major folding effects of mutation of Asp519 to either Glu or Ala. Analysis of substrates docked into the S1 site for each of the thrombin variants indicates that the Asp519Glu mutation clearly distorts the interaction between the guanidium group of the P1 Arg residue and residue 519, while the Asp519GluAla mutation results in a complete loss of essential interactions between enzyme and substrate.
Protein expression typically yielded 4–8 mg of purified prothrombin (> 95 purity as judged by SDS-PAGE analysis). Electrophoretic mobility of all prothrombin variants was identical as was their ability to bind to phospholipid membranes. All prothrombin variants were converted to thrombin by the snake venom activator. However, the amidolytic activities of purified wt thrombin and Asp519Glu and Asp519Ala thrombin were 100; 1.3% and 0.1%, respectively. The low but detectable activity of the Asp519Glu mutant as compared to the Asp519Ala mutant was also observed with other thrombin substrates (FV, fibrinogen and platelets).
Summary: We have shown that the Asp519Glu mutation in human prothrombin results in a serious bleeding phenotype and we hypothesize that the conservative Asp®Glu mutation allows for this mutation at position 519 to be compatible with life.

PA 2.13-2

Identifying novel thrombin interactions: alpha-synuclein binds to thrombin exosites and inhibits thrombin-mediated platelet aggregation

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Background: Alpha-synuclein is involved in the pathogenesis of Parkinson's disease (PD). It is expressed in the presynaptic neuron terminals and constitutes the major component of protein deposits in PD. Alpha-synuclein is essentially unfolded in solution and assumes a partially helical conformation when bound to phospholipid vesicles. The protein consists of three distinct regions: a positively charged N-terminal region (amino acids 1–60), a hydrophobic region encompassing the highly aggregation-prone Non-Amyloid sequence (65–90) and the C-terminal 103–140 region, rich in acidic amino acids and prolines. Alpha-synuclein is also expressed in T and B lymphocytes and platelets, where it is associated with the membrane of secretory alpha-granules. Thrombin, the final effector protease in the coagulation cascade, is the most potent activator of platelets and this function is exerted through cleavage of type-1 Protease Activated Receptor (PAR1), which results in alpha-granule release and platelet aggregation. Conversely, platelet degranulation is inhibited by alpha-synuclein (Park et al. 2002). Moreover, clinical evidences show that patients with Parkinson's disease are less susceptible to ischemic stroke and exhibit a significantly reduced tendency to aggregate platelets (Sharma et al. 1991).
Aims: Establish whether or not alpha-synuclein interacts with thrombin; study the effect of alpha-synuclein on thrombin procoagulant (fibrin generation and platelet aggregation) and anticoagulant (generation of active protein C) functions.
Methods: Wild-type and His-tag full-length alpha-synuclein were expressed in *E. coli* and purified. Thrombin mutants Arg73Aa and Arg101Ala were expressed in *E. coli*. Binding of alpha-synuclein to thrombin was studied by measuring the change of intrinsic fluorescence of thrombin and by Surface Plasmon Resonance on a Biacore X100 instrument. The effect of alpha-synuclein on fibrin generation was investigated by turbidimetric measurements, while the effect on platelet aggregation was evaluated on whole blood by Multiple Electrode Aggregometry (MEA). PC activation by thrombin was monitored by measuring the rate of S2366 hydrolysis.
Results: Fluorescence binding measurements yielded K_d = 2.6 ± 0.5 mM. Similar results were obtained after Biacore analysis (K_d = 4.2 ± 1.0 mM). Displacement experiments, carried out with specific binders of thrombin exosite-1 (hirugen and HD1 aptamer) or exosite-2 (fibrinogen g"- peptide and HD22 aptamer) and with thrombin mutants having exosite-1 (Arg73Ala) or exosite-2 (Arg101Ala) compromised, suggest that both exosites of the protease are involved in alpha-synuclein binding. Conversely, alpha-synuclein does not affect the affinity of the enzyme for some active-site inhibitors, like p-aminobenzamidine and the N-terminal domain 1–47 of hirudin, nor the efficiency of S2238 hydrolysis. Likewise, alpha-synuclein does not affect fibrin generation by thrombin and very preliminary results indicate that it is also ineffective on the thrombin-mediated PC activation. Strikingly, MEA measurements indicate that alpha-synuclein inhibits platelet aggregation by thrombin in a dose-dependent manner, whereas it only slightly reduces ADP-induced platelet aggregation.
Conclusion: alpha-synuclein binds to thrombin at both exosites, while leaving the protease active site accessible. These results rationalise previous work on the inhibitory effect of alpha-synuclein on thrombin-mediated platelet activation suggesting that alpha-synuclein impairs localization of thrombin on platelets by competing with the receptor GpIb-alpha for thrombin exosite-2. Our data also explain clinical evidences showing that patients with Parkinson's disease display lower incidence of thrombotic events.

PA 2.13-3

Amino acid sequence 473–487 of human prothrombin is required for timely activation by prothrombinase and optimal thrombin activity

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Background: Prothrombinase is the important enzymatic complex that catalyzes the proteolytic conversion of prothrombin (Pro) to thrombin with rates compatible with survival. Prothrombinase is composed of the enzyme factor Xa (fXa), and the cofactor factor Va (fVa) associated on a procoagulant membrane surface in the presence of divalent metal ions. Timely prothrombinase formation results in the suitable formation of a fibrin clot and the arrest of bleeding because the interaction of fVa with the members of prothrombinase leads to a fivefold increase in the catalytic efficiency of fXa as compared to the activity of fXa alone. Prothrombinase produces thrombin following two sequential cleavages of Pro (at Arg^{320} followed by cleavage at Arg^{271}) with formation of an enzymatically active intermediate called meizothrombin. Although membrane-bound fXa is capable of activating Pro, the overall rate of thrombin formation is not compatible with survival, and activation proceeds through the opposite pathway (cleavage at Arg^{271} is followed by cleavage at Arg^{320}) with formation of prethrombin-2. fXa is known to exhibit a strong interaction with Pro in the presence and absence of fVa. Previous studies have suggested that fXa interacts with Pro within amino acid region 473–487 in a fVa-dependent manner.
Aim: To investigate the functional importance of amino acid region 473–487 of Pro.
Methods: We used site-directed mutagenesis to create a recombinant Pro molecule with the region 473–487 deleted ($rPro^{\Delta 473–487}$). Wild type Pro ($rPro^{WT}$) and the deletion mutant were stably transfected in BHK-21 cells. The two recombinant molecules were purified according to an already established protocol. During the last step of the purification procedure, we used Mono-Q chromatography and a calcium gradient to isolate properly carboxylated $rPro^{\Delta 473–487}$ and $rPro^{WT}$. Both recombinant molecules were assessed by gel electrophoresis for their capability to produce active thrombin by fully assembled prothrombinase or fXa alone. The recombinant molecules were also assayed for clotting and chromogenic activity.
Results: Gel electrophoresis revealed that consumption of $rPro^{\Delta 473–487}$ by prothrombinase and thrombin formation was drastically impeded when compared with thrombin formation following cleavage of $rPro^{WT}$ by prothrombinase. In contrast, membrane-bound fXa alone, in the absence of fVa, displayed a marked increase in the rate of cleavage at Arg^{271} and activation of $rPro^{\Delta 473–487}$ as compared to cleavage at Arg^{271} and activation of $rPro^{WT}$. A similar cleavage pattern within the recombinant proteins was observed, implying that no structural alterations took place in $rPro^{\Delta 473–487}$ following the mutation. Additionally, while clotting assays revealed that $rPro^{WT}$ had clotting and chromogenic activities comparable to human plasma-derived Pro, $rPro^{\Delta 473–487}$ was devoid of clotting activity and severely impaired in its amidolytic activity.
Conclusion: Our data demonstrate that amino acid sequence 473–487 of Pro is required for optimal rates of activation by prothrombinase. In addition, our data suggest that amino acid region 473–487 is required for proper thrombin activity. These data are providing a rationale to the fact that to date there is no natural mutation within this region of Pro reported since any changes within this crucial amino acid region would lead to dire circumstances and are not compatible with survival.

PA 2.13-4

A novel prothrombin c.1787G>A mutation in Serbian family with recurrent thromboembolism- another case of antithrombin resistance

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Background: Family thrombophilia is considered a multifactorial and polygenic disorder in which gene-environment and gene-gene interactions play a pivotal role. The most frequent thrombophilic genetic risk factors are FV Leiden and FII G20210A mutations. The FII G20210A (c.*97G>A) variant in the 3′-untranslated region of the prothrombin gene is associated with elevated plasma prothrombin level and increased burst in thrombin generation. Recently, new mutation (c.1787G>A) affecting the prothrombin Arg596 residue, has been identified in a Japanese family with juvenile thrombosis. This Arg596Leu substitution leads to impaired inhibition of mutant thrombin by antithrombin, resulting in antithrombin resistance and increased thrombophila risk.

Aims: Here we report a novel prothrombin mutation detected in three sisters, suffering from a severe recurrent thrombosis from a young age.

Methods: Contrary to our expectations, the routine thrombophilia testing did not show any inherited risk factors. Additional genetic analysis revealed the presence of a new FII c.1787G>A mutation in heterozygous form in all three sisters.

Results: The mutation is located in the last exon of the prothrombin gene resulting in Arg596Gln replacement. Residue Arg596 is positioned within the region that contains one of the antithrombin-binding sites involved in thrombin inactivation by antithrombin. Considering the clinical findings and *in silico* protein structure prediction, we assume the same thrombophilic mechanism for the herein presented Arg596Gln as in previously described Arg596Leu.

Conclusion: Our study reports the first data for Caucasian population supporting the newly proposed mechanism of hereditary thrombophilia called antithrombin resistance.

PA 2.13-5

Effects of prothrombin Yukuhashi mutation on thrombomodulin-protein C system

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Background: We have reported antithrombin (AT) resistance in the prothrombin Yukuhashi substituted arginine for leucine at position 596 (p.Arg596Leu). The mutant thrombin, an active form of the prothrombin Yukuhashi, showed moderately lower clotting activity than that of wild-type prothrombin, while substantially impaired complex formation with AT. However, effects of the mutation on thrombomodulin (TM)-protein C (PC) pathway was not yet analyzed.

Aims: We evaluated effects of the prothrombin Yukuhashi mutation on TM-PC anticoagulation system.

Methods: We prepared expression vectors of wild type and mutant (p.Arg596Leu) prothrombins using pcDNA 3.1(+), and transfected them to HEK293 cells. We established respective stable transformants by G418 selection, and obtained clones highly expressing recombinant prothrombins. We cultured stable transformants highly expressing recombinant prothrombins in serum-free medium containing vitamin K, and collected and concentrated their conditioned media. Antigen levels of prothrombins were determined by an enzyme-linked immunosorbent assay (ELISA). We used *Oxyuranus scutellatus* venom (Ox), a factor Xa-like enzyme, as a prothrombin-to-thrombin converting enzyme together with cephalin and calcium chloride. We measured clotting time for Ox-activated recombinant thrombins using fibrinogen as a substrate in the presence or absence of recombinant TM (rTM). We determined relative fibrinogen clotting activities using a standard curve of clotting time of normal pool plasma. We also used a chromogenic substrate (S-2238) to evaluate thrombin activity. We measured APC generation activity with or without rTM for Ox-activated recombinant thrombins using S-2366 as an APC substrate in the presence of Pefabloc-TH. We expressed APC generation activity as changes in absorbance/min (delta A/min) at 405 nm

Results: In the fibrinogen clotting time assay, relative activity of the mutant thrombin was 21.8% of that of the wild type in the absence of TM. TM treatment reduced relative fibrinogen clotting activity of the wild type thrombin to 34.3%, whereas that of the mutant to 20.4%. Reduction rates of fibrinogen clotting activity were 65.7% for the wild type thrombin and 6.4% for the mutant, respectively. In the chromogenic assay using S-2238, relative activity of the mutant thrombin was 47.2% of that of the wild type in the absence of TM. In the absence of TM, APC generation activity (delta A/min at 405 nm) were fairly low; 0.0089 for the wild type and 0.0048 for the mutant. TM treatment enhanced APC generation activity to 0.13145 (14.8 folds) for the wild type and to 0.05735 (11.9 folds) for the mutant respectively, although relative APC generation activity of the mutant was 43.6% of that of the wild type.

Summary: We evaluated effects of the prothrombin Yukuhashi mutation on TM-PC pathway. We demonstrated that fibrinogen clotting activity of the mutant was low but resistant to TM compared with the wild type. TM enhanced APC generation activity of the mutant substantially, but that was still lower than that of the wild type. These data suggested that the prothrombin Yukuhashi mutation might cause not only AT resistance, but also TM resistance in terms of fibrinogen clotting activity, resulting in susceptibility to thrombosis.

PA 2.13-6

Evaluation of a new chromogenic method based upon the prothrombinase complex for determination of the functional activity of prothrombin

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Background: In clotting and chromogenic methods: for prothrombin (FII) activity based upon the use of Ecarin, FII activation occurs irrespective of the extent of g-carboxylation of Glutamic acid residues (Gla). One-stage clotting methods: involving FII activation by the extrinsic system reflect better biologically functional FII but are hampered by a limited resolution.

Aim: As an alternative to one-stage methods, a chromogenic method for FII activity has been developed.

Method: The method is based upon the prothrombinase (FII-ase) complex and utilizes purified FXa, FV, calcium ions and synthetic or highly purified phospholipids. Gamma-carboxylation of FII is mandatory for its binding and activation on phospholipid surfaces in the presence of calcium ions and accordingly no thrombin is generated in this FII-ase based method on analysis of acarboxy FII, which completely lacks Gla residues.

The method is intended for both plasma and FII containing factor concentrates. Plasma should be collected in citrate or EDTA and a dilution of 1:200 is used.

Results: The method offers a resolution of about 1.2 absorbance units between 0 and 1.5 IU/mL and the detection limit is 0.05 IU/mL. Due to the high dilution, which minimizes matrix interference, the method allows levels of Hb 10 mg/mL, triglycerides 10 mg/mL, bilirubin 0.8 mg/mL and heparin 4 U/mL.

The correlation vs. a chromogenic Ecarin based method on analysis of plasmas from 196 patients supposedly not receiving anticoagulant treatment was $FII_{FII\text{-}ase} = 1.14 \times FII_{Ecarin} - 0.15$ ($R^2 = 0.87$).
Analysis of 11 plasmas from individuals heterozygous for FII G20210A showed a complete agreement with FII-ase = 0.97 xEcarin + 0.03 ($R^2 = 0.99$) and a mean value (SD) for both methods of 1.33 (± 0.24) IU/mL. Thus, elevated FII levels in carriers of the G20210A mutation are verified also with the FII-ase based method.
Analysis of plasmas from patients on warfarin with an INR range of 2–4 ($n = 47$) showed 1.4–3.8-fold lower activities with the FII-ase based method vs. the Ecarin based method. Importantly, for plasmas with an INR between 2.0 and 2.2, the functional FII activity varied 2.6-fold as determined with the FII-ase method. In one of these cases, the FII activity was 0.18 IU/mL which is in the same range as for plasmas with an INR of 3.5, tentatively indicating an increased risk of bleeding. For three plasmas with INR 5, 5.4 and 6.5, the FII activities were 0.12, 0.11 and 0.075 IU/mL, respectively.
The proper applicability on analysis of FII containing factor concentrates with the FII-ase based method is illustrated from the calibration of the 4th IS FII, X concentrate (11/126) with the obtained activity 9.67 IU/ampoule being in good agreement with 9.53 IU/ampoule (GCV 2.9%) for PT-based FII methods.
Conclusion: In summary, this prothrombinase based method allows accurate determination of functional FII activity in plasmas and FII containing concentrates and it provides a new tool for evaluation of patients on antivitamin K treatment as well as on patients with compromised liver function.

PA2.14 – Regulation of Coagulation and Fibrinolysis – I

PA 2.14-1
Inhibitory effects of LDL-associated tissue factor pathway inhibitor

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Background: Tissue factor pathway inhibitor (TFPI) is present in plasma as full length TFPI (TFPI) and as a C-terminally degraded form associated with low-density lipoprotein particles (LDL-TFPI). Addition of TFPI to plasma induces a prominent dose-dependent prolongation of the clotting time (CT) when tested in a diluted prothrombin time (dPT) assay. In contrast, addition of LDL-TFPI or truncated recombinant TFPI ($TFPI_{1–161}$) was shown not to prolong CT when tested in a dPT assay. It was concluded that the anticoagulant function of TFPI in human plasma was limited to fl-TFPI. Since LDL-TFPI has preserved its factor Xa (FXa) inhibitory activity and also worked as an inhibitor of tissue factor/factor VIIa (TF/FVIIa) in FX activation assays it was unclear why LDL-TFPI failed to affect the dPT in a plasma environment; and it was suggested that a slower on-rate of LDL-TFPI in a rate limiting step might explain this phenomenon.
Aim: The aim was to further characterize kinetic properties of purified LDL-TFPI in plasma in a thrombin generation test (TGT) and in various chromogenic *in vitro* activity assays.
Methods: LDL-TFPI was purified from human plasma by sequential flotation ultracentrifugation. FXa activity was measured using a chromogenic assay. Inhibitor constant, K_I, was calculated using the Morrison tight binding non-linear curve fitting. Thrombin generation assay was performed in normal human plasma (NHP) or in FVIII-immuno depleted plasma using either 1 pM TF and 4 μM phospholipids (PLs) or 4 μM PLs and 0.5 nM FXa respectively.
Results: When TFPI inhibition was tested in a TF-induced TGT assay in NHP we observed, in line with findings by dPT experiments, that TFPI, but not LDL-TFPI prolonged the lag-phase of thrombin generation. The kinetics of thrombin generation were, however, not unaffected by LDL-TFPI. Similar to TFPI it decreased the peak height of thrombin generation. Steady state inhibition of FXa showed a faster inhibition with LDL-TFPI than TFPI ($K_I = 0.0071 \pm 0.003$ nM and $K_I = 0.15 \pm 0.006$ nM respectively). In addition, transient kinetic data on FXa inhibition showed thatLDL-TFPI was 2.5 fold faster than TFPI indicating that FXa inhibition was not rate determining for the lag phase, whereas it appeared to affect thrombin generation during the propagation phase. This was supported by FXa-inducedTGT in hemophilia A plasma showing thatLDL-TFPI more actively decreased the peak height of thrombin generation compared to TFPI.
Conclusions: Our results suggest that LDL-TFPI affects thrombin generation during the propagation phase. It may therefore play a more prominent physiological role *in vivo* than hereto anticipated from dPT measurements.

PA 2.14-2
The effect of fibrin(-ogen) on thrombin generation and decay

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Background: In clotting blood(-plasma) >95% of thrombin develops and decays after clot formation. Thrombin generation (TG) in clotting plasma can be assessed by measuring the conversion of an added thrombin substrate (eg. In calibrated automated TG measurement [CAT]). The removal of fibrinogen from plasma has been shown to reduce TG as assessed by CAT. On the contrary, in a subsampling procedure TG is increased. This is explained by the assumption that part of the thrombin is reversibly adsorbed onto fibrin and is protected from the action of antithrombins, and therefore remains longer amidolytically active. In human plasma, two variants of the fibrin(ogen) γ chain occur, γ' and γA, that both contain low affinity binding sites for thrombin. The γ' chain contains an additional exclusive high affinity binding site for thrombin. Plasma contains ~85% of fibrinogen that has two γA chains and ~15% with one γ' and one γA chain. Apart from slowing down thrombin decay, fibrin may also influence prothrombin conversion. We therefore investigated the effect of the presence of fibrin on thrombin decay and prothrombin conversion during thrombin generation (TG).
Aims: We wanted to quantify the effect of γA/γ'- and γA/γA fibrinogen on thrombin decay and to investigate whether or not the presence of fibrin also influences prothrombin conversion.
Methods: TG was measured with the CAT technique in normal pool plasma (NPP), defibrinated NPP, and defibrinated NPP resupplied with purified γA/γA fibrinogen, with γA/γ' fibrinogen or with the two types in their natural proportions. The rate constants of total thrombin decay and thrombin decay by α2Macroglobulin (α2M) were calculated from the TG curves and prothrombin conversion curves were constructed from the TG curves and these rate constants.
Results: The removal of fibrinogen significantly decreases TG and restoration of the original fibrinogen concentration reverses this effect; γA/γA fibrinogen does not restore TG completely (ETP 83% of normal), whereas γA/γ' fibrinogen increases TG to above its original level (ETP 141% of normal). Physiological fibrinogen has a significant dose-dependent attenuating effect on thrombin decay, which is primarily due to protection against the action of α2M (64% reduced, $P < 0.001$). Serpin dependent thrombin inactivation is significantly decreased by γA/γ' (24% reduced, $P < 0.001$), but not by γA/γA fibrin. The effects of fibrin on TG are not completely explained by these effects on thrombin inactivation alone. In the presence of fibrin prothrombin conversion is significantly higher than in its absence (127%, $P = 0.001$). This effect is independent of the type of fibrinogen added.
Conclusion: Fibrin(ogen) has an important positive effect on thrombin generation because it dose-dependently inhibits thrombin decay and fosters prothrombin conversion. Fibrinogen containing the γ' chain is more effective in protecting thrombin than the γA is and inhibition by α2M is more affected than that by other antithrombins. A high fibrin-

ogen concentration must be excluded as a trivial explanation for high TG values found in some diseased states.

PA 2.14-3

Role of the serpin protease Nexin-1, In tissue remodelling occurring during pulmonary fibrosis.

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Background: Idiopathic pulmonary fibrosis (IPF) is a chronic diffuse lung disease characterized by excess fibrous material which accumulates in the lung via the modulation of proteolytic degradation of extracellular matrix (ECM) and/or fibrin deposition. Protease Nexin-1 (PN-1), also named SerpinE2, is a tissue serpin produced by many cell types and involved in the regulation of coagulation and fibrinolysis. PN-1 inhibits thrombin, plasminogen activators (tPA and uPA) and plasmin. Its ability to regulate coagulation and fibrinolysis factors makes it a good candidate as a regulator of tissue remodelling.
Aims: Since PN-1 is expressed in lung, a fibrosis target organ where the regulation of proteolytic activity is of great importance, we aimed to test the hypothesis that PN-1 is a key regulator in lung remodelling occurring during IPF.
Methods: Human lung fibroblasts were cultured from IPF patient biopsies. In some experiments, cells were incubated with recombinant TGFbeta before analysis of PN-1 expression by real-time PCR, immunoblotting and antiprotease activity assay. Lung tissue extracts, and Brochoalveolar Lavages (BAL) from IPF patients were also analyzed by immunohistochemistry and biochemical techniques. mRNA expression of ECM proteins like collagen and fibronectin were also analyzed by real time PCR. For this purpose, mRNA was obtained from fibrotic fibroblasts or control lung fibroblasts whose PN-1 expression was up-regulated by transfection of a PN-1 expression vector.
Results: PN-1 (mRNA and protein) was significantly expressed in control lung fibroblasts and up-regulated in a dose- and time-dependent manner by TGFbeta; maximal overexpression was reached after 24 h stimulation. Moreover, PN-1 expression was 3.5 fold increased ($P < 0.01$) in fibrotic compared to control pulmonary fibroblasts. PN-1 was not only observed on cell membrane but also in the conditioned medium, demonstrating that it can be secreted when overexpressed by the fibroblasts. Both secreted PN-1 present in conditioned media and PN-1 present on cell surfaces, from TGFbeta-stimulated control fibroblasts and fibrotic fibroblasts, were functional since both could inhibit thrombin catalytic activity. Moreover, PN-1 overexpression could be observed in lung extracts and BAL from IPF patients (1.8 fold increase, $P < 0.05$). In parallel to PN-1 over-expression, an mRNA up-regulation of ECM proteins like collagen1-alpha2 (1.8 fold increase, $P < 0.05$) and fibronectin (2.9 fold increase, $P < 0.01$) was observed in fibrotic fibroblasts. Interestingly such an overexpression of mRNA of the ECM proteins could be mimicked when controls fibroblasts were transfected with a PN-1 overexpression vector.
Conclusions: PN-1 is overexpressed in lung fibroblasts under fibrotic conditions at the messenger and protein levels. Overexpressed PN-1 is secreted in the pericellular environment where it has been shown to be able to inhibit thrombin. Interestingly, overexpression of PN-1 is accompanied by an overexpression of ECM proteins favouring a fibrotic phenotype of cells. PN-1 thus represents a new actor in the evolution of tissue remodelling.

PA 2.14-4

Structural and functional studies of a monoclonal high affinity antibody against the human TFPI Kunitz-type protease inhibitor (KPI) 3 domain

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Background: Activated coagulation factor VIIa (FVIIa) in complex with Tissue factor (TF) initiates coagulation by proteolytically converting coagulation factors IX and X (FIX and FX) to their activated forms, FIXa and FXa. Tissue factor pathway inhibitor (TFPI) inhibits the initiation of the coagulation cascade by forming a proteolytically inactive FVIIa:TF:FXa:TFPI complex. The TF/FVIIa-mediated FXa generation is tightly regulated by TFPI in a process which, as a rate-limiting step, appears to involve TFPI inhibition of FXa, either when FXa is bound to the TF/FVIIa complex or in its near vicinity on the membrane.
Aims: Functional blocking of TFPI binding to FXa or the TF:FVIIa:FXa complex with a monoclonal antibody may facilitate haemostasis initiated by FVIIa:TF hereby compensating for impaired FIX/FVIII-dependent coagulation. We have studied high affinity monoclonal antibodies binding to the KPI-3 domain of human TFPI.
Methods: A murine monoclonal antibody, with high TFPI-KPI-3 affinity (K_D 260 pM), mAb 0001 and the corresponding Fab-fragment, Fab 0001, were cloned and expressed as mouse-human chimeric mAb/Fab and purified by affinity chromatography. A 1:1 complex of the Fab 0001 and TFPI-KPI-3 protein was purified and crystallized with the hanging-drop method using a precipitant solution containing 18% PEG 4000, 26% propanol and 100 mM sodium citrate, pH 5.5.
Thrombin activity was assessed by calibrated automated thrombin generation measurements (CAT) and was measured continuously after the conversion of the fluorogenic substrate Z-Gly-Gly-Arg-AMC.HCl. Fluorescence was measured in a microtiterplate Fluorskan Ascent fluorometer with excitation and emission wavelengths set at 368 and 460 nm, respectively.
Results: Binding of mAb 0001 to TFPI-KPI-3 shortened the clotting time in normal and hemophilia A plasma measured by a diluted prothrombin (dPT) assay. Binding also produced a prominent increase in tissue factor induced thrombin generation studied by CAT in FVIII immune-depleted plasma supplemented with 10 μM phospholipids (PS/PC; 25/75%).
The binding epitope of mAb 0001 on KPI-3 of TFPI was determined by solving the crystal structure at 2.1 Å resolution of the complex between KPI-3 and the Fab fragment, Fab 0001. There were two independent KPI-3/Fab 0001 complexes in the crystallographic asymmetric unit. Calculation of the areas excluded in pair-wise interactions gave for the soluble TFPI KPI-3/Fab 0001 molecular complex 680 and 669 Å^2 for TFPI KPI-3 and 532 and 536 Å^2 for Fab 0001 and the two crystallographic independent molecular complexes, respectively. Results from a site-directed mutagenesis Ala-scan study confirm the epitope determined by X-ray crystallography.
Summary: The epitope for mAb 0001 on TFPI KPI-3 was characterized. Activity data demonstrated that binding of a high affinity antibody to this epitope was capable of improving *in vitro* haemostasis under haemophilia A-like conditions.

PA 2.14-5

Apolipoprotein(a) inhibits the conversion of Glu-plasminogen to Lys-plasminogen on the surface of vascular endothelial and smooth muscle cells

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Lipoprotein(a) [Lp(a)] has been identified as an independent risk factor for cardiovascular diseases including coronary artery disease. Lp(a) has a complex structure consisting of a low-density lipoprotein (LDL) moiety and the unique glycoprotein apolipoprotein(a) [apo(a)]. Lp(a) possesses both proatherogenic and prothrombotic properties due to the LDL-like moiety and apo(a) component of the lipoprotein respectively. Molecular cloning of apo(a) revealed that it contains remarkable homology to the fibrinolytic zymogen plasminogen. Plasmin, generated through plasminogen activation, is a key component in cell migration and proliferation, angiogenesis, inflammation and wound healing. Binding of plasminogen to various cell surface receptors initiates the conversion of its native form, Glu-plasminogen, to its more readily activatable form, Lys-plasminogen. Conversion of Glu- to Lys-plasminogen is necessary for optimal stimulation of plasminogen activation on human umbilical vein endothelial cells (HUVECs). It has been previously shown that apo (a) can inhibit plasminogen activation, through the positive feedback Glu- to Lys-plasminogen conversion mechanism, on the fibrin surface. Current work from our group has shown that apo(a) can inhibit pericellular plasminogen activation on vascular cells. Therefore, we determined the potential role of inhibition by apo(a) of Glu- to Lys-plasminogen conversion.

A novel method was employed to detect plasmin-mediated Glu- to Lys-plasminogen conversion utilizing an inactive plasminogen mutant with a fluorescent tag. A recombinant apo(a) [r-apo(a)] variant, 17K, was found to inhibit Glu- to Lys-plasminogen conversion on the surface of HUVECs by 60%. A series of r-apo(a) variants containing deletions of specific domains or point mutations were utilized to asses which domains mediate the ability of apo(a) to inhibit conversion. Removal of the protease and kringle V domain of 17K results in a decrease in the ability to inhibit Glu- to Lys-plasminogen conversion on HUVECs. Removal of the strong lysine binding site (sLBS) in kringle IV type 10 abolishes the ability of 17K to inhibit conversion. Various isoforms of r-apo(a) (ranging in size from 3 to 21 kringle IV type 2 repeats) were utilized in order to assess whether isoform size contributes to the inhibitory effect of conversion. All isoforms were able to inhibit Glu- to Lys-plasminogen conversion to a similar extent.

The ability of apo(a) to inhibit Glu- to Lys-plasminogen conversion on smooth muscle cells (SMCs) was also evaluated. Apo(a) inhibited conversion to a greater extent (76%) on SMCs compared to HUVECs. Unlike HUVECs, removal of the sLBS in kringle IV type 10 only reduces, but does not abolish, the ability of apo(a) to inhibit conversion on SMCs. In the absence of cells, plasmin-mediated Glu- to Lys-plasminogen conversion is greatly reduced (by approximately 75%). Although apo(a) is able to further reduce this rate, the absolute magnitude of the decrease is correspondingly reduced, indicating that the effect of apo(a) in the presence of cells is in fact cell-dependent.

Our results indicate a novel mechanism in which apo(a) can inhibit pericellular plasminogen activation on HUVECs and SMCs. The kringle V domain, protease domain and sLBS in kringle IV type 10 of apo (a) are required to maximally inhibit Glu- to Lys-plasminogen conversion.

PA 2.14-6

TF/FVIIa mediated activation of FIX and PAR-2 escapes inhibition by TFPI under conditions of concomitant TFPI blockage of FX activation

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Background: The complex of FVIIa and TF triggers coagulation by recognizing its macromolecular substrates factors IX (FIX) and X (FX) through extended exosite interactions. In addition, TF mediates cleavage of protease activated receptor 2 (PAR-2) on e.g. MDA-MB-231 carcinoma cells. These activities are regulated by TF exposure, and various factors on the cell surface affecting its cofactor function. In addition TFPI works as a potent modulator of FX activation by TF/FVIIa. Initiation of coagulation is down-regulated by TFPI inhibition of TF/FVIIa in a feed-back mechanism requiring generation of FXa and subsequently formation of an inactive TF/FVIIa/FXa/TFPI complex. How TFPI inhibition affects TF-dependent FIX and PAR-2 activation reactions in absence and presence of concomitant TF/FVIIa/FXa/TFPI complex formation is less well described.

Aim: To study TFPI inhibition of the three TF/FVIIa substrates FX, FIX and PAR2 using MDA-MB-231 cells as a template.

Methods: FVIIa activation of FX and FIX on MDA-MB-231 cells was measured with chromogenic substrates. FVIIa activation of PAR-2 was determined as IL-8 expression measured by ELISA in the supernatant from MDA-MB-231 cells.

Results and Discussion: Measurements of TF-dependent activation of FIX and FX on the cells revealed that the two reactions were saturated at widely different concentrations of FVIIa with EC_{50} values of 4.4 ± 0.4 and 64 ± 7 pM. Maximal turnover of FX by TF/FVIIa was about 20 fold faster than that of FIX. Furthermore, FX and FIX activation were both subject to competitive inhibition by alternative substrates (IC_{50} approximately 200 nM).

TF/FVIIa mediated FX activation was inhibited by TFPI ($IC_{50} = 0.38 \pm 0.09$ nM) at markedly lover concentrations than FIX (IC_{50} approximately 26 nM). Inhibition by TFPI of FX activation was minimally affected by the presence of FIX in accordance with the importance of the interaction between FXa and TFPI for efficient inhibition of FX activation by TF/FVIIa. The presence of FX and concomitant TF/FVIIa/FXa/TFPI complex formation restricted the amount of TF available for FIX turnover without an appreciable effect on the IC_{50} for TFPI inhibition. This and data showing that FX and FIX activation were similarly neutralized by active site inhibited FVIIa seems to exclude that different pools of TF are involved in FX and FIX activation.

PAR-2 activation was saturated by FVIIa with $EC_{50} = 16 \pm 5$ nM. This decreased to $EC_{50} = 4.7 \pm 1.7$ nM with concomitant FX activation without an effect on turnover. Like FIX activation, inhibition of PAR-2 activation by TFPI required high concentrations of TFPI (IC_{50} approximately 26 nM). Formation of the TF/FVIIa/FXa complex increased TFPI inhibition of PAR-2 activation by decreasing IC_{50} to 3.7 ± 0.6 nM.

The data suggested that formation of the TF/FVIIa/FXa complex stimulated PAR-2 activation and its inhibition by TFPI. Still PAR-2 activation remained essentially unaffected by TFPI inhibition at physiological relevant sub-nanomolar TFPI concentrations where FX activation was fully blocked.

PA2.15 – Tissue Factor – II

PA 2.15-1

Increased mortality in SIRS patients with low levels of zymogen and high levels of activated coagulation factor VII

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Background: The Tissue Factor (TF)-Activated Factor VII (FVIIa) complex plays an important role in the inflammatory and coagulation responses in patients with Systemic Inflammatory Response Syndrome (SIRS) and sepsis. As zymogen FVII (FVII) and FVIIa compete for binding to TF, it is possible that the mutual relation of their plasma levels determines the extent of activity of the TF-FVIIa complex.
Aims: The objective of this research was to study the mortality in SIRS patients as a function of FVIIa and VII levels in plasma.
Methods: A prospective cohort study of 275 consecutive patients presenting with SIRS, aged 18 years or older and an anticipated Intensive Care Unit (ICU)-stay of at least 24 h. FVIIa was measured using a novel, quantitative ELISA that recognizes FVIIa but not its zymogen FVII. All-cause hospital mortality was followed over a period of 60 days.
Results: On admission, the percentage of circulating FVIIa over factor VII was significantly higher in non-survivors than survivors (2.8% vs. 1.5%, $P = 0.034$). High levels of FVIIa were associated with decreased survival (62% vs. 81%, $P = 0.030$) while high levels of FVII were associated with an increased survival (84% vs. 76%, $P = 0.039$). Patients with high FVIIa and low FVII levels had a three-fold increased mortality risk compared to the patients that had low FVIIa and high FVII levels (Hazard Ratio [HR] = 3.2, 95% Confidence Interval [CI] 1.4–7.3). This association persisted after adjustment for the APACHE IV score (adjusted HR = 2.7, 95% CI 1.2–6.2).
Conclusions: We found an increased mortality in SIRS patients with high FVIIa levels and low FVII levels, independently of the parameters included in the APACHE IV score. Our data indicate that FVIIa and FVII could be useful in stratifying SIRS patients with high mortality risk.

PA 2.15-2

Targeting protein C activation to tissue factor: *In vivo* studies in human tissue factor knock-in mice

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Thrombus formation by coagulation due to tissue factor (TF) exposure is associated with high mortality rates provoked by cardiovascular diseases such as ischemia, infarction, edema and inflammation. So far, the marketed antithrombotics do prevent thrombus formation but are also associated with an increasing risk of bleedings. By combining the anticoagulant properties of a TF antibody and a fragment of the protein C activator thrombomodulin (TM), substantial anti-thrombotic efficacy directly at the site of TF exposure is conceivable. Therefore, a genetic fusion was accomplished between the dimeric form of TF neutralizing single chain (Sc) antibody Fc fragment and the TM EGF-like domains 4–6. Constructive *in vitro* prothrombin time (PT) studies, *ex vivo* characterization as well as *in vivo* thrombosis experiments in human TF knock-in (hTFKI) mice were performed to investigate the antithrombotic and hemorrhagic properties of the fusion construct in comparison to the TF antibody or soluble TM individually.
PT studies with human plasma revealed a dose-dependent amplification of clotting time of the fusion construct compared to the TF antibody or TM individually. Further, to prove the efficacy of the fusion construct on human TF functionality, TF-rich brain extracts of WT (mouse TF) and hTFKI (human TF) mice were analyzed in a plasma clotting assay. Therein the fusion construct dose-dependently prevented clotting of human TF-activated plasma but not mouse TF-activated plasma. Inducing carotid or cremaster arterial thrombosis by ferric chloride or laser injury, respectively, the fusion construct prevented thrombosis in a dose-dependent manner. However, tail bleeding times, were similar in all treatment groups and did not show an improved therapeutic window compared to TF antibody alone.
In summary, the fusion construct outclasses the single components in *in-vitro* clotting assays. Further, the construct proved to be human TF specific and reduces thrombus growth in a dose-dependent manner similar to the TF antibody in two independent thrombosis models. However, due to the bleeding time prolongation which corresponds to the antibody, the benefit-risk ratio is comparable to the marketed drugs, therefore no superiority of targeting protein C activation at the site of TF exposure is achieved.

PA 2.15-3

Lung cancer chemotherapy agents induce protein disulphide isomerase dependent tissue factor decryption resulting in increased procoagulant activity *in vitro* and *in vivo*

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Background: Cancer patients undergoing chemotherapy have an elevated risk for thrombosis, with venous thromboembolism (VTE) being the second leading cause of death in cancer patients. However, the mechanism(s) by which chemotherapy increases the risk for thrombosis in lung cancer patients is not well understood. Previous studies have shown that endothelial cells treated with lung cancer chemotherapy agents promote coagulation through an increase in tissue factor (TF) procoagulant activity.
Aims: The purpose of this study was to determine the mechanism(s) by which anti-lung cancer therapy agents cisplatin, carboplatin, paclitaxel, and gemcitabine induce procoagulant effect *in vitro* and *in vivo* when used as single agents, or in clinically relevant combinations.
Methods: Human umbilical vein endothelial cells (HUVECs), blood monocytes, and A549 cells were treated for 24 h with clinically relevant concentrations of chemotherapy agents alone and in combination. Cell surface TF activity was measured by factor Xa (FXa) generation in the presence or absence of factor VIIa and $CaCl_2$ on treated cells. Cell surface TF antigen, phosphatidylserine (PS) levels, and protein disulphide isomerase (PDI) levels were measured by flow cytometry. Healthy Balb/C mice received intraperitoneal injections of lung cancer chemotherapy agents alone or in combination. Blood was collected 24 h post-treatment by carotid artery cannulation. Plasma levels of thrombin-antithrombin (TAT) complex were measured by ELISA.
Results: Treatment of HUVEC, A459 cells, and monocytes with single agent and combination chemotherapy significantly increased cell surface FXa generation. FXa generation was completely ablated in the absence of FVIIa or in the presence of HTF-1 (an anti-TF antibody), confirming that FXa generation is TF dependent. Flow cytometry demonstrated unchanged cell surface TF antigen levels suggesting increased FXa generation was due to TF decryption. There was a significant increase in PS levels on HUVEC treated with high-dose cisplatin and gemcitabine combinations, and significantly elevated levels of cell surface PDI on HUVEC and A549 cell surface treated with chemotherapy agents. Inhibition of PDI, but not PS resulted in a moderate decrease of FXa generation suggesting that TF decryption was PDI mediated. Treatment of cells with reducing

agents glutathione or dithiothreitol significantly reduced TF activity further suggesting that PDI mediated chemotherapy-induced TF decryption occurs through disulphide bond formation. Our *in vivo* results demonstrated that treatment of healthy mice with combination chemotherapy significantly increased plasma TAT levels in healthy mice.

Conclusions: Our studies are the first to explore the effects of lung cancer chemotherapy agents on monocyte and A549 cell TF activity levels, as well as the first to delineate the mechanisms by which lung cancer agents promote TF decryption on these cell lines. We are also the first to report *in vivo* increases in thrombin generation in response to treatment of healthy mice with anti-lung cancer agents. These studies reveal potential mechanisms by which lung cancer chemotherapy induce a hypercoagulable state.

PA 2.15-4

Tissue factor is not expressed in platelets activated by various agonists

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Introduction: Blood borne tissue factor (TF) has been implicated to be associated with monocytes as well as neutrophils and platelets. In humans monocytes may be the only blood cell expressing TF. When blood is stimulated, TF rich microvesicles may become fused with activated platelets. However, recent reports still claim that isolated activated platelets express TF activity and it has been suggested that the expression of TF is a result of rapid and dynamic process whereby TF activity may be detected as early as 3 min after stimulation and may be gone after 2 h.

Aim: To establish whether isolated platelets free of contaminating monocytes may generate TF activity after activation with TRAP, LPS, LPS/PMA, or complement (IgG antibody).

Methods: Platelet rich plasma (PRP) of blood collected in Fragmin was prepared by twice centrifugation at 150 ***g*** for 15 min. Contaminated monocytes were removed by using MS-columns with anti-CD14 antibody microbeads. Isolated platelets were re-suspended in plasma. The platelet samples were incubated with LPS/PMA (100 and 5 ng/mL) for 4 h or IgG (88/μg/mL final conc) from patient with Lupus anticoagulant for 3 h. Platelets activated with TRAP (10 μM final conc) was incubated for 10 min before testing for TF activity immediately after isolation and washed free of plasma.

Results: Platelet free of monocytes had a very small increase in TF like activity after being stimulated with LPS/PMA (0.2 ± 0.1 mU/10^8 plts), but this activity was not affected by anti-TF antibody. In contrast, platelets contaminated with monocytes had a 10 fold increase (2.0 ± 0.9 mU/10^8 plts) that was reduced to about the same TF activity as activated platelets free of monocytes. The IgG antibody had no TF activity inducing effect on platelets free of monocytes, but strongly induced TF activity in monocytes of whole blood (> 25 mU/10^6 cells). Platelets stimulated with TRAP for 10 min had no increase over the Background.

(0.9 ± 0.1 mU/10^8 platelets) for TF like activity in control. A rise in TF like activity was seen when anti-TFPI antibody was added (1.4 ± 0.4 mU/10^8 plts) and that was enhanced a little more when also anti-TF antibody was added (1.6 ± 0.4 mU/10^8 plts).

Discussion: Our results show clearly that TF activity is not expressed or associated with platelets activated in plasma by several platelet agonists, transiently or after longer stimulation. These results prove that platelets do not possess TF that can be converted to an active form or synthesized through a TF pre-mRNA system. It is mandatory to remove contaminating monocytes from platelet preparations when studying TF in platelets.

PA 2.15-5

PGE2 cigarette smoke-induced modulates endothelial tissue factor: role of EP1 receptor and SIRT1

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Background: Cigarette smoke exposure increases the risk of atherothrombosis, induces the expression of cyclooxygenase-2 (COX-2) and the release COX-2 derived eicosanoids, including Prostaglandin E2 (PGE_2). Moreover, cigarette smoke up-regulates the expression and activity of Tissue Factor (TF), the main initiator of the coagulation cascade. However, the relationship between PGE_2 and TF has not yet been elucidated.

Aims: In this study, we analysed the link between PGE_2 and TF, and the impact of their regulation on cigarette smoke-induced atherothrombosis.

Methods: The levels of PGE_2 and TF expression and activity in plasma were measured in 46 healthy active smokers (AS; 30 men and 16 women) and 19 non-smokers (NS; eight men and 11 women). In addition, PGE_2 and TF were also measured in aorta tissue of mice and in mouse cardiac endothelial cells (MCEC) treated with aqueous extracts of cigarette smoke (TS) plus IL-1β (TS/IL-1β) by EIA assay, real-time PCR and procoagulant activity, using one stage-clotting assay, and/or by Zymuphen MP-TF. Arachidonic acid-induced thrombosis was used to study the effect of TS/IL-1β in mice. The expression of SIRT1, the NAD^+-dependent protein deacetylase, was measured by Western blotting and by Immunofluorescence.

Results: Higher levels of both PGE_2 and TF were detected in plasma of AS compared to NS. Similar results were obtained in mice and in MCEC treated with TS/IL-1β. A highly significant correlation between PGE_2 and TF activity was observed in both human plasma and mouse tissue. Inhibition of PGE synthase (PGES) activity by CAY10526, or by specific PGES siRNA, markedly diminished both *in vitro* and *in vivo* the amount of TF and the mouse carotid artery thrombosis induced by TS/IL-1b. In contrast, treatment with exogenous PGE_2 increased both TF and arterial mouse thrombosis.

MCEC express three PGE_2 receptors: EP1, EP2 and EP4. In these cells, EP1 antagonists (AH6809 and SC51089) prevented the TF induced by TS/IL-1β. In contrast, an EP1 agonist (17-phenyl-trinor-PGE_2) increased TF. We excluded the involvement of other EP receptors because an EP4 antagonist (GW627368X) and EP2/EP4 agonists (misoprostol, butaprost) did not modify TF. The role of SIRT1 in the regulation of TF was also analysed. Both Sirtinol, an SIRT1 deactivator, and the specific SIRT1 siRNA increased TF expression and activity. In contrast, the SIRT-1 activators (resveratrol and CAY10591) reduced TF induced by both TS/IL-1β and EP1 agonist.

Finally, EP1 agonist or the selective PGES-1 siRNA prevented the inhibition of SIRT1 mediated by PGE_2 or byTS/IL-1β.

Summary/Conclusion: We conclude that PGE_2 increases TF expression and activity in both mouse carotid arteries and endothelial cells. The phenomenon involves the regulation of the EP1/SIRT-1 pathway. These findings suggest that EP1 may represent a target to prevent thrombotic events

PA 2.15-6

Proteomic analysis of tissue factor activation

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Background: Tissue factor (TF), initiator of blood coagulation, is also a signalling receptor. Signalling involves activation of protease acti-

vated receptor (PAR) 2, but the platelet derived growth factor β receptor has also been shown to be transactivated and other receptors have been suggested to be involved.
Aims: We investigated possible downstream activators when TF is stimulated by its ligand activated factor VII (FVIIa) using a proteomic approach.
Methods: A proteomic study was performed with the use of the refined technique of 2-D difference gel electrophoresis (DIGE) which has many advantages compared to traditional 2-D electrophoresis. The samples are pre-labelled with three different fluorescent dyes that are well separated on the wave length scale. This makes it possible to run two samples on each gel together with an internal standard run on every gel. Since the samples are compared to the same internal standard the detection of differences down to 10% is achievable with high accuracy. We used porcine aortic endothelial cells transfected with tissue factor and stimulated with 50 nM FVIIa for 20 min, 1, 3 h and catalytically inactive FVIIa (FVIIai) for 3 h. The proteins of interest were identified by mass spectrometry and we used both Western blot and a protein synthesis assay and a second cell line (human fibroblasts, 1137Sk) to further confirm our results for one of our interesting findings.
Results: Forty one spots were regulated differently over time or compared to non-stimulated cells, and 23 out of these were identified by mass spectrometry as 13 different proteins. One of them, elongation factor 2 (EF-2), an enzyme that promotes the translocation step in the elongation of the protein chain in protein synthesis, was investigated in more detail. The phosphorylation of EF-2 was increased when tissue factor was stimulated by FVIIa, and thereby more inactivated. Analyzing the effect of FVIIai and the PAR-2 agonist, SLIGKV, indicated that the inactivation is not PAR-2 dependent. In a protein synthesis assay we could confirm the results of decreased protein synthesis in two different cell lines when TF is stimulated by FVIIa.
Conclusions: We demonstrate that the phosphorylation of EF-2 is increased when FVIIa is bound to TF. This phosphorylation regulates the activity of the protein and inactivates EF-2, which in turn slows down the protein synthesis. Our data suggest that this effect is not mediated through the PAR-2 receptor since the phosphorylation effect on EF-2 was also seen after FVIIai incubation, which cannot cleave the PAR-2 receptor. Moreover, the PAR-2 agonist SLIGKV did not induce phosphorylation of EF-2 and when deleting the cytoplasmic domain of TF the effect is abolished. Instead, the effect may be mediated via a novel receptor that interacts with TF or directly by a TF cytoplasmic domain induced pathway. The reason and mechanism behind the decrease in protein synthesis need to be further explored, but speculating, it could be an initial stress response.

PA2.16 – Cancer and Thrombosis – IV

PA 2.16-1

Incidence and predictive factors of symptomatic venous thromboembolism related to port-a-cath in cancer patients

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Background: The use of indwelling central venous catheters, such as port-a-caths, has significantly improved the management of patients with malignancies receiving chemotherapy. Thrombosis is a common complication of catheters and is associated with significant morbidity. However, the true incidence of symptomatic port-a-cath-related deep vein thrombus (DVT) and its associated risk factors in cancer patients remains unclear.
Aim: To assess the incidence and risk factors associated with symptomatic venous thromboembolism (VTE) related to port-a-cath in cancer patients.
Methods: We performed a retrospective cohort study of consecutive cancer outpatients who have received an ultrasound guided port-a-cath insertion for the administration of chemotherapy between November 2010 and December 2011 in our institution. The primary outcome measure was symptomatic VTE. Venous thromboembolism was defined has symptomatic upper extremity port-a-cath related proximal (axillary vein or more proximal) DVT or pulmonary embolism (PE). Univariable and multivariable logistic regression analyses were used to identify risk factors for symptomatic VTE.
Results: A total of 400 cancer patients with a newly inserted port-a-cath for the deliverance of chemotherapy were included into the study. Median age was 58 years (range of 21–85 years) and 120 (30%) were males. All patients were followed for a median of 12 months and none received thrombophrophylaxis. Of the 400 patients included in the analysis, 34 patients (8.5%; 95% CI: 6.0–11.7%) had symptomatic VTE (16 DVTs, 16 PEs, and two with both). The median time from insertion of port-a-cath to VTE occurrence was 103 days (range of 13–371 days). In the univariate analyses, cancer type, metastatic disease, female gender and left sided port-a-cath insertion were significantly associated with the risk of VTE. The most common cancer types were GI tract cancer (62%) in patients with a symptomatic VTE and breast cancer (50%) was more frequent in patients without VTE. Ninety-four percent of patients with a symptomatic VTE had metastases compared to 60% in those without VTE. Other demographic factors including age, body mass index and smoking status were not significant contributors. Co-morbidities, including diabetes, coronary artery disease, hypertension, and chronic obstructive pulmonary disease (COPD) were also not significant contributors. In the multiple-variable analysis, female gender (OR 0.46, $P = 0.04$) and presence of metastases (OR 8.22, $P < 0.01$) were the two significant independent predictors of port-a-cath related VTE.
Conclusion: Symptomatic VTE is a frequent complication in cancer patients with port-a-cath receiving chemotherapy. Gender and presence of metastatic disease are independent risk factors for symptomatic VTE. Future trials assessing the role of thromboprophylaxis among these higher risk patients are needed.

PA 2.16-2

Prediction of venous thromboembolism in cancer patients by tissue factor dependent microparticle coagulant activity, biomarkers and a clinical score

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Background: Cancer patients receiving chemotherapy in an ambulatory setting are at risk of venous thromboembolism (VTE). Randomized controlled trials have shown that thromboprophylaxis with low-molecular-weight-heparin reduces the occurrence of VTE in cancer patients but the overall incidence of 4% is generally considered too low to justify thromboprophylaxis in all patients. A logical next step is to focus on tools for selecting cancer patients at the highest risk of VTE, in whom thromboprophylaxis is expected to have a more favorable risk-benefit ratio.

Aim: We aimed to determine the performance of tissue factor (TF)-dependent microparticle coagulant activity in predicting VTE, and to compare it to that of the Khorana score, and previously evaluated biomarkers.
Methods: In six hospitals blood was obtained from consenting ambulatory patients receiving chemotherapy for stage III or IV cancer. Patients were prospectively followed for 6 months for development of VTE. At inclusion, TF-dependent microparticle coagulant activity was measured in a fibrin generation test (FGT) on fresh plasma in the local laboratories. The outcome measure of this plasma recalcification test was the prolongation of the clotting time in the presence of antibody to factor VII/TF, expressed as percentage of the clotting time in the absence of antibody. The result was considered positive above 13%. Remaining plasma was frozen and stored for later central measurement of D-dimer, P-selectin, pro-thrombin fragment 1 + 2 (F1 + 2) and factor VIII; for these tests positivity cut-offs from the original publications were used. Clinical data were collected for calculation of the Khorana score, which assigns patients to three risk categories. Estimates of sensitivity and positive predictive value (PPV) were calculated, taking into account death as a competing risk, with 95% confidence intervals (CI) based on bootstrap re-sampling.
Results: The prospective cohort has recruited 443 patients, with a mean age of 61 years, of which 49% women. In total, 23 patients developed VTE, after a mean time of 2.1 months (5.2%); 77 patients died, after a mean follow-up of 3.3 months (18%). Sensitivity was estimated at 61% (95% CI: 31–84%) for FGT, 84% (61–97) for D-dimer, 85% (56–96) for P-selectin, 44% (61–97) for factor VIII, and 70% (36–89) for F1 + 2. The positive predictive value was 4.7% (1.9–9.3%) for FGT, 6.9% (3.5–12) for D-dimer, 14% (4.8–27) for P-selectin, 11% (4.5–21) for factor VIII and 5.9% (2.4–12) for F1 + 2. A high Khorana score had a sensitivity of 63% (28–83) and PPV of 5.1% (1.6–12). None of the differences were statistically significant.
Summary/Conclusions: There are no substantial differences in predictive performance between the studied biomarkers. None of the individual positive predictive values exceeded 15%. In this cohort of patients with advanced cancer, 18% died, which makes adjustment for death as competing risk of utmost importance. In our opinion single predictors will probably not solve the problem and combinations are likely to be helpful.

PA 2.16-3
High incidence of VTE despite electronic alerts for thromboprophylaxis in hospitalized cancer patients

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Background: Despite most cancer patients present high risk of venous thromboembolism (VTE) during hospitalization, thromboprophylaxis is frequently underused. Electronic alerts (e-alerts) have been associated with improvement in the use of thromboprophylaxis and a reduction of the incidence of VTE, both during hospitalization and after discharge, particularly in the medical setting. However, there are no data regarding the benefit of this tool in cancer patients.
Aim and Methods: Our aim was to evaluate the impact of a computer-alert system for VTE prevention on patients with cancer, particularly in those admitted in the Oncology/Hematology ward, comparing the results with the rest of inpatients at a university teaching hospital. The study included 32,167 adult patients hospitalized during the first semesters of years 2006–2010, 9265 (28.8%) of them with an active malignancy.
Results: Appropriate prophylaxis in medical patients, significantly increased over time (from 40% in 2006 to 57% in 2010) and maintained over 80% in surgical patients. However, while e-alerts decreased the incidence of VTE during hospitalization in patients without cancer (OR 0.31; 95% CI, 0.15–0.64), the impact was modest in cancer patients (OR 0.89; 95% CI, 0.42–1.86) and no benefit was observed in patients admitted for the Oncology/Hematology Departments (OR 1.11; 95% CI, 0.45–2.73). Interestingly, 60% of VTE episodes occurring in cancer patients in the last years developed despite appropriate prophylaxis.
Conclusion: Contrary to the impact on non-cancer hospitalized population, the implementation of e-alerts for VTE risk was insufficient to prevent the development of VTE during hospitalization among patients with malignancies.

PA 2.16-4
Clinical presentation, ADAMTS13-related measurements and outcomes in cancer-associated thrombotic thrombocytopenic purpura

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Background: Thrombotic thrombocytopenic purpura (TTP), a rare thrombotic disease of the microvasculature, may be idiopathic or secondary to various conditions. Idiopathic TTP is often characterized by plasmatic deficiency of von Willebrand factor (VWF) cleaving protease, ADAMTS13. Secondary TTP may be associated with autoimmune disorders, infections, drugs and cancer. Data on clinical features and ADAMTS13 levels in cancer-associated TTP are scanty.
Aims: To describe clinical presentation, outcomes and to analyze ADAMTS13- and VWF-related measurements in a cohort of cancer-associated TTP.
Methods: We analyzed clinical and laboratory data of 18 patients with cancer-related TMA referred to the Milan TTP Registry (URL: www.ttpdatabase.org) between 1999 and 2012. Inclusion criteria were: (i) clinical documentation of acute TTP and (ii) diagnosis of solid or hematologic malignancy, established by clinical, laboratory, radiological, histological findings. We measured ADAMTS13 antigen and activity, anti-ADAMTS13 antibodies by western blotting, VWF antigen and multimeric pattern on plasma samples collected before any transfusional therapy.
Results: Median age at presentation was 63 years (range: 29–83 years). Solid cancers affected stomach (n = 4, 22%), uterus (n = 2, 11%), liver (n = 1, 6%), prostate (n = 1, 6%), bladder (n = 1, 6%), skin (n = 1, 6%), rectum (n = 1, 6%); hematologic cancers were non-Hodgkin lymphoma (n = 2, 11%) and chronic myelomonocytic leukemia (n = 2.11%); three cases (16%) had metastatic tumors of unknown origin. TTP was the first sign of malignancy in 10 (55%) cases.
At presentation, nine patients (50%) had neurologic symptoms, 5 (28%) renal involvement, 13 (72%) bleeding diathesis. Median hemoglobin was 10.3 g/dL (range: 5.2–11.8 g/dL), platelets 16.5×10^9/mmc (range: 6–133 $\times$ 10^9/mmc), LDH 1664 IU/L (range: 286–7060 IU/L). Seventeen patients received plasma exchange or fresh-frozen plasma, associated with chemotherapy in two cases. Nine patients did not survive during the acute episode (data not available for four). ADAMTS13 and VWF values were tested in 14 patients: 28% had severe ADAMTS13 deficiency (activity below 10%), 21% had moderate deficiency (10–46%), 50% had normal ADAMTS13 activity (above 46%). Anti-ADAMTS13 antibodies were present in three out of four patients with severe ADAMTS13 reduction and in one out of three with moderate deficiency. VWF antigen resulted elevated in 64% of patients, independently of ADAMTS13 levels; 64% of patients had a consumption of ultralarge and large-size VWF multimers.
Summary/Conclusions: TTP may be the first manifestation of malignancy. ADAMTS13 activity may be normal or mildly reduced in can-

cer-related TMAs, suggesting that VWF-mediated thrombosis is not the only abnormality involved in this setting. Severely reduced ADAMTS13 levels associated with anti-ADAMTS13 antibodies do not rule out cancer-related TTP.

PA 2.16-5

TF expressed by microparticles is associated with mortality but not with thrombosis in cancer patients

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Background: A prothrombotic state is one of the hallmarks of malignancy and a major contributor to morbidity and mortality. Tissue factor (TF) is often overexpressed in malignancy and is a prime candidate in predicting the hypercoagulable state. Moreover, increased number of TF-expressing microparticles (MPs) in cancer patients may contribute to venous thromboembolism (VTE).

Aim: We have conducted a prospective cohort study to determine whether elevated TF antigen, TF activity and TF associated to MPs (MPs-TF) are predictive of VTE and mortality in cancer patients.

Methods and Results: The studied population consisted of 252 cancer patients and 36 healthy controls. TF antigen and activity and MP-TF were determined by ELISA and chromogenic assays respectively. During a median follow-up of 8.9 months, 40 thrombotic events were recorded in 34 patients (13.5%) and 73 patients (27.9%) died. TF antigen and activity were significantly higher in patients than in controls ($P < 0.01$) specially in patients with advanced stages whereas no differences were observed for MPs-TF activity. We did not find a statistically significant association of TF variables with the risk of VTE, but they were associated with mortality in univariate analysis. Multivariate analysis adjusting for age, sex, type of cancer and other confounding variables showed that TF and MPs-TF activity were independently associated with mortality ($P < 0.01$).

Conclusions: While TF variables were not associated with future VTE in cancer patients, we found a strong association of TF and MPs-TF activity with a poor outcome, thus indicating they might be good prognostic markers in cancer patients.

PA 2.16-6

Association of endothelial protein C receptor (A6936G) haplotype with thrombotic events as a risk factor in acute myeloid leukemia

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Background: Previously we presented evidence of the presence of endothelial protein C receptor (EPCR or PROCR) in a large group of leukemic cell lines and high plasma level of soluble EPCR (s-EPCR) in plasma of patients with human hematologic malignancies, giving a powerful insight into thrombotic risk. Haplotype characterization of HL-60, a myelomonoblastic cell line, shows single nucleotide polymorphisms (SNPs) originally described in A1 and A2 haplotypes and some additional new SNPs in the promoter and intronic regions.

Objective: To assess whether A3 (A6936G) haplotype, which is a genetic regulator of s-EPCR, can be involved in procoagulant activity of hematologic malignancies.

Patients/Methods: In a retrospective case-control study, haplotype of the EPCR gene was performed in 205 patients with hematologic malignancies from Saint Antoine Hospital [76 Chronic Lymphocytic Leukemia CLL (median age 67; 39 men, 37 women), 68 Acute Myeloid Leukemia AML (median age 62.5; 41 men, women), 33 Acute Lymphoblastic Leukemia ALL (median age 36; 18 men, 15 women) and 28 other malignant diseases (median age 64.5; s19 men, nine women)] and in 63 healthy donors (controls)(median age 25; 30 men, 33 women) provided by Hôtel-Dieu and Saint-Antoine Hospitals blood banks. DNA was purified using Qiagen DNA extraction Kit. All subjects were genotyped by Genoscreen using allele specific amplification for the three haplotypes of EPCR gene according to 6963A/G polymorphism (AA, AG and GG nucleotides). Clinical association of AG polymorphism with thrombotic events was studied in 75 patients (CLL, $n = 21$, AML, $n = 42$ and other, $n = 12$).

Results:

1 The distributions of AA, AG and GG nucleotides in all subjects were identical in healthy donors and in patients: the distribution of alleles in healthy donors and in patients are respectively 84.2% and 84.9% for AA, 14.2% and 14.1% for AG and 1.6% and 1% for GG.
2 The percentages of the three haplotypes (AA, AG and GG) were 80.1%, 17.1% and 2.8% for AML-90.9%, 9.1% and 0% for ALL-85.5%, 14.5% and 0% respectively for CLL – 85.6%, 14.4% and 0% for the other malignant hematological diseases, and 84.2%, 14.2% and 1.6% for controls.
3 In 75 patients tested for thrombotic events, thrombosis occurred in 38.1–14.3–4.8% of patients with AML, CLL and others respectively. In addition, the incidence of thrombotic events is much higher in AG allele-patients than in AA-ones: 45.5% of AML patients and 20.% of CLL patients with AG phenotype had thrombotic event, whereas thrombosis occurred only in 7.5% of AML patients and in 12.5% of CLL patients with AA phenotype, suggesting a high incidence of thrombosis in patients with EPCR-AG haplotype.

Conclusion: In this pilot study, the results demonstrate a striking association of AG heliotype of EPCR with thrombotic events in AML. The presence of AG allele in AML patients may be a risk factor for thrombosis and its determination in patients can be crucial for thrombosis prevention in this malignancy.

PA2.17 – Arterial Vascular Disorders II

PA 2.17-1

TM5441, a novel PAI-1 antagonist, prevents hypertension and arteriosclerosis in an experimental model for vascular aging

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Background: Long-term inhibition of nitric oxide synthase (NOS) by L-arginine analogues such as N^{ω}-nitro-L-arginine methyl ester (L-NAME) has previously been shown to induce systemic hypertension and arteriosclerosis in animal models. NOS inhibition is also known to augment the expression of plasminogen activator inhibitor-1 (PAI-1) in vascular tissue. Our laboratory previously reported that PAI-1 deficient mice (PAI-$1^{-/-}$) are protected against hypertension and vascular fibrosis due to long-term L-NAME treatment. These findings led to the hypothesis that pharmacological inhibition of PAI-1 activity is protective against the L-NAME-induced hypertension in wild-type (WT) mice. Addtionally, L-NAME has been shown to induce vascular senescence and aging. However, PAI-1's role in this process has yet to be investigated.

Aims: The aim of this study are to determine whether pharmacological inhibition of PAI-1 using the novel inhibitor TM5441 can protect mice from L-NAME induced hypertension, cardiac hypertrophy, arteriosclerosis, and vascular aging.
Methods: WT C57BL/6 mice were either fed a normal chow diet or TM5441 chow (20 mg/kg/day) starting at 6 weeks of age. These same animals were given either regular or L-NAME water (1 mg/mL). Animals were placed on their respective treatments for 8 weeks. Systolic blood pressure (SBP) measurements were taken every 2 weeks. Echocardiograms were performed at both baseline and after 8 weeks. After 8 weeks, the mice were sacrificed and organs were collected for histology and qRT-PCR analysis.
Results: After 8 weeks of treatment, we found that TM5441 attenuated the development of hypertension compared to animals that had received L-NAME alone (SBP 162.62 ± 21.18 vs. 182.99 ± 13.21 mm Hg, $P = 0.009$). We also observed significant reductions in left ventricle wall thickness ($P = 0.03$) and mass ($P = 0.02$). Additionally, TM5441-treated mice had a 34% reduction in periaortic fibrosis relative to WT animals on L-NAME ($P = 0.003$). Finally, we looked at the senescence marker $p16^{Ink4a}$ in the aorta and found that L-NAME-treated animals had a 2-fold increase in $p16^{Ink4a}$ expression over WT, and that this upregulation was prevented by TM5441.
Conclusions: Pharmacological inhibition of PAI-1 is protective against the development of hypertension, cardiac hypertrophy, and periaortic fibrosis in mice treated with the L-NAME. Furthermore, PAI-1 inhibition attenuates the arterial expression of $p16^{Ink4a}$. PAI-1 appears to play a pivotal role in vascular senescence, and these findings suggest that PAI-1 inhibitors may provide a novel approach in preventing vascular aging and hypertension.

PA 2.17-2

Vascular smooth muscle cells confer a prothrombotic phenotype within the vessel wall of spontaneously hypertensive rats

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Background: Clinical and experimental evidence point towards an impairment of the hemostatic balance in blood conferred by change in individual coagulation factors in the presence of elevated blood pressure. The vascular wall is one of the elements that determine the thrombotic response. However, it is still unclear whether hypertension confers a prothrombotic state in blood and within the vessel wall.
Aims: This study investigated whether hypertension-induced changes in coagulation proteins cause an increase thrombin generating capacity both in blood and within the vascular wall and tested the respective contribution of vascular smooth muscle cell (VSMC) and endothelial cells (ECs).
Methods: We used 12-week-old spontaneously hypertensive rats (SHR) compared with age-matched Wistar rats. Two-millimeter ring segments, VSMCs or ECs were isolated form the descending thoracic aorta of both SHR and Wistar rats. Thrombin generation was monitored in platelet-rich PRP and platelet-poor plasma (PPP) as well as the surface of adherent VSMCs or endothelial cells (ECs) using calibrated automated thrombography (CAT) and quantified by the endogenous thrombin potential (ETP).
Results: The plasma of SHR had lower thrombin-forming capacity in response to a stimulation with 0.5 pM of tissue factor (TF) compared to Wistar rats (246 ± 16 vs. 338 ± 27 nM/min, $P = 0.007$), which was increased in the presence of platelets (594 ± 29 vs. 785 ± 51 nM/min, $P = 0.003$). This was related to lower prothrombin (58 ± 4 vs. 83 ± 4%, $P = 0.0004$) and higher tissue factor pathway inhibitor (TFPI) levels (6.9 ± 0.2 vs. 4.5 ± 0.4 U/mL, $P = 0.0002$) in SHR plasma. The addition of thoracic aorta rings to Wistar plasma pool increased thrombin generating capacity compared to 50 pM TF alone. These effects were more pronounced for SHR than Wistar rings (699 ± 19 vs. 637 ± 7 nM/min, $P = 0.007$ and 529 ± 17 nM/min for TF alone). Whereas no difference was observed for ECs, thrombin formation was higher at the surface of cultured SHR aortic SMCs than from Wistar (787 ± 5 vs. 824 ± 9 nM/min, $P = 0.003$). Exposure of negatively-charged phospholipids was higher on SHR than on Wistar rings (3.4 ± 0.4 vs. 1.9 ± 0.2 nM equivalent phosphatidylserine/g of ring, $P = 0.0007$) as well as on SMCs (747 ± 33 vs. 649 ± 222 nM equivalent phosphatidylserine/mL, $P = 0.03$). TF and TFPI activities were higher in SHR SMCs whereas no differences were observed for ECs between the two strains. In young, 5-week-old rats, before blood pressure increase, similar differences between SHR and Wistar are observed in plasma while there was no difference at the surface of aortic rings and SMCs.
Summary/Conclusion: These results show opposite thrombin generating capacity of plasma and vessel walls in SHR compared to Wistar. The higher prothrombotic phenotype of the SHR vessel wall was due to the ability of SMCs to support thrombin generation. These findings suggest that the hypertension-induced membrane phospholipid reorganization and synthesis of procoagulant molecules in SMCs provide substrates for increased thrombin formation within the vessel wall.

PA 2.17-3

Effect of hyperglycemia and hyperinsulinemia on platelet insulin signaling and coagulation pathways

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Background: Type 2 diabetes mellitus (T2DM) is a prothrombotic and proinflammatory state with elevated plasma coagulation factors and evidence of platelet and monocyte activation. We previously showed that in healthy non-diabetic subjects that infusion clamp-generated hyperglycemia (HG) and hyperinsulinemia (HI) and the combination of HG+HI increased circulating membrane-bound tissue factor-procoagulant activity (TF-PCA). In addition, markers of thrombin generation and platelet/monocyte activation were induced.
Aims: To understand the mechanisms and pathways underlying these cellular perturbations.
Methods: We performed whole genome expression profiling (U133 Plus 2.0 Affymetrix GeneChips) of platelets (leukocyte-depleted) and monocytes before and after 24 h of HG+HI infusion clamping in a healthy non-diabetic subject. HG was maintained at approximately 200 mg/dL by glucose infusion, which elevates endogenous insulin to induce HI.
Results: We defined time-dependent differential mRNA expression [24 vs. 0 h fold change (FC) ≥ 2] common to both platelets and monocytes using Partek Genomics Suite. Ingenuity Pathways Analysis revealed alterations in canonical insulin receptor signaling and coagulation pathways in both cell fractions. For example, alterations were seen in platelet expression of insulin signaling pathway genes: *IRS1* (insulin substrate receptor 1, FC (24 vs. 0 h) = 0.14), *INSR* (insulin receptor, 0.23), *GSK3B* (glycogen synthase kinase 3 beta, 5.5), *VAMP2* (vesicle-associated membrane protein 2, 14.3), *STXBP4* (syntaxin binding protein 4, 0.13), *PIK3C3* (Phosphoinositide-3-kinase class 3, 0.31) and *PTPN11* (protein tyrosine phosphatase, non-receptor type II, SHP2, 13.0). In the coagulation pathway *F3* (tissue factor, 3.7) and *TFPI* (tissue factor pathway inhibitor, 4.5) were altered. These were selected for qRT-PCR confirmation. For additional validity in platelets the 24 h sample was compared to the 0 h sample plus four normal platelet controls. Notably, eight out of the nine selected transcripts listed above were confirmed in platelets and/or monocytes. In platelets, these include ↑*GSK3B*, ↓*STXBP4*, ↑*PTPN11* in insulin signaling, and ↑*F3*

and ↑*TFPI* in coagulation. In monocytes, *STXBP4, PIK3C3, PTPN11* and *TFPI* were downregulated. Due to the importance of F3 (TF) in T2DM related cardiovascular complications, we measured TF antigen in platelets and monocytes by ELISA. TF antigen increased 2- and 5-fold over 24 h in platelets and monocytes, respectively. Further, TF-PCA in whole blood increased from 7.9 to 69.7 μ/mL. Immunoblot analyses for platelet GSKb3 (↑ approximately 34%) and PTPN11 (↑ approximately 42%) showed increases. Finally, amplification (qRT-PCR) of platelet mRNA using F3 intron 4-specific primers showed that HG+HI leads to splicing of TF pre-mRNA *in vivo*.
Conclusions: HG+HI, even in the non-diabetic state, induces demonstrable changes in platelets, including alterations in insulin-signaling and coagulation pathways, with upregulation of *GSK3B* and *PTPN11* (important in insulin signaling) and in *F3* and *TFPI* in coagulation. Upregulation of platelet *F3* (i.e. TF) is an important finding associated with upregulation of TFPI (the principal TF inhibitor) and *GSK3B,* shown to be a negative regulator of TF synthesis. Further studies in healthy subjects and DM patients will better define the pathways altered in platelets and monocytes by HG, HI and HG+HI, and leading to the prothrombotic and proinflammatory state in diabetes mellitus.

PA 2.17-4
FVII-activating protease (FSAP) confers protection to primary astrocytes and neurons exposed to oxygen-glucose deprived conditions

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Background: Factor VII activating protease (FSAP) is a circulating plasma serine protease that is activated by factors released by dead cells such as histones, nucleosomes and nucleic acids. It inactivates tissue factor pathway inhibitor (TFPI) and activates single chain urokinase thus having potential roles in both the coagulation and fibrinolytic system. FSAP antigen and activity are increased in patients with ischemic stroke and single nucleotide polymorphisms (SNP) in the gene are risk factors for stroke. In this study, we investigated the role of endogenous FSAP in brain ischemia using an *in vivo* mouse model transient middle cerebral artery occlusion (tMCAO) and *in vitro* using neuronal-astrocytic cell culture model for oxygen-glucose deprivation injury (OGD).
Aim: To analyze the pathophysiological significance of FSAP in ischemic brain.
Methods: $FSAP^{-/-}$ mice were subjected to tMCAO for 90 min and reperfusion injury was assessed after 24 h using MRI scans and neurological scores. *In vitro*, primary cortical neurons and astrocytes were isolated from E16 mice and exposed to OGD. Neurons were treated with tissue plasminogen activator (tPA)/*N*-Methyl-D-aspartic acid (NMDA) under OGD conditions to analyze excitotoxic injury. LDH release, cellular metabolic activity assay, TUNEL assays and Caspase 3/7 activity assays were carried out to analyze cell death in the presence of exogenous FSAP. Casein zymography was used to analyze endogenous urokinase (uPA) and tPA activity while gelatin zymography was performed to analyze the MMP-2 and MMP-9 activity. Total RNA was isolated and expression for BCL-2, BCL-XL, tPA, uPA, MMP's and their inhibitors was analyzed. Protein lysates were prepared and analyzed for Akt, ERK phosphorylation and BCL-2.
Results: In the mouse stroke model, no difference was observed between the $FSAP^{+/+}$ and $FSAP^{-/-}$ mice in infarct volume or neuroscore. After stroke there was a 1.4-fold elevated FSAP activity in $FSAP^{+/+}$ mice. In primary cortical neurons FSAP treatment reversed NMDA mediated excitotoxicity by approximately 50% (P-value < 0.0001). FSAP increased endogenous tPA (approximately 1.5-fold) and uPA (approximately 2.5-fold) activity while decreased MMP-9 activity (approximately 1.5-fold) in primary cortical neurons. FSAP-treated astrocytes showed decreased TUNEL positive cells (approximately 2.9-fold) (P-value = 0.0019) and decreased Caspase 3/7 activity (approximately 1.5-fold) (P-value < 0.0001). Decreased LDH release (approximately 56%) and increased cellular metabolic activity (approximately 22%) were indicative of improved cell survival in FSAP treated astrocytes. FSAP up-regulated Akt phosphorylation (approximately 2.5-fold), up-regulated MMP-2 activity (approximately 1.5-fold), increased endogenous tPA (approximately 10-fold) and urokinase activity (approximately 15-fold) in primary astrocytes under OGD conditions. FSAP increased BCL-2 and BCL-XL mRNA expression by 2-fold and 2.5-fold respectively in astrocytes exposed to OGD.
Conclusions: Endogenous FSAP does not influence infarct volumes or functional recovery in the mouse tMCAO model. On the other hand a SNP that leads to reduced FSAP activity is associated with higher stroke-related mortality in humans. In neurons and astrocytes, FSAP protects from hypoxic and excitotoxic injury. These novel results provide insight into the protective role of FSAP in ischemic stroke.

PA 2.17-5
Antithrombin deficiency increases the severity of arterial thrombosis and promotes resistance to platelet antiaggregation *in vivo*

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Background: Despite genetic risk factors for venous thrombosis are also associated with arterial complications, antithrombin deficiency has only been considered in the framework of venous thrombosis. However, the key role of thrombin in atherothrombosis and platelet reactivity sustains a relevant function in this setting for antithrombin, the main endogenous inhibitor of thrombin.
Aim: To evaluate the role of antithrombin in arterial thrombosis and its potential effect on outcomes of antiplatelet therapy in a mouse model.
Methods: Wild type (WT) and antithrombin deficient (AT+/−) mice (with 50% plasma levels of antithrombin) were treated with vehicle, aspirin (single i.p. dose of 100 mg/kg of body weight) or clopidogrel (two doses of 2 mg/kg of body weight by oral gavage). At least nine animals per genotype and treatment were used for two *in vivo* studies: $FeCl_3$ induced-thrombosis in carotid artery and tail bleeding times (BT). In the last procedure, the quantitation of blood loss during bleeding was done by estimating of total hemoglobin content as arbitrary units of absorbance (UA). Moreover, *in vitro* light transmission platelet aggregation analysis induced by collagen, ADP and arachidonic acid were also carried out.
Results: AT+/− mice showed a significantly shorter occlusion time following $FeCl_3$ injury of the carotid artery when compared to WT animals (5.01 ± 1.25 vs. 6.91 ± 2.50 min, respectively; $P = 0.025$). In contrast, both AT+/− and WT mice displayed similar BTs (< 5 min) and total blood loss as assessed by haemoglobin content. Aspirin and clopidogrel treatment inhibited platelet aggregation induced by arachidonic acid and ADP, to a similar extent in WT and AT+/− mice. As expected, aspirin and clopidogrel treatment increased bleeding tendency in WT mice (6/9 with aspirin and 7/9 with clopidogrel had BT > 10 min). Interestingly, among AT+/− mice only two out of 10 aspirin -treated and four out of nine clopidogrel-treated showed BT > 10 min. In agreement, the total haemoglobin content of the blood loosed during the BT procedure was 0.81 ± 0.27 and 1.87 ± 0.62 UA for aspirin and clopidogrel, respectively, in WT mice, compared to 0.11 ± 0.04 and 1.02 ± 0.34 for aspirin and clopidogrel, respectively, in AT+/− mice. We also observed that aspirin efficiently delayed the occlusion time of the carotid flow in the $FeCl_3$ model of-thrombosis on WT mice (no carotid occlusion in 8/9 animals), while this drug has milder effects on AT+/− mice (5/11 animals showed a patent open carotid after $FeCl_3$ injury). In contrast, clopidogrel treatment impaired thrombus formation in this model similarly in both groups.

Conclusions: Our findings in a mouse model support a relevant effect of antithrombin in both arterial thrombosis and in the efficacy of aspirin and clopidogrel. Deficiency of this key anticoagulant might increase thrombin generation under physiological or pathological activation of the clotting cascade, thus resulting in faster clot formation and reduced efficacy of current antiplatelet drugs.

PA 2.17-6

The hypercoagulable profile of stent thrombosis patients

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Background: Stent thrombosis (ST) is a devastating complication after percutaneous coronary interventions (PCI), with major clinical impact due to the high risk of myocardial infarction and death, as well as a high recurrence rate. The incidence of ST is approximately 1–5%, despite long-term dual antiplatelet therapy with aspirin and clopidogrel. The underlying pathophysiological mechanisms of ST are multifactorial. Recent studies showed that a low degree of platelet inhibition, despite antiplatelet therapy, is a risk factor for ST. Whether the coagulation system is also involved in the pathophysiology of ST is unknown.

Aim: In this study we assessed the coagulation profile of ST patients by means of thrombin generation (TG).

Methods: A single-center case-control study was performed, including 63 patients who underwent PCI with stent implantation. All subjects provided written informed consent. Cases ($n = 23$) had a history of an angiographically confirmed ST (Academic Research Consortium criteria) during follow-up. Controls ($n = 40$) had a previously implanted coronary stent but no ST $\geq$ 12 months after the index PCI. The time between the index PCI (controls) or ST PCI (cases) and venous blood collection was $\geq$ 3 months. Subjects using oral anticoagulants at the time of blood collection were excluded. TG in human platelet-poor plasma was measured using the Calibrated Automated Thrombogram (CAT) method with 0, 1, and 5 pM tissue factor (TF) triggers. Active site-inhibited FVII (ASIS) was added to determine the contribution of the contact system, and the protein C pathway was analyzed by addition of recombinant thrombomodulin (TM).

Results: TG was significantly increased in plasma from cases as compared to controls for all TF triggers (0, 1 and 5 pM TF). Results showed a significantly enhanced contact activation in cases compared to controls; peak height: 241 vs. 183 nM, time to peak: 12.1 vs. 16.1 min, velocity index: 98 vs. 63 nM/min, all $P < 0.05$ (0 pM TF trigger with ASIS). These data were confirmed by the 1 pM TF trigger assay and even at the highest TF trigger (5 pM) TG was elevated in cases compared to controls; peak height: 263 vs. 233 nM, time to peak: 5.5 vs. 6.0 min, velocity index: 101 vs. 80 nM/min, all $P < 0.05$. Inhibition of TG by addition of TM reduced the endogenous thrombin potential (ETP) with 22.6% in cases and by 30.6% in controls ($P < 0.05$), suggesting alterations in the protein C pathway for patients with ST.

Conclusions: This is the first study demonstrating the involvement of the coagulation system in ST, as well as an enhanced thrombogenic profile. Patients with a history of ST showed a shift towards a hypercoagulable state, most likely caused by enhanced contact activation and attenuation of anticoagulation by the protein C pathway, as shown by the thrombin generation results. Therefore, oral anticoagulants might be useful in reducing the recurrence rate in ST patients.

PA2.18 – Intravascular Devices and Interventions

PA 2.18-1

Survival effects of inferior vena cava filter in patients with acute symptomatic venous thromboembolism: a subgroup analysis from the RIETE registry

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Background: The effectiveness of inferior vena cava filter use among patients with acute symptomatic venous thromboembolism (VTE) and contraindication to anticoagulation remains unclear.

Methods: In this prospective cohort study of patients with objectively confirmed symptomatic acute VTE and absolute or relative contraindications to anticoagulation, identified from the multicenter, international, prospective, Registro Informatizado de la Enfermedad TromboEmbólica (RIETE registry), we assess the association between filter insertion and all-cause mortality, pulmonary embolism (PE)-related mortality, and recurrent VTE rates through 30 days after initiation of anticoagulant treatment or filter insertion. We used propensity score-matching to adjust for the likelihood of receiving a filter.

Results: A total of 40,142 patients were treated for acute VTE (20,503 with deep vein thrombosis [DVT] and 19,639 with PE). Filter-treated patients ($n = 371$) had significantly greater comorbidity and laboratory abnormalities, with a higher frequency of cancer, immobilization, recent major bleeding, anemia, thrombocytopenia, and renal failure than those not treated with a filter ($n = 39{,}771$). The 344 patients presenting with any acute VTE and treated with a filter (198 also anticoagulated) were matched with 344 patients treated without a filter (328 anticoagulated). Propensity score-matched pairs showed a trend toward lower risk of death for filter insertion compared with no insertion (6.6% vs. 10.2%, $P = 0.12$). The 30-day, risk-adjusted PE-related mortality rate was lower for filter insertion than no insertion (1.7% vs. 4.9%, $P = 0.03$). Risk-adjusted recurrent VTE rates were higher for filter insertion than no insertion (6.1% vs. 0.6%, $P < 0.001$).

Conclusions: In patients presenting with VTE and absolute or relative contraindication to anticoagulation, filter insertion was associated with a lower risk of PE-related death and a trend toward a lower risk of all-cause mortality than no filter insertion. Filter insertion was associated with a higher risk of recurrent VTE.

PA 2.18-2

Improving inferior vena cava (IVC) filter retrieval rates using wristband identification in a tertiary care and trauma centre

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Background: IVC filter use has been rising dramatically since the introduction of retrievable models but published retrieval rates are suboptimal. We conducted a prospective, quality-improvement study to increase IVC filter retrieval rates in our institution.

Aims: To improve filter retrieval rates at our institution by (i) the application of a labeled wristband to identify patients with filters (ii) educational pamphlets for patients and medical staff and (iii) referral

of patients with filter *in situ* at the time of discharge to our thrombosis clinic for follow-up.

Methods: All consecutive patients who have a filter placed by the Interventional Radiology Department at Vancouver General Hospital from November 2011 to December 2012 were approached for study enrolment within 48 h of filter insertion. Consenting patients were issued a wristband identifying them as IVC filter recipients (intervention cohort). Educational pamphlets regarding retrieval and follow-up were provided to patients and the primary care team. Data on indication for filter insertion, frequency of removal, documentation of a follow-up plan, reasons for non-retrieval, and all-cause mortality were extracted from chart review using standardized forms. Primary outcome of attempted filter retrieval rate is compared with historical data from January 2007 to December 2010 (historical cohort).

Results: During the study period, 50 of 58 patients who had retrievable IVC filters placed were enrolled. Mean age of the intervention cohort was 56 years (range 18–88 years); 57.1% were male and the mean length of hospital stay was 37 days (range 1–879 days). In the historical cohort of 275 patients, mean age was 59 years (range 14–93 years); 55.5% were male with a mean length of hospital stay of 39 days (range 3–166). The most common indications in the intervention cohort and the historical cohort for IVC filter were: a contraindication to anticoagulation (78% vs. 72.4%, respectively), high risk for pulmonary embolism (10% vs. 11.3%, respectively), and primary prophylaxis of pulmonary embolism (12% vs.14.9%, respectively). All baseline patient characteristics (age, sex), length of hospital stay, thrombotic risk factors and indications for IVC filter placement were similar between both cohorts. In our intervention cohort, 78% had a retrieval attempt, with an associated success rate of 94.7%. This was a significant improvement from the attempted retrieval rate of 60% observed in our historical cohort ($P = 0.015$). One patient in the intervention cohort and 28 patients in the historical cohort did not have an attempted retrieval despite no clear reason for the filter to remain permanent (2% vs. 10.2%, respectively, $P = 0.04$). A trend towards increased documentation of a follow-up plan was also noted (86% vs. 73.8%, $P = 0.124$).

Conclusions: In our institution, a simple intervention using a labeled wristband, provision of educational material and referral for outpatient follow-up was associated with a significant improvement in observed IVC filter retrieval rates.

PA 2.18-3

Effect of ethanol locks on occlusion of central venous catheters used for administration of total parenteral nutrition

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Background: Central venous catheters (CVCs) are widely used to administer medications and take blood samples. Long term CVC retention in patients gives a risk of catheter-related infections that can evolve serious septic consequences. To prevent infection, some clinics instill 70% ethanol into CVCs during periods between injection of drugs or sampling. Unfortunately, in our pediatric clinic, we have observed blockage in CVCs with an ethanol lock in children receiving total parenteral nutrition (TPN). This has resulted in difficult treatment management for these patients and must be overcome to optimize care.

Aim: To investigate the ability of ethanol to induce aggregation of plasma or blood in catheters used for TPN.

Methods: Initial experiments assessed effects of temperature and increasing ethanol on plasma protein precipitation (measured as loss of 280 nm absorbance in the soluble phase). Next, model CVC catheters were used for incubations in various media and conditions. Citrated blood was obtained from healthy donors with ethical consent and commercial citrated plasma was purchased. CVCs were filled with 70% ethanol and clamped to keep liquid inside. Ethanol-filled CVCs were placed in tubes of plasma and precipitates at the catheter tip collected. Precipitates were resuspended in reducing SDS sample buffer, electrophoresed on polyacrylamide gels and stained for protein. Similar experiments were performed with ethanol-containing catheters in recalcified plasma and blood ± recalcification. Finally, tests were done whereby CVCs were loaded with ethanol containing a small residue of TPN at the catheter tip. Aggregates that were blocking CVC ports in patients were examined to compare with precipitates in the model CVC experiments.

Results: Ethanol-containing CVCs gave plasma protein precipitation that was directly affected by temperature. As expected, increasing ethanol content in plasma gave greater protein precipitation up to a plateau level. In plasma, ethanol-containing CVCs caused slow induction of protein aggregates at the tip. Gel analysis of these precipitates revealed a number of protein bands, including fibrinogen chains. Similar experiments with ethanol-loaded CVC's in citrated blood yielded the same protein bands as with plasma plus some strong low molecular weight species. When calcium was added so plasma or blood samples could clot, only minor differences in bands of proteins precipitated by CVCs with ethanol were observed relative to those of non-recalcified plasma or blood. The presence of TPN residue at the tip of CVCs with ethanol had little additional effect on aggregation and clotting for citrated plasma or blood and recalcified plasma. However, massive clots that strongly adhered to the CVC were induced by TPN with ethanol-loaded catheters in recalcified blood. Comparisons confirmed that banding in gels loaded with samples from patient catheters most closely resembled that from TPN-coated ethanol-containing CVCs in recalcified blood.

Summary/Conclusions: We have shown that the combination of TPN and ethanol induces dense, adhering clots that mimic the occluding aggregates found in catheters of patients receiving TPN that have an ethanol lock. Given the seriousness of CVC function loss, we recommend consideration of a change in protocol for ethanol locks in CVCs delivering TPN.

PA 2.18-4

A multidisciplinary pulmonary embolism response team (PERT). Initial clinical experience at Massachusetts general hospital

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Background: The optimal care of patients (pts) with severe pulmonary embolism (PE) involves several specialties. In order to provide timely, organized, evidence-based recommendations and therapy, a multidisciplinary team is required. We established an innovative Pulmonary Embolism Response Team (PERT), with an infrastructure for rapid, multidisciplinary consultation and to facilitate comparative-effectiveness research. Understanding the types of cases seen by our PERT will help other specialists assess the impact of creating similar teams elsewhere.

Methods: The MGH PERT includes specialists in cardiology, emergency medicine, pulmonary/critical care, interventional radiology, vascular medicine and thoracic surgery. We launched the PERT Oct. 22, 2012 and asked clinicians who diagnosed massive or submassive PE to call a 24-h emergency number to activate the PERT. Upon activation, PERT members used commercially available online meeting software (GoToMeeting®) to discuss the case, view data and radiologic images and develop a treatment plan. For each case, prospective clinical data are collected in a research registry. For the current report, we describe our initial 12 week experience with team activation (10/22/12–01/12/13), including demographics, clinical characteristics, treatments and

outcomes. Descriptive statistics (means and proportions) were analyzed using SAS v9.2.

Results: *Activations:* In 12 weeks, there were 30 unique PERT activations, plus two duplicate activations. Twenty seven (90%) occurred during day or evening hours. Most (17, 57%) originated in the emergency department, 7 (23%) in intensive care units and 6 (20%) on hospital floors. Median time from activation to PERT meeting was 54 min.

Patients: Mean age was 57 ± 17 years and 19 (63%) were male. Comorbid illness included: cardiopulmonary disease (n = 10, 33%), recent trauma or surgery (n = 8, 26%); cancer (n = 8, 26%); prior venous thromboembolism (n = 3, 10%). Twelve (40%) patients had a contraindication to thrombolysis or high-risk of bleeding.

PE Severity: Twenty-five (83%) pts had diagnosed PE, 5 (17%) had suspected but unconfirmed PE. Two (6%) were in cardiac arrest. Seven (28%) PE were saddle, 8 (32%) main pulmonary artery, 9 (36%) lobar, and 1 (3%) segmental. Twenty (80%) were bilateral. Sixteen (64%) pts with PE had elevated troponin-t and 13 (52%) had elevated NT-proBNP. Twenty (80%) had right heart strain on echocardiography or computed tomography. Eight (32%) were hypotensive (< 90 mmHg) and 9 (36%) were intubated. Sixteen (64%) had concomitant deep vein thrombosis.

Treatment and Outcomes: After consultation, the PERT considered 5 (20%) PE low risk, 18 (72%) submassive and 2 (8%) massive. Twenty-three (92%) pts received unfractionated heparin, 1 (4%) enoxaparin, and 1 (4%) no anticoagulation. Two (8%) pts had catheter directed thrombolysis and 5 (20%) had a vena cava filter placed. One (4%) pt had hematuria after anticoagulation. Seven (23%) pts overall and 3 (12%) with confirmed PE died prior to hospital discharge.

Discussion: We describe an innovative multidisciplinary Pulmonary Embolism Response Team (PERT). Our initial experience suggests that the PERT was activated frequently and appropriately. Most pts had massive or submassive PE, suggesting clinicians are highly efficient at selecting pts with severe PE. The PERT provided recommendations in < 1 h in most cases.

PA 2.18-5

CHA2DS2-VASc score and risk for reobstruction after endovascular treatment of the superficial femoral artery: differences between balloon angioplasty and stenting

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Background: The CHA_2DS_2-VASc (congestive heart failure, hypertension, age > 75 years (doubled), type 2 diabetes, previous stroke, transient ischemic attack, or thromboembolism (doubled), vascular disease, age 65–75 years, and sex category) score was published as a predictive scoring model for stroke in atrial fibrillation patients. As multiple vascular risk factors are included in this score we evaluated the occurrence of reobstruction after endovascular treatment (percutaneous transluminal angioplasty (PTA) and stent) of the superficial femoral artery (SFA) in peripheral arterial occlusive disease (PAOD) patients according to their CHA_2DS_2-VASc score independent of a coexisting atrial fibrillation.

Methods: We evaluated 773 PAOD (529 PTA and 244 stent) patients treated at our institution from 2005 to 2010. CHA_2DS_2-VASc score was calculated and the occurrence of a symptomatic reobstruction during a median follow up of 58 months was investigated. Furthermore all constituents of the score were individually investigated concerning their association with reobstruction.

Results: In PTA patients reobstruction rate increased with increasing CHA_2DS_2-VASc Score (P = 0.009). Arterial hypertension was associated with an increased risk for reobstruction (OR 2.4; 95% CI 1.7–3.4), as was type 2 diabetes (OR1.9;95%CI1.5–2.3). In stent patients reobstruction rate was high, but independent of the CHA_2DS_2-VASc Score (P = 0.5). Its constituents were not associated with an increased risk for reobstruction.

Conclusion: A high CHA_2DS_2-VASc score was associated with a high risk for reobstruction after PTA of the SFA. On the other hand the reobstruction risk in stent patients was high but independent of the CHA_2DS_2-VASc score, indicating that the pathophysiology of instent restenoses is different from reobstruction after PTA.

PA 2.18-6

Longitudinal investigation of the effect of centrifugal continuous flow left ventricular assist devices (cfLVADS) on haemostatic parameters

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Background: Patients implanted with cfLVADS routinely receive aspirin and/or clopidogrel and anticoagulation. Therapy related bleeding is therefore common, however may also reflect changes secondary to shear forces arising from the implanted device.

Aims: This study aims to assess changes to haemostatic parameters in patients with cfLVADS in order to determine if they can ultimately predict clinical bleeding.

Methods: Twenty-eight patients (24M/4F), implanted with HeartWare cfLVAD were recruited. Baseline blood samples were collected pre-implant and at 1, 7, 30, 90 and 180 days post-implant. Platelet counts were measured by routine methods. Soluble P-selectin (sP-Selectin), soluble GPVI (sGPVI), von Willebrand Factor Antigen (vWF-Ag) and vWF Collagen Binding Activity (vWF-CBA) were measured using ELISA. Microparticle procoagulant phospholipid activity was measured using the Procoag-PL assay. Platelet aggregation was measured using a Multiplate Impedence Aggregometer and platelet function analysis performed using a PFA-100.

Results: Compared to baseline, median platelet counts were significantly lower at day 1 (206 ± 16–159 ± 15, $P < 0.001$), and higher at 30 and 90 days (301 ± 27 × 10^9/L, $P < 0.001$ and 257 ± 16 × 10^9/L, $P < 0.001$ respectively). sP-Selectin levels were significantly elevated at day 1 (29.1 ± 1.9–35.9 ± 2.9 ng/mL) but were not significantly elevated on subsequent days. sGPVI levels were persistently elevated above reference ranges for all timepoints (including baseline). vWF-Ag levels were significantly lower at days 30, 90 and 180 in comparison to baseline (239 ± 18–184 ± 14%, P = 0.024, 165 ± 13%, P = 0.003 and 164 ± 19%, P = 0.001, respectively). vWF-CBA was also significantly decreased at day 1 when compared to baseline (225 ± 18–187 ± 14%, P = 0.003) and remained decreased at all timepoints up to 180 days (99 ± 18%, $P < 0.001$). This corresponded to a decreased vWF ratio (CBA:Ag) for up to 90 days post implant. vWF multimers are pending. Closure times for the PFA-100 were significantly prolonged from day 1 and aggregation to ADP and arachidonic acid was decreased, which was consistent with dual antiplatelet therapy, however further Multiplate testing revealed significantly decreased aggregation to collagen at days 7, 90 and 180 and to ristocetin from day 1 onwards. Microparticle procoagulant activity was significantly increased at day 90.

Summary/Conclusion: Our data shows a persistent elevation in sGPVI levels and decline in vWF-Ag, vWF-CBA and vWF ratio (CBA:Ag) over time in patients supported with HeartWare cfLVAD. Interestingly, levels of sP-selectin are not increased. Platelet aggregation is impaired in addition to that due to the effects of anti-platelet therapy. Overall, these changes may increase the risk of bleeding in these patients and likely reflect the effects of shear forces from the device itself. Microparticle procoagulant activity was increased at only one timepoint – the significance of this finding and its contribution to the overall haemostatic state of these patients remains to be determined.

PA2.19 – Paediatric Thrombosis II

PA 2.19-1

Antiphospholipid antibody syndrome in monozygotic twins

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Background: Antiphospholipid antibody syndrome (APLAS) is an autoimmune disorder defined by continued presence of antiphospholipid antibodies (aPLa) in association with thrombosis or pregnancy loss. APLAS has a low prevalence in pediatrics. Compared to adults, the condition is unique due to developmental hemostasis, high rates of aPLa related to infections, different laboratory normal values, and prevalence of underlying diseases. In addition, the rarity of teen presentation of thrombosis often leads to delayed diagnosis. APLAS has not been previously described in pediatric monozygotic twins. There is only one reported case in adult monzygotic twins.
Aims: To describe APLAS in pediatric monozygotic twins with similar case presentations.
Methods: Chart reviews were performed to obtain clinical data. A literature review of pediatric APLAS and familial antibody syndrome was performed. Informed consent and ethics review was obtained.
Results: Twin A, 15 year old previously well male presented to multiple healthcare sites with a history of a painful and swollen right arm prior to diagnosis. Family history was significant for a great grandfather with early onset (< 30 years) arthritis. Doppler ultrasound revealed a thrombus in the right subclavian, axillary, and lateral brachial vein. VQ scan demonstrated mismatched perfusion in the right lower lobe. Laboratory Results INR 1.6 (Normal range (NR) 0.8–1.2), PTT 93 s (NR29–43 s) D-Dimer 0.57 mg/L, NR (< 0.5 mg/L), Cardiolipin 74GPL (NR 0–14GPL) at presentation, 85GPL and 83GPL and 3 and 6 months, respectively. Lupus Anticoagulant was positive as per dilute venom viper test on presentation and at 6 months. ANA, anti-centromere antibodies, anti-dsDNA, ENA, ANCA were negative and IgG, IgM, and IgA were normal.
He was commenced on anticoagulation. He had visual disturbances and was diagnosed with transient ischaemic attacks (MRI brain normal) and started on aspirin.
His asymptomatic twin, Twin B, had cardiolipin antibodies of 22GPL and a positive lupus anticoagulant.
Six months later, Twin B presented clinically with a painful, swollen right calf. He was assessed and diagnosed immediately as the presence of aPLa was previously confirmed. Doppler ultrasound demonstrated a right superficial femoral to popliteal vein thrombus. VQ scan revealed bilateral pulmonary emboli. His laboratory investigations demonstrated a D-dimer 3.66 mg/L, APCR 3 (NR ratio > 2.3), Protein C 1.4 U/mL, Protein S 1.02 U/mL, Factor V Leiden and Prothrombin G20210A mutation not detected, Cardiolipin 14GPL, Lupus anticoagulant positive on repeat testing. He was commenced on anticoagulation. Twin B had the similar visual disturbances and was also started on aspirin.
Both patients had no evidence of vasculopathy.
Summary: This is the first case report describing monozygotic pediatric twins with primary APLAS. The first adult case report was recently published. The combination of these 2 reports demonstrates rationale for screening of monozygotic twins if one is diagnosed with APLAS.

PA 2.19-2

In vitro assessment of the effect of dabigatran on thrombosis of adult and neonatal plasma using thromboelastography

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Background: Thromboelastography (TEG) is a global assay used for evaluating features of clot formation. Although its clinical use is limited, TEG has long been used to assess thrombosis *in vitro*. Dabigatran is an anticoagulant that acts as a reversible direct inhibitor of thrombin. Specific clotting tests have shown a linear relationship between dabigatran plasma concentration and its effects. However, none of these studies used a global assay, such as TEG, to demonstrate this relationship in neonates. Neonatal hemostasis differs from adults. Specifically, most coagulation factors & natural anticoagulants at birth are < 50% of adult levels and there are several structural and functional differences. The effect of varying dabigatran concentrations on characteristics of clotting in neonates compared to adults is unclear.
Aims: To compare the clotting profile of neonatal and adult platelet poor plasma when exposed to different concentrations of dabigatran
Methods: Adult pooled plasma in buffered citrate was purchased from Affinity Biologicals (Ancaster, ON, Canada). Neonatal cord blood was collected from placentas of healthy full term newborns using citrated syringes. The study was IRB approved and informed consent was obtained prior to collection. Platelet-poor plasma was isolated, pooled and frozen. Prior to experiment, plasma was thawed and filtered (using a 0.2 μm filter) to remove microparticles. Optimal tissue factor (TF) levels were predetermined and corn trypsin inhibitor (CTI) was used to inhibit the contact coagulation pathway. Dabigatran was prepared by chemical conversion of the pro-drug. Its structure & activity was verified via standardized testing. Experiments were performed as follows: 30 μL reaction mixture of 0.8 M $CaCl_2$, 30 μg/mL CTI, 2 pM TF and dabigatran (0, 40, 110, 180, 250 and 320 ng/mL) in imidazole buffer (pH = 7.4) was mixed with 330 μL of plasma in a TEG cup. Four fundamental TEG parameters, R, K, α and Maximum Amplitude (MA), were measured for a maximum of 180 min with Haemoscope TEG 5000. Scanning electron microscopy (SEM) was performed to evaluate fibrin clot structure for both neonatal and adult plasma, in the presence and absence of dabigatran.
Results: Without dabigatran, time to clot initiation (R) was similar between neonatal and adult samples. There was a significant delay in clotting of neonatal samples with increasing dabigatran concentrations ($P < 0.05$). Speed of clot strengthening (α) in both samples without dabigatran also showed no difference, whereas neonatal samples with dabigatran were > 4-fold slower in clotting ($P < 0.05$). Additionally, while there were no differences in maximum clot strength (MA) without dabigatran, neonatal samples with dabigatran were weaker ($P < 0.05$), especially at higher dabigatran concentrations. SEM showed minimal differences between neonatal and adult fibrin clot structure in the absence of dabigatran. However, with dabigatran, both neonatal and adult clots contained less small-diameter fibers, thicker large-diameter fibers and greater porosity. Moreover, these dabigatran-induced differences were intensified in neonatal clots.
Conclusions: Neonatal plasma clotting with dabigatran has slower onset, slower speed and weaker clots that are more porous, compared to adult plasma clotting. Thus, neonatal plasma may be more sensitive to dabigatran as assessed by our *in-vitro* TEG study.

PA 2.19-3

A deep look at superficial vein thrombosis in hospitalized children

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Background: Superficial vein thrombosis (SVT) has been studied in adults and there is scarce information about this condition in children.
Aims: To report the spectrum of SVT in hospitalized children.
Methods: Retrospective chart review of children diagnosed with a radiologically-proven SVT between January/2007 and October/2012 at The Hospital for Sick Children, Toronto. Research Ethics Board was obtained. Descriptive and Fisher's exact statistics were utilized.
Results: Eighty-six patients 0–18 years ([median: 12.2 years; IQR: 4.0–15.2]; M/F: 1.2/1]), had 101 SVTs identified. Of 101 SVTs, 54/101 (53%) were exclusive SVT. In those the most common underlying conditions included infection/sepsis [(29%), local infection (n = 5)], cancer (29%), surgery [(29%), post-liver transplant (n = 6)], systemic inflammation (11%) and trauma (11%). The most frequent identified risk factors were catheter-related issues [41/54, 76%: peripherally intravenous inserted central catheters (PICC: 22/41), peripheral intravenous (14/41), catheter insertion attempts (3/41), central venous line (2/41)], and TPN (7/54, 15%). Distribution encompassed upper extremity veins [(46/54, 85%): basilic (33/54), cephalic (11/54), antecubital (2/54)], or lower extremity veins [(8/54, 15%): greater saphenous (5/54), lateral marginal (1/54), other (2/54)]. Initial clinical findings were swelling (72%), tenderness/pain (44%), redness (39%), phlebitis (31%), warmth (20%), and line malfunction (7%). Time between initial clinical findings and imaging was median 0 days [IQR: 0–1].
Treatment modalities included, in isolation or combined, unfractionated heparin (UFH)/low molecular weight heparin (LMWH) (27/54), antibiotics (15/54), catheter-removal (11/54), no therapy (9/54), aspirin/NSAID's (9/54), warfarin (1/54), and mixed (12/54). Radiological follow up was available in 51/54 (94%) (median f 85.5 days [IQR: 20–184]). Radiological resolution rates were: total (35%), partial (13%), unchanged (33.5%), progression (11%), missing (7.5%). SVT progression documented radiologically occurred in six patients, while on no therapy (n = 2), full dose warfarin (n = 1), full dose (n = 1) or prophylaxis (n = 1) UFH/LMWH or antibiotic alone (n = 1); none developed deep vein thrombosis (DVT). There was no significant difference in radiological resolution amongst treatment modality (P = 0.23).
47/101 (47%) SVTs occurred concomitantly with a DVT: 39/47 (83%) were treated with UFH/LMWH (median duration: 109 days [IQR: 89–194]) with complete radiological resolution in 29/39 (74%), partial in 5/39 (13%), unchanged status in 3/39 (8%), and unknown in 2/39 (5%). In the group of SVT without (17/54) and with concomitant DVT (39/47) treated with UFH/LMWH alone, the radiologic resolution rates for the composite total/partial resolution were 6/17 (35%) vs. 34/39 (87%), respectively (P = 0.001). There were two patients with documented radiological SVT progression on: a) prophylaxis UFH (n = 1); and b) full dose LMWH (n = 1).
Summary/Conclusion: Image-proven SVT in hospitalized children is commonly associated with lines, and almost half concurrently with a DVT. Currently, a variety of therapies are used to treat hospital-based SVT in children and a few underlying conditions may particularly predispose patients to those events (i.e. liver transplantation). While progression to DVT may be rare, the role of SVT-related therapy remains to be defined. Further prospective paediatric studies are required to better understand the true impact of this entity in children.

PA 2.19-4

Portal vein thrombosis in neonates: results of an anticoagulation protocol

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Background: Portal vein thrombosis (PVT) is a common thrombotic event in neonates. The majority of patients will have resolution of thrombus with a minority going on to develop complications such as portal hypertension. Due to the scarcity of published studies, the role of anticoagulation (ACT) in neonatal PVT is unclear.
Aims: The aim was to describe the treatment and outcomes following portal vein thrombosis in a neonatal cohort treated according to an institutional anticoagulation protocol.
Methods: The study was approved by the hospital research ethics board and written consent waived. A retrospective chart review of neonates presenting with PVT to the Hospital for Sick Children from January 2008 to September 2010, identified from the clinical thrombosis database, was conducted. All patients were < 30 days at the time of diagnosis and treated according to the institutional protocol for anticoagulation for neonatal portal vein thrombosis. In the protocol, neonates with non-occlusive PVT are not treated with ACT unless thrombotic extension occurs or there is a concomitant other indication for ACT, and neonates with occlusive PVT without contraindication to ACT are treated. Clinical and radiologic data were collected. Poor outcome was defined as portal hypertension, hypersplenism, liver atrophy on follow-up. Descriptive statistics and Fisher exact testing were completed to compare the neonates by presenting features, treatment and outcome.
Results: There were 94 patients identified. The mean age (± SD) was 10 days (± 5). The mean gestational age was 35 weeks (± 5). Fifty-three (56%) patients received ACT and 41 (44%) patients did not receive ACT. Of the patients treated with ACT 6/53 (11%) received standard heparin, 34/53 (64%) received low molecular weight heparin and 13/53 (25%) received both standard and low molecular weight heparin. Follow-up occurred for a mean of 344 days (± 315). ACT was continued for a mean 41 days (± 55). Complete resolution of thrombus occurred in 48/94 (51%), partial resolution in 15/94 (16%) and progression in 25/94 (27%). Progression of PVT was associated with initial non-occlusive thrombus occurring in 15/36 (42%) of patients with non-occlusive thrombus vs. 9/57 (16%) of patients with initial occlusive thrombosis (P = 0.008). Thrombosis involving the inferior vena cava (IVC) was associated with thrombotic progression occurring in 8/12 (67%) of patients with IVC involvement compared to 16/83 (67%) of patients without IVC involvement (P = 0.001). There was no association of anticoagulation with thrombus resolution or decreased rates of poor outcome. Liver atrophy occurred in 25/94 (27%). Portal hypertension or hypersplenism occurred in 6/94 (6%) patients. Major bleeds occurred in 7/53 (13%) of patients who received ACT.
Conclusions: The majority of neonates had a good outcome, and anticoagulation was not associated with decreased rates of poor outcome in this cohort of patients treated according to an institutional protocol for neonatal PVT. Prospective studies may identify a subset of neonates who benefit from anticoagulation.

PA 2.19-5

Clinical presentation and molecular basis of congenital antithrombin deficiency in children: a cohort study

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Background: Antithrombin (AT) deficiency is a rare autosomal-dominant thrombophilia. The natural history of AT deficiency in children is

poorly understood. Furthermore, the genotype-phenotype correlation in AT deficiency remains debatable.

Aims: To describe the clinical manifestation and molecular basis of congenital AT deficiency in children managed at the Hospital for Sick Children (HSC) over a 13-year period.

Methods: The study was approved by the HSC research ethics board. Pediatric patients (ages 0–18 years) diagnosed with congenital AT deficiency between January 1, 2000 and December 31, 2012 were identified from the thrombosis database at HSC. Clinical and laboratory characteristics were abstracted from the medical records. Identified patients were prospectively solicited for antithrombin (*SERPINC1*) gene sequencing. Written consent was obtained. Genomic DNA was extracted from EDTA-anticoagulated blood. The *SERPINC1* gene was analyzed by PCR followed by sequencing of both DNA strands of the entire coding region and the highly conserved intron-exon splice junctions. Reflex multiplex ligation-dependent probe amplification analysis was done to detect deletions or duplications. Descriptive statistics were used to summarize parameters [Mean (± SD)]. Baseline characteristics were tested for an association with venous thromboembolism (VTE) using t-test and Fisher's exact test.

Result: Twenty five patients (10 females and 15 males) from 16 unrelated pedigrees were identified. Mean age at diagnosis was 9.2 (± 6.2) years. Eight patients were diagnosed after VTE [mean age at diagnosis: 8.7 (± 8.3) years] and 17 patients were diagnosed secondary to a family history of AT deficiency [mean age at diagnosis: 9.4 (± 5.2) years]. Mean duration of follow up for the cohort was 4.7 (± 4.4) years. Of 18 subjects who underwent additional thrombophilia testing, one was found to be heterozygous for prothrombin gene (PG) mutation. Most recent mean AT-activity (n = 25) and AT-antigen (n = 17) were 0.53 (± 0.08) and 0.58 (± 0.12) IU/dL respectively. Of the eight patients with VTE, seven had additional risk factors (three anatomical anomalies, two neonatal age, one oral contraception, one dehydration, one immobilization, one PG mutation). All patients with VTE were treated with anticoagulation and two required supplemental antithrombin-concentrate. Four patients were left on long term anticoagulation (three with anatomical anomalies and one with concurrent PG mutation). No patient has had recurrent VTE with 2.2 (± 2.6) years of follow up. None of the 17 patients diagnosed secondary to a positive family history have had VTE, with 5.8 (± 4.8) years of follow up. Mutation analysis has been completed on 15 subjects from 13 pedigrees. Eight mutations (including four novel mutations) were identified: four on exon 7, three on exon 5 and one on exon 2 of the *SERPINC1* gene. Deletions, frameshift and nonsense mutations had a greater association with VTE as compared to missense mutations (P = 0.007).

Summary/Conclusion: In our institutional cohort of pediatric AT deficient patients, there were often additional risk factors at the time of VTE. Patients with congenital AT deficiency, who developed VTE in the presence of transient-acquired risk factors were taken off anticoagulation after treatment of acute event, with no recurrence. The genotype-phenotype correlation observed in this study needs validation in larger cohorts.

PA 2.19-6

Characteristics and long-term outcome in children with essential thrombocythemia: an analysis of 61 cases from a single Chinese center

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Background: Essential thrombocythemia (ET) is a rare thrombotic disorder in pediatric patients, with a risk of progressing to myelofibrosis and leukemia.

Aims: In this retrospective study, we evaluated the clinical and laboratory features and long-term outcome in pediatric patients with ET at our center.

Methods: We reviewed 61 pediatric patients (age ≤ 18 years) who were consecutively diagnosed with ET between July 1983 and October 2012. This study was approved by the hospital based medical ethic committee. Informed consent was obtained from the guardians of the patients.

Results: The median age was 12 years (range 2–18 years), with 41 males and 20 females. Microcirculatory and systemic symptoms occurred in 43 cases (70.5%), of which 3 (4.92%) showed transient syncope. Hepatosplenomegaly was found in 34 patients (55.7%). Minor bleeding occurred in nine cases (14.8%), but none suffered from major bleeding or thrombosis either at diagnosis or during follow-up. JAK2 V617F mutation was analyzed in 41 patients, with nine positive (22.0%) and 32 negative (78.0%). There were no significant differences in white blood cell (P = 0.925), neutrophil (P = 0.969), hemoglobin (P = 0.930), platelet count (P = 0.529), microcirculatory disturbance (P = 0.556), systemic symptoms (P = 0.204) or hepatosplenomegaly (P = 0.402) at diagnosis between JAK2 V617F positive and negative cases. Platelet count was not statistically related to hepatosplenomegaly (P = 0.930) or bleeding (P = 0.222), but positively related to microcirculatory disturbance (P = 0.002). First-line treatment included platelet apheresis (six cases, 9.8%), antiplatelet agents (35 cases, 57.4%), interferon (32 cases, 52.5%) and hydroxyurea (41 cases, 67.2%). Clinical intolerance to hydroxyurea was found in two patients, reversible liver enzymes increased in four cases and skin ulcer in one patient. The median follow-up was 30.5 months (range 1–180 months). None of the patients died. Two patients (3.28%) progressed to overt myelofibrosis, but none developed secondary hematological or non-hematological malignant tumors.

Conclusions: The mutated rate of JAK2 V617F is lower in children than in adults. Most pediatric ET patients have microcirculatory disturbance and the risk is elevated as platelet count increases. Transient syncope is not rare in pediatric patients. The side effects of hydroxyurea should be closely monitored. Major thrombosis and bleeding events are extremely scarce, but some may progress to myelofibrosis. The underlying pathogenesis is to be further studied to develop targeted therapy in the future.

PA2.20 – Thrombophilia II

PA 2.20-1

Thromboelastometric parameters heritability and their relation with thromboembolic disease. Results from GAIT-2 project

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Background/Aims: Thromboelastometry (TEM) can provide comprehensive information on the mechanism of clot formation. At present, it is useful for diagnosing hemorrhagic diseases and for the therapeutic use of blood products in transfusion. We investigated the utility of TEM parameters as intermediate phenotypes that might correlate with the risk of thrombosis. With any complex phenotype, usually the first step is to calculate the heritability (estimate of the genetic contribution to the observed variability of the phenotype). The next step is to determine the role of environmental covariates, and the potential relation with thrombophilia.

Methods: The GAIT-2 (Genetic Analysis of Idiopathic Thrombophilia) project included a total of 1113 individuals, belonging to 35 extended pedigrees recruited through a propositus suffering from idiopathic venous thrombosis. A total of 175 subjects suffered from thrombotic disease; 101 of them had venous and 93 arterial thrombosis (19 suffered from both types). The ages ranged from 2 to 101 years old. A TEM test was performed in 935 individuals without adding any exogenous inducer of coagulation (NATEM test). We used the 4-chanels ROTEM^R device (Pentapharma, GmbH, Munich, Germany). We analyzed the following phenotypes: CT (clotting time), CFT (time to a

clot firmness of 20 mm), alpha angle (measured between the midline of the tracing and a line drawn from 1 mm wide point tangential to the curve), MCF (maximum clot-firmness) and MCF-t (time to MCF).
The analyzes used the Sequential Oligogenic Linkage Analysis Routines (SOLAR). This program allows the partitioning of the total variance, into the variance due to genetics (heritability) and the environmental effects. SOLAR also allows the analysis of the correlation with the risk of thrombosis. Age, sex, smoking and oral contraceptives were analyzed as covariates.
Results: The heritability of thromboembolic disease (including venous and arterial thrombosis) was 46% ($P = 0.00009$). Age was the only covariate that significantly influenced the risk of disease. The TEM phenotypes heritabilities were 12%, 13%, 24%, 34% and 37% of the total variance for CFT, alpha angle, MCF, CT and MCF-t, respectively. All of them had statistical significance. Age, sex, contraceptives and smoking influenced on the TEM parameters. In addition, CFT, alpha angle and MCF showed a strong absolute value of genetic correlation [rhoG of 0.88 ($P = 0.006$), 0.89 ($P = 0.012$) and 0.51 ($P = 0.026$), respectively] and environmental correlation [rhoE of 0.44 ($P = 0.0009$), 0.48 ($P = 0.004$) and 0.44 ($P = 0.0008$), respectively] with the risk of disease.
Summary/Conclusions: We detected genetic factors that influence both, the risk of thrombotic disease and the TEM parameters. Also, we determined the influence of environmental effects on the TEM phenotypes. These results confirm the hypothesis that the parameters of the TEM are influenced genetically, and that they can be considered as intermediate phenotypes in the search for genes that influence thrombotic risk

PA 2.20-2
Evaluation of a genetic analysis service for antithrombin deficiency: experience of Sheffield diagnostic genetics service

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Background: Antithrombin (AT) is a natural anticoagulant that inhibits thrombin and factor Xa (FXa). Inherited autosomal dominant AT deficiency occurs in up to one in six hundred of the population and may increase risk of venous thrombosis by 10–50 fold. The activity of AT as an inhibitor of thrombin and FXa is enhanced over a thousand fold through binding to heparin.
Sheffield Diagnostic Genetics Service (SDGS) has offered a service for the investigation of antithrombin deficiency since 2006 providing both identification of point mutations and large deletions and duplications.
Aims: To evaluate mutation detection rate, mutation spectrum and the profile of mutations in types I and II AT deficiency among index cases (IC) referred to SDGS for confirmation of heritable antithrombin deficiency.
Methods: Genomic DNA was extracted from peripheral blood and individual exons of the *SERPINC1* gene amplified and sequenced. Multiplex ligation-dependent probe amplification (MLPA) was performed, when requested, where point mutations were not identified.
Results: Samples were received over 7 years from 117 families. Of these, point mutations were identified in 77 (66%) of the IC. MLPA was requested in only 10 of the 40 IC where no point mutation was identified and large deletions were identified in two (2% of cohort): a deletion of exons 3–6 and of exons 1–6 (exons numbered sequentially from exon 1), both in patients with type I deficiency.
A wide range of point mutations was identified, both previously reported and novel, with ten mutations identified in more than one IC. Of 79 cases with mutations, 15 (19%) were heterozygous for the p.Ala416Ser mutation (Type II(RS), Cambridge II, legacy numbering A384S), 4 (5%) each for p.Pro73Leu (II(HBS), Basel, P41L), p.Ala414Thr (II(RS), Hamilton, A382T) and p.Asn437Lys (II(PE), La Rochelle, N405K) and 3 (4%) for p.Met283Val (II(RS), M251V). Four further mutations were found in 2 (2.5%) cases each. Two were novel (p.Glu227Lys and c.400delC) and two previously reported (p.Glu227* and c.1154-14G>A). Lastly, two cases of homozygosity for p.Leu131Phe (II(HB), Budapest III, L99F) were identified.
Twenty-five patients had 23 novel mutations including five small deletions, an insertion, acceptor splice site mutation and 14 missense mutations; notable mutations were p.Met1?, affecting the methionine initiation codon, a new heparin binding defect (p.Arg164Leu) and a substitution with pleiotropic effects (p.Val447Asp).
Analysis of AT activity (AT:Ac) and antigen (AT:Ag) levels in IC where levels were supplied by referring clinicians revealed that 33 patients without mutations identified had mean AT:Ac of 68 IU/dL and AT:Ag of 73 IU/dL. This compared with 15 patients with null mutations, mean AT:Ac of 48 IU/dL and AT:Ag of 51 IU/dL and 15 patients with AT Cambridge II, mean AT:Ac of 76 IU/dL and AT:Ag of 103 IU/dL.
Summary/Conclusion: Mutations were identified in 68% of cases referred to SDGS for investigation confirming the cause of their deficiency. The spectrum of mutations included a high proportion of both novel and common mutations. The low rate of follow-up requests for MLPA may result in large deletions or duplications not being identified; such mutations have been reported in up to 10% of patients with antithrombin deficiency.

PA 2.20-3
Who is being screened for hereditary and acquired thrombophilia? A single center experience

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Background: Testing for thrombophilia is expensive and time-consuming, and the results have an uncertain role in formulating treatment decisions. Evidence-based and expert opinion criteria for thrombophilia testing are available, but we observe that thrombophilia testing at our institution is frequently performed independent of these guidelines and may not add value to patient care. The aim of our study is to assess the proportion of thrombophilia testing at our institution that is appropriate according to published guidelines and to determine the impact of abnormal test results on medical decision making.
Methods: We reviewed the medical literature for evidence-based and expert opinion guidelines and used these data to synthesize a set of indications for thrombophilia testing. We then retrospectively reviewed all thrombophilia test panels conducted at Dartmouth-Hitchcock Medical Center between January 1 and December 31, 2006, the first year for which complete data are available, to determine if testing was consistent with contemporaneous guidelines. We used our electronic medical record to review patient data to assess testing appropriateness based on these guidelines and to determine what proportion of treatment decisions were altered by abnormal test values. Results are presented descriptively using median values and ranges and percentages where appropriate.
Results: During the study period, 479 patients underwent screening for hereditary and/or acquired thrombophilia. Most were female ($N = 313$; 65%), and the median age was 45 years (range 1 month–87 years). Based on a loose interpretation of guidelines available at the time, we identified 147 patients with a personal history of venous thromboembolism (VTE; 31%), 77 patients with pregnancy complications (16%) and 26 individuals with a family history of VTE or hereditary thrombophilia (5%) in whom we judged testing for thrombophilia to possibly be appropriate. Inappropriate testing was performed in 120 patients with a personal or family history of arterial thrombosis or atherosclerosis (25%) and in 109 (23%) with conditions for which an association with thrombophilia is either unlikely or irrelevant (e.g., confusion, bleeding, seizures, headache) or to determine a pre-test probability of a thrombotic event at the time of presentation with acute symptoms (e.g., rule out pulmonary embolism in a patient with dyspnea). At least

one abnormal test was identified in 176 subjects (37%). Of the abnormal test results, only 9 (5%) were repeated to confirm the abnormality, and four of these were subsequently normal. We identified 16 patients with an abnormal test result whose medical management was then altered, accounting for 3% of the total study population.

Conclusion: Even when applying a flexible interpretation of testing guidelines, we discovered nearly half of all thrombophilia testing at our institution may be inappropriate. Our data suggest that abnormal test results rarely impact treatment decisions for individuals who have undergone testing. Moreover, we identified the potential for misdiagnosis of thrombophilia based on a one-time spurious test value. Our results expose a void and point to a need for increased clinician education regarding indications for and limitations of thrombophilia testing and offer a rationale for placing restrictions on ordering thrombophilia test panels.

PA 2.20-4

Role of ABO blood group and of thrombophilic abnormalities on the presence of residual vein obstruction after deep-vein thrombosis of the lower limbs

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Background: The presence of residual vein obstruction (RVO) has been consistently associated with an increased risk of post-thrombotic syndrome in patients with a previous deep vein thrombosis (DVT) and there is some evidence suggesting an increased risk of DVT recurrence. Only few studies have assessed potential risk factors for RVO.

Aims: In this study, we evaluated whether ABO blood group with or without associated thrombophilic abnormalities is associated with RVO after a standard course of anticoagulation for a first DVT.

Methods: Patients with a first DVT who underwent screening for thrombophilic abnormalities were eligible for this study. Information was collected on ABO blood group and on risk factors for DVT. Each patient underwent compression ultrasonography of lower limbs for the detection of RVO at least after 6 months of a standard course of anticoagulant treatment.

Results: A total of 268 patients (mean age 50.3 years, 120 women) were included. After 8.3 ± 2.9 months of anticoagulant treatment, 126 (47.0%) patients had RVO. At multivariate analysis, active malignancy (Odds Ratios [OR] 5.54, 95% confidence interval [CI] 2.17, 14.13), non-O blood group (OR 3.71, 95% CI 1.61, 8.56), and femoral involvement (OR 3.35, 95% CI 1.94, 5.78) were significantly associated with an increased RVO risk, whereas an unprovoked index event was only marginally significant (OR 1.81, 95% CI 0.98, 3.36 P 0.06) and severe thrombophilia was not associated with RVO (OR 1.32, 95% CI 0.56, 3.11).

Conclusions: After a standard course of anticoagulation for a first DVT, non-O blood group is predictor of RVO.

PA 2.20-5

Genetic determinants of thrombin generation and their relation to thrombosis. Results from GAIT-2 Project

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Background and Aims: There is evidence to support the hypothesis that there is an association between thrombin generation and the risk of thrombosis. From the technology that measures the dynamics of thrombin generation, we studied three distinct quantitative phenotypes that should help identify genes that affect the susceptibility to thrombotic disease.

Methods: We used the families from the Genetic Analysis of Idiopathic Thrombophilia Project (GAIT-2). This sample consisted of 1113 individuals in 35 extended families selected through a proband with idiopathic thrombophilia. The sample had 175 subjects with thromboembolism (101 of them had venous thrombosis, 93 arterial thrombosis and 19 had both venous and arterial events). Of the 1113 subjects, 935 were phenotyped. Thrombin generation was evaluated in plasma according to the method described by Hemker et al. by means of Fluoroskan Ascent (ThermoLab systems, Helsinki, Finland). The quantitative phenotypes were lag time, thrombin peak and endogenous thrombin potential (ETP). The heritabilities, the effects of measured covariates (age, sex, contraceptives and smoking) and the correlations between the risk of thrombosis and the three quantitative phenotypes were estimated by a variance component method, employing the computer package Sequential Oligogenic Linkage Analysis Routines (SOLAR).

Results: Genetic factors accounted for 46% ($P = 9.4 \times 10^{-05}$) of the variation in the risk of thrombosis, with age as its only statistically significant covariate. The heritabilities of lag time, thrombin peak and ETP were 52% ($P = 1.4 \times 10^{-16}$), 48% (1.1×10^{-14}) and 45% (5×10^{-14}), respectively. All the covariates influenced the lag time. The thrombin peak was influenced by age, sex and contraceptives and the ETP was influenced by age, contraceptives and smoking. The ETP was phenotipically ($\rho_p = 0.16$, $P = 0.014$) and genetically ($\rho_g = 0.35$, $P = 0.028$) correlated with the risk of thrombosis. In addition, the thrombin peak showed a weaker genetic correlation ($\rho_g = 0.30$, $P = 0.051$) with risk of thrombosis.

Conclusions: The relatively high heritabilities indicate that genetic factors play a significant role in the risk of thrombosis and thrombin generation. The significant genetic correlations suggest that there are pleiotropic effects of genes. Our studies should help to detect candidate genes responsible for thrombophilia.

PA 2.20-6

Coagulation factor XIII TYR204PHE gene variation and the risk of ischemic stroke

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Background: The presence of gene variant Tyr204Phe in subunit A1 of the coagulation factor XIII (Tyr204Phe factor XIII) was reported to be strongly associated with ischemic stroke (IS). This risk was further increased in contraceptives users. These findings, however, have never been confirmed in other studies.

Aims: To investigate the Tyr204Phe factor XIII variant as a risk factor in a case-control study of IS Brazilian patients.

Methods: Two hundred and twenty IS patients referred to our service and 220 controls without IS history and not genetically related to the patients were included. Exclusion criteria were transient ischemic attack, cardioembolic events, malignancy or chronic diseases. All cases were tested for Tyr204Phe factor XIII variant by polymerase chain reactions with fluorescent allele-specific oligonucleotide probes. Other IS risk factors, such as hypertension, diabetes mellitus, dyslipidemia, smoking, obesity and estrogen contraceptive use were also recorded.

Results: FXIII Tyr204Phe variant was found in heterozygosis in five patients (2%) and in eight controls (3.6%) with an odds ratio (OR) of 0.6 (95% confidence interval 0.2–1.9), $P = 0.4$. The homozygous form (Phe204Phe) was not found neither in IS patients nor in controls subjects. Also, no evidence for a thrombotic risk related with the presence of the FXIII Tyr204Phe variant was observed in regard to gender, age

(< 50 years), race and estrogen use, when comparing IS patients to controls.
Other risk factors were significantly more prevalent in IS patients than in controls, such as hypertension (OR 4.3), diabetes mellitus (OR 3.4) dyslipidemia (OR 1.7), and smoking (OR 1.9). However, obesity (OR 0.9) and estrogen contraceptive use (OR 1.2) were not associated with a higher risk.
Conclusion: The presence of Tyr204Phe factor XIII variant was not a risk factor for IS in this population. The role of the Tyr204Phe variant as a the risk for IS requires further study.

PA3.01 – Platelet Activation: Receptor Changes – II

PA 3.01-1

Tracking of GPVI-dimer cluster formation at the membrane level with live-cell imaging of platelet binding to various collagenous substrates by TIRF (total internal reflection fluorescence) microscopy

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Background: Dimers of platelet collagen receptor GPVI are the collagen-binding form of this receptor, possessing much higher affinity for collagen than monomers. Quantitation of dimers using dimer-specific antibodies 204-11 Fab and m-Fab-F (Jung et al., *J Biol Chem*, 275: 30000) demonstrated that dimers are constitutively present in resting platelets and increase upon platelet activation by collagen-related-peptide or thrombin. However, although clustering of GPVI-dimers on transfected cells is evident and GPVI-dimer-specific antibodies induce platelet activation, there has been no direct visualization of this in live platelets.
Aims: To determine if clustering of GPVI-dimers occurs on platelets upon interaction with collagenous substrates and whether different collagen substrates support characteristic clustering patterns.
Methods: Platelets labeled with Alexa-488-204-11 Fab (GPVI-dimer-specific) and/or Alexa-588-1G5 Fab (reacts with both GPVI forms) were reacted with collagenous substrate immobilized on glass-bottomed culture dishes (MatTek) and imaged by a Nikon A1R Inverted Confocal/total internal reflection fluorescence (TIRF) microscope. Alternatively, biotinylated $(GPO)_{10}$ or $(GPP)_{10}$ was bound to lipid bilayers on glass coverslips mounted in a flow chamber and fluorescent-antibody-labeled platelets were flowed over this surface and monitored by TIRF microscopy.
Results: Immobilized collagen type III demonstrated two patterns of cluster formation: (i) Cluster formation initiated immediately upon platelet-collagen engagement, with rapid increase in clusters from 0 to 48 s, with well-separated clusters. (ii) Centralized large cluster: initial cluster of GPVI-dimer is formed, which is then enlarged by subsequent recruitment of other GPVI-dimers. Immobilized $(GPP)_{10}$ (inactive peptide): very few platelets adhere and these are still moving, extending filopodia, with few dimer clusters formed. When Collagen Toolkit peptide III-30, a sequence from collagen type III with high affinity for dimer, was immobilized, many platelets adhered with rapid formation of well-separated distinct small clusters. When platelets interacted with $(GPO)_{10}$ attached to a mobile lipid bilayer, freely mobile GPVI-dimer clusters were formed, in some instances coalescing with other clusters to form a larger cluster; this markedly contrasts with the very weak platelet binding on $(GPP)_{10}$.
Conclusions: TIRF microscopy enabled specific observation of interactions of GPVI-dimer or total GPVI with surface-immobilized or lipid-bilayer-bound collagenous substrate. Platelet adhesion to all collagenous surfaces was concomitant with GPVI-dimer-cluster formation. Upon contact with the collagenous surface, platelets form GPVI-dimer clusters, which facilitate adhesion, showing filopodia formation until they are fully activated and spread. Cluster formation patterns were characteristic of the immobilized collagenous substrate. On immobilized collagen type III, 40–60% of platelets showed initial formation of a GPVI-dimer cluster that increased in size without forming other clusters. Other platelets formed numerous independent GPVI-dimer clusters. Platelets formed many small GPVI-dimer clusters on immobilized III-30. Since peptide immobilized at much higher density than collagen, these differences may depend on the density and affinity of GPVI-dimer for the substrates. On lipid-bilayer-bound GPO peptides, the formed GPVI-dimer clusters were mobile. Clustering of GPVI-dimers is an initial and requisite step for platelet adhesion to a collagenous surfaces.
*SMJ and AYP are co-first authors and this work was performed at SPW's lab, with TIRF microscopy performed at the BALM facility, at the University of Birmingham.

PA 3.01-2

Ligand- and force-induced allosteric shift of the platelet integrin aIIbβ3 to a higher affinity state

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Background: The platelet integrin αIIbβ3 and fibrinogen form the receptor-ligand pair essential for platelet aggregation. Previously, we found that αIIbβ3 exists in at least two active forms that we have designated as lower and higher affinity states. These states, in turn, form complexes with fibrinogen that differ in their mechanical stability. Moreover, these states are interconvertible and their relative populations likely determine overall aIIbb3-mediated platelet adhesiveness.
Aims: To elucidate the conditions and mechanisms of the allosteric transition between the lower and higher affinity states of αIIbβ3.
Methods: We studied the nanomechanics of the interactions between fibrinogen and αIIbβ3 using an optical trap to measure the force-free association of individual surface-attached fibrinogen and αIIbβ3 molecules and the forced dissociation of αIIbβ3-fibrinogen complexes and applied a novel approach named Binding-Unbinding Correlation Spectroscopy (BUCS) that combines force-clamp measurements of bond lifetimes with the binding mode to quantify the dependence of the binding probability on the interaction time. Experimental data were modeled using the joint probability distribution $P(T,t)$ for the ligand-receptor bonds formed over time T and that survived until time t, to account for internal dynamics of formation and rupture of a αIIbβ3-fibrinogen complex.
Results: Fibrinogen-reactive αIIbβ3 states differ in their zero-force on-rates ($kon1 = 1.4 \times 10^{-4}$ and $kon2 = 2.3 \times 10^{-4}$ μm^2/s), off-rates ($koff1 = 2.42$ and $koff2 = 0.60$/s),and dissociation constants ($Kd1 = 1.7 \times 10^4$ and $Kd2 = 2.6 \times 10^3$ L/μm^2). The strength of αIIbβ3-fibrinogen interactions was time-dependent due to a progressive increase in the fraction of higher affinity αIIbβ3-fibrinogen complexes that were characterized by a faster on-rate. In other words, the mechanical resistance of the complex increased with the duration of contact between the αIIbβ3- and fibrinogen-coated surfaces, a result of a time-dependent ligand-induced structural rearrangement in the αIIbβ3-fibrinogen complex that favors the formation of a higher affinity state. Unexpectedly, forced off-rates for the higher-affinity form were significantly smaller that their force-free counterpart, implying that application of pulling force also induces a conformational transition from lower to higher affinity αIIbβ3-fibrinogen complexes. We hypothesized that the shift between the active αIIbβ3 conformations from its lower affinity to its higher affinity states resulted from perturbation of sites on αIIbβ3 away from its ligand-binding site. Measurements of the rupture force distribution of αIIbβ3-fibrinogen bonds on ADP-stimulated mouse and human platelets showed that the lower force regime (corresponding to the lower affinity state) was attenuated

and the higher force regime (corresponding to the higher affinity state) was enhanced in mouse platelets. These data support the notion that alterations in the sites of αIIbβ3 responsible for allostery can change the relative distribution of its active affinity states.

Summary/Conclusion: The two-dimensional kinetic rates obtained for the lower and higher affinity αIIbβ3 and fibrinogen interactions at the single-molecule level offer direct evidence for the ligand- and force-dependent shift in αIIbβ3 conformation and activity, underlying the dynamics of fibrinogen-mediated platelet adhesion and aggregation. The identification of allosteric sites that alter the mechanical stability of αIIbβ3-fibrinogen complexes may provide therapeutic targets to alter the distribution of αIIbβ3 activation states.

PA 3.01-3

Disulfide bond exchange is required for integrin activation and post-ligation signaling during fibrin clot retraction

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Background: Integrin αIIbβ3 plays a major role in thrombosis by mediating platelet adhesion, aggregation and fibrin clot retraction. These processes require activation of αIIbβ3 and post-ligation signaling. We previously showed that disruptions of specific disulfide bonds in the β3 subunit activate αIIbβ3 and αvβ3 involving disulfide bond exchange.

Aims: To investigate the role of disulfide bond exchanges in αIIbβ3- and αvβ3-mediated fibrin clot retraction.

Methods: We co-expressed in baby hamster kidney cells human WT-β3 or β3 containing cysteine substitutions that disrupt the C523-C544 bond (C523S, C544S and C523S/C544S) or the C560-C583 bond (C560S) together with human WT-αIIb, giving rise to WT or mutated human αIIbβ3 complexes as well as chimeric complexes between human β3 and the endogenous hamster αv (αvβ3). Activity of αIIbβ3 was determined by measuring the binding of PAC-1 antibody to WT or mutated αIIbβ3 using flow cytometry. The time-course of fibrin clot retraction was recorded in the presence or absence of the thiol blocker dithiobisnitrobenzoic acid (DTNB), the integrin activating antibody anti-LIBS6 and/or an αvβ3 blocker.

Results: All mutants displayed significant PAC-1 binding compared to WT αIIbβ3 that bound PAC-1 only in the presence of anti-LIBS6. Cells expressing WT αIIbβ3 and αvβ3 displayed fibrin clot retraction. Presence of an αvβ3 blocker decreased the rate of retraction, indicating that αvβ3 contributes to clot retraction. The constitutively active mutants and WT cells pre-treated with anti-LIBS6, retracted the clot faster than untreated WT cells. This effect was more pronounced in the presence of an αvβ3 blocker. DTNB significantly inhibited clot retraction by WT cells but had almost no effect on cells expressing the C523S, C544S or C560S mutations; however, cells expressing the double C523S/C544S mutation that has no free thiols were inhibited by DTNB to the same extent as WT cells, either in the presence or absence of αvβ3 blocker. DTNB significantly inhibited anti-LIBS6-enhanced clot retraction of WT cells but only in the presence of αvβ3 blocker. This inhibition was more pronounced when DTNB was added prior to anti-LIBS6 than when it was added after it.

Conclusions: Cells expressing WT αIIbβ3 can retract fibrin clots without prior integrin activation. However, αIIbβ3 activation significantly enhances clot retraction. Both αIIbβ3 activation and post-ligation signaling during clot retraction depend on disulfide bond exchange, suggesting a regulatory role for disulfide bond exchange in this process. In our cell model, αvβ3 significantly contributed to clot retraction in a disulfide bond exchange dependent manner and was not affected by prior activation, suggesting that αvβ3 on platelets and perhaps endothelial cells contribute to clot retraction.

PA 3.01-4

Inhibition of platelet aggregation does not prevent endocytosis of tissue factor-rich microvesicles by platelets: involvement of cytoskeleton, scavenger receptor CD36 and serotonin transporter

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Platelets possess innate ability to interact and internalize a wide variety of molecules and circulating particles. We have previously demonstrated that under experimental conditions, platelets internalize and store tissue factor-rich microvesicles from human origin (hTF + MVs) and that this process causes reversible aggregation of platelets, returning to their original resting state. There is limited knowledge on the mechanisms involved in the endocytosis, traffic and redistribution of molecules, particles or microvesicles by platelets.

We have investigated the mechanisms implied in the endocytosis and traffic of hTF+MVs. Implications of membrane receptors GPIIb-IIIa, CD36 and the 5-HT transporter (SERT) were investigated. The involvement of the small GTPase RhoA, PI3K, and modifications of cytoskeletal organization during vesicular trafficking were analyzed.

Isolated platelets were incubated with hTF+MVs for up to 10 min. Inhibitory strategies to the GPIIbIIIa (Abciximab), the scavenger receptor CD36 (anti-CD36), SERT (S-Citalopram, SCit), and PI3-kinase (Wortmannin, Wo) were used. Aggregometry and ultrastructural microscopy were applied to assess platelet activation and vesicle uptake. Cytoskeletal assembly and activation of RhoA and PI3K were analyzed by ELISA and western-blot.

All the inhibitory strategies prevented platelet aggregation induced by hTF+MVs. Ultrastructural studies revealed that endocytosis of hTF+MVs was efficiently prevented by anti-CD36, SCit and Wo, but was minimally affected by blockade of GPIIb-IIIa. Endocytosis caused reversible actin polymerization and association of contractile proteins (alpha-actinin, actin binding protein and myosin) to the cytoskeleton, being maximal at 1 min. Maximal activation of RhoA occurred at 1 min with translocation to the polymerized cytoskeleton, decreasing gradually afterwards, with similar kinetics being observed for activation of PI3K.

We conclude that endocytosis of hTF + MVs by platelets involves reversible cytoskeletal assembly, activation of PI3K and association of the small GTPase RhoA to the cytoskeleton. Mechanisms of endocytosis are not fully prevented by powerful inhibitors of platelet-platelet interactions, such as abciximab, but are effectively interferred by receptorial inhibition of the scavenger receptor (CD36) and the 5HT transporter (SERT). Our results indicate that mechanisms of aggregation and endocytosis by platelets are two independent processes that involve differential receptorial and activating pathways. Further studies should be preformed to assess the involvement of additional endocytosis mechanisms such as Rab GTPases, and toll-like receptors.

PA 3.01-5

First identification and biological characterization of an IgM type platelet cold agglutinin causing temperature-dependent activation, secretion and aggregation of human platelets

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Background: Platelet cold agglutinins (PCA) are a rare phenomenon mostly recognized by pseudothrombocytopenia (PTCP), or spontaneous *in vitro* platelet clumping, in blood anticoagulated with EDTA (80–90%) or with citrate or other anticoagulants (10–20%). It is considered clinically irrelevant and not associated with specific diseases or drug use. Both immunoglobulin (Ig) IgG and IgM classes can be PCA, but only IgM has been found in few EDTA-independent PTCP. Glycoproteins (GP) IIb, IIb/IIIa, and GP78 were shown as target epitopes of PCA. To the best of our knowledge, no PCA has been reported to induce platelet activation, secretion or aggregation.

Objective: We investigated an atypical IgM PCA type causing activation, secretion and aggregation of both autologous and allogeneic platelets.

Methods and Results: A woman (37 year) with lifelong mild bleeding diathesis and no analytical abnormalities other than chronic moderate thrombocytopenia ($\approx 100 \times 10^9$pl/L), was referred after casual pathological finding in testing with PFA-100 (TO > 300 s, with both col-adp and col-epi cartridges) and Multiplate (null aggregation with all agonist). Blood sampling revealed spontaneous platelet clumping in citrate, ACD-A and heparin, but not in EDTA. Clumping was severe (> 90% platelet drop), rapid (< 15 min), and temperature-dependent (not observed in blood kept at 37 °C). It was prevented by blood collection in the presence of anti-GP IIb/IIIa antibody (LJ-CP8), but not by anti-GP Iba (LJ-Ib1) or anti-vWF (NMC-4). Flow cytometric (FC) analysis of patient's EDTA-platelet rich plasma (PRP), showed an abnormally high degree of basal platelet activation (> 80% CD62+ platelets). While plasma levels of Igs were normal, a FC antiplatelet assay both in plasma and serum distinguished a low titre of antiplatelet IgM. Consecutive blood sampling of the propositus during the last 4 years has consistently demonstrated moderate thrombocytopenia ($\approx 100 \times 10^9$pl/L), maintenance of the atypical pattern of PTCP, high spontaneous platelet activation, and low titre antiplatelet IgM.

In a wide series of experiments incubating patient's plasma or serum with allogeneic platelets (from healthy volunteers, blood-bank leukodepleted platelet concentrates [PCs], or particular patients), we consistently found that: (i) patient's PCA induced temperature-dependent platelet clumping (> 90% drop) in allogeneic citrated blood, platelet-rich plasma or PCs; (ii) it caused no platelet lysis as assessed by LDH measurement; (iii) this PCA promoted full platelet activation as assessed by FC demonstration of surface expression of CD62, ^{14}C-serotonin release (> 50%), TxA_2 production (≥ 100 ng/mL), and thin section electron microscopy and immunofluorescence imaging; (iv) the PCA-induced platelet clumping could be blocked with either EDTA, LJ-CP8 and anti-human IgM antibody, but only anti-human IgM impaired PCA-mediated platelet activation; (v) depletion of IgM from the patient's serum by affinity chromatography removed the PCA activity; (vi) the patient's serum containing PCA, but not fibrinogen, promoted full activation but not clumping of PRP from two different Glanzmann subjects and from one case of afibrinogenemia. All these results demonstrate that the *in vitro* platelet clumping induced by this atypical IgM-PCA represents real platelet aggregation mediated by platelet activation and fibrinogen binding to GP IIb/IIIa.

Summary: This is the first report of an IgM autoantibody causing temperature-dependent platelet activation and aggregation of human platelets.

PA 3.01-6

Phosphospecific flow cytometry enables large-scale signaling profiling and compound screening in human platelets

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Background: Cyclic nucleotide-stimulated signaling cascades represent the most potent endogenous inhibitors of platelet activation and therefore may offer targets for the development of novel antiplatelet agents. Mapping of cyclic nucleotide-dependent phosphorylation events could facilitate the identification of novel compounds that inhibit platelets through these pathways. Identification of phosphorylated proteins by immunoblot techniques are not conducive to large-scale signaling profiling or compound screening. Flow cytometric analyses of protein phosphorylation overcome the limitations of traditional immunologic approaches. Cell-based multiplexing techniques can dramatically increase the throughput of flow cytometric analyses, enabling fast acquisition of large sample sets and rapid screening of entire compound libraries.

Aim: To develop and validate a multiplexed phosphospecific flow cytometry platform for the analysis of cyclic nucleotide signaling events in platelets.

Methods: Flow cytometric analyses of intraplatelet protein phosphorylation were performed using phosphospecific antibodies for VASP. These analyses employed amine-reactive fluorophores to create a multiplexing protocol.

Results: Stimulation of platelets with the nitric oxide donor, S-nitrosoglutathione, or prostaglandin I_2 (PGI_2) led to the concentration-dependent phosphorylation of VASP on serines 157 and 239. Flow cytometric analyses of phosphorylation correlated with Western blotting, with both methods able to discern intermediate levels of phosphorylation. We used a fluorescent cell barcoding protocol to facilitate multiplexing, which enabled large-scale signaling profiling and compound screening through the simultaneous staining and acquisition of 24–72 samples in a single analysis tube. Using this approach to signaling profiling, we were able to demonstrate comprehensive differences in the phosphorylation kinetics of VASP following stimulation with several different cyclic nucleotide-elevating agents including adenosine, forskolin, PGE_1, and PGI_2. Subsequent analyses implicated phosphodiesterases 2, 3, and 5 in the regulation of VASP phosphorylation downstream of different receptor systems. Having established that the method was sufficiently robust to perform simultaneous analyses of single phosphorylation events in multiple samples, we applied the protocol to screen a library of prostaglandin compounds. Initial screening revealed three previously uncharacterized, structurally novel, antiplatelet compounds, which signaled through prostanoid receptors, elevated intraplatelet cAMP, and inhibited collagen-induced platelet aggregation in a concentration-dependent manner.

Conclusions: Phosphospecific flow cytometry is a rapid quantitative method for the measurement of intraplatelet protein phosphorylation, enabling large-scale signaling profiling and compound screening in human platelets. Using this approach, we have identified three potential lead compounds that can modulate platelet activity.

PA3.02 – Thrombus Formation – I

PA 3.02-1

Serotonin contributes to platelet calcium signalling and thrombus formation in flowing blood

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Background: Serotonin (5-hydroxytryptamine, 5-HT) is an important mediator of cell-to-cell signalling both in neuronal and non-neuronal systems. Platelets contain three serotoninergic components – the $5HT_{2A}$ receptor, SERT and VMAT2 – and 5-HT has previously been shown to influence platelet function. Intact platelets take up 5-HT through SERT and store the monoamine in vescicules through VMAT2. After platelet activation, secreted 5-HT binding to $5\text{-}HT_{2A}$ elicits Ca^{2+} signalling through PLCβ activation.
Aim: The aim of this work was to study the role of platelet 5-HT in eliciting intracellular and intercellular Ca^{2+} signalling and regulating platelets thrombus growth under flow.
Methods: Platelet adhesion and activation onto a VWF or collagen type I substrate were monitored under flow at an initial wall shear rate of 1500/s using real-time video microscopy. We analyzed concurrently the instantaneous velocity and intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in single platelets loaded with FLUO 3-AM and treated with ketanserin or R-96544 (two different $5\text{-}HT_{2A}$ antagonists; 5 μM or 50 nM, respectively), aspirin (1.5 mM) or ADP receptors antagonists (MRS 2179 and ARC-69931 MX). Thrombus formation and volume measurements were obtained by real-time confocal microscopy (UltraVIEW, Perkin Elmer) analyzing series of confocal sections collected at 0.2 μm interval in the *z* axis using Andor™ TECHNOLOGY iQ software.
Results: Platelets adhering to VWF demonstrated $[Ca^{2+}]_i$ elevation and several Ca^{2+} flashes propagating along the flow direction with decreasing intensity as the distance from the origin increased. We identified 5-HT as a key mediator of intercellular calcium communication (ICC) since ketanserin completely abolished its occurrence; similar results were obtained with R96544 and aspirin, while P2Y1 and P2Y12 had apparently no effect. In addition, $5\text{-}HT_{2A}$ blockage was accompanied by decreased initial platelet recruitment and subsequent ratio of thrombus growth. Platelet thrombus volume in the presence of ketanserin significantly decreased from 41.2×10^3 to 28.2×10^3 μm^3 on VWF ($P = 0.012$) and from 83.3×10^3 to 56×10^3 μm^3 on type I collagen ($P = 0.038$).
Conclusion: Our results demonstrate a novel mechanism by which released serotonin, through binding to the $5\text{-}HT_{2A}$ receptor, plays a relevant role in the generation of Ca^{2+} signals that, under high shear stress, contribute to platelet crosstalk through ICC. The resulting amplification and spatio-temporal modification of Ca^{2+} signals can regulate platelet adhesion as well as the rate and extent of thrombus growth.

PA 3.02-2

Advanced multi-parameter assessment of microspot thrombus formation: a systems biology approach

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Background: In whole-blood flow assays the roles of glycoprotein (GP)Ib complex, GPVI and integrins α2β1 and αIIbβ3 in thrombus formation on collagen/vWF have been well established. However, platelets can also interact with other components of damaged vessel walls via several adhesive receptors, which can also support the thrombus-forming process. We reasoned that a systematic comparison of thrombus formation at multiple adhesive substrates is needed to fully assess and characterize this process.
Aims: To develop a microspot-based whole-blood perfusion test for assessment of shear-dependent thrombus formation combined with a systematic analysis of a panel of output parameters. To use this test to identify different types of thrombi and to determine how thrombus formation is regulated by different receptor-substrate interactions.
Methods: Arrays of different extracellular matrix proteins and derived peptides were micro-spotted on glass coverslips. In total, 52 (combinations of) substrates were spotted, and their presence was confirmed. Whole blood from healthy controls or patients with a specific platelet function disorder was perfused over the spotted arrays in a new parallel-plate flow device at shear rates of 150–1600/s. Brightfield and multicolor confocal microscopic images of platelets were recorded in real-time and at end-stage, resulting in measurements of parameters reflecting (stable) platelet adhesion, platelet activation, platelet secretion, platelet procoagulant activity, thrombus size, volume and morphology. Values obtained were normalized for comparative analysis, using systems biology techniques. Reproducibility of key parameters was analysed.
Results: The 52 micro-spotted substrates supported thrombus formation at low or high shear rate to a different extent, from weak (adhesion of few discoid platelets) to strong (large, semi-occlusive thrombi with highly activated platelets). Unsupervised hierarchical clustering analysis revealed cohesion of the following outcome parameters: (i) αIIbβ3 activation and α-granule secretion; (ii) thrombus size, morphology and stable adhesion; (iii) thrombus volume and phosphatidylserine exposure. Clustering analysis furthermore indicated three types of thrombus formation, distinguishable in > 6 outcome parameters: type I consisting of adhered single platelets; type II of densely distributed small platelet aggregates; and type III of more disperse distributed large platelet aggregates. Type I thrombi were formed on substrates binding to integrins, GPVI, CLEC-2 or CD36 alone. Type II thrombi were formed on combinations of these substrates with VWF or laminin (binding to GPIb or α6β1, respectively). Type III thrombi were obtained with combined substrates binding to GPIb, α2β1 and GPVI, or binding to GPIb, CLEC-2 ± α2β1 (α6β1). The suitability of this multi-substrate, multi-parameter test to detect platelet functional deficiencies was confirmed with blood from patients with bleeding, i.e. Glanzmann's thrombasthenia and type 2A von Willebrand disease. In these cases, 15–32% of the thrombus scores were reduced.
Conclusions: Advanced multi-parameter detection of microspot-based thrombus formation provides new insight into the combined substrate-receptor interactions that are required for this process. Deficiency in formation of type III thrombi may be a good indicator of major platelet function deficiency, associated with an increased risk of bleeding.

PA 3.02-3

Platelet aggregometry under flow conditions: the rate of change of surface distribution of platelets or thrombi quantifies changes in aIIbβ3 and a2β1 activity in real time

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Background: Thrombus formation in flowing blood requires synergy between platelet receptors GpVI, integrin $\alpha_2\beta_1$, GpIb/V/IX and integrin $\alpha_{IIb}\beta_3$. Previously, we used triple-helical collagen-mimetic peptides and confocal microscopy to investigate collagen receptor-mediated platelet thrombus formation under flow conditions *in vitro*. End-point measurements of thrombus formation such as surface coverage and

ZV_{50} were used to quantify platelet adhesion and activation. However, as these parameters do not quantify platelet behaviour in real time, valuable information about dynamic platelet receptor activity is lost.

Aims: To develop image analysis techniques to quantify $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$ activity in flowing human blood, thus providing a dynamic assessment of integrin activity in real time.

Methods: Whole blood was perfused over different thrombogenic substrates at a shear rate of 1000/s during which images of thrombus formation were acquired using confocal microscopy. End-point parameters of thrombus formation were calculated after 5 min. The rate of change of surface distribution of platelets or thrombi (designated platelet mobility, PM) was calculated from image sequences taken at 0.2 Hz. Thrombogenic substrates consisted of collagen, VWF or combinations of collagen-mimetic peptides CRP, GFOGER and VWF-III (ligands for GpVI, $\alpha_2\beta_1$ and VWF respectively). Additionally, the inert peptide GPP_{10} and a panel of $\alpha_2\beta_1$-adhesive peptides of varying affinities were used. GR144053 was used to antagonise $\alpha_{IIb}\beta_3$, thus isolating $\alpha_2\beta_1$-dependent platelet responses. DM-BAPTA-AM was used to block calcium-dependent receptor activation. The role of secondary signalling pathways in thrombus formation was examined using specific antagonists.

Results: PM quantifies the proportion of platelets that have undergone stable adhesion on a thrombogenic surface under flow conditions in real time. On VWF-III or coated VWF, PM was high, indicating continuous platelet rolling. On collagen and CRP/GFOGER/VWF-III, PM decreased exponentially, indicating rapid stable adhesion. Omission of GFOGER from a replete surface abrogated the decline of PM with time, and further treatment with GR144053 abolished stable adhesion. Aspirin, NF449 and MRS2179 had no effect on stable adhesion or thrombus formation. DM-BAPTA-AM, PGE_1 and 2-MeS-AMP abrogated thrombus formation, but had no effect on $\alpha_{IIb}\beta_3$-dependent stable adhesion. PM profiles reflected the affinity of $\alpha_2\beta_1$-adhesive peptides, when coated alongside CRP and VWF-III. DM-BAPTA-AM abrogated PM on low affinity $\alpha_2\beta_1$-adhesive peptides, but not on GFOGER.

Summary/Conclusions: Use of $\alpha_2\beta_1$-adhesive peptides demonstrates that differences in PM profile reflect the activation state of $\alpha_2\beta_1$. $\alpha_2\beta_1$ plays a significant role in platelet adhesion in the early phases of platelet recruitment to thrombogenic collagenous surfaces. On thrombogenic surfaces lacking an $\alpha_2\beta_1$-adhesive ligand, PM quantifies $\alpha_{IIb}\beta_3$ activity in real time and is thus analogous to light transmission platelet aggregometry, albeit under physiologically relevant flow conditions. Stable adhesion and thrombus formation are $\alpha_{IIb}\beta_3$-dependent processes that require engagement of GpVI. However, thrombus formation but not stable adhesion is regulated by $P2Y_{12}$ signalling and is calcium dependent, indicating that these are two functionally distinct processes.

PA 3.02-4

Murine strain differences in platelet adhesion and thrombus formation under flow conditions

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Background: Genetically modified mice have provided significant advances in our knowledge of haemostasis and thrombosis and new targets for anti-thrombotic drugs. However, the use of experimental arterial thrombosis models both *in vivo* and *ex vivo* in several mice strains showed specific differences that reflect differential responses to vascular thrombosis. These could include demonstrated differences in plasmatic proteins (pro-coagulant, anti-coagulant proteins or proteins involved in fibrinolytic system) and in platelet number and/or function (expression of adhesive receptors modulating platelet activation and aggregation). Moreover recent study have reported that platelet adhesion and aggregation in mice still occur in the absence of fibrinogen and VWF, suggesting that other molecules are involved in these processes.

Aim: The aim of our study was to investigate differences among two mouse strains in platelet aggregation and thrombus formation in *ex vivo* experiments and whether these differences can be related to plasmatic and/or platelet features.

Methods: We used confocal videomicroscopy to measure the volume of platelet thrombi forming during blood perfusion over fibrillar collagen type I at wall shear rate of 1500/s in two different mice strains: C57BL/6 (C57) and Balb/C (BalbC). Rate and extent of thrombus growth were calculated in reconstituted blood and in mixed blood by analyzing a series of confocal sections at 0.2 μm intervals in the z axis. Reconstituted blood stands for C57 or BalbC blood with only platelet count normalized ($600 \times 10^3/\mu L$), while mixed blood is constituted by one strain platelets added to the opposite strain plasma: in other words C57 platelets were resuspended in BalbC plasma and viceversa.

Results: In experiments with reconstituted blood the average volume of thrombi was significantly smaller in C57 than in BalbC demonstrating the most thrombotic phenotype of BalbC mice (mean ± CI: 32.125 ± 3277 vs. 135.886 ± 28.784 μm^3). Measurement of total platelet surface coverage in each z-plane of a series of confocal sections demostrated that C57 mixed blood elicits aggregate growth in height and the first layers of platelets contacting collagen were more extended than in C57 reconstituted (mean ± CI: 116.588 ± 46.800 vs. 32.125 ± 3277 μm^3). Otherwise, reconstituted BalbC blood shows thrombi bigger than the ones formed with the same strain type of platelets (BalbC) in C57 plasma (mean ± CI: 135.886 ± 28.784 vs. 92.205 ± 14.182 μm^3).

Conclusions: Measuring platelet thrombus formation in flowing blood we confirm that there are considerable strain differences in thrombosis process: BalbC mice are more thrombotic than C57 mice, as previously evaluated in tail bleeding model as well as in FeCl3-induced model of arterial thrombosis. Platelet and plasmatic features together contribute to the differences observed in our mice strains model of arterial thrombosis. In summary, to fully exploit the opportunities presented by mice models, it becomes important that mice hemostatic system will be defined and standardized in order to have a better comparison with human hemostatic system.

PA 3.02-5

A role of platelet C-type lectin like receptor-2 (CLEC-2) in thrombus formation *in vivo*

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Background: C-type lectin like receptor-2 (CLEC-2) induces platelet aggregation by binding to the snake venom rhodocytin or the membrane protein podoplanin. We have reported that CLEC-2 stabilizes thrombi formed on the surface of collagen through homophilic interactions with CLEC-2 under flow conditions. However, an internal ligand of CLEC-2 that induces thrombus formation in the artery is not known.

Aim: To clarify the role of platelet CLEC-2 in thrombus formation at the sites of damaged vessel walls.

Methods: The femoral artery of CLEC-2-chimera (KO) or wild type-chimera (WT) was injured by $FeCl_3$ and the occlusion time was measured. The binding of CLEC-2 rabbit Fc2 fusion protein (hCL2-rFc2) to thin sections of atherosclerotic plaques was detected by immunohistochemical staining. Direct binding of hCL2-rFc2 to coronary artery smooth muscle cells (CASMC) was examined by flowcytometry and immunohistochemistry. Whole blood was flowed over CASMC and platelet adhesion to CASMC was analyzed. Secretion of 5-TH and PDGF from platelets mediated through CLEC-2 by binding to CASMC was examined by ELISA.

Results: The occlusion time of femoral arteries of KO was significantly prolonged compared with that of WT in $FeCl_3$ injury model (979.5 ± 387.2 in WT, 1479.7 ± 549.0 in KO, $P < 0.05$). We hypothesized that there is an unidentified ligand for CLEC-2 in the deep layer of the arterial wall. In the plaque, binding of hCL2-rFc2 was observed along with smooth muscle actin staining. The binding of hCL2-rFc2 to CASMC was observed both flowcytometry and immunohistochemistry. When murine blood was perfused onto CASMC monolayer, platelet aggregate formation was observed under flow conditions and thrombus volume was greatly reduced in the absence of CLEC-2. Granule secretion was observed upon stimulation by CASMC in wild type platelets, however, it was absent in the CLEC-2 deficient platelets.
Conclusion: We propose that CLEC-2 induces thrombus formation at the site of severely damaged vessel walls where smooth muscle cells are exposed to blood flow by binding to unidentified CLEC-2 ligand(s) on the surface of smooth muscle cells. The binding of platelets to CASMC via CLEC-2 may also contribute to the repair of damage and/or facilitate neointimal formation through granule secretion. A ligand for CLEC-2 on CASMC is now under investigation by MS/MS.

PA 3.02-6
Lack of non-neuronal serotonin is associated with reduced ischemia-reperfusion injury in myocardial infarction in mice

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Background: Cardiac tissue serotonin levels are increased during myocardial ischemia, probably with significant contributions from platelet serotonin release. The role of cardiac serotonin in ischemia-reperfusion (I/R) injury is still incompletely understood. Tryptophan hydroxylase-1-deficient mice (Tph1−/−) lack serotonin in platelets and other non-neuronal tissues and were described to develop dilated cardiomyopathy and cardiac dysfunction. We found recently that platelet serotonin promotes inflammatory cell recruitment.
Aims: To study the effect of non-neuronal serotonin on myocardial I/R injury *in vivo* and in an *ex vivo* working heart model.
Methods: Twelve week-old C57Bl/6 (WT) and Tph1−/− mice underwent heart surgery to induce myocardial infarction by ligation of the left anterior descending artery (LAD). After 30 min the ligation was opened to allow reperfusion of the affected myocardium. Hearts were excised 24 h afterwards and infarct size in relation to the area at risk (AAR) was determined by double staining with monolite blue and triphenyl tetrazolium chloride using a Langendorff perfusion system. Hearts of untreated WT and Tph1−/− mice were excised and perfused in working mode using Krebs-Henseleit buffer containing 0.4 mM palmitate and 5 mM glucose. In addition, working hearts were subjected to 20 min of global no-flow ischemia with subsequent reperfusion for 25 min.
Results: Myocardial infarction was reduced in Tph1−/− compared to WT after occlusion and reperfusion of the LAD: Mean infarct size was 53.5 ± 13% AAR in WT and 36.9 ± 10% AAR in Tph1−/− mice ($n = 6$; $P < 0.05$). In isolated working hearts of five WT and Tph1−/− mice cardiac power (30.7 ± 3 vs. 32.9 ± 4 mW/gHW; n.s.) and developed aortic pressure (24.1 ± 3 vs. 23.6 ± 2 mmHg; n.s.) were similar. Isolated working hearts subjected to global no-flow ischemia failed gradually, but recovered similarly in both groups after reperfusion (recovery of cardiac power 63 ± 19% vs. 55 ± 8% and recovery of developed aortic pressure 78 ± 18% vs. 86 ± 30% in WT and Tph1−/−, respectively; $n = 5$; n.s.).
Conclusion: Lack of non-neuronal serotonin decreased tissue damage after myocardial infarction with subsequent reperfusion in an *in vivo* mouse model. Isolated, i.e. denervated working hearts from Tph1−/− mice were equally susceptible to global I/R injury as hearts from WT mice, suggesting that the effect observed *in vivo* depended on circulating blood. In conclusion, blood- and hence most likely platelet-derived serotonin is involved in the regulation of myocardial I/R injury.

PA3.03 – Platelet Signal Transduction – II

PA 3.03-1
Thrombin induces phosphorylation of key components of the mTORC1 pathway via a novel PKC/P2Y12 dependent pathway in human platelets

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The immunosuppressive and anti-proliferative drug rapamycin (Sirolimus) is currently used in the clinic to prevent transplant rejection, restenosis and as a cancer treatment. It has been suggested that rapamycin has antiplatelet and antithrombotic properties as it can block platelet spreading, clot retraction and thrombus remodelling. However, little is known about how activation of its target the mammalian target of rapamycin complex 1 (mTORC1) is regulated in platelets.
Stimulation of platelets with thrombin, PAR-1, collagen-related peptide or U46619 all induced phosphorylation of the mTORC1 regulator tuberous-sclerosis complex-2 (TSC2) at both S939 and T1462 and the mTORC1 substrate p70S6K at T389. Thrombin induced phosphorylation of TSC2 was rapid and sustained whereas p70S6K phosphorylation was delayed peaking at 15 min. Surprisingly, inhibition of PI3K/Akt with wortmannin/Akti did not block TSC2 phosphorylation and only a small reduction in p70S6K was observed. In contrast, inhibition of PKC resulted in significant loss in both thrombin-mediated TSC2 and p70S6K phosphorylation. One of the mechanisms by which PKC regulates platelet function is by promoting granule secretion and subsequent autocrine activation of P2Y receptors. We observed that antagonism of P2Y12, but not P2Y1, blocked TSC2 and p70S6K phosphorylation. Furthermore, addition of ADP rescued PKC blockade of thrombin-mediated TSC2 and p70S6K phosphorylation. Pretreatment of platelets with the Src inhibitor PP1 strongly reduced both TSC2 and p70S6K phosphorylation, suggesting that the Src family of kinases regulates mTORC1 downstream of P2Y12.
These results demonstrate a significant role for PKC and subsequent autocrine activation of P2Y12 receptors in the activation of the mTORC1 pathway.

PA 3.03-2
Functional responses and molecular mechanisms involved in platelet activation triggered by histones

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Background: Histones are highly alkaline proteins found in cell nuclei that can be released by dying or inflammatory cells. The recent observation that histones are major components of neutrophil extracellular DNA traps and promote platelet aggregation and platelet-dependent thrombin generation has highlighted these proteins as potent prothrombotic molecules.
Aim: Since the mechanism(s) of platelet activation by histones is not completely elucidated, we explored the ability of individual human recombinant H1, H2A, H3 and H4 to induce platelet activation as well as the molecular mechanisms involved.
Methods: Platelet adhesion and spreading were determined by confocal microscopy. Binding of fibrinogen, expression of P-selectin and forma-

tion of platelet-leukocytes aggregates were evaluated by flow cytometry. The release of von Willebrand factor (vWF) was analyzed by ELISA. Phosphorylation of platelet ERK, AKT, P38 and NFκB was studied by Western blot.

Results: All histones (10–50 μg/mL) were substrates for platelet adhesion and spreading (C:1 ± 1; H1: 1 ± 2; H2A: 2 ± 0.7; H3: 16 ± 3; H4:43 ± 6; number of cells per field, 10 μg/mL, $n = 3$), and triggered fibrinogen binding (C: 12 ± 6; H1: 74 ± 24; H2A: 150 ± 35; H3: 332 ± 50; H4: 510 ± 57, mean fluorescence intensity (MFI), $n = 5$), von Willebrand factor release (C: 17 ± 6; H1: 28.7 ± 1; H2A: 118 ± 33; H3: 144 ± 35; H4: 180 ± 22, ng/mL $n = 3$), P-selectin exposure (C: 9 ± 3; H1: 18 ± 6; H2A: 26 ± 10; H3: 76 ± 24; H4: 123 ± 46 MFI, $n = 5$) and formation of platelet-leukocyte aggregates (C; 17 ± 3; H1: 22 ± 10; H2A: 37 ± 13; H3: 135 ± 69; H4: 215 ± 81, MFI $n = 5$). Histones synergize with thrombin in triggering fibrinogen binding and P-selectin expression. Western blot assays showed that histones induce the phosphorylation of platelet ERK, AKT, P38 and NFκB. Accordingly, platelet activation induced by histones was significantly impaired by pretreatment of platelets with two non-structurally related NFκB inhibitors (BAY 11-7082 and Ro 106-9920), ERK (U0126), PI3K (LY 294002) or P38 (SB 203580) blockers. Pre-incubation of platelets with aspirin or dexamethasone markedly decreased fibrinogen binding and adhesion mediated by histones without modification of P-selectin exposure. Functional platelet responses induced by H3 and H4, but not H1 and H2A, were partially mediated through interaction with Toll like receptors 2 and 4.

Conclusions: Our data identify histones as important triggers of hemostatic and inflammatory-mediated platelet responses, which are partially inhibited by anti-inflammatory drugs.

PA 3.03-3

Regulation of Rap1b activation by cyclic nucleotide-dependent protein kinases in human platelets

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Introduction: In agonist stimulated platelets, Rap1b is activated by Calcium and Diacylglycerol regulated-Guanine nucleotide Exchange Factor I (CalDAG-GEFI). Rap1b is critical for integrin activation and subsequent platelet aggregation. Cyclic nucleotide-dependent protein kinases were shown to prevent platelet aggregation by inhibiting Rap1b activation. The mechanism of Rap1b inhibition is poorly understood. Though, Rap1b is phosphorylated by both Protein Kinase A (PKA) and Protein Kinase G (PKG), the phosphorylation of Rap1b per se has no effect on its activation.

Aims: The aim of the study is to identify PKA/PKG phosphorylation sites in CalDAG-GEFI and the effect of CalDAG-GEFI phosphorylation on Rap1b activation.

Methods: Phosphorylation site(s) in CalDAG-GEFI was identified by the radio-active phosphate incorporation assay in HEK293 cells transfected with wild-type and phospho-mutant CalDAG-GEFI. The phosphorylation site(s) in platelet CalDAG-GEFI was determined by mass spectrometry. Phospho-antibody (P-CalDAG-GEFI^{S587}) was developed to determine the phosphorylation of CalDAG-GEFI in Western blots. Rap1b activation was detected by pull-down assay using RalGDS coupled sepharose beads. Intracellular calcium measurements were performed with Fura-2/AM loaded platelets in luminescence spectrometer.

Results: Radio-active phosphate incorporation assay and mass spectrometry revealed that S587 is the major PKA phosphorylation site in CalDAG-GEFI. In human platelets, PKA stimulation leads to rapid phosphorylation of S587 and the phosphorylation was 35 fold higher compared to unstimulated control. PKG stimulation also leads to S587 phosphorylation, but to a lesser extent than PKA. PKA pre-activation prevents intracellular calcium increase in thrombin stimulated platelets, which is necessary and sufficient for Rap1b activation and platelet aggregation. Surprisingly, PKA pre-activation prevented Rap1b activation and platelet aggregation also in calcium ionophore stimulated platelets, where PKA did not affect the intracellular calcium increase. This indicated that PKA mediated phosphorylation of CalDAG-GEFI could prevent Rap1b activation. In HEK293 cells transfected with CalDAG-GEFI, calcium ionophore induced Rap1b activation, and PKA pre-activation prevented the Rap1b activation. In phospho-mutant (S587A) transfectants, PKA pre-activation did not inhibit Rap1b activation as wild-type, while the phospho-mimetic (S587D) mutant failed to activate Rap1b following calcium ionophore stimulation. These results confirmed that PKA phosphorylation of S587 in CalDAG-GEFI prevents Rap1b activation.

Conclusion: CalDAG-GEFI is phosphorylated at S587 by both PKA and PKG in human platelets. Using *in vitro* experiments, we demonstrated that CalDAG-GEFI phosphorylation prevents Rap1b activation. We propose that this is one of the important mechanisms of platelet inhibition mediated by cyclic nucleotide signaling.

PA 3.03-4

CGMP signaling pathway regulates platelet shape change through modulation of myosin light chain phosphatase – the role of CPI-17 signalling

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Background: Platelet shape change requires Rho family GTPase-dependent inhibition of myosin light chain (MLC) phosphatase and MLC phosphorylation. MLCP is a heterotrimer that consists of a 37 kDa catalytic subunit (PP1δ), a 110 kDa regulatory subunit termed myosin phosphatase targeting subunit (MYPT-1) and a 20 kDa protein (M20). Thrombin inhibits MLCP activity through ROCK and protein kinase C (PKC) dependent pathways. Nitric oxide (NO) inhibits shape change through cGMP dependent mechanism although the mechanisms underlying these actions of cGMP are unclear.

Aim: Here we investigated the molecular regulation of MLCP activity by cyclic GMP signaling.

Results: Platelet stimulation with thrombin induced Rho Kinase (ROCK) dependent inhibitory phosphorylation of MTYP1 on threonine^{853}, leading to diminished phosphatase activity and a net increase in phosphoMLC^{Ser19}. RhoA formed a signaling complex with ROCK-II and MYPT-1, which resulted in the disassociation of the catalytic phosphatase unit. Treatment with NO inhibited thrombin induced phosphoMYPT1^{hr853} and increased phosphatase activity to inhibit MLC phosphorylation. The mechanism of action of NO included inhibition of RhoA was through its phosphorylation on Serine^{188} in a cGMP-dependent manner. This phosphorylation event inhibited thrombin induced membrane association and GTP-loading of RhoA preventing its participation in the activation of ROCK.

Interestingly inactivation of MLCP by thrombin was prevented under conditions where PKC was inhibited. Since inhibition of PKC had no effect on thrombin-induced phosphoMTPY-1^{Thr853}, it suggested an alternative mechanism of regulation. Since MLCP activity is also modulated through association of the catalytic subunit PP1δ with the protein CPI-17, a PKC substrate, we examined how these pathways were affected by NO/cGMP signaling. Stimulation of platelets with thrombin led to phosphorylation of CPI-17^{Thr38}, which required PKC but was independent of ROCK activation. The phosphorylation of CPI-17 stimulated its association with PP1δ and loss of phosphatase activity. Pretreatment of platelets with NO blunted thrombin induced CPI-17 phosphorylation, CPI-17/PP1δ complex formation and PP1δ inactivation in a cGMP dependent manner. Importantly, NO/cGMP signalling had no effects on the activation of PKC in response to thrombin.

Conclusion: These data demonstrate that cGMP signaling targets at least two different pathways, ROCK and CPI-17, in platelets to prevent the inhibition of MLCP. The disinhibition of MLCP by NO/cGMP prevents MLC phosphorylation and contributes to the inhibition of shape change. These data demonstrate a completely new mechanism by which cGMP signaling regulates platelets.

PA 3.03-5
Small molecule inhibitors of PDK1 inhibit platelet functional responses

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Background: PDK1 (3-phosphoinositide-dependent protein kinase 1) has become a valid drug target for anticancer agents to treat tumors that possess elevated PKB/Akt and S6K activity. Many small molecule inhibitors of PDK1 have been described with the ultimate goal of using them in a clinical environment. Their possible influence on platelets and haemostasis has not been addressed.
Objective: The purpose of this study was to investigate the effect of two small molecule inhibitors of PDK1, BX795 and BX912, on platelet functional responses.
Methods: We assessed the agonist-induced phosphorylation state of Akt by Western blotting and measured aggregation, dense granule secretion, thromboxane formation and clot retraction in the presence and absence of BX795. Akt activity was also assessed using an *in vitro* kinase assay.
Results: Both these compounds inhibited agonist-induced phosphorylation of Akt at Thr308 in a concentration-dependent manner. The phosphorylation of two downstream substrates of Akt, GSK3 and PRAS40, was also reduced. However, BX912 also inhibited agonist-induced phosphorylation of Akt at Ser473. BX795 inhibited PAR4-, 2-MeSADP- or collagen-induced aggregation, dense granule secretion, thromboxane formation and clot retraction in a concentration-dependent manner. Using an *in vitro* kinase assay, we demonstrated that Akt activity was completely abolished if Thr308 on Akt was not phosphorylated.
Conclusions: Taken together, this study demonstrates the importance of PDK1 as its inhibition influences platelet physiological responses.

PA 3.03-6
Gas6 regulates thrombin-induced expression of VCAM-1 through a FoxO1 in endothelial cells

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Background: Venous thrombosis is a leading cause of morbidity and mortality in medicine. Increased concentrations of the growth-arrest specific gene 6 (Gas6) in patients suffering from venous thrombosis suggest that Gas6 plays a clinically relevant role in the pathogenesis of this disease. Gas6 is a vitamin K-dependent protein that has been shown to stabilize thrombi, protect endothelial cells from apoptosis and promote endothelial cell activation *in vivo*. In Gas6 deficient (−/−) mice, it has been demonstrated that the expression of VCAM-1 is blunted. The above data suggest that the expression of VCAM-1 induced by Gas6 is critical in the pathogenesis of venous thrombosis; however, the intracellular signaling mechanisms remain largely unknown. We previously demonstrated that the anti-apoptotic effect of Gas6 was mediated partially through Forkhead box O1 (FoxO1). FoxO1 is an effector of the PI3K/Akt signaling pathway and its phosphorylation results in its translocation from the nucleus to the cytoplasm, thereby regulating its transcriptional activity.
Aims: It is hypothesized that Gas6 promotes thrombin-induced VCAM-1 expression through the regulation of FoxO1 in endothelial cells.
Methods: Endothelial cells were isolated from the lungs of wild type (WT) and $Gas6^{-/-}$ mice. Thrombin was used as a pro-thrombotic stimulus. Phosphorylation and cellular localization of FoxO1 was analyzed by western blot analysis. VCAM-1 expression was evaluated by quantitative RT-PCR and western blot analysis in WT and $Gas6^{-/-}$ endothelial cells.
Results: VCAM-1 mRNA expression of was significantly increased after 30 min of stimulation with thrombin in WT cells ($P < 0.05$) but thrombin-mediated induction of VCAM-1 mRNA was blunted in $Gas6^{-/-}$ endothelial cells. At the protein level, thrombin induced a time-dependent expression of VCAM-1 with a maximum at 2 h ($P < 0.05$) in WT but not in $Gas6^{-/-}$ endothelial cells.
In WT endothelial cells, thrombin treatment resulted in maximal phosphorylation of FoxO1 at 30 min ($P < 0.05$) and translocation of FoxO1 from the nucleus into the cytoplasm ($P < 0.05$). None of these effects were observed in $Gas6^{-/-}$ endothelial cells. Finally, FoxO1 siRNA treatment of the endothelium resulted in increased VCAM-1 expression in WT but not $Gas6^{-/-}$ cells.
Summary/Conclusions: Taken together, these data demonstrate that Gas6 promotes VCAM-1 expression through the regulation of FoxO1. This pathway may be involved in the pro-thrombotic role of Gas6. These data also suggest that interfering with Gas6-induced signaling could be exploited clinically to treat thrombotic disease.

PA3.04 – Microparticles and Tissue Factor

PA 3.04-1
Human tears and sweat trigger clotting of blood: the role of tissue factor-exposing vesicles

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Background: The epithelium provides not only a barrier to microorganisms, but also produces fluids as saliva, tears (lacrima) and sweat to trap, kill and remove microorganisms. Tears contain a thus far unidentified coagulant activity. Recently, we observed high levels of coagulant TF-exposing vesicles in human saliva and urine (Blood 2011;117:3172–80), suggesting that such vesicles may be involved in host defence by supporting clot formation and thus maintenance of the barrier integrity.
Aim: To investigate whether the procoagulant activity of tear fluid and sweat is associated with vesicles.
Methods: We collected tear fluid and sweat from healthy subjects and studied the presence of vesicle associated tissue factor (TF) antigen (flow cytometry and Western Blot) and activity (fibrin generation assay).
Results: Tear fluid from all subjects ($n = 13$) shortened the clotting time of normal pool plasma from ≥ 3600 to $1332\ s \pm 502$ ($P < 0.0001$). This shortening was completely TF-mediated ($P < 0.0001$). TF in tear fluid is exclusively associated with cell-derived vesicles. Sweat contains detectable levels of TF-exposing vesicles ($n = 8$) also, albeit on average 3–4 fold lower than in tear fluid. Sweat from two subjects triggered TF-mediated clotting of plasma.
Conclusions: This study demonstrates for the first time that tears and sweat can trigger the clotting of blood via coagulant TF that is associated with cell-derived vesicles. Thus, clotting of blood can be initiated or facilitated by exposure of blood to sweat or tears.

PA 3.04-2

Characterisation of the procoagulant properties of microparticles derived from different cell types

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Background: Microparticles (MPs) are shed by all vascular cells upon activation or apoptosis. They vary in size and inherit antigenic features from their parent cells. Increased numbers of MPs in the circulation carry increased risk of thrombosis. The thrombotic nature of MPs comes from negatively charged phospholipids and tissue factor (TF) on their surface.

Aims: We compared the relative procoagulant activity, in terms of procoagulant phospholipid (PPL) and TF activities, of MPs generated from platelets, endothelial cells and macrophages.

Methods: Washed platelets isolated from five healthy individuals were activated with cross-linked collagen-related peptide to generate Platelet-derived MPs. MPs were generated from the human Eahy926 endothelial cells by stimulation with TNF-α, and from the pro-monocytic THP-1 cell line by LPS (following transformation into macrophages using phorbol-12-myristate-13-acetate) ($n = 3$ for both). MPs were isolated by centrifugation and their PPL and TF activities were measured by Calibrated Automated Thrombogram, using 1 pM TF and 4 μM PL reagents respectively, using peak thrombin (PT) as the measure of PPL and lag time (LT) as the measure of TF activity. The PPL and TF concentrations in the preparations were also measured using the Zymuphen MP-TF and MP-activity ELISAs. The MP count in each sample was measured by nanoparticle tracking analysis (NTA) using the Nanosight NS500 and data was normalized to 10^{12} MP/mL.

Results: Analysis by the CAT assay showed that MPs from all three cell types had significant PPL activity with macrophage-derived MPs > platelet-derived MPs > endothelial cell-derived MPs (PT of 338 ± 63; 297 ± 33 and 200 ± 27 nM respectively). All three types of MPs also showed PPL activity in the ELISA but although macrophage-derived MPs still had the highest activity (36.2 ± 17.9 nM), endothelial cell-derived MPs showed higher levels of PPL activity than platelets in this assay (6.90 ± 1.46 vs. 1.85 ± 0.52 nM respectively). The TF activity of the particles also was different for each cell types. MPs derived from macrophages and endothelial cells showed significant TF activity in both assays (LT of 19 ± 3.1 and 90 ± 8.9 min, and TF concentration of 37.0 ± 3.4 and 3.80 ± 1 pM respectively). In contrast to the two other cell types platelet derived MPs showed no TF activity in the CAT assay and negligible levels of TF by ELISA (0.09 ± 0.08 pM).

Conclusion: These data demonstrate (i) that MPs from different cell types have variable activities of PPLs and TF, (ii) that assays for PPL and TF may vary in their sensitivity and (iii) that platelet-derived MPs do not inherently express TF.

PA 3.04-3

Microparticle-associated tissue factor activity correlates to plasma levels of bacterial lipopolysaccharides in meningococcal sepsis

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Background: Meningococcal disease, i.e. meningitis and sepsis, is caused by *Neisseria meningitidis (Nm)*, a gram negative bacteria with lipopolysaccharides (LPS) in the outer membrane. The levels of LPS from *Nm* in blood are closely associated with clinical presentation and outcome. Patients with meningitis have undetectable levels of LPS and low grade of coagulation disturbances, whereas patients with sepsis may have very high levels of LPS and often develop systemic septic shock and subsequent disseminated intravascular coagulation (DIC). In meningococcal sepsis, LPS generate tissue factor (TF), the main initiator of coagulation, on circulating monocytes and monocyte-derived microparticles (MPs), which both appear to contribute to the pathogenesis of DIC.

Aims: To measure MP-associated TF activity and relate it to the levels of bacterial LPS in samples from patients with meningococcal disease.

Methods: Citrated plasmas from patients with sepsis or meningitis were centrifuged (2000 *g*, 15 min, room temperature [RT]) and MPs isolated by pelletation (17,000 ***g***, 30 min, RT). MP-associated TF-dependent activity was measured with a plasma-based thrombin-generation assay (CAT) and whole blood rotational thromboelastometry (ROTEM), both assays using contact activation inhibition (corn trypsin inhibitor (CTI), 18.3 μg/mL). LPS levels were measured using a chromogenic Limulus amebocyte lysate assay.

Results: MPs obtained from sepsis patients ($n = 13$) initiated a significantly more efficient thrombin generation in the CAT assay compared to MPs from meningitis patients ($n = 10$), with lagtime = 18 ± 11 vs. 41 ± 9 min, time to peak = 29 ± 17 vs. 64 ± 12 min and peak = 87 ± 66 nM thrombin vs. 21 ± 7 nM thrombin (mean ± SD and $P < 0.05$ for all parameters). In comparison with meningitis patients, clotting time (CT) was reduced from 684 to 382 s. When MPs from sepsis patients were subjected to whole blood rotational thromboelastometry (ROTEM) (mean of two donors in each group). Preincubating the MPs obtained from the sepsis patients with anti-TF antibodies prolonged the lagtime in the CAT assay from 8/6 to 49/52 min ($n = 2$) and clotting time in the ROTEM from 478/286 to 1103/837 s ($n = 2$), indicating a TF-dependent thrombin generation and whole blood clot formation. The levels of plasma LPS in patients with sepsis (range 2–2100 EU/mL) were correlated with all four thrombogram parameters in the CAT assay; lagtime ($r^2 = 0.67$), time to peak ($r^2 = 0.67$), peak ($r^2 = 0.61$) and ETP ($r^2 = 0.50$).

Conclusion: Our results show a close association between bacterial LPS levels and MP-associated TF activity in samples from patients with meningococcal disease.

PA 3.04-4

Thrombin generation in plasma measured with a commercial reagent for the detection of microparticle-derived tissue factor is heavily influenced by contact activation

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Background: Since the discovery of circulating microparticles (MPs) exposing tissue factor (TF), various assays for the detection and characterization of TF-exposing MPs have been established. A commercially available reagent (MP-reagent, Thrombinoscope®,The Netherlands) containing synthetic phospholipids has been developed to allow for quantification of the contribution of TF-exposing MPs to thrombin generation in plasma using the Calibrated Automated Thrombogram (CAT) method. However, it is known that a plethora of materials, including plastics, are capable of activating Factor XII (FXII), and previous work from our group has suggested that synthetic phospholipids might augment the coagulation response to contact activation. Thus, the possibility that contact activation of FXII on synthetic surfaces, assisted by the presence of synthetic phospholipids, and not TF-exposing MPs, could be the main contributor to thrombin generation in the abovementioned assay, deserves investigation.

Aims: To investigate how FXII-dependent and TF-dependent coagulation contributes to thrombin generation using commercially available reagents for microparticle detection.

Methods: Plasma samples were obtained from healthy subjects and treated according to the instructions of the manufacturer. The effects

of increasing concentrations of synthetic phospholipids on FXII-dependent coagulation and thrombin generation were assessed in platelet-free plasma in which the microparticle content had been reduced by filtration (0.2 μm) using the Reorox instrument (Medirox AB, Nyköping, Sweden) and the CAT method, with and without CTI (100 μg/mL). This experiment was conducted either without exogenously added FXII-activator to investigate the effect of contact activation, or in the presence of increasing concentrations of kaolin. The contributions of FXII- and TF-dependent coagulation on thrombin generation using the MP-reagent or the PRP-reagent supplied by the manufacturer were evaluated in the presence of the FXII-inhibitor corn trypsin inhibitor (CTI) or antibodies directed against tissue factor (TF-Ab, shown to block tissue factor-induced coagulation).

Results: It was shown that synthetic phospholipids dramatically enhanced Factor XII-dependent coagulation and thrombin generation in filtrated platelet-free plasma. Even without exogenously added factor XII-activator (kaolin), 0.5–2.0 μM synthetic phospholipids was sufficient to produce robust thrombin generation within 15 min, which was almost completely inhibited by CTI. We then compared CATs from normal plasma samples pre-treated with either CTI or TF-Ab. Using the MP-reagent, thrombin generation was dramatically delayed or completely absent in the presence of 100 μg/mL CTI, while being virtually unaffected by the addition of 100 μg/mL of TF-blocking Ab. When using the PRP-reagent supplied by the same manufacturer, which contains tissue factor but only very small amounts of phospholipids, the reverse held true, i.e. thrombin generation was significantly delayed in the presence of TF-Ab, but unaffected by the presence of CTI.

Conclusions: We conclude that results from thrombin generation experiments conducted with reagents containing synthetic phospholipids are heavily influenced by spontaneous contact activation of FXII. Efforts to investigate the contribution of TF-exposing MPs to thrombin generation using such reagents should be interpreted cautiously, unless being performed in the presence of inhibitors that block the intrinsic coagulation pathway.

PA 3.04-5

Microparticle-associated tissue factor activity in patients with metastatic pancreatic cancer and its effect on fibrin clot formation

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Background: Tissue factor (TF) is a transmembrane receptor and the main initiator of the blood coagulation cascade. Microparticles (MPs) are negatively charged membrane vesicles that are defined by their size (0.1–1 μm) and a procoagulant phosphatidylserine (PS)-rich surface that strongly increases the proteolytic activity of TF. Highly elevated MP-associated TF activity was found in patients with pancreatic cancer, one of the most prothrombotic malignancies.

Aim: To investigate whether MP-TF activity reflects the prothrombotic state in pancreatic cancer patients.

Methods: MP-TF activity was determined with a chromogenic endpoint assay. The TF-dependent and -independent effect of MPs on fibrin clot formation was determined with a plasma clotting assay. Expression of PS on MPs was quantified with a prothrombinase assay. Measurements were performed in patients with metastatic pancreatic cancer, in lipopolysaccharide-stimulated blood (LPS-plasma), which is rich in monocyte-derived TF-bearing MPs, and in healthy individuals. The study was approved by the institutional ethics committee and all participants signed informed consent.

Results: The median MP-TF activity (pg/mL) was 1.06 (range: 0.19–10.34) in 27 pancreatic cancer patients, 0.61 (range: 0.36–0.79) in 10 LPS plasma samples and 0.18 (range: 0.04–0.39) in 10 healthy individuals (each pair-wise comparison: $P < 0.001$).

When MP pellet derived from pancreatic cancer patients was added to normal pooled plasma, the median time to fibrin clot formation was 273.4 s (range: 146.6–354.4), $P < 0.001$). The fibrin clot formation time was significantly shorter when MP pellet from LPS plasma was added to normal pooled plasma (median: 157.4 s, range: 149.5–170.4). The time to fibrin clot formation was longest after the addition of MP pellet from healthy controls (median: 300.6 s [range: 261.1–417.9], each pair-wise comparison: $P < 0.001$). Only in MPs derived from LPS-plasma the fibrin clot formation time strongly depended on TF (median prolongation after TF blockade: 68% in LPS-plasma, 15% in pancreatic cancer patients and 4% in healthy individuals).

The median PS exposure (nM PS equivalent) on MPs was 6.5 (range: 2.5–66.2) in pancreatic cancer patients, 29.7 (range: 28.0–36.4) in LPS-plasma and 17.6 (range: 6.1–49.3) in healthy controls (each pair-wise comparison: $P < 0.05$).

Summary/Conclusions: In the present study we investigated MP-TF activity levels, the procoagulant potential of TF-bearing MPs and PS exposure on MPs. Elevated MP-TF activity levels were found in patients with metastatic pancreatic cancer and in LPS-plasma compared to healthy controls. MPs derived from LPS-plasma but not MPs from patients with metastatic pancreatic cancer had a strong TF-dependent impact on fibrin clot formation. Exposure of PS was high on MPs from LPS-plasma but low on MPs from pancreatic cancer patients, which might explain the different effect on fibrin clot formation.

In conclusion, our results indicate that the prothrombotic potential of TF-bearing MPs is limited in patients with metastatic pancreatic cancer and that elevated MP-TF activity might not be the major trigger for the increased risk of thrombosis in these patients.

PA 3.04-6

Cell-derived microparticles as novel tools for thrombolytic therapy?

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Background: Fibrinolysis using recombinant tissue type plasminogen activator (r-tPA) remains the only approved treatment for acute ischemic stroke. The limits of r-tPA treatment include a narrow therapeutic window, a low recanalization rate and direct neurotoxic effects. Therefore, safer and more efficient fibrinolytic therapies are needed. Recent data indicate that besides their procoagulant components, microparticles (MPs) may be a major source of plasminogen activators *in vivo*. Therefore, MPs could be not only clot triggers but also active fibrinolytic agents.

Aims: The purpose of this study was to investigate whether tPA-enriched MPs could trigger efficient fibrinolysis without exerting deleterious effects on neuronal survival.

Methods: tPA-MPs obtained from human HEK293 cells, were isolated by a sequential ultracentrifugation procedure and were finely characterized by high-resolution laser-scanning confocal microscopy. The plasminogen activator activity of these MPs was measured by western blot, fibrin-zymography and tPA-specific chromogenic assay. Their fibrinolytic efficiency was determined by clot lysis assays and on fibrin plaques using a pool of human or murine plasma.

Results: tPA-enriched MPs were able to dissolve fibrin clots and reduced the time for clot lysis as a function of their concentration. The fibrinolytic effect of MPs was two-fold more effective than the corresponding amount of soluble r-tPA. The same results were observed using fibrin plaques. Our results suggest that tPA-MPs may be more resistant to soluble inhibitors than r-tPA. Moreover, unlike r-tPA, tPA-MPs did not exert neurotoxic effects in cultures of murine cortical neurons.

Conclusions: In summary, our results suggest that tPA-enriched MPs develop higher and more efficient fibrinolytic capacity and with fewer sides effects than soluble r-tPA. However *in vivo* studies are required to confirm this potential approach for thrombolytic therapy.

PA3.05 – Genetic Platelet Disorders – I

PA 3.05-1

Characterization of the second patient with Hermansky-Pudlak Syndrome type-7 (HPS7), and a novel HPS1 mutation. Value of an autozygosity mapping approach to prioritize mutation screening in HPS

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Background: Hermansky-Pudlak syndrome (HPS) is an autosomal recessive disorder characterized by abnormalities of lysosome-related organelles. HPS patients present with variable oculocutaneous hypopigmentation, bleeding diathesis and some with granulomatous colitis and pulmonary fibrosis. Mutations in nine genes (*HPS1-HPS9*) cause HPS in humans. The large number of HPS culprit genes (> 118 exons) and lack of genotype-phenotype correlation, complicate the molecular diagnosis of HPS.

Aims: We aimed to confirm the clinical suspicion of HPS in two unrelated patients, by means of platelet function assessment and identification of alterations in *HPS* genes. The UK patient was recruited to the Genotyping and Platelet Phenotyping (GAPP) study (ISRCTN 77951167).

Methods: Case 1 is a 30 year old Spanish man displaying oculocutaneuos albinism, nygstamus and hemorrhagic history including nose bleeding requiring cauterization, excessive bleeding from small wounds, and abnormal bleeding upon surgery. His parents were related by blood. Case 2 is a 77 year old English woman, with pale skin and hair, reduced visual acuity, nystagmus, as well as menorrhagia from menarche. This led to hysterectomy, prolonged bleeding from minor injuries, long bleeding after dental extractions, and heavy post partum hemorrhage after her three vaginal deliveries, among other lifelong bleeding complications. Her parents were first cousins. None of the two cases had coagulopathy or other recognized cause of bleeding. Platelet function studies included light transmission aggregometry (LTA) with different agonists and ATP secretion by lumiaggregometry (Case 2), ^{14}C-serotonin uptake and whole mount electron microscopy (Case 1). DNA for molecular studies was obtained from both patients. In Case 1, the *HPS1* gene was first chosen for PCR amplification and sequencing, as it is the most frequently mutated in HPS. By contrast, in Case 2 we first performed autozygosity linkage mapping by genotyping microsatellite markers flanking HPS genes, in order to identify a candidate causative gene.

Results: As compared to a parallel control, and to our historical data from healthy subjects, Case 1 platelets displayed impaired aggregation toward ADP (5 mM), epinephrine (5 mM), ristocetin (1.2 mg/mL) and collagen (2 mg/mL) (25%, 85%, 40%, and 95% reduction). Case 2 platelets also showed reduced aggregation with PAR-1 (30 mM) and collagen (1 mg/mL). Deficiency of platelet dense granules was evidenced in Case 1 by 50% reduction of ^{14}C-serotonin uptake and absence of dense bodies in whole mount, and in Case 2 by lack of ATP secretion in lumiaggregometry test with agonists such as PAR-1 (100 mM) and PAR-4 (500 mM). In Case 1 *HPS1* sequencing revealed a novel homozygous nonsense mutation c.844G>T [p.Glu204Stop] in exon 7. In Case 2, only the HPS7 locus displayed autozygosity for an extended genetic distance, prompting us to select *HPS7*/*DTNBP1* as the potential causative gene. Sequencing of *DTNBP1* identified a homozygous mutation c.177 G>A [p.Trp59Stop] in exon 4.

Summary: Here, demonstration of platelet dysfunction, dense granule deficiency, and genetic analysis confirms HPS in two unrelated pedigrees. One is a new variant of HPS type1, while the other represents the second reported variant of HPS7. This work illustrates that autozygosity mapping in consanguineous pedigrees is a useful tool to prioritize mutation screening of HPS genes.

PA 3.05-2

Aberrant mRNA processing in compound heterozygote with glycoprotein IIb gene mutations causing Glanzmann thrombasthenia

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Background: Glanzmann thrombasthenia (GT) is a rare autosomal recessive bleeding disorder caused by quantitative or qualitative abnormalities of glycoprotein (GP) IIb/IIIa complex in platelet. While many GT mutations acting at the protein level have been investigated, only fewer data on genetic defects affecting GPIIb or GPIIIa mRNA were available.

Aims: The aim of this study was to characterize the molecular mechanism underlying GT in a Japanese patient.

Patients/Methods: The patient was a 8-years old Japanese female who had suffered from hemorrhage and purpura. GPIIb/IIIa expression on the platelet was analysed by flow cytometry analysis. We analyzed the patient by DNA sequencing of all exons and splicing junctions of GPIIb gene (*ITGA2B*) and GPIIIa gene (*ITGB3*). Platelet mRNA was analyzed by quantitative real-time RT-PCR (reverse-transcription polymerase chain reaction) assay.

Results: Flow cytometry analysis revealed undetectable levels of platelet GPIIb/GPIIIa complex. Sequencing of genomic DNA revealed presence of two mutations in the *ITGA2B*, a compound heterozygote for a novel splicing mutation at the acceptor site in intron 5 (c.625-1G>A) and a previously reported nonsense mutation (c.1750C>T, p.Arg584X) in exon 17. RT-PCR assay on the patient's platelet RNA revealed the expression of GPIIb mRNA was about 3% of normal control (N = 6), and some populations in each defect GPIIb mRNA. The former showed the presence of two populations of mRNA, one with the skipping of exon 6 and the first 14-bp of exon 7 (result in deleted 60-bp) and the other with the retention of intron 5 (result in inserted 74-bp). The latter was identified two different mRNA populations, one with the nonsense mutation and the other with lacking of the last 75-bp of exon 17 including the nonsense mutation (resulting in 75-bp deletion).

Conclusions: We supposed that these mRNA populations were caused by splicing error or nonsense-mediated mRNA decay pathway. The elucidation of the genetic basis of GT in the patient suggested that premature terminations seemed to be associated with allele-specific mRNA degradation, and that several aberrant splicing events occurred.

PA 3.05-3

Identification of two novel mutations leading to MYH9-related disease and its correlation of genotype/phenotype

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Background: MYH9-related macrothrombocytopenias are among the causes of hereditary thrombocytopenias. They are associated with mutations in the non-muscle myosin gene (MYH9) and are characterized by thrombocytopenia, large platelets and leukocyte inclusions. Depending on the diagnosis, non-hematologic alterations such as cataracts, deafness and renal failure may also be present.

Aims: Evaluate thrombocytopenic patients with large platelets for the diagnosis of MYH9-related macrothrombocytopenia and identify the mutational profile of MYH9 gene.

Patients and Methods: We selected patients with thrombocytopenia and high mean platelet volume (MPV > 10.6 fL). Presence of leukocyte inclusions and large platelets was confirmed by analysis of peripheral blood smear. All cases were assessed for the presence of other non-hematological manifestations such as cataracts, hearing loss, impaired renal function, and also for family history of thrombocytopenia.

Results: Twenty-one patients with a mean age of 31.3 years old (10–57 years old), belonging to 17 families were included in this study. Ten cases (48%) had the previous diagnosis of ITP and had undergone immunosuppressive therapy. Mean platelet count was 50×10^9/L ($11–107 \times 10^9$/L) and MPV was 15.1 fL (8.2–17.7 fL). In morphologic evaluation, it was observed that all patients had large platelets, and in 13 cases more than 20% of the counted platelets were larger than 6 μm. In nine patients (five families) leukocyte inclusions were observed, and in two of them, belonging to the same family (FAM1), renal failure was also present, which features Fechtner syndrome. Another patient presented hearing loss without leukocyte inclusions, rendering the diagnosis of Epstein syndrome probable. Thus, we considered for molecular investigations 10 cases (48%), distributed in six families, in whom the most likely diagnosis was MYH9-related macrothrombocytopenia. The mutation was confirmed in five unrelated families (eight patients). E1841K mutation (exon 38) was found in three families (FAM 1, 4 and 5), and R1933Stop mutation (exon 40) was found in FAM 2. These two mutations had been previously described in several families in Asia, Europe and the USA, and had been associated with May-Hegglin anomaly (consistent with FAM 2, 4 and 5 diagnosis), but not with other macrothrombocytopenias (such as that observed in FAM1). In depth analysis of FAM 1 provided the finding of a novel mutation F636L (exon 15) in MYH9 gene of the two affected subjects (mother and son). Three unaffected daughters were negative for both E1841K and F636L mutations, confirming by segregation that both mutations were present in the same allele and were responsible for the phenotype compatible with Fechtner syndrome. Another not previously described mutation in exon 15 (T664R) was detected in a patient from FAM3. None of these novel mutations was found in a screening of 100 blood donors (200 alleles).

Conclusion: Careful clinical and family history assessments as well as systematic evaluation of peripheral blood smear are key points for correct diagnosis of MYH9-related macrothrombocytopenias. Identification of novel mutations in MYH9 gene can help to gain insights into the pathophysiology of these diseases, since location of molecular alterations reflects in the clinical manifestations.

PA 3.05-4

Variant glanzmann thombasthenia from a newly described Ile282Thr mutation in ITGB3 with functionally defective alpha IIb beta 3 integrin and mild phenotypic presentation

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Background: Glanzmann Thrombasthenia is a rare disorder of platelets resulting from defective platelet aggregation and characterized clinically by mucocutaneous bleeding. The mechanism of platelet dysfunction is attributed to defects in the ITGA2B and ITGB3 genes, which encode for the fibrinogen receptor, leading to either quantitative or qualitative deficiencies of the platelet integrin alpha IIb beta 3. Many genetic variants and their clinical significance have been described, although phenotypic heterogeneity exists even among individuals with the same mutation.

Aims: Here we report a novel point mutation in the ITGB3 gene that renders the alpha IIb beta 3 complex unstable.

Case History: The patient had a history of bruising, petechiae, and epistaxis in childhood, which became less pronounced with age. Platelet aggregation studies initially revealed markedly decreased responses to collagen and absent responses to ADP and arachidonic acid. Repeat testing performed in adulthood revealed only mildly decreased responses to ADP and arachidonic acid. Morphologically, the platelets were normal in size and granularity. Flow cytometric analysis showed the presence of alpha IIb beta 3 integrin on platelets and at normal levels.

Results: Sequence analysis of genomic DNA detected heterozygosity for the c.845T>C, p.(Ile282Thr) variant in exon 6 of the ITGB3 gene. No other mutations or unclassified variants were identified, and no mutations were detected in the ITGA2B gene. The possibility of a large deletion or rearrangement was ruled out by the detection of heterozygosity for several polymorphisms. This mutation occurs in an area of beta 3 involved with fibrinogen binding that influences the stability of binding with alpha IIb.

Summary/Conclusion: Despite this mutation and a clinical presentation consistent with variant Glanzmann Thrombasthenia, both the patient's laboratory and clinical findings appeared to demonstrate improvement over time. We conclude that the Ile282Thr mutation in ITGB3 can result in a phenotypically mild form of variant Glanzmann Thrombasthenia.

PA 3.05-5

Identification of three novel mutations in TUBB1 gene in patients with macrotrombocytopenia

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Background: Congenital macrothrombocytopenias represent a genetically heterogeneous group of platelet disorders among which MYH9 and Bernard Soulier syndroms are the most frequent forms. Unfortunately the pathogenesis remains unknown for half of the cases identified.

Beta 1 tubulin is a protein specific of platelets and megakaryocytes. By assembling with alpha tubulin, beta 1 tubulin forms heterodimers and microtubule polymers that are essential during thrombocytopoiesis. A recent study revealed for the first time a mutation, p.R318W, in *TUBB1* gene resulting in macrothrombocytopenia (Kunishima *et al.* 2009; Blood, 113, 458–461).

Aim: To screen *TUBB1* gene in our french cohort of patients with congenital macrothrombocytopenia of unknown etiology after having

excluded MYH9, Bernard Soulier, gray platelet and platelet von Willebrand syndroms.
Methods: In 73 propositi with macrothrombocytopenia, we amplified the entire coding sequence of exons and exons-introns boundaries of *TUBB1* gene, and sequenced the PCR products.
Results: An alteration of *TUBB1* gene was identified in three propositi. The platelet count (G/L) was 77, 83 and 96 and the mean platelet volume (MPV) was not given by the analyzer for the first patient and 15.8 and 14.5 fl for the two last patients respectively. Platelet size was larger than normal on May Grünwald Giemsa stained blood smears in the three cases. The platelet agregation was normal in the three cases. Electron microscopy for the patient bearing the mutation p.F260S in *TUBB1* gene (hereafter) revealed the presence of round platelets with an enlarged size.
We identified three different missense mutations in *TUBB1* gene, one in exon 3, c.185 G>A predicting the substitution of arginine for a glutamine p.R62Q, and two in exon 4 c.779 T>C and c.1041 C>G predicting the substitution of a phenylalanine for a serine p.F260S and an asparagine for a lysine p.N347K, respectively. The patients were heterozygous for the mutations. These mutations were not found in 100 healthy controls and in the NCBI SNP database. In addition, these mutations are different from the first one published.
Conclusion: The beta 1 tubulin residues affected were highly conserved among species indicating their functional impact on the protein. These new results highlighted the role of an alteration of beta tubulin 1 in the mechanism of macrothrombocytopenia.

PA 3.05-6
Exome sequencing for causal gene discovery in three patients with primary platelet secretion defect

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Background: Platelet secretion defects (PSD) are heterogeneous platelet functional defects characterized by reduced granule secretion upon stimulation by different platelet aggregation agonists. Autosomal dominant inheritance has been described in PSD, making it a phenotype amenable to genetic mapping by exome sequencing (i.e. sequencing of protein-coding areas of the genome). However, the clinical and laboratory heterogeneity of PSD warrants a tailored approach.
Aims: To undertake a pilot study in order to develop analysis pipelines for gene discovery and compile a list of candidate causal genes for PSD.
Patients/Methods: We sequenced the exome of three unrelated Italian patients with PSD and seven controls. Sequencing was carried out by Illumina HiSeq after DNA target capture by NimbleGen SeqCap EZ Exome Libraries v2.0. SOAPaligner, SOAPsnp and BWA were the software of choice for read alignment and mapping onto the reference genome sequence and for single nucleotide variant and indel calls. Annotation on databases of common (and likely neutral) genetic variation and exclusion of variants found in healthy controls were used as criteria to identify potentially disease-related genes. Databases included dbSNP132, 1000Genomes Project and exome variant server repositories.
Results: Sequencing was successful with > 95% of the 44-megabase target DNA being covered by at least 10 sequence reads. On average, approximately 25,000 single nucleotide variants and 450 indels were found in the 10 sequenced exomes. After removal of variants found in dbSNP, 1000Genomes databases or seven control exomes, 608, 635 and 592 genes had at least one nonsynonymous or splice-site SNV or coding indel in the three PSD patients. Intersection of the three gene-lists yielded eight candidate genes. After exclusion of large, highly-variable genes and genes with little or no gene-expression in platelets, *ANKRD36* and *NPRL3* were the strongest candidate genes.
Conclusions: This study illustrates the power of exome sequencing for the identification of genes underlying PSD and identifies candidate causal genes for this condition.

PA3.06 – Fibrinolytic System: Clinical – III

PA 3.06-1
The effect of factor XIII and other regulators of fibrinolysis on the outcome of thrombolysis in ischemic stroke patients

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Background: Thrombolysis by recombinant tissue plasminogen activator (rtPA) is among the most effective pharmacological strategies in acute ischemic stroke (IS). Little is known why in some patients thrombolytic therapy is less efficient and which are the factors promoting intracranial hemorrhage in others. In most cases, these complications cannot be foreseen at the initiation of therapy and their occurrence remains unexplained. Effectiveness as well as adverse effects of the thrombolytic agent are likely to depend on factors influencing fibrinolysis and clot structure.
Aims: To investigate whether levels of factor XIII (FXIII) and of other major regulators of fibrinolysis affect the outcome of thrombolysis (the opening of the closed vessel) and the risk of hemorrhagic complications. To find out whether known polymorphisms of FXIII and of fibrinolytic proteins have an effect on the outcome of thrombolytic therapy in IS patients.
Methods: The study population included 100 IS patients, who underwent thrombolytic therapy within 3 h of the onset of clinical symptoms. Blood samples were taken at admission, 1 and 24 h after the initiation of rtPA infusion. FXIII activity and antigen levels, α_2-plasmin inhibitor (α_2PI) activity and antigen levels and thrombin activatable fibinolysis inhibitor (TAFI) antigen were measured from all blood samples. FXIII-A Val34Leu and Tyr204Phe, FXIII-B His95Arg and intron K (IVS11 + 144) polymorphisms as well as α_2PI Arg6Trp and TAFI Thr325Ile polymorphisms were genotyped. Clinical data of patients using the National Institutes of Health Stroke Scale (NIHSS) score and results of imaging tests were registered at admission and on day 1 and 7 after therapy. Unfavorable short-term outcome was defined as an increase in NIHSS score by at least four points on day 7. Long term functional outcome was assessed at 3 months after the event by the modified Rankin scale. The study was approved by the Regional Ethics Committee and informed consent was obtained in all cases.
Results: FXIII levels were significantly lower after thrombolytic therapy. The decrease was more pronounced in the case of severe IS (NIHSS > 5), but the levels did not correlate with the outcome of thrombolysis. α_2PI levels at admission were significantly lower in patients with severe IS but were not associated with the outcome. The decrease in FXIII and α_2PI levels after therapy were more pronounced in atherothrombotic than in cardioembolic stroke. FXIII-B intron K and α_2PI Arg6Trp polymorphisms showed protective effect against the development of an ischemic lesion, other FXIII polymorphisms were without effect. TAFI levels significantly decreased after thrombolytic therapy but were not associated with stroke severity or with thrombolysis outcome. Thr325Ile polymorphism increased stroke severity (OR:2.6; 95% CI:1.1–6.6), but did not influence the outcome.

Conclusions: FXIII, α_2PI and TAFI levels decreased after thrombolytic therapy but did not influence the outcome of thrombolysis. Decreased levels of α_2PI before the initiation of rtPA infusion, which might be due to increased α_2PI consumption, predict more severe stroke. For the first time we have shown that the outcome of thrombolysis is influenced by FXIII-B intron K and α_2PI Arg6Trp polymorphisms.

PA 3.06-2

Quality of life outcomes in a randomized trial of tenecteplase versus placebo for submassive pulmonary embolism

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Background: Acute submassive pulmonary embolism (PE) can cause persistent symptoms that degrade quality of life (QOL). Possible causes of poor QOL include persistent right ventricular (RV) dysfunction and complications from deep venous thrombosis (DVT). This study was designed to test the hypothesis that intravenous tenecteplase would improve measurements of QOL 3 months after submassive PE.

Methods: Multicenter randomized double-blind, placebo controlled trial. Eligible patients were ambulatory at baseline, had image-proven acute PE, SBP >90 mm Hg and evidence of RV dysfunction (hypokinesis on echocardiography (echo), elevated serum troponin or brain natriuretic peptide). Patients received anticoagulation with low molecular weight heparin (LMWH) and either tiered-dose tenecteplase or saline, followed by anticoagulation with oral warfarin sodium. Patients were assessed 3 months later as follows: 1. RV function on resting echocardiography, 2. exercise capacity with the 6 min walk distance (6MWD), 3. dyspnea severity with a validated instrument to estimate NYHA functional class, 4. self-perception of wellness, assessed by the Physical Component Summary [PCS] and Mental Component Summary [MCS] scores from the SF-16®, and 5. For PTS symptoms with the VEINES-QOL. Unfavorable indexes of QOL were predefined as poor functional capacity (RV dysfunction and NYHA score >2 or 6MWD < 330 m), and SF16 or VEINES-QOL scores <2SD below the mean for this population.

Results: 83 patients with PE were enrolled with mean age 55 ± 14 years, including 38 with concomitant DVT; 43 were randomized to placebo and 40 to tenecteplase. In hospital, one patient treated with placebo died from PE and one patient treated with tenecteplase died of intracranial hemorrhage. No other patient had a significant hemorrhagic adverse event and the frequency of minor bleeding was not different between groups. Within 5 days, two patients, both treated with placebo, had circulatory deterioration, both requiring rescue thrombectomy. Within 3 months, five (11%) placebo patients had recurrent VTE vs. one (3%) treated with tenecteplase. The mean values (±SD) for the 76 patients with complete follow up for the 6MWD, SF36® PCS and SF36® MCS scores and VEINES-QOL scores were 405 ± 105 m, 44 ± 12, 53 ± 10 and 91 ± 15, respectively. The 6MWD values correlated significantly with PCS scores ($r^2 = 0.35$, $P < 0.001$) but not with MCS scores ($r^2 = 0.04$, $P = 0.1$). RV dysfunction (dilation or hypokinesis) was present in 27/76 (36%) and no QOL scores were significantly different between patients with and without RV dysfunction. Comparison of patients treated with placebo vs. tenecteplase at 3 month follow-up revealed poor functional capacity in 8/39 vs. 4/37 ($P = 0.23$). On the SF36®, a higher proportion of placebo patients had poor PCS scores (12/39 vs. 4/37, $P = 0.03$) but not MCS scores (3/39 vs. 2/37, $P = 1$). Among the 37 patients with DVT, the VEINES-QOL scores were poor in 2/19 placebo patients vs 0/18 tenecteplase patients ($P = 0.25$).

Conclusions: In this randomized, double-blinded study, the primary benefit of fibrinolytic treatment for submassive PE to patients was a significantly improved self-perception of their quality of physical health, which was correlated with exercise capacity.

PA 3.06-3

Fibrinolysis impairment in obstructive sleep apnoea syndrome is driven by increased PAI-1 and TAFI activity

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Background: Obstructive sleep apnoea syndrome (OSAS) is a common disorder associated with a hypercoagulable state. Prothrombotic alterations have been suggested to explain in part increased cardiovascular risk, including risk of myocardial infarction and stroke, in OSAS patients. Little is known about fibrinolysis in OSAS.

Aim: We sought to investigate fibrinolysis and coagulation parameters in OSAS.

Methods: One hundred and sixty-seven consecutive subjects with suspected OSAS underwent a polysomnography. There were 35 subjects with severe OSAS (apnoea/hypopnoea index (AHI) > 30 events/h), 26 with moderate OSAS (AHI = 15–30 events/h), and 49 with mild OSAS (AHI = 5–15 events/h). Fifty-seven individuals in whom OSAS was ruled out (AHI < 5 events) served as a reference group. Prior to polysomnography, fasting blood samples were obtained from all subjects to determine clot lysis time (CLT), plasminogen activator inhibitor (PAI)-1 antigen, activated thrombin-activatable fibrinolysis inhibitor (TAFIa), plasmin and antiplasmin.

Results: OSAS patients and the reference group had similar clinical characteristics, including the prevalence of smoking habit, chronic obstructive pulmonary disease, asthma, hypertension, coronary artery disease, history of myocardial infarction or stroke. Both groups differed with regard to age (OSAS vs. reference group: 59 [49.0–64.0] vs. 49 [42.0–58.0] years), BMI (31.6 [28.1–37.4] vs. 28.3 [26.3–30.6] kg/m^2), diagnosed diabetes (23.6% vs. 7.0%), total cholesterol (5.1 [4.4–5.8] vs. 5.4 [4.6–6.3] mM), C-reactive protein (2.3 [1.1–3.9] vs. 1.2 [0.8–2.6] mg/L), and fasting glucose (5.4 [5.0–5.9] vs. 5.2 [4.9–5.5] mM; all $P < 0.05$). Both groups had similar plasma fibrinogen. OSAS patients compared with the reference group had higher plasma levels of PAI-1 antigen (31.8 [23.0–36.6] vs. 21.4 [19.3–24.6] ng/mL; $P < 0.000001$), TAFIa (28.4 [22.8–33.9] vs. 24.0 [20.0–30.7] ug/mL; $P = 0.002$), and prolonged CLT (98 [84–111] vs. 76 [70–80] min; $P < 0.000001$). All these parameters increased with the severity of OSAS (from mild through moderate to severe OSAS: PAI-1, 26.4 ± 6.0 vs. 30.4 ± 6.8 vs. 39.3 ± 8.5 ng/mL; $P < 0.000001$; TAFIa – 27.2 (IQR, 21.9–32.5) vs. 28.1 (20.8–32.9) vs. 32.5 (25.8–40.1) ug/mL; $P = 0.04$; CLT, 88.0 (79.0–98.0) vs. 98.0 (88.0–106.0) vs. 111.0 (101.0–128.0) min; $P < 0.0001$). There were no differences in plasmin and antiplasmin activity between OSAS subjects and reference group. There were positive correlations in OSAS patients between PAI-1, TAFIa, CLT and polysomnographic parameters such as AHI ($R = 0.66$, $P < 0.001$; $R = 0.29$, $P = 0.002$; $R = 0.55$, $P = 0.001$, respectively), apnoea index ($R = 0.55$, $P < 0.001$; $R = 0.28$, $P = 0.004$; $R = 0.42$, $P < 0.001$), hypopnoea index (HI) ($R = 0.54$, $P < 0.001$; $R = 0.20$, $P = 0.038$; $R = 0.49$, $P < 0.001$), desaturation index ($R = 0.66$, $P < 0.001$; $R = 0.32$, $P = 0.001$; $R = 0.56$, $P < 0.001$), time of snoring ($R = 0.34$, $P < 0.001$; $R = 0.28$, $P = 0.003$; $R = 0.33$, $P < 0.001$), and inverse correlations with blood oxygen saturation ($R = -0.36$, $P < 0.001$; $R = -0.24$, $P = 0.012$; $R = -0.40$, $P < 0.001$). Antiplasmin activity showed inverse correlations only with HI ($R = -0.21$, $P = 0.031$) and time of snoring ($R = -0.23$, $P = 0.016$). No correlation was observed between plasmin activity and polysomnography parameters.

Conclusions: OSAS is associated with impaired fibrinolytic capacity which is largely driven by increased plasma PAI-1 antigen and TAFIa. A novel observation is that prolonged fibrin clot lysis along with PAI-1 and TAFIa increase with the progression of OSAS, which could contribute to increased rate of thromboembolic cardiovascular events among severe OSAS patients.

PA 3.06-4

Type 1 plasminogen activator inhibitor (PAI-1) and risk of acute coronary syndrome in the european prospective investigation into cancer (EPIC)-Italy cohort

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Background: PAI-1 levels were associated with arterial disease in multiple studies. However, whether high PAI-1 levels independently increase the risk remains to be elucidated.

We evaluated the association between PAI-1 and risk of acute coronary syndrome (ACS) in four out of five centers of the European Prospective Investigation into Cancer (EPIC)-Italystudy.

Materials and Method: We conducted a case-cohort study on 1454 (702 men; 752 women) volunteers of the EPIC-Italy cohort, by comparing subjects who developed ACS (MI fatal or nonfatal, coronary revascularization, sudden death) in a mean follow-up of 11.93 years.

Using a nested case-cohort design in the EPIC-Italy study (n = 34,148), we identified a random subcohort (n = 820) and incident acute coronary syndrome cases (n = 634 plus 13 cases originated from the random subcohort) occurring between baseline (1993–1997) and end of 2006 (Varese and Naples), of 2007 (Ragusa) or 2008 (Turin); PAI-1 levels were measured in citrated plasma collected at recruitment by ELISA (Hyphen Zymutest PAI-1, IL, Milano). The relative risk (RR) and 95% confidence interval (CI), adjusted by relevant confounders and stratified by center, were estimated by aCoxregression model using Prentice method.

Results: Individuals in the highest compared with the lowest quartile of PAI-1 had significantly increased risks of ACS (RR: 4.11; 95% CI: 2.90–5.82; P for trend < 0.001) in univariate analysis, after adjustment for sex, age and recruitment center the risk decreased to 2.67 (1.82–3.92). Additional adjustment for education, smoking habit, BMI, total physical activity, hypertension, diabetes and hyperlipidemia treatment, further decreased the risk to 1.97 (1.28–3.03) (P for trend < 0.001). The risk of ACS increased by 34% for each increase in 1 standard deviation of PAI-1 levels.

Conclusions: Our data provide the first evidence for a link between high PAI-1 levels and an increased risk of ACS in the population.

PA 3.06-5

N-terminal heterogeneity of alpha-2-antiplasmin is associated with plasma clot lysis time and risk of arterial thrombosis

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Background: A reduced fibrinolytic potential is associated with risk of arterial thrombosis, although the exact mechanism underlying this association is yet unknown. The main function of alpha-2-antiplasmin (a2AP), the inhibition of plasmin, is influenced by the proteolytic modifications the protein undergoes in the circulation. Approximately 70% of circulating a2AP is N-terminally cleaved between residues Pro12 and Asn13, turning the native Met-a2AP into Asn-a2AP. Additionally, approximately 35% of circulating a2AP is cleaved at its C-terminus, resulting in a form of a2AP that has lost most of its activity by losing its ability to bind to plasmin(ogen). Non-plasminogen binding a2AP can still inhibit plasmin, but kinetically very slow.

Aims: We investigated the association of N-terminal heterogeneity of a2AP and the risk of arterial thrombosis. Additionally, with a2AP being a fibrinolysis inhibitor, we investigated the association of N-terminal heterogeneity of a2AP and the lysis times of plasma clots.

Methods: We focused on the active plasminogen-binding form of a2AP, PB-a2AP, and on individuals homozygous for the common R-allele of polymorphism Arg6Trp, as this polymorphism strongly influences N-terminal cleavage of a2AP. New ELISA assays were set up to measure the antigen levels of both total PB-a2AP and N-terminally intact Met-PB-a2AP. With these data we calculated the percentage Met-PB-a2AP, reflecting N-terminal heterogeneity. a2AP antigen levels were measured in the plasma samples of 404 patients with arterial thrombosis (myocardial infarction, ischemic stroke or peripheral artery disease) and 318 healthy control individuals from the ATTAC study. Informed consent was obtained and the study was approved by a recognised medical ethics committee. Risk estimates were determined by using the 75th percentile (P75) as calculated in control individuals and were adjusted for age and sex. Plasma clot lysis times (PCLT) were measured previously.

Results: Total-PB-a2AP levels (mean ± SD) did not differ between patients and control individuals, 47.3 ± 11.1 vs. 46.7 ± 11.9 μg/mL respectively, P = 0.416. Although individuals with Total-PB-a2AP levels above P75 had a slight increase in arterial thrombotic risk (OR:1.19, 95% CI: 0.83–1.71), this did not reach statistical significance. We did find that patients had significantly higher Met-PB-a2AP levels compared to the Met-PB-a2AP levels in control individuals, 14.8 ± 5.2 vs. 13.4 ± 4.8 μg/mL respectively, P < 0.001. Individuals with Met-PB-a2AP levels above P75 had a 1.76-fold increased risk of arterial thrombosis (95% CI: 1.25–2.49). This was also observed in the percentage Met-PB-a2AP, which was increased in patients (31.7 ± 10.0%) compared to the percentage Met-PB-a2AP in control individuals (29.3 ± 9.8%, P = 0.002), with a 1.56-fold increase in risk for individuals with a percentage Met-PB-a2AP above P75 (95%CI: 1.10–2.21). Furthermore, PCLT is known to be increased in patients with arterial thrombosis compared to the PCLT in control individuals (99.7 ± 30.4 vs. 92.6 ± 20.9 min, P = 0.005 in our study). We additionally found a positive correlation (Spearman's rho) of the percentage Met-PB-a2AP with plasma PCLT in the control individuals (0.172, P = 0.020).

Conclusions: We found that an increase in Met-PB-a2AP, i.e. a decrease in N-terminal cleavage of a2AP, is associated with an increase in PCLT and an increased risk of arterial thrombosis.

PA 3.06-6

Polymorphisms of matrix metalloproteinase gene and adiposity indices in European children: results of the IDEFICS study

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Objective: We investigated the relationship between matrix metalloproteinase 3 (*MMP3*) polymorphisms and adiposity indices in European children of the IDEFICS (Identification and Prevention of Dietary- and Lifestyle-Induced Health Effects in Children and Infants) project.

Subjects: Sixteen thousand two hundred and twenty-four Caucasian children (2 ± 9 years) were recruited into a population-based survey from eight European countries. Four thousand five hundred and forty children were randomly selected for genetic studies (T0); 3238 children were re-examined 2 years later (T1). Anthropometric measures were collected by standardized protocols at T0 and T1.

Results: Six variants of *MMP3* gene were genotyped. Homozygotes for the variant A allele of rs646910 and for the H3 haplotype had higher hip circumference ($P = 0.002$ and 0.001; age, sex and country adjusted) at T0. The association remained significant after false discovery rate (FDR) correction. At T1, subjects carrying rs646910 A/A genotype or H3/H3 diplotype showed significantly higher values of BMI, waist and hip circumference and sum of tricipital and subscapular skinfolds, all associations remaining significant after FDR correction ($P = 0.020$–0.048).

Conclusions: We showed for the first time an association between the *MMP3* rs646910 variant and indices of adiposity in European children, highlighting the involvement of metalloproteinase genes in adipose tissue remodeling and growth.

PA3.07 – Haemophilia A: Basic – III

PA 3.07-1

Concomitant low doses of activated prothrombin complex concentrate (APCC) and recombinant activated factor VII are efficacious in hemophilic mice and exhibit additive joint action *in vitro*

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Background: Treatment of hemophilia A is complicated by development of FVIII neutralizing antibodies, occurring in approximately 30% of patients with severe hemophilia A. In these cases, acute bleeds are often treated with bypassing agents, APCC or recombinant factor VIIa (rFVIIa), which act independently of FVIII to achieve hemostasis. However, in 10–20% of bleeding events, patients are refractory or non-responsive to monotherapy. Sequential combined bypass therapy (SCBT) or concomitant combined bypass therapy (CCBT) of APCC and rFVIIa are one of the last resorts to arrest bleeding.

Aim: We developed a translational *in vitro*-to-*in vivo* approach to study the combined effect of APCC and rFVIIa (BAX817) in human global hemostasis assays, and the efficacy of CCBT in a hemophilia A mouse model.

Methods: The activity of APCC and rFVIIa was assessed in a calibrated automated thrombograph (CAT) assay in FVIII-inhibited normal human plasma at high phospholipids and without tissue factor. We tested clinically relevant plasma concentrations of APCC (20–1000 mU/mL) and rFVIIa (0.11–5.25 μg/mL), alone and in combination, to select a ratio that normalized thrombin generation. For the efficacy study, FVIII knockout mice ($n = 16$ per group) received a single intravenous injection of 200–2700 μg/kg rFVIIa, 60–250 U/kg APCC, concomitantly administered combinations of 60 U/kg APCC + 200–2000 μg/kg rFVIIa, or buffer. Tail clips were performed and blood loss measured over 60 min.

Results: APCC and rFVIIa, alone and in combination, showed a concentration-dependent response under the given CAT conditions. The total effect of the combination on thrombin generation was consistenty lower or equal to the sum of the individual effects, suggesting an additive effect of the joint action of rFVIIa and APCC. Several combinations reached activity equivalent to 1000 mU/mL APCC; we selected 200 mU/mL APCC + 0.88 μg/mL rFVIIa as the starting point for the efficacy study. Based on previous efficacy data from rFVIIa and APCC monotherapy studies, we translated these *in vitro* plasma concentrations into doses for the mouse tail clip model. Statistical analysis of blood loss showed that the minimally effective doses were 2000 μg/kg rFVIIa, 250 U/kg APCC, and for the combination therapy, 1200 μg/kg rFVIIa + 60 U/kg APCC. Importantly, the effectiveness of 1200 μg/kg rFVIIa and 60 U/kg APCC alone was not significantly different from that of buffer. Thus, we demonstrate that suboptimal doses of rFVIIa and APCC when administered alone, correct a bleeding phenotype in combination.

Summary/Conclusions: To our knowledge, this is the first efficacy study evaluating CCBT with low doses of rFVIIa and APCC in a hemophilia animal model. We show that combined doses of rFVIIa and APCC – inefficacious on their own – reduce blood loss in FVIII knockout mice after tail clipping. Interestingly, the minimally effective combination dose in mice translates into human doses of 40 μg/kg rFVIIa + 20 U/kg APCC, which fall into the dose ranges of CCBT as applied in the clinic. We further provide new *in vitro* evidence that the joint action of rFVIIa and APCC is additive rather than synergistic.

PA 3.07-2

A single residue in tissue factor pathway inhibitor (TFPI) determines the specificity of an inhibitory peptide to TFPI and guides selection of preclinical models

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Background: TFPI is a potent inhibitor of the extrinsic factor X activation complex and efficiently regulates coagulation initiation. We developed *de novo* peptide inhibitors of TFPI that enhance coagulation in hemophilia models. Direct inhibition of TFPI in hemophilia models alleviates bleeding complications and may become an important approach in hemophilia treatment. TFPI inhibitors are currently under investigation for inhibitory antibodies and aptamers. Recently, we demonstrated that an optimized linear peptide JBT-A7 bound to the Kunitz-1 (K1) domain of human (h)TFPI with a Kd < 1 nM and efficiently inhibited TFPI in human test systems.

Aims: We characterized target cross-reactivity of TFPI inhibitors including a fusion peptide of a linear (JBT-A7) and a cyclic (JBT-B5) peptide, to efficiently select animal species for preclinical studies.

Methods: We co-crystallized JBT-A7 with human K1 and JBT-B5 with K1-K2 produced by *E. coli*. Critical residues for JBT-A7 binding to K1 were identified from the structure and compared to animal TFPI by

sequence alignment. Species selective TFPI inhibitory activity of peptides was tested in TF-triggered thrombin generation in mouse, rat, rabbit, dog, minipig, macaque and marmoset plasma, which was supplemented with anti-FVIII inhibitors to mimic hemophilia A conditions and to be more sensitive to TFPI inhibition. Recombinant TFPI1-160 from several animal species was used to show direct inhibition of TFPI by peptides in a FXa inhibition model assay. The interaction of JBT-A7 with recombinant TFPI1-160 was studied using Biacore.

Results: Crystallographic measurements showed that the specificity of JBT-A7 to K1 is determined by electrostatic interactions at distinct charged hot spots, e.g. Lys29 and Asp32 interact with an Asp and Arg-Lys, respectively, in JBT-A7. Extended steric surface complementarities of the binding partners explain both the high affinity and specificity. JBT-B5 interacts with K1, K2 and the linker region between these Kunitz domains. Activity screening in plasma indicated cross-reactivity of JBT-A7 to mouse and marmoset TFPI only. Macaque TFPI did not react with JBT-A7 despite a sequence identity of 92.5% to human TFPI1-160. Sequence alignment revealed a single mutation A30P, impacting the binding site and resulting in a complete loss of cross-reactivity. Reduced binding to macaque TFPI was confirmed by Biacore studies. Although marmoset TFPI is less related to human TFPI than macaque TFPI is, it did not show the A30P exchange. JBT-A7 blocked marmoset TFPI with activity similar to human TFPI. The cyclic JBT-B5 does not cross-react to any animal TFPI tested. Nevertheless, fusion of both peptides results in TFPI inhibitory activity in marmoset plasma similar to that in human plasma.

Summary/Conclusions: The data show that detailed knowledge of a compound guides the selection of an appropriate animal species, and that a change of only one critical residue within the protein target may determine species specificity for preclinical studies. This structure-guided *in vitro* approach aids in reducing the number of animal experiments.

PA 3.07-3

Regulation of plasminogen activator inhibitor-1 promotes immune tolerance to factor VIII in murine hemophilia A

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The development of inhibitory antibodies against factor VIII (FVIII) is the enormous impact on hemophilia A patients, who are treated with FVIII products. Plasminogen activator-plasmin system is associated with not only thrombolysis but also various reactions including inflammation. However, little is known about how fibrinolytic components coordinate immune response. Here we show that plasminogen activator inhibitor-1 (PAI-1), a regulator of fibrinolytic system, is a key player in controlling immune response in a mouse model of hemophilia A. Genetic deletion of PAI-1 prevented FVIII deficient mice from development of inhibitor formation after repeated loading of FVIII. PAI-1 depletion was associated with decreased major histocompatibility complex class II expression by bone marrow F4/80 positive cells and reduced T cell proliferation. Transplantation of mouse bone marrow cells transduced with a short hairpin RNA sequence targeting PAI-1 as well as administration of synthetic PAI-1 inhibitor caused a significant reduction of anti-FVIII antibody titer in FVIII deficient mice. These results suggest that PAI-1 regulation before FVIII exposure may represent a novel therapeutic option for preventing the development of FVIII inhibitors in hemophilia A patients.

PA 3.07-4

The effect of FVIII deficiency on the dynamics of thrombin and fibrin generation under flow on tissue factor-rich surfaces

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Background: Blood flow affects coagulation by determining the rate of zymogen delivery to, and enzyme transport away from a growing thrombus. While it is known that FVIII deficiencies diminish the rate of thrombin generation under static conditions, there is little known about how this deficiency influences thrombus formation under flow on well-defined TF-rich surfaces.

Aims: The objective of this study was to measure the effect of FVIII deficiencies on fibrin formation and thrombin generation under flow in a whole blood system, using a microfluidic flow assay (MFA). We compared how the MFA compared to static assays in terms of their sensitivity to FVIII levels. Finally, we used the MFA to measure the response to replacement therapies and bypassing agents.

Methods: Whole blood from individuals with severe ($n = 7$), moderate ($n = 8$), mild ($n = 5$) hemophilia or healthy controls ($n = 8$) was perfused over a collagen-tissue factor surface at a shear rate of 100/s for 5 min. Transient fibrin and platelet accumulation were measured by fluorescence microscopy. Thrombus morphology was measured by scanning electron microscopy. To aid in the interpretation of the experiments, thrombin generation as a function of FVIII levels was simulated using computational models of thrombus formation. Pre- and post-treatment assays were run on samples from individuals receiving recombinant FVIII replacement therapies or FVIIa bypassing agents. Results from the MFA were compared to turbidity and thrombin generation (TG) assays to gauge the relative sensitivity of a flow based approach to FVIII deficiency.

Results: The rate of fibrin formation and final fibrin accumulation was a strongly correlation to FVIII plasma levels. ($r = 0.81$, $P < 0.001$). The FVIII levels also influenced the magnitude and the distribution of thrombin generation as predicted by computational models. The average thrombin concentration in the thrombus was 50 nM at 100% FVIII, 5 nM for 10% FVIII and 1 nM for 1% FVIII. Integrated fibrin density in the MFA was able to discriminate between healthy controls (39.03 ± 6.1 RFU), mild (22.34 ± 2.86 RFU) and severe (11.4 ± 1.5 RFU) hemophilia. The MFA was equally or more sensitive to FVIII levels compared to maximum absorption in a turbidity assay ($r = 0.69$, $P < 0.004$) and TG assay ($r = -0.18$, $P = 0.50$). Fibrin accumulation following replacement and bypass therapy showed a marked increase in fibrin density before (10.0 ± 0.7 RFU) and 1 h after treatment (52.8 ± 2.3 RFU).

Conclusions: These results show that a collagen-TF based microfluidic flow assay is sensitive to FVIII levels. This assay could also potentially be used to predict bleeding severity, as well as for preventive dosing and response to new coagulation products.

PA 3.07-5

Characterization of large deletions in the F8 gene using multiple competitive amplification and genome walking technique

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Background: Large deletions in the F8 gene are responsible for approximately 3% of severe hemophilia A (HA) cases. However, only a few breakpoints in large deletions have been characterized.

Aims: To identify large deletions in the F8 gene and to characterize the molecular mechanisms leading to these deletions.

Methods: We used the AccuCopy technology, a copy number variation (CNV) genotyping method based on multiplex competitive amplification, to confirm deletions in index patients and to screen potential female carriers in 10 HA families. Also, breakpoints of these large deletions were characterized by a primer walking strategy and genome walking technique.
Results: Ten large deletions and four female carriers were identified by AccuCopy. The extents of deleted regions ranged from 1.3 to 68.5 kb. Exact breakpoints of these deletions were successfully characterized. Eight of them presented microhomologies at breakpoint junctions and several recombination-associated elements (repetitive elements, non-B conformation forming motifs and recombination-associated sequence motifs) were also observed in close proximity to the junctions.
Conclusions: The AccuCopy technology is a reliable and efficient tool for detecting large deletions in the F8 gene and for identifying HA female carriers. Genome walking technique is a highly specific, efficient and versatile method for characterizing the deletion breakpoints. Molecular characterization of deletion breakpoints revealed that non-homologous end joining and microhomology-mediated replication-dependent recombination were the major causative mechanisms of the ten large deletions in the F8 gene.

PA 3.07-6

Role of mannose-ending glycanes in the immunogenicity of FVIII in FVIII-deficient mice

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Background: Ten to thirty percent of patients with hemophilia A develop anti-factor VIII (FVIII) inhibitory antibodies after FVIII replacement therapy. The reasons for the elevated immunogenicity of FVIII are still poorly understood. A mandatory step for developing an anti-FVIII immune response is the endocytosis of the exogenously administered FVIII by antigen presenting cells (APCs), such as dendritic cells or macrophages, and the presentation of FVIII-derived peptides by APCs to CD4+ T lymphocytes. Previously, using human monocyte-derived dendritic cells (Mo-DCs) as a model of APCs, we have demonstrated that endocytosis of human recombinant FVIII by Mo-DCs leads to presentation of FVIII peptides to T cells *in vitro*, and that endocytosis is mediated at least in part through the macrophage mannose-receptor (CD206). However, we also showed that the latter mannose-sensitive pathway is not involved in the uptake of FVIII by murine bone marrow-derived dendritic cells (BM-DCs).
Aim: The aim of this study was to evaluate the importance of mannose-sensitive pathway in the immunogenicity of FVIII *in vivo* using FVIII-deficient (FVIII°) mice.
Methods: To investigate the importance of mannose-ending glycans in the immunogenicity of FVIII, we used different strategies. (i) We produced a demannosylated (DM-FVIII) human B domain-deleted FVIII lacking oligomannose carbohydrates at Asn239 and Asn2118, and a wild-type B domain-deleted FVIII (WT-FVIII, Δ2FVIII, LFB, Les Ulys) by transducing CHO-S cells with lentiviral vectors. To evaluate FVIII immunogenicity, FVIII° mice were injected intravenously with 1 UI of WT-FVIII or DM-FVIII 4 times at weekly interval. (ii) We saturated mannose-sensitive receptors in FVIII° mice with mannan, or mannose-ending glycans of FVIII with soluble CD206 (sCD206), prior to FVIII injection. (iii) We also compared the immunogenicity of FVIII in wild-type (WT) mice and CD206-deficient (CD206°) mice. In all cases, the anti-FVIII immune response was evaluated by measuring anti-FVIII IgG titers by ELISA and FVIII inhibitory titers by chromogenic assay.
Results: Purified WT-FVIII and DM-FVIII had similar specific activities (2007 and 2783 IU/mg, respectively) and presented the same capacity to bind VWF and activated platelets. Surprisingly, we observed a significantly increased immune response in FVIII° mice treated with DM-FVIII as compared to mice treated with the WT-FVIII: anti-FVIII IgG titers of 21,732 ± 12,349 and 2546 ± 1219 μg/mL, respectively ($P = 0.015$); FVIII inhibitory titers of 685 ± 335 and 84 ± 26 BU/mL, respectively ($P = 0.004$). Saturation of mannose-sensitive receptors *in vivo* or *ex vivo* quenching of mannose-ending sugars on FVIII using sCD206, prior to injection to FVIII° mice, both showed the same tendency. Likewise, CD206° mice developed a significantly higher anti-FVIII immune response than wild-type mice.
Conclusion: In contrast to our hypothesis based on results generated using human cells *in vitro*, blocking the interaction between FVIII and mannose-sensitive receptors in FVIII° mice increases FVIII immunogenicity. Whether this is due to intrinsic differences in CD206 expression in mice as compared to human, or to the fact that Mo-DCs are not an appropriate surrogate to mimic mannose-dependent antigen endocytosis *in vivo* is being investigated.

PA3.08 – Heparin-Induced Thrombocytopenia (HIT): Clinical – II

PA 3.08-1

Off-label use of fondaparinux in suspected acute heparin-induced thrombocytopenia (HIT) – final results from the GerHIT multicentre, retrospective registry study

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Background: Life-threatening HIT is treated with the non-heparin anticoagulants argatroban (A), lepirudin (L), or danaparoid (D). Frequently, fondaparinux (F) is used off-label.
Aims: The aim was to obtain data on therapy efficacy and safety of the different anticoagulants.
Methods: In a national multi-centre registry study, patients diagnosed with HIT between 01/2005 and 10/2009 were included when the retrospectively determined 4T's scores were ≥ 4 points (intermediate or high clinical risk for HIT), and when they were treated with at least one dose of A, L, D, or F. Outcome measures were: incidences of thromboembolic complications, amputations, skin lesions, thrombocytopenia, skin lesions, platelet recovery, bleeding and fatal complications.
Results: Ninety-six (49.2%) of 195 patients had an intermediate, and 94 (48.2%) patients had a high 4T's score (median: five points; six points in the F therapy group). Immunogenic and functional heparin-induced activation assays were positive in 54.6% and 32.8%, respectively. Of the patients, 57 (24.2%) were exposed to A, 9 (3.8%) to L, 70 (29.7%) to D, and 98 (41.5%) to F. The composite endpoint (thrombosis, amputation, skin necrosis) occurred with A in 8.8%, with L in 11.1%, with D in 12.9%, and with F in 0.0% of patients. Platelet counts recovered in all but 9 (4.6%) patients within 9.0 days (median). Of these, only one patient, who was also refractory to A/D, did not respond to F. All cause in-hospital mortality rates were 10.5% for A, 22.2% for L, 17.1% for D, and 0.0% for F. Bleeding complications occurred with A in 8.8%, with L in 22.2%, with D in 5.7%, and with F in 4.1%.

Summary/Conclusion: Fondaparinux seems to be an effective and safe alternative therapy option in suspected acute HIT although currently not licensed; no HIT-specific complications occurred even in patients with a high clinical probability for HIT. Further data are needed urgently because Lepirudin has been retrieved off the market, Danaparoid access has been limited due to manufacturing problems, and argatroban is contraindicated in patients with impaired hepatic function.
The Ethics Committee of the State Chambers of Physicians (Landesaerztekammer), Bavaria, confirmed that ethics approval was not required due to the complete anonymisation of the retrospective data that were acquired from the patients' individual medical records. The provision of study information to the patients and an informed consent form were not required. The study was registered at ClinicalTrials.gov (NCT01304238).

PA 3.08-2

Laboratory testing for heparin induced thrombocytopaenia (HIT); current practice amongst UK NEQAS and ECAT participants

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Background: Diagnosis of Heparin Induced Thrombocytopaenia (HIT) is based on a combination of clinical assessment, usually employing a 4T's score or similar, and laboratory testing. Therefore to ensure an accurate diagnosis, sensitive and specific laboratory assays are required.
Method: UKNEQAS initiated proficiency testing surveys for Heparin-Induced Thrombocytopenia (HIT) screening in 2008, to explore diagnostic accuracy amongst laboratories and to support improved laboratory performance of HIT screening. In the most recent exercise carried out in July and August 2012, three lyophilised citrated plasma samples were sent to 90 centres, of which approximately half were UK-based. One sample was a pooled normal plasma (coded HIT 12:02 below), one was a sample from a patient with positive results in several laboratory tests for HIT (Coded HIT 12:03), the third sample (HIT 12:01) was a dilution of the same positive sample constructed using normal plasma.
Results: A number of trends in relation to laboratory testing have occurred in the period between 2008 and 2012. Platelet aggregation tests are less frequently used and none of the four centres currently using conventional aggregometry reported use of washed platelets recommended in guidelines based on improved sensitivity relative to unwashed platelets. Amongst users of ELISA techniques an increasing proportion utilise assays detecting only IgG antibodies as recommended in UK Guidelines. For sample HIT12:03 (undiluted positive) all centres using either IgG or IgG, A & M-based assays reported an abnormal result. For sample HIT12:02 (normal plasma) all ELISA user reported a negative result. For sample HIT12:01 (diluted positive sample) there was a lack of concordance in interpretations – 4/16 (25%) of centres using a IgG/A/M ELISA reported a positive screen, and 6/16 reported an equiviocal result, whilst 12/14 centres using IgG-only ELISAs reported a negative screen, with the remaining two centres reporting equivocal results. For HIT12:02 (normal plasma) false-positive results were reported by five of 21 users of the Diamed technique with five more considering results to be equivocal or weakly positive. This technique recommends the use of fresh serum, and the effect of using lyophilised citrated plasma as a quality control material is being investigated. One of 12 IL HIT latex assay users reported an equivocal result on this sample, with the remainder reporting negative results, as did all users of ELISA, Stago STIC lateral flow assay, platelet aggregation assays and two Multiplate users. Samples HIT12:01 and HIT12:02 were also distributed to participants in the ECAT quality assurance programme. Two hundred and nine centres reported results; in general, ECAT results were comparable to those of NEQAS. Amongst ECAT participants, 49% of centres employing the Diamed method reported a positive screen on sample HIT12:02 (normal plasma) whilst all those using an ELISA-based method reported a negative result. For sample HIT12:01, a split of interpretations was observed amongst centres employing the same methods.
Conclusion: These survey data indicate that variability in laboratory test results is sufficient to influence patient management decisions, and confirms the continued need for proficiency testing in this area to help improve laboratory testing in the investigation of possible HIT.

PA 3.08-3

Detection and measurement of anti-IL8 IgG antibodies in cardiac surgery patients and heparin-induced thrombocytopenia using a multiplexed fluorimetric assay

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Background: Antibodies (Abs) to interleukin-8 (IL8) have been detected alone or in combination with Abs to PF4/heparin (H) complexes in few patients with heparin-induced thrombocytopenia (HIT). However, their pathogenicity remains uncertain such antibodies to IL8 have also been detected in healthy subjects. Cardiac surgery (CS) is a clinical situation associated with a high risk of Abs to PF4/H and HIT. In addition, CS induces a systemic inflammatory response with strong release of IL8.
Aims: To develop a sensitive and specific method for investigating whether Abs to IL8 may exist after cardiac surgery and in HIT patients, or not.
Methods: We developed a cytometry-based immunoassay using color-coded paramagnetic beads (Bio-plex Protm Magnetic COOH Beads) on which a murine monoclonal antibody to human CXCL8/IL8 was covalently coupled. Beads were then incubated without (w/o) or with recombinant human IL8 (rhIL8) and after washings, 'test beads' (i.e. with rhIL8) or 'control beads' (i.e. w/o rhIL8) were incubated with diluted plasma samples. After washings, a conjugated anti-human IgG was incubated with 'test beads' and 'control beads'. Beads were then washed and analyzed with a Bioplex100tm. Mean fluorescence intensity (MFI) was measured and results were expressed as 'MFI ratio' (=MFI with 'test beads' divided by MFI with 'control beads') and as 'Differential MFI' (=MFI with 'test beads' minus MFI with 'control beads'). To define the cut-off values, experiments were done with plasma samples from 54 healthy subjects. The cut-off values of 'MFI ratio' and 'differential MFI' were defined as > the 90th percentile and three of the 54 controls (5.5%) were identified as having significant levels of Abs to IL8. The presence of Abs to IL8 was then studied in 196 consecutive CS patients treated by heparin post-operatively and in 52 patients having definite HIT.
Results: Significant levels of Abs to IL8 were measured in 15 CS patients (7.6%) on day 7 postoperatively and in six HIT patients (11.6%). Results obtained were highly specific since in every case no significant fluorescence signal could be recorded when an excess of free IL8 was added in diluted plasma before incubation with 'test beads'. Abs to PF4/heparin complexes were also detected in 15 of the 21 plasma samples containing anti-IL8 Abs (six from HIT and nine from CS patients). No inhibition of anti-IL8 Abs binding to 'test beads' was obtained after spiking diluted plasma samples with purified PF4. Interestingly, anti-IL8 Abs were also detected preoperatively in every positive CS patients, with similar levels compared to those measured postoperatively. In addition, SRA performed with CS plasma samples and variable concentrations of rhIL8 and unfractionated heparin was consistently negative.

Summary/Conclusion: IgG Abs to IL8 may be detected by a specific assay in few patients with HIT and after cardiac surgery, but with an incidence that is not significantly higher than in healthy subjects. Moreover, our data obtained with pre-operative samples and with SRA do not support a role of heparin treatment in the synthesis of Abs to IL8 and their pathogenicity that remains to be demonstrated.

PA 3.08-4

Prospective evaluation of a rapid and IgG specific nanoparticle-based lateral flow immunoassay (Stic Expert HIT®) for the diagnosis of heparin-induced thrombocytopenia

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Background: The diagnosis of Heparin Induced Thrombocytopenia (HIT) is based on clinical suspicion and biological assays detecting antibodies to PF4/H but many require some delay before obtaining a result. Recently, a rapid and IgG-specific lateral flow immunoassay (LFIA) using PF4/polyanion complexes linked to biotin as antigens and gold nanoparticles coated with antibodies specific to biotin has been developed (Stic Expert HIT®, Stago).
Aims: To evaluate the performances of this assay in a prospective cohort of patients with suspected HIT.
We also compared the results obtained with serum and plasma and evaluated the inter-reader reproducibility of the assay.
In addition, results obtained with the Stic expert HIT® were compared to those collected in five centers with the rapid particle gel immunoassay (H/PF4-PaGIA®, Bio-rad).
Methods: Three hundred and thirty-seven consecutive patients were enrolled from February to October 2012 in 10 French centers. The pre-test probability of HIT was evaluated using the 4T's score. The LFIA was performed in each center on plasma and serum. IgG specific ELISA (Asserachrom HPIA IgG®, Stago) and serotonin release assay (SRA) were performed in our lab. PaGIA was performed in five centers in 124 patients.
Results: Definite HIT was diagnosed in 42 patients with positive ELISA and SRA (12.5%). The 4T's concluded to a low risk (LowR), intermediate risk (IR) and high risk (HR) of HIT in 28.8%, 61.4% and 9.8% of patients, respectively.
The interpretation of LFIA results is visual but the inter reader reproducibility was excellent with both plasma and serum (kappa ratio values higher than 0.9).
The negative predictive value (NPV) of LFIA performed on plasma was 99.6% with a negative likelihood ratio (LR) of 0.03. The LFIA was positive in 88 patients including 41 with definite HIT. Therefore, the PPV and positive LR of the Stic Expert were 46.6% and 6.06, respectively. Results obtained with serum were comparable.
According to the Bayes' equation, the post-test probability of HIT was 0.4% when the Stic Expert was negative in IR patients (pre-test probability = 11.6%), and equalled 43.3% when the test was positive.
The agreement between results obtained with the LFIA and PaGIA was not good, with 37.1% of discrepant results (kappa ratio < 0.4).
The NPV of PaGIA was 100% with a negative LR < 0.001 but PPV and positive LR were 22.2% and 2.24, respectively. Therefore, the post-test probability was < 0.1% when PaGIA was negative in IR patients, but it increased from 11.6% to 22.7% only, when the result was positive.
Summary/Conclusions: The LFIA Stic Expert HIT® is a rapid assay, which reliably allows to exclude HIT in < 40 min when used in combination with the 4T's score. This test can be performed on both serum and plasma and is particularly useful in LowR or IR patients for whom heparin treatment can be continued safely if the result is negative.
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PA 3.08-5

The clincal impact of anti-protamine/heparin antibodies in patients undergoing cardiac surgery

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Background: Protamine, routinely used post-cardiac surgery to reverse the anticoagulant effects of heparin, is immunogenic and a cause of anaphylaxis. We observed an otherwise unexplained rapid platelet count decrease directly after protamine adminstartion post cardiac-surgery.
Aim: To determine the incidence and clinical relevance of protamine-reactive antibodies in cardiac-surgery patients.
Methods: We tested 591 cardiopulmonary-bypass surgery patients for anti-protamine and anti-protamine-heparin IgG antibodies by enzyme-immunoassay (days 0, 6, 10, > 120) and by a functional assay to identify platelet-activating antibodies. We compared platelet counts and thromboembolic events for patients with platelet-activating anti-protamine-heparin antibodies against patients with and without non-platelet-activating antibodies. An animal model was used to assess antibody pathogenicity *in vivo*.
Results: Of 591 cardiopulmonary-bypass surgery patients, 57 (9.6%) patients tested positive for anti-protamine-heparin antibodies at baseline and 154 (26.6%) tested positive at day 10. Diabetes was identified as a risk factor for the development of anti-protamine-heparin antibodies. Fifty-two (8.8%) and 64 (10.8%) patients seroconverted until day 6 and 10 post CPB surgery, respectively, of which only 13/52 (25.0%) and 12/64 (18.8%) developed IgM antibodies. In the majority of patients these antibodies were transient and titers decreased substantially after 4 months ($P < 0.001$).
In vitro studies showed that these antibodies activate platelets in the presence of heparin and protamine in a FcgRIIa dependent fashion. Seven patients had platelet-activating, anti-protamine-heparin antibodies at baseline. They showed a greater and more prolonged decline in platelet counts compared to antibody-negative patients ($P = 0.003$), in addition, two of those patients developed early arterial thromboembolic complications vs. 9/584 controls (multivariate analysis: odds ratio 21.58; 95% confidence interval 2.90–160.89, $P = 0.003$). Using a NOD/SCID mouse model anti-protamine/heparin antibodies activated human platelets via FcgRIIa and induced thrombocytopenia only when protamine and heparin were present but not with protamine alone.
Summary/Conclusion: Our studies identify a novel post-protamine prothrombocytopenic disorder in post-cardiac surgery patients associated with platelet-activating anti-protamine-heparin antibodies, characterized by increased risk for early arterial occlusions.

PA 3.08-6

Impact of heparin-induced thrombocytopenia on acute coronary artery thrombosis in patients undergoing percutaneous coronary intervention

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Background: Percutaneous coronary intervention (PCI) has drastically reduced mortality in patients with acute coronary syndrome.

Anticoagulation with heparin during the procedure and concomitant (and subsequent) intensive antiplatelet therapy help to greatly reduce the recurrence of coronary artery thrombosis (CAT). Nonetheless, acute CAT remains associated with high mortality rates in patients who undergo PCI, despite receiving such intensive antithrombotic treatment. One possible cause is heparin-induced thrombocytopenia (HIT), a severe thrombotic side effect of heparin, because heparin is frequently administered to treat acute coronary syndrome in addition to anticoagulation during PCI. However, there are very few comprehensive reports on HIT-associated CAT during or soon after PCI, although some case reports have described HIT-associated CAT in PCI patients. Extensive analysis to elucidate the impact of HIT on acute CAT can potentially provide insights into how to decrease mortality in PCI patients.

Aims and Methods: To clarify the characteristics of HIT-associated CAT, we retrospectively investigated 21 patients who suffered from acute CAT during or soon after PCI and were clinically suspected of having HIT at 20 hospitals between August 2008 and January 2012. HIT was ultimately diagnosed by the detection of anti-platelet factor 4 (PF4)/heparin IgG with platelet-activating properties (HIT antibodies) at a therapeutic heparin concentration, but not at a high heparin concentration or with anti-FcγRIIa antibodies. The assay was performed using washed platelets prepared from whole blood of HIT antibody-sensitive healthy volunteers. The HIT and non-HIT groups were compared to identify characteristics associated with HIT.

Results: Of the 21 patients, five were diagnosed with HIT (23.8%). Optical density values of anti-PF4/heparin antibodies detected by ELISA were significantly higher in HIT patients than in non-HIT patients (2.88 ± 0.71 vs. 0.22 ± 0.51 in ELISA for IgG/A/M, $P < 0.01$; 2.02 ± 0.77 vs. 0.11 ± 0.012 in ELISA for IgG, $P < 0.01$). The decrease in platelet count was significantly greater in the HIT group ($74.9 \pm 14.3\%$ vs. $35.6 \pm 21.1\%$; $P < 0.001$). All the patients in the HIT group underwent PCI using unfractionated heparin within the past month (but not in the last 10 days) ($P = 0.014$), which led to the presence of HIT antibodies when the next PCI was performed. Within 90 min of heparin administration for PCI, acute CAT occurred concomitantly with a precipitous (> 50%) fall in platelet count, a clinical picture consistent with rapid-onset HIT. Among the five HIT patients, two had other thrombotic complications (stroke or pulmonary embolism), and one died even with immediate cessation of heparin and administration of a nonheparin anticoagulant. There were no significant differences between the groups in terms of sex ($P = 0.517$), age ($P = 0.734$), hyperlipidemia ($P = 0.34$), body mass index ($P = 0.972$), current tobacco use ($P = 0.469$), and renal dysfunction ($P = 0.554$).

Conclusions: Our results suggest that the contribution of HIT to acute CAT should be considered in patients who will undergo PCI if they underwent interventions involving heparin such as PCI within the past month (but not in the last 10 days). In such high-risk patients, routine screening for HIT antibodies before PCI or use of a nonheparin anticoagulant for PCI may be effective for reducing the incidence of acute CAT.

PA3.09 – Von Willebrand Factor: Clinical – II

PA 3.09-1

Re-evaluation of sequence variation in type 1 von Willebrand disease in the MCMDM-1VWD cohort

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Background: The MCMDM-1VWD study investigated involvement of the von Willebrand factor (*VWF*) gene in the pathogenesis of type 1 von Willebrand disease (VWD1). Previously published mutation analysis on index cases (IC) (Goodeve et al, Blood 2007;109:112) identified 124 candidate mutations in 105 IC (70%), with 87 IC having one mutation, 17 having two and one having three mutations.

Aims: (i) To use current technology to seek further candidate mutations in IC. (ii) To re-evaluate the cohort in light of current knowledge of *VWF* sequence variation.

Methods: Samples from 150 IC with a historic VWD1 diagnosis along with their affected and unaffected family members were available. *VWF* PCR primers were redesigned where necessary to eliminate single nucleotide polymorphisms within their sequence. Twenty-eight IC in whom candidate point mutation(s) had potentially been missed in previous analysis were PCR amplified and sequenced. Exon deletions/duplication were sought in 104 IC using multiplex-ligation dependent probe amplification. One thousand genomes and African-American/white American VWF sequence variant data was compared with candidate mutations (Wang et al JTH 2012 doi: 10.1111/jth.12093; Bellissimo et al Blood 2012;119:2135). Data was available on bleeding score (BS), linkage analysis and multimeric profile along with several phenotypic parameters.

Results: Among 150 IC, an additional 11 candidate mutations were identified; five through sequence analysis (two splice, two missense, one silent) plus six large deletions (exons 3, 33–34, 32–34; 1 IC each and exons 4–5; 3 IC). Comparison of candidate mutations in IC with published sequence variant data plus pedigree inheritance suggested that candidate mutations in 7 IC; p.M740I, p.P2063S, p.P2145S, p.R2313H and p.R2663P were likely polymorphic. Previously identified variants in the *VWF* promoter were also discounted ($n = 8$). Following re-evaluation, 119 candidate mutations were present in 106 IC (71%), with 93 IC having one mutation and 13 having two mutations (10 compound heterozygotes, three allelic mutations). Previous multimer analysis identified slight multimer abnormalities (AbM) in57 IC. Mutations were identified between exons 24–45 in all these 57 IC and 34 mutations (60%) were fully penetrant. IC were divided into four groups based on 20 IU/dL VWF:RCo increments. Among 48 IC with ≤ 20 IU/dL VWF:RCo, 98% had a mutation identified and 88% had AbM; 63% families had fully penetrant mutations. Figures decreased to 81% with mutation(s) and 29% AbM among those with > 20–≤ 40 IU/dL VWF:RCo, 45% with mutation(s) and 2% AbM with > 40–≤ 60 IU/dL VWF:RCo, and 35% with mutation(s), 10% AbM with > 60 IU/dL VWF:RCo. Only 18% of IC with VWF:RCo > 40 IU/dL had fully penetrant mutations. Forty-four IC (29%) had no mutation identified. Among these, 30 IC were unlikely to have VWD1 as no *VWF* mutation was identified and linkage analysis did not support *VWF* gene involvement. Twenty-one of these IC had BS 3–19; VWF:Ag and VWF:RCo were both ≥ 40 IU/dL.

Conclusions: Further analysis of *VWF* sequence variation has enhanced understanding of VWD pathogenesis in the MCMDM-1VWD cohort. Patients diagnosed with VWD1, no *VWF* mutation but

high BS are likely misdiagnosed and further defects in for example, platelet function may be responsible and should be investigated.

PA 3.09-2

NHLBI guidelines and the percentile 2.5th of plasma VWF for the diagnosis of type 1 VWD. Comparison in 4517 referred patients and 280 selected patients with conclusive bleeding history

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Background: Global consensus regarding laboratory criteria for diagnosing type 1 VWD are still lacking. The overlapping of lower cut-off values of plasma VWF between non-bleeder population and patients with pathologic bleeding, as well as the relative high frequency of bleeding symptoms in otherwise healthy individuals constitute major diagnostic difficulties. To transitorily circumvent this problem, the 2008 NHLBI Guidelines recommended diagnosing definitive VWD in patients with plasma VWF < 30 IU/dL and those patients with VWF levels between 30–50 IU/dL would be labeled as 'possible VWD' or 'low-VWF'. A recent survey found that only 27% of North American Laboratories followed the NHBLI guidelines. An alternative criterion is to use the 2.5th percentile of plasma VWF as cut-off value for the diagnosis of VWD, also including the VWF:CBA.

Aims: To analyze and compare the yield of both approaches to diagnose VWD.

Methods: We diagnose type 1 VWD when VWF is < 2.5th percentile in at least two of these three measurements: VWF:Ag, VWF:RCo and VWF:CBA, using cut-off values of < 42, < 37 and < 39 IU/dL, respectively, obtained from 299 non-bleeders healthy controls. Types 2 are diagnosed as usual (discrepant VWF antigen and function, RIPA, multimers..). We reviewed retrospectively 4517 patients tested in our Lab for diagnosis of VWD in a five-year period (2008–2012). They were referred by their physicians and we had no access to the bleeding history of most of them. Also, we compared these approaches in a different group of 280 selected patients interviewed by one of us (TQ) and judged as pathological bleeders, matched for age, gender and ABO blood type with the controls (Haematologica 2007;92:357)

Results: Applying NHLBI guidelines, a total of 158/4517 patients were diagnosed as VWD (3.5 ± 0.4% per year) and 802/4517 (17.9 ± 1.9% per year) were classified as Possible VWD, whereas using the percentile 2.5th criterion we diagnosed VWD in 312/4517 (6.9 ± 0.27% per year). In the selected 280 patients the NHLBI criterion diagnosed 39 (13.9%) and 67 (23.9%) of the patients as VWD and Possible VWD, respectively, whereas the percentile 2.5th criterion diagnosed VWD in 50 (17.9%) patients. Among the 299 Controls, 46 (15.4%) would have been considered as Possible VWD with NHLBI recommendations.

Conclusions: (i) Independent of the criterion, a very small proportion of the patients referred end up with VWD diagnosis. (ii) As expected, stringent clinical criteria to select patients for study increase the proportion of diagnosed patients. (iii) Patients considered Possible VWD are 5-fold more than those with Definitive VWD (NHLBI), and most of them have VWF in the range of 42–50 IU/dL, which is higher than the percentile 2.5th. This is why more than 15% of the non-bleeder Controls qualify as 'laboratory' Possible VWD. (iv) NHLBI Guidelines appear too restrictive and might miss the diagnosis of VWD in a significant proportion of patients. (v) Patients with type 1 VWD do not differ clinically from those with other Mild Bleeding Disorders; specific treatment for each disorder demands further refinement and universal consensus on the laboratory criteria for VWD diagnosis. (FONDECYT 1130853).

PA 3.09-3

Comparison of pediatric bleeding questionnaire (PBQ) and ISTH/SSC bleeding assesment tool (BAT) scores in pediatric patients referred with mucocutaneous bleeding

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Background: The accurate assessment of hemorrhagic symptoms in pediatric subjects referred with mucocutaneous bleeding to determine the presence of an underlying bleeding disorder is essential, but can be difficult; epistaxis and bruising are common in childhood, and major hemostatic challenges may not yet have occurred. The PBQ, based on the MCMDM-1VWD Bleeding Questionnaire, incorporates pediatric-specific bleeding symptoms to quantify bleeding severity in children, scoring symptoms in a −1 to +4 range. An abnormal score has been determined to be ≥ 2 (Bowman et al, JTH 2009;7:1418). The ISTH/SSC BAT, in which bleeding symptoms are scored in a 0 to +4 range, has been developed to standardize reporting of bleeding symptoms in adults and children (Rodeghiero et al, JTH 2010;8:2063). Criteria for scoring each symptom are similar between the questionnaires, but differences exist.

Aim: To perform a detailed comparison between PBQ and ISTH/SSC BAT scores in a prospective study of children < 18 years of age referred to our tertiary-care Bleeding Disorders Clinic with mucocutaneous bleeding and/or a family history of von Willebrand disease or a platelet function disorder.

Methods: Bleeding scores were determined by face-to-face interview of 100 subjects/parents.

Results: The mean age of the study population was 9.7 years (range: 0.5–17.8 years), and 58% were female.

The median PBQ and ISTH/SSC BAT scores were 3, with ranges of 0–12 and 0–13, respectively. The two scores were identical in 52% of children. In the 25% of children with a lower ISTH/SSC BAT score, it was lower by 1 (in 92%) or 2 (in 8%), mainly due to a lower score for cutaneous bleeding symptoms. In the 23% of children with a higher ISTH/SSC BAT score, it was higher by 1 (70%), 2 (26%) or 3 (4%), mainly due to the lack of a −1 score for a child who did not bleed with at least two tooth extractions or surgeries and/or a higher score for menorrhagia.

Fifteen percent of children received scores for pediatric-specific symptoms, specifically macroscopic hematuria, post-circumcision bleeding, cephalohematoma, umbilical stump bleeding, post-venipuncture bleeding, or conjunctival hemorrhage. A normal PBQ score (i.e., 0 or 1) was observed in 11% of children. In these subjects, the ISTH/SSC BAT ranged from 0 to 3, with 5 children scoring 2 or 3.

Summary/Conclusions: In this population of children referred with mucocutaneous bleeding symptoms and/or a family history of a mucocutaneous bleeding disorder, PBQ and ISTH/SSC BAT scores were similar, but not identical. Although pediatric-bleeding symptoms have not been observed by others using the PBQ and/or ISTH/SSC BAT (Bowman et al. JTH 2009;7:1418; Bidlingmaier et al, JTH 2012;10:1335), they were reported in the present study, indicating that inclusion of these symptoms in the standardized questionnaires is important. Since many children with a normal PBQ score had an ISTH/SSC BAT score higher than the PBQ cut-off, it will be essential to determine the cut-off for a normal ISTH/SSC BAT bleeding score in pediatric subjects if it is to be adopted for general use in evaluating whether a child has an underlying bleeding disorder.

PA 3.09-4

Previously missed mutations in the MCMDM-1VWD type 1 von Willebrand disease study

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Introduction: Three studies conducted in Europe, the UK and Canada provided details about the molecular genetic basis of type 1 von Willebrand disease (VWD1), but failed to detect mutations involved in 30–40% of cases. Reasons for this failure may include SNP within primer annealing sites leading to lack of mutant allele amplification or insensitivity of mutation analysis methods. The EU study MCMDM-1VWD undertook mutation scanning and DNA sequencing of the von Willebrand factor (*VWF*) gene in 154 index cases (IC) with a historic diagnosis of VWD1 but failed to detect mutations in 30%.

Aim: (i) To reanalyse *VWF* in IC where phenotype suggested that mutations may have been missed. (ii) To determine mechanisms by which mutations identified affect VWF synthesis and secretion.

Methods: *In silico* analysis was performed to screen previously used MCMDM-1VWD PCR primers for presence of SNP within primer binding sites and when necessary new primers were designed. The 52 *VWF* exons and flanking regions were amplified and sequenced. Eighteen IC (four where a previously identified heterozygous mutation did not fully explain phenotype and 14 with no mutation identified) having low VWF levels and significant bleeding were investigated. Site-directed mutagenesis was used to create recombinant mutant cDNA expression vector, then transiently transfected into HEK293T cells. Media and cell lysates were analysed after 48 h for VWF:Ag by ELISA.

Result: Heterozygous mutations were identified in four families comprising missense mutations p.W2271G (in compound heterozygosity with p.R854Q) and p.C1157R (in compound heterozygosity with p.S539Lfs*38; IC subsequently classified as VWD3) silent mutation p.L1382 = and splice mutation c.1432+1G>T (in compound heterozygosity with p.R816W). p.W2271G and p.C1157R, predicted to be damaging by *in silico* analysis, affect fully conserved residues. Unlike p.R854Q and p.R816W, p.W2271G and c.1432+1G>T segregated with disease phenotype and their presence alone or in compound heterozygosity with p.R854Q (VWF:Ag 37–38 IU/dL, VWF:RCo 32–43 IU/dL, FVIII:C (p.[W2271G;R854Q]) 28–34 IU/dL, FVIII:C (p.W2271G) 85 IU/dL) and p.R816W (VWF:Ag 47–49 IU/dL, VWF:RCo 52–67 IU/dL, FVIII:C (c.1432+1G>T;p.R816W) 14 IU/dL, FVIII:C (c.1432+1G>T) 113 IU/dL) explain phenotype differences in the affected families. Expression studies of recombinant mutant VWF (rVWF_G2271 and rVWF_R1157) showed reduced secretion levels in the homozygous state ($P < 0.0001$) by 96.7% and 98.2% respectively compared to rVWF_wt with significantly increased intracellular retention (30%; $P = 0.03$ and 90%; $P < 0.0001$ respectively). In the heterozygous state, both also resulted in significantly reduced secretion ($P < 0.05$) by 77% and 55% respectively while intracellular retention was observed only in rVWF_R1157 by 47% ($P = 0.001$).

Conclusion: *In vitro* expression data indicates that mutations located within D3 and D4 domain can lead to intracellular retention. Mutations were missed due to insensitivity of mutation analysis method (p.W2271G) and SNP within primer annealing sites (p.C1157R and c.1432+1G>T). For the remaining IC, no mutations were found but presence of other genetic defects in deep intronic/regulatory *VWF* regions, intronic or non-coding copy number variation or mutation within other genes may influence disease phenotype.

PA 3.09-5

Molecular characterization of 14 italian patients with type 3 von Willebrand disease

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Background: Type 3 von Willebrand disease (VWD) is a rare autosomal recessive bleeding disorder characterized by a quantitative defect of the von Willebrand factor (VWF).The genetic analysis of type 3 is required to identify patients at possible risk of inhibitor occurrence in presence of homozygous partial or complete gene deletions.

Aim of the Study: We investigated the molecular basis of VWD in 14 patients affected by type 3 VWD, after carrying out the complete sequence of the VWF gene coding region.

Results: A causative mutation as identified in 27/28 disease-associated alleles. Six novel mutations and 10 already described were found. Six homozygous null mutations were found in eight patients: a splicing mutation in intron 13 (c.1534-3C>A); a novel insertion in exon 11 (c.1209insA, Y403 fs); a novel small deletion in exon 36 (c.6187DelC, P2063 fs); two nonsense mutations (Cys2533X and Gln2544X); finally a novel splicing mutation in intron 21 (c.2820+5G>C) that by *in vitro* analysis seems to reduce significantly the donor splice site score. One patient shows a novel homozygous missense variation (Cys2325Ser). Three other patients were compound heterozygous. One has two null mutations, Arg1659X and a novel small deletion in exon 28 (c.4470delC), which forms a premature stop codon 97 codons downstream. The second patient presents a c.4944delT and a splicing mutation in intron 18 (c.2443-1G>C). Finally one patient shows the Gln2544X mutation (already found in homozygosis) and a splicing variation in intron 15 (c.1946-4C>T). One patient shows three mutations: a deletion in exon 18 (c.2435DelC) in one allele, and the missense variation Ser85Pro together with a potential splice variation c.5170+10C>T in the other allele. Only in a single patient a single heterozygous mutation was found, a novel small deletion in exon 24 (c.3179delG) with creation of a premature stop codon 58 amino acids downstream; no other variations were found after complete sequencing of entire VWF coding region.

Conclusions: The molecular bases of type 3 VWD in Italy show a wide heterogeneity, at variance with what is observed in Great Britain or in North Europe where homozygous partial or complete gene deletions are rather common. mRNA studies are ongoing to verify the pathophysiological mechanisms associated with splicing mutations and to study the nonsense mediated mRNA Decay (NMD) process possibly associated with null mutations.

PA 3.09-6

Orthopaedic surgery in patients affected by von Willebrand disease and rare bleeding disorders

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Background: Patients affected by VWD and severe RBDs may suffer from disabling chronic arthropathy as a consequence of recurrent intra-articular bleeding or may require orthopaedic surgery independently of their coagulation disorder. Orthopaedic surgery in patients with clotting disorders represents a surgical challenge and information

regarding the management of orthopaedic surgery in such patients are limited.

Aim: Description of orthopaedic surgery management in patients affected by VWD or RBDs.

Methods: Retrospective data collection regarding orthopaedic surgery between January 1981 and November 2012. Seventy-one procedures were made in 48 patients affected by VWD (25 patients) or RBDs (23 patients). In RBDs patients, the classification in severe, moderate or mild factor deficiency was defined on the basis of RBDs project [JTH 2012].

Results: Of the 71 orthopaedic procedures, 12 were minor surgery (seven hand surgery, five others) and 59 were major surgeries (19 arthroprosthesis, 19 arthroscopic procedures, 21 others procedures). The median age of the patients at surgery was 47 years (range: 10–78) and the median follow-up time was 37 months (range: 1–384). The most frequent RBDs were FVII (11/22) and FXI (7/22) deficiency. Sixty procedures were performed using replacement therapy (RBDs: 16 severe, two moderate, three mild; VWD: eight type 1, eight type 2, seven type 3, two AWVS), replacement therapy and desmopressin (three severe RBDs, one AVWS), desmopressin (VWD: five type 1 and two type 2), tranexamic acid (RBDs: three mild and 1 severe). Eight procedures (seven mild RBDs, one VWD type 1) were performed without prophylaxis, with a bleeding complication reported in one case of mild FVII deficiency that required treatment with replacement therapy after surgery. Bleeding complications occurred in five more procedures (overall 8%): three patients affected by VWD (one type 1, one type 2 and one AVWS), one patient affected by severe FVII deficiency and one patient affected by moderate FXI deficiency. In two cases (VWD type 1 and AVWS) the bleeding complication was associated with low molecular weight heparin (LMWH), started 12 h after surgery. In one patient affected by type 3 VWD, the presence of anti-VWF inhibitor was diagnosed after surgery (ankle arthroscopy). This patient was treated with high dosage and frequent administration of FVIII/VWF concentrate (70–90 U/kg every 6–8 h), with no bleeding complications. Post-operative rehabilitation was started immediately after surgery, when indicated, with anti-haemorrhagic prophylactic treatment in patients affected by severe/moderate deficiencies. No late bleeding problems were recorded. Only three patients received prophylaxis with LMWH.

Conclusion: Good control of haemostasis can be achieved with an appropriate substitutive therapy in severe bleeding disorders. In mild deficiency replacement therapy can be avoided before surgery in some cases, but needs to be available and used in case of excessive bleeding. The control of the bleeding disorder combined with the surgical technique is the reason for a high success rate. Post-operative rehabilitation begins immediately after surgery under treatment coverage when indicated. In conclusion, orthopaedic surgery performed in a specialised haemophiliac centre by a highly experienced surgeon is a safe procedure.

PA3.10 – Anticoagulant Agents – XII

PA 3.10-1

DATA III survey. Ten-year follow-up on Dutch orthopedic thromboprophylaxis

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Background: Our two previous surveys in the Netherlands have revealed that guidelines regarding orthopedic thromboprophylaxis were not followed and that a wide variation in protocols exists.

Aims: The DATA III survey (Dutch Antithrombotic Treatment for Arthroplasties) was performed to assess the current use of thromboprophylactic modalities and to compare it with the results of two previous surveys and with national guidelines.

Methods: All departments of orthopedic surgery in the Netherlands were sent a follow-up survey on venous thromboprophylaxis, and the data obtained were compared to the results of two surveys performed 5 and 10 years earlier.

Results: The response rate was 94 out of 108 departments (87%). All used pharmacological thromboprophylaxis following arthroplasties of the hip and knee. Low molecular weight heparin (LMWH) was used most frequently (77% of the departments), followed by rivaroxaban (15%), fondaparinux (6%), Vitamin K Antagonists (VKA) (6%), the combined use of VKA and LMWH (5%) and dabigatran (3%). Ten years earlier, VKA treatment was the predominant prophylaxis (79%). All departments prescribed pharmacological prophylaxis after femoral and tibia fractures during admission. Ninety percent of the departments used LMWH. Prophylaxis was continued for 6 weeks in 78% of cases. Only two departments did not use extended prophylaxis after hip fracture surgery and in one department, no extended prophylaxis after proximal knee fracture surgery was given. After hip and knee arthroplasty, LMWH treatment was initiated on the day before surgery in 21% of cases (compared to 65% 10 years ago), perioperatively in 57%, and in the evening following surgery in 22%.

In general, for daycare surgery and arthroscopies either no prophylaxis was given (68% and 56% respectively) or a single shot of LMWH. After anterior cruciate ligament reconstruction, 89% of the departments used prophylaxis. Twenty-nine percent administered prophylaxis once or during admission, whereas 71% used extended prophylaxis, varying from 10 until 42 days.

During plaster cast immobilization, more departments used thromboprophylaxis nowadays. Patients treated with a below knee plaster cast, received thromboprophylaxis in 88% of the departments (compared to 50% 10 years ago). Thirty-three percent of the departments only used thromboprophylaxis when the patient is not allowed to bear weight on the below knee plaster cast. Patient with a plaster cast above the knee were given thromboprophylaxis in 96% of cases. Prophylaxis was most frequently given by means of LMWH. VKA are not used nowadays, in contrast to 2002, when 68% of the departments used VKA for this indication.

Summary/Conclusions: The use of pharmacological prophylaxis after arthroplasty of the hip and knee and also after fracture surgery around the hip and knee is common practice in the Netherlands. Five years ago, the widely used VKA had been largely replaced with LMWH. In 2012 low molecular weight heparin remains the most commonly used thromboprophylactic agent. The new oral anticoagulant rivaroxaban is used more frequently than fondaparinux after hip and knee arthroplasty. There is a significant increase in the use of thromboprophylaxis during below knee plaster cast immobilization. In general, national guidelines are properly adhered to.

PA 3.10-2

First year experience with the German external quality assessment scheme for the new oral anticoagulants (INSTAND e.V.)

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Background: The new oral anticoagulants (NOAC) dabigatran and rivaroxaban are assigned for use without laboratory monitoring. However, in clinical situations as (major) bleeding events or renal failure drug monitoring might be required. Especially for dabigatran, the manufacturer recommends specific drug level monitoring in situations with an assumed bleeding risk.

Aims: An external quality assessment scheme (EQAS) of the German proficiency testing organization INSTAND e.V. was established in 2012 to investigate the suitability of different monitoring methods for dabigatran and rivaroxaban.

Methods: In two trials in May and October 2012 lyophilized and stabilized plasma samples containing two concentration levels of dabigatran and rivaroxaban, respectively, were sent to the participants.

Overall 38 laboratories participated with eight different methods for dabigatran monitoring and 34 laboratories with seven different methods for rivaroxaban monitoring. Target levels were 0.12 and 0.30 mg/L for the dabigatran samples and 0.05 and 0.25 mg/L for the rivaroxaban samples, respectively.

Results: The same samples were used for the two trials to test reproducibility. Results are shown as: (all samples/results of first trial/results of second trial).

Dabigatran level 1 samples showed the following results mean ± SD (0.12 ± 0.026/0.12 ± 0.019/0.12 ± 0.032), coefficient of variation, CV (22.1%/15.9%/27.1%), median (0.11/0.11/0.11), interquartile range, IQR (0.10–0.12/0.11–0.12/0.10–0.13), min-max (0.08–0.23/0.08–0.18/0.08–0.23) and level 2 samples: mean ± SD (0.31 ± 0.041/0.32 ± 0.040/0.30 ± 0.042), CV (13.3%/12.6%/13.9%), median (0.31/0.31/0.30), IQR (0.28–0.33/0.30–0.33/0.28–0.33), and min-max (0.23–0.45/0.25–0.45/0.23–0.43).

Rivaroxaban level 1 samples: mean ± SD (0.10 ± 0.014/0.10 ± 0.016/0.10 ± 0.013), CV (14.4%/16.3%/13.2%), median (0.10/0.09/0.10), IQR (0.09–0.10/0.09–0.10/0.09–0.10), min-max (0.06–0.14/0.06–0.14/0.06–0.14), and level 2 samples: mean ± SD (0.28 ± 0.035/0.28 ± 0.048/0.29 ± 0.022), CV (12.4%/17.2%/7.6%), median (0.29/0.29/0.29), interquartil rage (0.27–0.30/0.27–0.31/0.27–0.30), and min-max (0.14–0.34/0.14–0.34/0.25–0.34).

Summary: Accuracy, precision and reproducibility in this first German NOAC EQAS were better than expected as most of the participants just established their assays and several different (new) methods were used. Calibration of the dabigatran methods seems to be slightly better than calibration of the rivaroxaban methods as target values were very good reproduced in both sample levels with the dabigatran methods. Overall, available methods for measurement of plasma concentrations of the new oral anticoagulants are suitable for drug monitoring purposes.

PA 3.10-3

Case fatality of antithrombotic therapy associated major bleeding or major trauma

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Background: Antithrombotic therapy (antiplatelet drugs [AP] and oral anticoagulants[OAC]) is associated with an increased incidence of haemorrhagic complications. The outcome of bleeding complications is equally important as the incidence itself. With the introduction of new oral anticoagulants the outcome may become even more important as these new drugs do not have a specific agent that can restore normal haemostasis in case of life-threatening bleeding. Most available data on the impact of bleeding is derived from large randomized trials. It is uncertain to what extent these data apply to real-world patients who generally have more pre-existent comorbidities than a standard trial population.

Aims: To compare the outcome of bleeding complications in real-world patients treated with oral anticoagulants, antiplatelet drugs or both.

Methods: The medical records of all patients admitted to three academic hospitals in Ontario, Canada with major bleeding (selected with ICD-10 codes) or major trauma (injury severity score of 12 or higher) between October 2010 and March 2012 were reviewed. Transfers from community hospitals were excluded. Patients were divided into groups according to pre-admission antithrombotic therapy: OAC only, AP only, and combined OAC and AP therapy. Primary outcome was in-hospital mortality for all patients and for subgroups of type of admission (intracranial haemorrhage [ICH], gastro-intestinal [GI] bleeding, or major trauma). Secondary outcomes were length of hospital stay and transfusion requirements.

Results: Of 1834 screened patients, 289 were eligible and included in the analysis (mean age 76 years, mean duration of admission 12 days, SD 21 days): 74 were treated with OAC only (group 1; 70 patients used vitamin K antagonists [VKA], three used dabigatran etexilate), 180 were treated with AP only (group 2), and 35 received combined OAC and AP therapy (group 3; 34 VKA, 1 dabigatran etexilate). The proportions of patients admitted with spontaneous ICH, GI bleeding and major trauma were 18%, 41% and 27% in group 1, 11%, 57% and 32% in group 2, and 14%, 60% and 26% in group 3. The case-fatality rate for groups 1, 2, and 3 respectively were 28%, 8% and 17% for all patients combined ($P < 0.001$), 69%, 30% and 40% for spontaneous ICH ($P = 0.08$), 3%, 2% and 10% for GI bleeds ($P = 0.21$), and 41%, 10% and 22% for patients with major trauma ($P = 0.005$). None of the patients treated with dabigatran etexilate died. There were no differences in the duration of admission between groups. Erythrocyte transfusion was given in 47%, 57% and 69% of patients in groups 1, 2 and 3 respectively ($P = 0.02$). In patients treated with VKA, 41% of patients received prothrombin complex concentrate (PCC) and 59% of patients received either PCC or fresh frozen plasma.

Conclusions: The case-fatality rate of haemorrhagic complications associated with oral anticoagulants seems higher for real-world patients treated with oral anticoagulants than for patients treated with antiplatelet therapy only. The number of patients treated with dabigatran etexilate was too small to draw any conclusions.

PA 3.10-4

Tissue factor-induced hypercoagulability in rats is increased by low doses of the direct thrombin inhibitor dabigatran but decreased by the factor Xa inhibitor rivaroxaban

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Background: Hypercoagulability has been reported with low doses of the direct thrombin inhibitor melagatran in preclinical studies. Melagatran increased thrombin generation in human plasma *in vitro* and aggravated disseminated intravascular coagulation at low plasma concentrations *in vivo* in rats. This phenomenon was not observed with the direct Factor Xa inhibitors DX-9065a or edoxaban.

Aims: The purpose of this study was to compare the effects of rivaroxaban – a direct Factor Xa inhibitor, with those of dabigatran – a direct thrombin inhibitor, in a rat model of tissue factor (TF)-induced hypercoagulability by measuring thrombin-antithrombin (TAT) and fibrinogen levels.

Methods: Fasted male Wistar rats were anaesthetized and received intravenous rivaroxaban (0.0009–0.9 mg/kg) or dabigatran (0.001–1.0 mg/kg), or the appropriate vehicle. Disseminated intravascular coagulation was induced by TF. TF (RecombiPlasTin, Instrumentation Laboratory; 8 mg/kg) or vehicle was given at 0.3 mL/min as an intravenous bolus 5 min later; a control sham group received appropriate vehicle only. Blood was withdrawn 10 min later by puncture of the abdominal aorta for the measurement of TAT and fibrinogen levels using ELISA kits (Enzygnost, Dade Behring, and Rat Fibrinogen Elisa, Immunology Consultants Laboratory, respectively), and the plasma levels of rivaroxaban and dabigatran were determined by liquid chromatography-tandem mass spectrometry.

Results: Rivaroxaban dose-dependently inhibited TF-induced TAT generation over the dose range of 0.009–0.9 mg/kg (corresponding to plasma levels of 24–1952 μg/L) compared with the TF-control group. Rivaroxaban normalized TAT generation completely at a dose of 0.27 mg/kg (481 ± 21 μg/L) from 133 ± 6 to 21 ± 1 μg/mL ($P < 0.001$) but did not affect TAT generation at lower doses (0.0009–0.0027 mg/kg; 4–14 μg/L). Rivaroxaban had no effect on plasma

fibrinogen levels at any dose tested. At a dabigatran dose of 0.3 mg/kg (390 ± 23 μg/L), TF-induced TAT generation was completely normalized to 31 ± 2 μg/L ($P < 0.001$). In contrast, dabigatran potentiated TF-induced TAT generation at low doses. TAT increased to 144 ± 12 μg/L at a dabigatran dose of 0.01 mg/kg (< 25 μg/L) and to 133 ± 10 μg/L at 0.03 mg/kg (40 ± 3 μg/L), compared with 105 ± 7 μg/L in the TF-control group ($P < 0.01$, $P > 0.05$, respectively). Similarly, plasma fibrinogen levels were significantly reduced by 0.01 and 0.03 mg/kg dabigatran from 2.4 ± 0.1 to 1.9 ± 0.1 g/L ($P < 0.05$).

Conclusions: Both rivaroxaban and dabigatran potently reduced TF-induced hypercoagulation in this rat model. However, direct thrombin inhibition with dabigatran increased hypercoagulability at low plasma concentrations (which was not seen with rivaroxaban). These results are consistent with the findings in previous studies comparing the direct thrombin inhibitor melagatran and the direct Factor Xa inhibitors DX-9065a and edoxaban.

PA 3.10-5

Relative lack of effect of steady-state erythromycin on the pharmacokinetics and pharmacodynamics of a single dose of rivaroxaban in subjects with renal impairment or with normal renal function

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Background: Rivaroxaban is eliminated through renal excretion and drug metabolism, with cytochrome P450 (CYP) 3A4 enzymes and P-glycoprotein (P-gp) transporters both playing significant roles. Separate assessments of renal impairment (RI) and concomitant administration of erythromycin (a moderate inhibitor of CYP 3A4 and P-gp) have shown that each, individually, can result in increases in rivaroxaban exposure. Although these individual increases were not considered clinically relevant, the previous studies did not discount the potential for a complex drug-drug and disease interaction that, in theory, may lead to a synergistic increase in exposure to rivaroxaban as suggested previously in a physiologically-based pharmacokinetic (PK) model from the FDA.

Aims: The aim of this study was to investigate the PK and pharmacodynamics (PD) of rivaroxaban when co-administered with steady-state (SS) erythromycin in subjects with mild/moderate RI relative to subjects with normal renal function (NRF).

Methods: The PK and PD of rivaroxaban were assessed in subjects with NRF ($n = 8$), mild RI ($n = 8$) or moderate RI ($n = 8$) who were otherwise healthy, after a single 10 mg dose of rivaroxaban either alone or with SS erythromycin (i.e. after 5 days of a 500 mg three-times-daily regimen).

Results: Rivaroxaban t_{max} and $t_{½}$ were similar between renal function groups and treatments; C_{max} was reached 2–4 h post-administration, and elimination half-lives ranged between 7.4 and 10 h in all groups. When assessing the effects of RI, administration of rivaroxaban 10 mg to subjects with mild/moderate RI increased rivaroxaban C_{max} by 23% and 36%, and AUC_{∞} by 15% and 17%, respectively, relative to subjects with NRF. When assessing the effects of erythromycin, co-administration of rivaroxaban 10 mg with SS erythromycin to subjects with NRF increased rivaroxaban C_{max} by 40% and AUC_{∞} by 39%. When assessing the effects of RI plus erythromycin, co-administration of rivaroxaban 10 mg with SS erythromycin in subjects with mild/moderate RI increased rivaroxaban C_{max} by 56% and 64%, and AUC_{∞} by 76% and 99%, respectively, relative to subjects with NRF. Changes in PD parameters displayed similar trends to those seen for PK. RI and concomitant erythromycin use had generally similar independent effects on Factor Xa inhibition and prothrombin time prolongation. When RI and erythromycin use were combined, the effects on Factor Xa inhibition and prothrombin time prolongation were approximately additive in most cases. No serious adverse events occurred during the study and no subjects reported persistent adverse events at the end of the study or at the time of the last follow-up visit.

Summary/Conclusions: Considered separately, RI and erythromycin co-administration each increased rivaroxaban PK and PD as expected. When the effects of RI and erythromycin were combined, approximately additive effects on PK and PD were observed, in contrast to the synergistic effect predicted in the previous model. These data suggest that clinically relevant interactions of rivaroxaban with erythromycin in patients with mild to moderate RI are unlikely. All treatments in all renal function groups examined were well tolerated, and no new safety concerns were identified.

PA 3.10-6

Effects of rivaroxaban and dabigatran on hemostasis and reversion of their antithrombotic effects by different coagulation factors: evidence raised from a clinical study in healthy volunteers

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Background: There is a lack of information on therapies that could reverse the effects of new oral anticoagulants in patients that require an urgent restoration of their impaired hemostatic mechanisms.

Aims: (i) To assess the effects of new oral anticoagulants rivaroxaban and dabigatran on hemostasis, and (ii) To evaluate the effects of different coagulation factor concentrates (prothrombin complex concentrates (PCCs), activated PCCs (aPCCs) and rFVIIa) to reverse the alterations of hemostasis induced by these new anticoagulants.

Methods: The study was approved by our institutional medical ethics committee (Eudra CT2010-022985-29; ClinicalTrials.gov Identifier: NCT01478282). Studies were performed using blood samples from 10 healthy individuals subjected to 5 days of treatments with 20 mg/day for rivaroxaban and 150 mg/12 h for dabigatran doses separated by a washout period of 14 days. Blood samples were spiked *in vitro* with: a) PCCs (50 IU/kg); b) aPCCs at 75 IU/kg, or c) rFVIIa at 270 μg/kg. Several laboratory approaches were applied to explore modifications on overall hemostasis including: (i) thrombin generation (TG), (ii) thromboelastometry parameters (TEM), (iii) standard coagulation parameters, and (iv) studies under flow conditions to evaluate modifications on platelets and fibrin deposition on damaged subendothelial surfaces.

Results: Rivaroxaban and dabigatran treatments resulted in alterations of the different laboratory tests related to their recognized anticoagulant action. Standard coagulation parameters (PT, INR and APTT) were variably affected by rivaroxaban and dabigatran. aPCCs and rFVIIa totally reversed the effects of rivaroxaban on these routine tests, whereas only aPCC were capable to reverse partially the effects of dabigatran on APTT. Rivaroxaban and dabigatran caused alterations of TG with delayed time to peak, reduced maximum thrombin peak and decreased velocity index ($P < 0.05$), though alterations in TG were clearly dependent on the reagents used for activation. Modifications in TG caused by rivaroxaban were corrected by the different coagulation factors with efficacies following this order aPCC>rFVIIa = PCC. Reversion of alterations in TG after dabigatran were accomplished according to the following order of efficacy aPCC = PCC>rFVIIa. Clot viscoelastic parameters (TEM) were significantly altered by rivaroxaban or dabigatran treatments. While all concentrates (rFVIIa, aPCC and PCC) were able to reverse the effects of rivaroxaban ($P < 0.05$) on this test, only aPCCs seemed effective at reversing alterations induced by dabigatran ($P < 0.05$). Treatment with rivaroxaban or dabigatran did not cause marked alterations on platelet reactivity towards damaged vascular surfaces, but both anticoagulants demonstrated a consistent effect decreasing fibrin formation

on the subendothelium ($P < 0.01$). Reductions in fibrin formation observed after rivaroxaban were significantly improved ($P < 0.01$) by rFVIIa and aPCC, whereas alterations in fibrin formation following dabigatran treatment were only reversed by aPCCs ($P < 0.05$).
Conclusions: Reversal strategies may differ depending on the new anticoagulant agent and its specific mechanism of action. Our results indicate that while alterations in hemostasis induced by rivaroxaban were reversed to a variable extent by all the concentrates tested, reversion of dabigatran actions were predominantly achieved with aPCCs. Finally, TEM technologies may be useful as a point of care for a rapid evaluation of effects of new anticoagulants.

PA3.11 – Blood Coagulation Tests – X

PA 3.11-1

Capillary electrophoresis improves the selection of DNA-aptamers against activated coagulation factors

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Background: The development of enzyme-capture assays for direct measurement of activated coagulation factors in plasma samples requires enzyme-specific ligands that bind their targets with high affinity even in complex matrices. Due to their high affinity and specificity, DNA-aptamers, proved to be valuable tools for the development of such assays (Angew Chem Int Ed Engl. 2011; 50: 6075–8; J Thromb Haemost. 2012; 10: 390–8).
Aims: The aim of this study was to establish a highly efficient method based on capillary electrophoresis (CE) for the separation of unbound ssDNA from protein targets to expedite the selection of DNA-aptamers against activated coagulation factors.
Methods: A PA800 CE-System and silica-fused capillaries (Beckman Coulter) were used for all experiments. The system was run in normal polarity under electroosmotic flow (EOF). The used ssDNA-pool comprised a randomized region of 40 nucleotides at a total length of 81 nucleotides. For initial assay optimization, activated protein C (APC) and activated factor XIIIA (FXIIIAa) were chosen as targets. Binding affinities of selected aptamers were assessed by filter retention analysis.
Results: After optimization of system settings and buffer conditions, the retention time of the random library was found to be at least two times longer than that of the target proteins. Thus, the protein fraction, also containing bound aptamers, could be collected separately. In total, six selection cycles, including (i) incubation of ssDNA with target, (ii) CE for separation, (iii) amplification of selected sequences, (iv) single-strand separation for next round, were performed for both targets. In both cases, highest binding affinity of enriched pools was found after only five rounds of selection (Kd: APC: approximately 10 nM; FXIIIAa: approximately 30 nM).
Summary/Conclusions: The use of CE for the selection of DNA-aptamers targeting activated coagulation factors was successfully established. In comparison to previously applied methods for aptamer-selection, the CE-based method described here proved to be more efficient, resulting in a decrease of needed selection cycles by a factor of 2. The future goal is to apply this method for the identification of a variety of ligands against activated coagulation factors.

PA 3.11-2

Endogenous thrombin potential measured in the presence of thrombomodulin as a tool in prediction of thombotic events

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The Calibrated Automated Thrombogram (CAT) could be more effective in assessing thrombotic risk than standard coagulation tests, but clinical experience in this field is insufficient and association between the endogenous thrombin potential (ETP) and phenotype remains to be established. Predictive values of ETP obtained in the presence and in the absence of thrombomodulin (TM) may differ. Aim of our study was to assess the relationship between venous thromboembolism (VTE) recurrence and ETP measured by CAT with and without adding TM. The study involved 48 VTE patients on VKA in the extended-treatment phase (M/F 24/24, mean age 54.6 VTE ± 15.8 years, duration of VKA 1.5–4 years), among them 19 patients at low intensity treatment i.e. international normalized ratio (INR) 1.5–1.9; 15 patients with therapeutic INR range of 2.0–3.0 and 14 patients on gradual discontinuation of VKA therapy with INR 0.9–1.4. The last subgroup was followed up for 18 months. Control group ($n = 28$) was age matched. ETP was measured according to Hemker et al., 5 pmol concentration of tissue factor was used. STATISTICA 6.1 was used, data are given as mean ± SD. In controls ETP was 1731.4 ± 253.7 nM/min without added TM and 932.8 ± 272.6 nM/min with TM. Two-fold significant ($P < 0.001$) decrease of ETP compared to controls was measured even in patients with INR 1.5–1.9: 642.8 ± 129.7 nM/min in the absence of TM and 481.2 ± 106.8 nM/min in the presence of TM. In patients with INR 2.0–3.0 the corresponding parameters were 358.1 ± 210.4 nM/min and 293.2 ± 167.4 nM/min respectively. ETP in patients with INR 0.9–1.4 was much less decreased compared to the above mentioned subgroups of patients, and significantly differed from controls only when measured without TM (1487.4 ± 425.3 vs. 1731.4 ± 253.7 nM/min, $P = 0.02$). In the presence of TM ETP was 896.9 ± 296.0 vs. 932.8 ± 272.6 nM/min in controls, $P = 0.6$. Increased ETP was defined as > 2114 nM/min without TM and > 1433 nM/min with TM (i.e. above 95th percentile in controls). Two patients from the subroup with INR 0.9–1.4 had abnormal values of ETP. In one case increased ETP in the absence of TM (2257 nM/min) was accompanied by normal result (1210 nM/min) in the presence of TM, so VKA's therapy was discontinuated and during the following 18 months of observation patient had not recurrent thrombosis. In the other patient with ETP 2343 nM/min in the absence of TM and importantly 1603 nM/min in the presence of TM (i.e. both above 95th percentile measured in controls) thrombosis took place 12 months later in spite of prolonged VKA therapy. We conclude that though ETP determined without TM demonstrate strong positive correlation with the ETP obtained with adding of TM ($R = 0.93$, $P < 0.001$), the last one seems to be more sensible in detecting a degree of hypercoagulobility that predicts thombotic event. Our data suggest that CAT may allow individualized treatment based on global haemostatic response in patients on VKA, but predicting clinical events with the help of ETP needs further elucidation.

PA 3.11-3

Evaluation of the performance characteristics of a diluted thrombin time assay for dabigatran determination using a new thrombin time liquid reagent

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Background: DG-TT L Human is a new ready-to-use liquid reagent developed by Grifols, consisting of human thrombin for thrombin time (TT) determination in citrated human plasma samples. Additionally, the reagent DG-TT L Human may be used in a quantitative assay for dabigatran determination in samples from patients treated with the new oral anticoagulant dabigatran etexilate. This assay consists in the addition of the thrombin reagent to a plasma sample that has been previously diluted in a buffer and supplemented with normal lyophilized plasma (DG-Ref, Grifols) to normalize fibrinogen. Results are not interfered by the presence of heparins in plasma samples. Calibrator plasmas with known dabigatran concentrations are used to construct a four point calibration curve that allows the calculation of the dabigatran concentration present in a sample.
Aims: The aim of this study is to evaluate the performance of a procedure for dabigatran quantification with a new thrombin time liquid reagent.
Methods: Tests were performed in the optical coagulometer Q Hemostasis Analyzer (Grifols). A precision study was performed over 10 days using plasma containing two different levels of dabigatran (110 and 310 ng/mL). Linearity and limits of detection and quantification of the assay were determined using dabigatran calibrator and control plasmas from Hyphen Biomed. Accuracy was estimated at two different concentrations of dabigatran (130 and 320 ng/mL). The performance of the assay was tested using samples from patients treated with dabigatran ($n = 60$, collected at different time points of treatment). Results with the new assay were compared with the data obtained with the commercially available kit Hemoclot Thrombin Inhibitors (Hyphen).
Results: Precision results showed appropriate coefficients of variation (CV) of 9.34% and 4.70%, for dabigatran concentrations of 110 and 310 ng/mL respectively. The accuracy was satisfactory at the dabigatran concentrations tested (130 and 320 ng/mL, maximum bias of minus 2.6%). The assay measurement range obtained was from 30 to 760 ng/mL of dabigatran (usual plasma concentrations range between 20 and 400 ng/mL). In the method comparison study, the measurement limit of the new assay was taken into consideration and samples containing dabigatran concentrations lower than 30 ng/mL were not used for calculations. Good correlation of the new assay and the results obtained with the Hemoclot kit (30–328 ng/mL) was observed in this subset of samples ($y = 0.96x + 0.00$; $r = 0.90$) and no significant differences were observed in a Passing Bablok fit and a difference plot ($n = 26$; allowable bias of 20%). Comparison between methods in samples with dabigatran from 0 to 30 ng/mL ($n = 34$) showed a significant difference, as expected in samples with dabigatran levels under the measurement range of both assays.
Conclusion: The new DG-TT L Human is a reagent that allows both, thrombin time determination and dabigatran quantification when using a calibration curve with plasma containing dabitragan. This assay shows an adequate range of measurement and performance features comparable to the specific kit for dabigatran determination currently available in the market.

PA 3.11-4

A simplified assay for quantification of circulating activated protein C levels

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Background: The protein C pathway plays a crucial role in the regulation of blood coagulation, as it controls the generation of thrombin. Protein C circulates in plasma as an inactive zymogen and is activated on the vascular endothelial cell membrane by the thrombin-thrombomodulin complex, a process further enhanced when protein C binds to its membrane receptor, the endothelial-cell protein C receptor. Activated protein C (APC) is an enzyme with anticoagulant and cytoprotective functions. Accumulative data suggest that the number of situations in which *in vivo* circulating APC will need to be measured will increase over the next few years. Therefore, the availability of a rapid, sensitive and simple assay for quantifying circulating APC is crucial. Available assays are difficult to apply in routine laboratory. Our previously reported assay required an excessive pre-analytical handling: drawing of blood into two citrate tubes; immediate addition of a fresh APC inhibitor to one tube in order to block complexation of circulating APC to protein C inhibitor (PCI) (tube 1), and of heparin to the other tube to force all circulating APC to bind to PCI (tube 2), measurement of APC:PCI complexes in both tubes by ELISA, and subtraction of APC:PCI complexes (tube 2−tube 1) (method A).
Aims: To set up a simplified method based on the measurement of APC:PCI in a unique blood tube anticoagulated with heparin (method B).
Methods: We measured APC levels, with both methods, in 187 plasma samples, 117 from patients with venous thrombosis (VT) and 70 from healthy controls. Informed consent was obtained from all subjects of the study and it was approved by the medical ethics committee of our Institution.
Results: The mean APC level in the 125 samples was 1.09 ± 0.40 ng/mL (method A) and 2.07 ± 0.71 ng/mL (method B). The coefficient of correlation between both methods was $r = 0.789$ ($P < 0.001$). The mean APC level in the 117 VT patients was 0.95 ± 0.36 (method A) and 1.73 ± 0.55 (method B), significantly lower (as previously reported) than those in the 70 controls, 1.33 ± 0.36 and 2.64 ± 0.74, respectively, ($P < 0.001$). In both groups there was a significant correlation between the levels obtained by the two methods (VT, $r = 0.730$; controls, $r = 0.731$) ($P < 0.001$). Both Passing & Bablock regression and Bland & Altman plot analyses showed that both assays are equivalent.
Conclusions: These results confirm that the APC level is lower in VT patients than in healthy individuals. As both assays are equivalent, the new simplified assay, which measures the sum of circulating free APC and APC complexed to PCI, may be used to estimate the level of circulating APC, and will allow its use in routine laboratories.

PA 3.11-5

The use of the new Siemens Innovance VWF activity assay in monitoring treatment for von Willebrand disease

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Background: Von Willebrand disease (VWD) is caused by the dysfunction of, or reduction in concentration of, von Willebrand factor (VWF). An accurate measure of the functional activity of VWF is essential for both the classification of VWD and response following replacement VWF therapy. Traditional activity assays, utilising ristocetin, such as automated platelet aggregometry or manual visual agglutination (MVA) are laborious to perform and notoriously subject

to a high degree of assay variation. A new Siemens (Marburg, Germany) activity assay, Innovance VWF Activity, has recently been introduced which uses polystyrene particles coated with a monoclonal antibody against GP1b to bind to recombinant GP1b and the VWF in the test sample. Agglutination is measured turbidometrically.

Objectives: To evaluate the new automated Innovance VWF activity assay on the CS2100i analyser and compare it to the manual visual agglutination (MVA) assay in normal donors and VWD patients prior to and following treatment with DDAVP or VWF concentrates.

Results: Thirty-eight normal subjects (21 female and 17 male) were used to establish the reference range; all blood groups were included. The mean Innovance activity was 101 IU/dL (49–167 IU/dL) median 98 IU/dL, SD 0.30.

Inter and intra assay imprecision, at normal (88 IU/dL) and low levels (30 IU/dL) was < 2.7% and at very low levels (4 IU/dL) was 10%. The imprecision of our current MVA method is 7%.

Thirty-three patient samples were tested prior to and following treatment. Thirteen patients with type 1 VWD were treated with either DDAVP ($n = 12$) or Haemate P ($n = 1$). The mean activity prior to treatment was 29 IU/dL (median 27 IU/dL) for the MVA method and 34 IU/dL (median 37 IU/dL) for Innovance whilst following treatment was 112 and 136 IU/dL (medians 104 and 126 IU/dL) respectively.

Twenty patients with types 2 or 3 VWD were treated with DDAVP ($n = 4$), Haemate P ($n = 12$), Wilate ($n = 3$) or Alphanate ($n = 1$). The mean activity prior to treatment was 12 IU/dL (median 7 IU/dL) by the MVA method and 14 IU/dL (median 10 IU/dL) using Innovance and following treatment was 77 and 80 IU/dL (medians 77 and 81 IU/dL) respectively. Although no difference was found in normal subjects, a statistically significant difference between MVA and Innovance in pre treatment samples ($P < 0.05$) was observed due to some very low levels. Following treatment, the difference was not statistically significant ($P > 0.05$).

In type 1 VWD, with all treatment types, there was a mean 3.8 fold (median 3.9 fold) increase in MVA activity and 3.9 fold (median 3.4) increase for Innovance activity following treatment. In types 2 or 3 VWD with all treatment types, there was a mean 6.5 fold (median 11.8 fold) increase in MVA activity and 5.9 fold (median 8.1) increase in Innovance activity.

Conclusion: The Innovance activity assay is a rapid, easy to use automated test with improved imprecision compared to our manual VWF activity assay. The Innovance assay is suitable for monitoring treatment of both DDAVP and concentrates in patients with VWD.

PA 3.11-6
Effekt of rivaroxaban on clot formation kinetics

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Background: Patients with thrombophilia show increased and accelerated thrombin formation. Antithrombotic therapy reduces thrombin generation or directly inhibits thrombin. Rivaroxaban, a direct fXa inhibitor, reduces the prothrombinase activity and subsequently thrombin generation velocity, expressed as peak. Thrombin generation assays are expensive and time consuming. Clot waveform analysis bases on an aPTT measurement, which is inexpensive, fast and fully automated. Using an optical detection system, the fibrin formation curve and its subsequent derivatives provide further information about the clot formation process.

Aim: The aim of the study was to investigate the effect of rivaroxaban on clot formation with different methods: in patients with thrombophilic conditions. The focus is laid on clot waveform analysis to investigate the kinetics of fibrin generation.

Methods: More than 200 patients with hereditary thrombophilia or pregnancy in comparison with non-pregnant healthy controls were included in the study after an informed consent was obtained. The effects of rivaroxaban were determined in plasma samples spiked with different amounts of the anticoagulant (between 0 and 500 nM) using thrombin generation assays ETP (Siemens Healthcare Diagnostics, Marburg, Germany) and Technothrombin TGA (Technoclone, Vienna, Austria). Clot waveform analysis is measured using an ACL TOP (Instrumentation Laboratory, Kirchheim, Germany), using a Synthasil-activated aPTT assay (Instrumentation Laboratory). Measured optical data were processed using the Savitzky-Golay algorithm. The concentration of rivaroxaban was determined using an anti Xa-assay.

Results: All used methods showed a clear dose-response relationship for rivaroxaban. Healthy controls needed less rivaroxaban to reduce thrombin generation than patients with thrombophilia or pregnancy. This difference increased with the severity of the underlying prothrombotic state. Nevertheless, rivaroxaban reduced thrombin formation significantly also for patients with extraordinary high thrombin generation.

The reduction of the prothrombinase activity can be observed using clot waveform analysis, and showed an almost linear relationship over the concentration range of the anticoagulant.

Conclusions: It is possible to estimate the amount of Rivaroxaban to reduce thrombin generation to a certain value. As expected from higher initial thrombin generation values, patients with thrombophilia or pregnancy need more fXa inhibitor to reduce TG compared to healthy controls. This effect was consistent using thrombin generation or clot waveform analysis. However, these estimations lie within clinically relevant rivaroxaban levels. Clot waveform analysis might emerge as a inexpensive und convenient alternative to thrombin generation measurements. The second derivative of the fibrin formation curve is a good measure for anti Xa activity.

PA3.12 – Blood Coagulation System – III

PA 3.12-1
Impact of BDNF Val66Met polymorphism on thrombosis

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Background: Brain-derived neurotrophic factor (BDNF) is a neurotrophin that plays a key role in neuron plasticity and vascular development. A single nucleotide polymorphism in the BDNF gene (BDNF Val66Met) has been associated with depression, and recently, it has been proposed as a genetic risk factor for cardiovascular disease. Intriguingly, reduced BDNF levels are detected in subjects with depression, and they seem associated with increased in patients affected by acute coronary syndrome. No Information, however, is available concerning the potential influence of the BDNF Val66Met polymorphism in thrombotic processes.

Aims: In this study we have assessed the impact of BDNF Val66Met polymorphism on haemostatic system and its relationship to thrombosis experimentally induced.

Methods: To assess the role of BDNF Val66Met polymorphism in thrombosis, ferric Chloride ($FeCl_3$) carotid arterial injury and thromboembolism, induced by injection of collagen and epinephrine, were carried out in BDNF knock-in mice generated with a homozygous BDNF variant (Met/Met), and then compared to Val/Val control mice. Thromboelastometry analyses (by ROTEM®) and platelet/leukocyte interactions (by cytofluorimetry) were performed in citrated blood. Levels of fibrinogen, and activities of plasminogen activator inhibitor 1 (PAI-1), tissue plasminogen activator (tPA), thrombospondin-1 (TSP-1), and coagulation factors were measured in plasma. TF activity of circulating leukocytes and carotid artery was performed by

one-stage plasma recalcification time assay. Finally, clot retraction test, an indicator of platelet function, was carried out in platelet rich plasma.

Results: Genetic BDNF Met/Met variant accelerated carotid artery thrombus formation in response to $FeCl_3$ and increased mortality after collagen/epinephrine injection. Recalcification tests, carried out in whole blood of Met/Met mice by NATEM thromboelastography test, showed a significant increase both in clot firmness and clot elasticity, and a reduction in the clot formation time compared to Val/Val mice. Activation of the extrinsic or intrinsic pathways (EXTEM and INTEM respectively) suggested that BDNF Knock-in mice have hyper reactivity of platelets and higher levels of fibrinogen, but normal levels of coagulation factors. Indeed, specific analyses indicated that Met/Met mice have higher levels of TSP-1, a major secretory product of activated platelets, a shorter clot retraction time, and an increase in circulating platelet/leukocyte aggregates compared to control mice. Levels of factors VIII, IX, XI, XII, PAI-1 activity and antigen, and tPA activity were similar in the two groups. In addition, platelet and withe blood cell counts were increased in BDNF Knock-in mice. Importantly, TF, the key activator of blood coagulation, was elevated both in circulating leukocytes and in carotid arteries of Met/Met mice.

Summary/Conclusion: The increased basal activation of haemostatic system associated with increased platelet activation and vascular TF activity observed in BNDF Met/Met mice may partly explain the predisposition of this mouse model to thrombosis. Therefore, genetic BDNF Val66Met variant may thus play a key role to increase the susceptibility to thrombotic events.

PA 3.12-2

Mutation of Thr-211 to Pro in the activation peptide of factor X in a bleeding patient is associated with a molecular defect in the intrinsic pathway

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Background: Thrombin is the final serine protease of the clotting cascade which is responsible for cleaving fibrinogen to form insoluble fibrin clots at the vascular injury sites. Thrombin can be generated by either intrinsic or extrinsic pathway in the clotting cascade. In both pathways, the vitamin K-dependent coagulation zymogen in plasma factor X (FX) needs to be activated to its active form (FXa) which is followed by the assembly of FXa into the prothrombinase complex and activation of prothrombin to thrombin. FX can be activated by either one of the physiological activators: the factor VIIa-tissue factor complex or the factor IXa-factor VIIIa complex in the extrinsic and intrinsic pathways, respectively. A deficiency in either one of these activation pathways can be associated with bleeding disorders. We recently identified a bleeding patient who exhibited clotting defect only in the intrinsic pathway.

Aim: The aim of this study was to elucidate the molecular basis of the clotting defect in the bleeding patient and to identify the coagulation factor responsible for the clotting defect specifically in the intrinsic pathway.

Methods: Exome sequencing was used to determine whether the patient carries a genetic defect in one of the known clotting factor genes and if so to use recombinant DNA methods to express the cDNA carrying the identified genetic defect in a mammalian expression system.

Results: Exome sequencing revealed that the patient carries a novel homozygous FX mutation that results in the substitution of Thr-211 with a Pro on the activation peptide of the FX zymogen. The residue Thr-211 is the site of an O-linked glycosylation in the activation peptide of FX. Thus, we postulated that the elimination of carbohydrate residues of Thr-211 due to a Pro substitution may specifically impacts the activation of FX by FIXa in the intrinsic Xase complex, thereby impairing thrombin generation in the subject. To test this hypothesis, we expressed a FX cDNA containing this mutation in HEK-293 cells and following the purification of the zymogen to homogeneity characterized its biochemical properties in both purified and plasma based assay systems. The results suggested that the activation of the physiological concentration of the FX mutant by the FIXa-FVIIIa complex, but not by the FVIIa-TF complex, has been markedly impaired.

Conclusions: Analysis of the results suggests that the loss of Thr-211 glycosylation site renders the FX mutant specifically a poor substrate for FIXa in the intrinsic Xase complex, thus explaining the bleeding phenotype of the patient carrying this mutation.

PA 3.12-3

Additional effect of BβArg448Lys polymorphism on fibrin clot structure/fibrinolysis and cardiovascular complications in subjects with type 2 diabetes

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Background: Fibrinogen BβArg448Lys polymorphism affects clot structure and fibrinolysis, potentially explaining the association with coronary artery disease and stroke. Despite the increased risk of atherothrombosis in diabetes, the role of this polymorphism on clot structure and ischaemic events in this condition has not been studied.

Aims: This work investigates the potential additional effects of BβArg448Lys polymorphism on fibrin clot structure/fibrinolysis and predisposition to vascular events in individuals with diabetes.

Methods: Genotyping for BβArg448Lys was carried out in 822 individuals with type 2 diabetes participating in the population-based Edinburgh Type 2 Diabetes Study (mean age 68 ± 0.14, 422 males) using standard techniques. Clot maximum absorbance and lysis time were measured by validated turbidimetric assays, with confocal and scanning electron microscopy used to visualise the fibrin networks. Both individual plasma samples and pooled plasma purified fibrinogen were investigated. T-test and Mann–Whitney analysis were used for normally distributed and non-parametric data, respectively. The interaction between the polymorphism and ischemic events was investigated using regression models, with and without adjustment for traditional risk factors.

Results: Bβ448Lys variant was present in 31.5% of subjects with similar frequency in men and women. Mean age of subjects with Bβ448Lys was lower compared with Bβ448Arg (67.5 ± 0.26 and 68.3 ± 0.18 years, respectively, $P = 0.02$). Clot density in Bβ448Lys subjects was higher than Bβ448Arg (0.37 ± 0.007 vs. 0.35 ± 0.004 au, respectively, $P < 0.05$) and lysis time was longer (761 ± 20 vs. 719 ± 15 s, respectively, $P < 0.05$). These changes were still significant after adjusting for fibrinogen levels. Bβ448Lys was associated with a previous history of stroke and transient ischaemic attack (TIA) in the whole group [OR 1.93 (1.15, 3.24)], which was still evident after adjusting for traditional vascular risk factors [OR 1.98 (1.16, 3.37)]. The predictive value of Bβ448Lys for stroke/TIA was only significant in females, suggesting a possible interaction between gender, polymorphism and disease [OR 4.18 (1.56, 11.22)]. Purified fibrinogen showed differences in clot density for Arg/Arg, Arg/Lys and Lys/Lys variants at 0.046 ± 0.0007, 0.040 ± 0.0005 and 0.036 ± 0.0002 au, respectively ($P < 0.001$), accompanied by changes in clot lysis time at 13.9 ± 0.5, 17.0 ± 0.3 and 18.6 ± 0.4 min, respectively ($P < 0.001$). Differences in clot structure between these genetic variants of fibrinogen were further confirmed by both confocal and scanning electron microscopy.

Discussion: Bβ448Lys variant of fibrinogen is present in around a third of people with diabetes and confers additional risk in these subjects, probably related to the formation of more compact fibrin clots, which

are resistant to fibrinolysis. People with diabetes and fibrinogen Bβ448Lys may have reduced life expectancy and women have a higher risk of cerebrovascular disease. These data may have important clinical implications for patient clinical management and future prospective studies are warranted to fully understand the role of BβArg448Lys in predisposition to ischaemic events in diabetes.

PA 3.12-4

Acute and severe coagulopathy in adult mice following silencing of hepatic antithrombin and protein C production

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Background: Mice deficient in the natural anticoagulants antithrombin (*Serpinc1*) or protein C (*Proc*) display early lethality due to thrombosis-related coagulopathy, thereby precluding their use in gene function studies or thrombosis models.
Methods: RNA interference was used to silence antithrombin (si*Serpinc1*) and/or protein C (si*Proc*) in normal adult mice (dose siRNA 3.5 or 7 mg/kg body weight). Mice were sacrificed within 2–5 days after siRNA administration. Liver *Serpinc1* and *Proc* transcript level, plasma antithrombin, protein C, and fibrinogen levels, and tissue fibrin deposition, were analyzed. Full necropsy was performed, tissues were formalin fixed, and microscopical analysis was performed on HE-stained 5-μm sections.
Results: Severe coagulopathy was observed following combined 'knockdown' of antithrombin and protein C. Two days after si*Serpinc1*/si*Proc* injection, (occlusive) thrombi were observed in (large) vessels in multiple tissues (head, leg and liver), and hemorrhages were prominent in the ocular, mandibular, and maxillary areas. In total 19 out of 19 si*Serpinc1*/si*Proc* (sum of animals for 3.5 and 7 mg/kg dose) vs. 0 out of 11 siNEG-treated animals featured abnormalities ($P < 0.0001$, Fisher's exact test). si*Serpinc1*/si*Proc*-treated animals at 3.5 or 7 mg/siRNA/kg demonstrated evident fibrin deposition (111 ± 129 $P < 0.0001$ and 1574 ± 1364 ng/mg $P = 0.0005$ vs. 5.4 ± 2.7 ng/mg in control siNEG injected animals). This coincided with reduced plasma fibrinogen levels in si*Serpinc1*/si*Proc*-treated mice (−43% $P = 0.012$ and −96% $P = 0.002$ for 3.5 or 7 mg/siRNA/kg group, respectively). The thrombin inhibitor dabigatran etexilate fully prevented coagulopathy in si*Serpinc1*/si*Proc*-treated mice (3.5 mg siRNA/kg), including formation of thrombi in head, livers and legs, liver fibrin deposition, lowering of plasma fibrinogen. Silencing *Serpinc1* alone yielded a comparable but milder phenotype with a later onset. The phenotype was absent when targeting *Proc* alone.
Conclusion: We conclude that RNA interference of *Serpinc1* and/or *Proc* allows studying the function of these genes *in vivo*, and provides a novel, controlled mouse model for spontaneous venous thrombosis.

PA 3.12-5

Platelet-derived microparticles attenuate the procoagulant nature of platelet-associated prothrombinase

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Background: Due to their procoagulant nature, platelet-derived microparticles have been implicated in several thrombotic processes. Their increased expression of procoagulant activity is due, in part, to the enrichment of anionic phosphatidylserine headgroups on their outer membrane leaflets that mediate the binding of several coagulation enzyme complexes including Prothrombinase. The prothrombinase complex, composed of the serine protease factor Xa and its protein co-factor factor Va assembled on an appropriate membrane, activates the zymogen prothrombin to the serine protease α-thrombin 'the key enzyme of the hemostatic process as it lies at the crossroads of both the pro- and anti-coagulant pathways. The activation of prothrombin is the result of two proteolytic cleavages at Arg271 and Arg320. Initial cleavage at Arg271 results in the formation of the non-catalytically active intermediate prethrombin-2, while cleavage at Arg320 results in the formation of the catalytically active species meizothrombin. In contrast to α-thrombin, meizothrombin is an anticoagulant, having considerably decreased activity towards platelets, fibrinogen, factor V, and factor VIII, while activating protein C more readily when complexed with thrombomodulin.
Aims: Platelet-associated prothrombinase maximizes procoagulant activity as it activates prothrombin via the more procoagulant prethrombin-2 pathway, as opposed to Prothrombinase assembled on phospholipid vesicles of defined content that utilize the more anticoagulant meizothrombin pathway. Given these differences, the goal of the current study was to determine if platelet-derived microparticles support Prothrombinase that promotes the prethrombin-2 or meizothrombin activation pathway.
Methods: Platelet-derived microparticles were formed using calcium ionophore A23187 or by extruding thrombin-activated platelets through a 0.2 μm filter. The activation pathway utilized by Prothrombinase assembled on each surface was monitored by gel electrophoresis.
Results: When Prothrombinase was assembled on microparticles derived from platelet activation effected by calcium ionophore A23187, it utilized both the prethrombin-2 and meizothrombin pathways of thrombin generation, suggesting that upon microparticle formation, the less procoagulant meizothrombin pathway is upregulated. We hypothesize that this is a direct consequence of increased phosphatidylserine expression on the platelet's outer leaflet resulting in a surface that is akin to phospholipid vesicles of defined content. However, as microparticles contain most of the membrane proteins of their parent cells, the continued use of the prethrombin-2 pathway of prothrombin activation is likely due to the presence of intact membrane microenvironments surrounding factor Va and factor Xa receptors, which define the unique structural features of platelet-associated Prothrombinase. In contrast, when thrombin-activated platelets were extruded through a 0.2 μm filter, the microparticles generated almost exclusively supported Prothrombinase that utilized the meizothrombin pathway of prothrombin activation. The lack of utilization of the prethrombin-2 pathway, suggests that extrusion of the platelets alters the membrane microenvironment of the factor Va and Xa receptors, such that the only available pool of microparticle-associated Prothrombinase stems from the increased phosphatidylserine present on the outer leaflet.
Summary/Conclusions: These data suggest that, depending upon the precipitating events leading to microparticle formation, populations can be formed that express a wide range of procoagulant activity, the degree of which may impact their role in pathological, as well as physiological, processes.

PA 3.12-6

Contraction of whole blood clots: platelets and fibrin are on the exterior and compress erythrocytes into close-packed polyhedra on the interior

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Background: Contraction of blood clots is necessary for hemostasis, wound healing and to restore flow past obstructive thrombi. However, little has been known about the structure of contracted clots and mechanisms of contraction. Erythrocytes, biconcave cells that are

highly deformable to allow their passage through the microvasculature, are abundant in venous thrombi, and to a lesser extent in arterial thrombi. Erythrocytes promote hemostasis, but their participation in clot contraction has not been reported.

Aims: To understand the mechanisms of clot contraction and the roles of erythrocytes, platelets and fibrin.

Methods: We examined the structure and composition of contracted whole blood clots by scanning electron microscopy and confocal light microscopy. Whole blood was clotted by recalcification and addition of thrombin or kaolin, while following the process of clotting, including contraction, with a new technique using T2 magnetic resonance.

Results: Contracted clots display a remarkable structure, with a tessellated array (or mosaic tiling of space) of compressed polyhedral erythrocytes within and a meshwork of fibrin and platelet aggregates on the exterior. Little fibin and few platelets were found on the interior of the contracted clots. The same results were obtained with both thrombin and kaolin as activators of clotting and also with reconstituted human blood and clots prepared from mouse blood. Confocal microscopy of hydrated clots confirms the results of scanning electron microscopy. Platelets (with their cytoskeletal motility proteins) and fibrin(ogen) (as the substrate bridging platelets for contraction) are required to generate the forces necessary to segregate platelets/fibrin from erythrocytes and to compress erythrocytes into a closely packed polyhedral array. To assess the density of packing of the polyhedral erythrocytes, we replaced the water surrounding the clots with D_2O and observed by T2 magnetic resonance that hydrogen/deuterium exchange for the contracted clots was very slow, consistent with their very tightly packed, almost impermeable structure.

Summary/Conclusions: We have observed a new erythrocyte morphology, closely packed polyhedra, in contracted clots, and an unexpected spatial redistribution of platelets and fibrin that occurs during contraction. Clot contraction is an essential part of hemostasis, since both human genetic disorders of platelet myosin IIA and megakaryocyte myosin IIA-knock out mice show a bleeding phenotype. These observations on contracted clots imply that they are stiff, rigid structures that can form an impermeable, watertight seal. On the one hand, contraction of clots within the vasculature may relieve obstruction of blood vessels and allow recanalization, especially in the venous system. On the other hand, these results account for long-standing clinical observations that fibrinolysis is greatly prolonged following clot contraction, since perfusion or diffusion of lytic enzymes into these tightly packed polyhedral erythrocytes would be nearly impossible (NIH grants HL030954/HL090774 to JWW, and HL090697 to DBC, and HL110860 to DBC/LR/JWW).

PA3.13 – Acquired Coagulation Disorders – IV

PA 3.13-1

Thrombin generation in obese patients

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Background: It was found that obesity is associated with a hypercoagulable state either due to an increase of clotting factors plasma levels and/or the impairment of the fibrinolytic pathway. Several studies reported that elevated levels of coagulation factors such as PAI-1, prothrombin, factors VII and VIII, and fibrinogen were observed in patients suffering from obesity. These investigations were based on a limited number of subjects and did not specifically look at differences in fat amount and distribution. The measurement of capability of generating thrombin is very useful as an expression of prothrombotic phenotypes compared to conventional coagulation tests. The thrombin activity can be measured in plasma by continuous cleavage of chromogenic or fluorescent substrates, and can be registered in a curve (Thrombin generation Test [TGT]). Various parameters can be considered, including the time until thrombin burst (lag phase), the peak amount of thrombin generation (peak thrombin) and the total amount of thrombin generated [endogenous thrombin potential (ETP)]. The ETP defines the pro- and anticoagulant balance as a global function test of clotting. Increased ETP levels were seen in obesity associated myocardial infarction, stroke, and pulmonary embolism. However, ETP in asymptomatic obese subjects remains to be clarified.

Aim: The aim of this study is to determine whether any differences in the coagulation system between obese patients with different degree of obesity and normal weight subjects exist with the use of TGT.

Methods: Eighty consecutive patients referred to our Department, of whom 20 were overweight [BMI = 25–29.9 kg/m^2], 20 with I degree obesity [BMI = 30–34.9 kg/m^2], 20 with II degree obesity [BMI = 35–39.9 kg/m^2] and 20 with III degree [BMI > 40 kg/m^2]) were enrolled. Eighty age (± 3 years) and gender-matched normal weight healthy individuals acted as controls. TGT was determined in Platelet Poor Plasma (PPP) by means of CAT method, using 80 μL of PPP together with 20 μL of PPP-Reagent, a mixture of Tissue Factor (5 pM) and synthetic phospholipids (4 μM). TG curves were described in terms of Lag time (min), C max (Peak, nM) and ETP (nM*min), where shortened lag-times and elevated values of both peak thrombin and ETP are indexes of hypercoagulability.

Results: TG test showed that obese patients had shorter lag time (2.55 ± 0.3 min; $P = 0.04$), higher peak thrombin (300.9 ± 84.7 nM; $P < 0.001$) and ETP (1791.2 ± 362.0 nM*min; $P = 0.04$) than healthy controls (2.84 ± 0.55 min, 1411.5 ± 174.1 nM, 254.3 ± 58.3 nM*min, respectively). In particular, patients suffering from 3rd degree obesity showed a significantly shorter lag time ($P = 0.047$) and a significantly higher peak thrombin ($P < 0.0001$) and ETP ($P = 0.018$) than healthy controls.

BMI and waist circumference inversely correlated with the lag time ($r = -0.40$ and -0.49; $P < 0.001$, respectively) and positively with the Peak and ETP values ($r = 0.52$ and 0.58; $P < 0.0001$, respectively).

Conclusions: TGT seems to be a reliable test to identify the hypercoagulable state observed in patients suffering from obesity. Significant correlation between degree of obesity and TGT parameters suggested a growing prothrombotic potential with the increase of fat mass. Larger studies are needed to confirm our observation.

PA 3.13-2

Predictors of vascular thrombotic events in pediatric liver transplantation

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Background: Liver transplantation (LT) is increasingly performed in children with liver failure. With state of the art multidisciplinary and comprehensive care, the survival rates in pediatric LT (PLT) patients have increased to 88% at 10 years. However, vascular thrombotic events (VTE) occur in up to 30% of PLT patients. These VTE often result in significant morbidity and mortality. The identified risk predictors for VTE in PLT population are inconsistent and vary according to the study methodology and reporting, and have not adequately and systematically studied.

Aim: To identify the predictors for VTE in PLT.

Methods: A retrospective cohort study was performed on all pediatric patients undergoing first LT at University of Alberta, Canada between January 2001 and July 2012. Demographic data, clinical data, graft, donor and recipient parameters, surgical details and serial radiological imaging data were recorded in a comprehensive predesigned proforma and analysed. The KIDCLOT© pediatric thrombosis group at Stollery Children's Hospital introduced an anticoagulation protocol in Novem-

ber 2006. Thus, for the purpose of analysis, patients were evaluated in two groups (before and after November 2006) and as a whole group.
Risk factors associated with VTE were analysed by univariate and multivariate regression analysis. A '*P*' value of < 0.05 was considered significant.
Results: Over the last 10 years, 91 (era 1: 30, era 2: 61) patients underwent first LT. The mean age at LT in the entire cohort was 4.5 ± 4.9 years. Forty-four patients were < 10 kg at the time of PLT. Biliary atresia (BA) with failed Kasai procedure was the most frequent (39.6%, *n* = 36) indication in both era 1 (43.3%) and era 2 (37.7%).
There were 30 VTE events in 25 (27.5%) patients: 11 hepatic artery thrombosis (HAT), 15 portal venous thrombosis (PVT), four hepatic venous thrombosis (HVT). In era 1, 10 (33.3%) patients had 10 VTE: five HAT, four PVT, 1 HVT. In era 2, 15 (24.6%) patients had 20 VTE: six HAT, 11 PVT, three HVT.
Comparative analysis was done between each of the clinical parameters, laboratory features, graft parameters and surgical factors in patients with and without VTE in Era 1, 2 and the entire group. No significant predictors of VTE were identified in era 1 patients, potentially due to small numbers. In era 2, age ≤ 2 years ($P = 0.01$), weight ≤ 10 kg ($P = 0.002$) and diagnosis of BA ($P = 0.01$) were significantly associated with VTE on univariate analysis. In the entire group, age ≤ 2 years ($P = 0.007$), weight ≤ 10 kg ($P = 0.001$), diagnosis of BA ($P = 0.001$) were significantly associated with VTE.
In multivariate analysis weight ≤ 10 kg was the only independent predictor of VTE ($P = 0.020$, OR: 4.251 (95% CI: 1.254–14.4).
Conclusion: This study demonstrates conclusively, that weight < 10 kg is the sole predictor of VTE in PLT, despite a multitude of clinical, graft and surgical factors potentially contributing to VTE. This observation indicates the critical need of close clinical and radiological monitoring and suggests more aggressive prophylactic postoperative anticoagulation in PLT patients ≤ 10 kg.

PA 3.13-3
A prospective study to evaluate early clauss fibrinogen and fibtem as predictors of progression of major obstetric haemorrhage

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Background: Postpartum haemorrhage (PPH) accounts for 75% of severe maternal morbidity in the UK. The normal range for fibrinogen at the time of delivery is about 4–6 g/L. Clauss fibrinogen has been shown to be a useful biomarker for predicting progression of early PPH to the need for transfusion and invasive procedures. Our retrospective data confirms that clauss fibrinogen is predictive for the need for ≥ 4 units red blood cell (RBC) with a receiver operator characteristics (ROC) curve AUC (95% CI) of 0.85 (0.78–0.93). Clauss fibrinogen often takes too long to be clinically useful in the context of treating PPH. Fibtem® measured on a ROTEM® machine can provide a rapidly available near patient estimate of fibrinogen.
Aims: To establish whether Fibtem analysis had a similar utility to clauss fibrinogen for predicting progression of PPH.
Methods: After ethical approval consecutive women admitted toCardiffwho had either a PPH of ≥ 1500 or ≥ 1000 mL in association with a precipitant were enrolled. On identification of PPH, a paired clauss fibrinogen and Fibtem assay were taken. Data recorded included measured blood loss, units of RBC transfused and the need for invasive procedures. ROC analysis was used to examine the utility of fibrinogen and Fibtem maximum clot firmness (MCF) to predict progression to transfusion with any RBC, ≥ 4 units of RBC, or the need for invasive procedures.
Results: From April to September 2012, PPH fitting the entry criteria was identified in 179/3200 births (5.6/100), progressing to ≥ 1500 mL in 84 and ≥ 2500 mL in 17 patients. Forty-two women received RBC transfusion and 10 receiving ≥ 4 units. The ROC curve AUC values were significant for all three outcomes as predicted by both clauss fibrinogen and Fibtem®. The AUC (95% CI, *P* value) for progression to any RBC transfusion was 0.72 (0.6–0.84, $P < 0.01$) for fibrinogen and 0.74 (0.63–0.86, $P < 0.01$) for Fibtem, ≥ 4 units of RBC transfusion was 0.84 (0.7–1.0, $P < 0.01$) for fibrinogen and 0.80 (0.6–1.0, $P < 0.01$) for Fibtem®, and for progression to invasive procedures 0.93 ($P < 0.01$) and 0.89 ($P < 0.01$) respectively. A Fibtem MCF < 18 mm, equivalent to fibrinogen about 2.5–3 g/L, associated with ongoing bleeding, had a positive predictive value (95% CI) of 89 (67–98)%, and a negative predictive value of 84 (78–90)% for the need for any RBC transfusion.
Conclusions: In this prospective observational study, Fibtem was found to be as useful as laboratory fibrinogen for predicting the need for any RBC, ≥ 4 units RBC transfusion and the need for invasive procedures. Fibtem, however, was available substantially sooner than laboratory fibrinogen and might therefore be more useful to trigger intervention. These data provide further evidence for the utility of fibrinogen as an early biomarker for progression of PPH and demonstrate that the Fibtem assay can be used in a similar way. Given the value of low peripartum Fibtem for predicting progression of PPH, the study team are undertaking an interventional trial to establish whether the correction of Fibtem to normal peripartum levels reduces bleeding and the need for transfusion.

PA 3.13-4
Early cytokine secretion may predict response to treatment in acquired hemophilia

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Acquired hemophilia (AH) is a rare, life-threatening, bleeding disorder caused by autoantibodies that inhibit the clotting function of FVIII (Inhibitors). A major challenge in AH management is that patients vary in their responses to treatments (immunosuppressive agents such as cyclophosphamide, corticosteriods, azothioprine, and rituximab; alone or in combination). To date, no definite biomarker exists that can predict responsiveness of AH patients to a specific therapy, and study of this population has been hampered by the low incidence of the disease ($1{:}1 \times 10^6$) as well as the presence of co-morbidities including autoimmune disorders and malignancies.
The aim of the study was to determine immune factors associated with outcome of anti FVIII inhibitor antibody treatment in AH patients. After IRB approval and informed consent, blood samples were collected at the start of therapy and at various time points during treatment and follow-up (up to 806 days post treatment). B-cell activating factor (BAFF) levels were determined by ELISA (R&D Systems). The remaining cytokine and chemokine levels were determined using a 18-plex Milliplex MAP assay (Millipore) and analyzed using a Bio-plex 200 (Biorad). Inhibitor levels were determined by Bethesda assay and anti-FVIII IgG subclasses were determined by ELISA. Data were stratified according to time post-treatment and plasma measurements of cytokines and inhibitor levels were compared between non-responders (NR) and complete responders (CR) using logistic regression analysis. Statistical significance was confirmed via two-sample Mann–Whitney test (Stata/IC).
Fifty percent of the patients were male and the average age was 62.5 (range 51–88). The underlying disorder was idiopathic in 6/12 of patients, autoimmune in 4/12, and cancer in 2/12. Of 12 patients, 11 were treated with rituximab, either alone or in combination with cyclophosphamide and/or corticosteroids. One patient achieved spontaneous remission. Six patients demonstrated a complete response to treatment, and their inhibitor level returned to 0 BU/mL at an average of 153 days (± 61 days). IgG4 anti-FVIII antibody was found in 9/11

patients. Within the group of treated CR patients, titers of all subclasses of anti-FVIII Ab decreased following treatment, surprisingly, detectable anti-FVIII IgG titers returned in four patients at their last follow-up, despite a remission in inhibitor and remaining asymptomatic. A higher baseline secretion of plasma GCSF was associated with NR (median 66.1 vs. 34.6 pg/mL, $P = 0.04$). Within the first 30 days of treatment, an increase in the chemokine MCP-1(CCL2) was associated with NR (median 361 vs. 268 pg/mL, $P = 0.04$). Comparison of plasma cytokine secretion between patients post-treatment revealed that only BAFF concentrations at later time points (> 1 year) were increased in NR (median 3397 vs. 1890 pg/mL, $P = 0.02$).

Conclusions: Though the overall trends in kinetics and levels of cytokine secretion were similar between CR and NR patients, the combination of specific cellular signals within 30 days following the initiation of treatment may determine final outcome. Tracking of plasma GCSF and MCP-1 secretion in patients may provide a realistic predictor of outcome.

PA 3.13-5

Real time assessment of hemostatic alterations induced by cardiopulmonary bypass surgery

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The heterogeneity of hemostatic alterations, which cause hemorrhage in cardiopulmonary bypass surgery (CPBS), is still poorly understood. In search of clarification, different hemostatic components were systematically assessed by point-of-care methods. The hemostatic capacity in the reperfusion phase (RP) of CPBS was compared to preoperative conditions at surgery preparation. To focus on hemostatic changes induced by CBPS, the time of reperfusion (rather than end of surgery) was chosen in order to exclude any effects of pronounced bleeding and subsequent hemotherapy at the end of surgery.

Methods: A total of 69 patients was investigated. The extrinsic coagulation pathway was tested by use of the thrombelastometric clotting time (CT-EXTEM, ROTEM®). High-dose heparin during CPB hinders the assessment of the intrinsic pathway, but FVIII was determined by a chromogenic assay (Siemens®). Both fibrinogen levels (Clauss method; Siemens Multifibren®) and maximal clot formation (MCF-FIBTEM, ROTEM®) together with FXIII activity (Siemens Berichrom®) were used to assess clot substrates. Primary hemostasis was examined by platelet aggregometry (Multiplate® ADP, ASPI, TRAP), von Willebrand factor (vWF) activity (Siemens BC®) and vWF antigen (Instrumentation Laboratories®). Fibrinolytic activity was probed by D-dimers (Siemens Innovance®).

Results: Mean clotting time (CT-EXTEM) from start to reperfusion increased by 20 ± 22 s (mean + SD), but pathologic prolongation occurred in only eight of 67 patients (11.9%) at RP (0% at starting point). vWF activity was reduced in three of 65 patients (4.6%) at starting point and normalized or remained normal in all. Similar results were found regarding FVIII activity. Fibrinogen levels decreased by 29% (Clauss method) and 33% (MCF-FIBTEM), respectively. At start, eight of 64 patients (12.5%) exhibited pathologically low MCF-FIBTEM values, as compared to 28 of 64 patients (43.8%) in RP. Reduced FXIII levels were observed in 19 of 61 patients (31.2%) at start and in 54 of 61 of patients (88.5%) in RP, respectively. Increased fibrinolysis as defined by a D-dimer cut-off of 5 mg/L was present in only six of 64 patients (9.4%) in RP, all of whom had elevated values already at surgery preparation. The pattern of platelet aggregation (PA) in response to ADP/TRAP was variable: (i) in 25 of 47 patients (53.2%) PA was normal at start and remained unaffected during CPB, (ii) in nine of 47 patients (19.1%), PA was normal and became pathologic, (iii) in four of 47 patients (8.5%), PA was pathologic at start and became normal and (iv) in nine of 47 patients (19.1%), PA was pathologic throughout.

Conclusion: Hemostatic alterations associated with CPBS are predominantly characterized by changes in fibrinogen and FXIII levels. In contrast, the extrinsic pathway is affected to a lesser degree. Fibrinolysis does not appear to contribute to CPB coagulopathy, which may result from the application of heparin and tranexamic acid during CPB. Likewise, changes in FVIII or vWF appear to be irrelevant. Platelet function in CPBS reveals different patterns including a subgroup of patients in whom reduced platelet aggregability is restored during CPB. The data of this pilot study provide a rationale for differential hemotherapy of bleeding in CBPS.

PA 3.13-6

Novel detection of heparin-like substance as a cause of activated partial thromboplastin time prolongation in dengue hemorrhagic fever

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Background: Dengue hemorrhagic fever is a major public health concern in tropical and subtropical countries. Hemorrhagic complication due to dengue virus infection can result in significant morbidity and mortality of the patients. In addition to thrombocytopenia, other hemostatic parameter derangements, for example, activated partial thromboplastin time (aPTT) prolongation has been reported in dengue hemorrhagic fever. However, the mechanism of abnormal aPTT observed in dengue virus infection is unclear. According to our previous observation in bleeding patients with dengue infection, aPTT prolongation was not corrected by a mixing study. Moreover, the fibrinogen activity was low. Therefore, we hypothesized that heparin-like substance could be responsible for this finding.

Aims: To investigate the presence of heparin-like substance in dengue hemorrhagic fever as a cause of aPTT prolongation.

Methods: Six plasma samples from five patients with dengue hemorrhagic fever and aPTT prolongation were obtained. Mixing study and fibrinogen activity assays were performed in all samples. In addition, protamine titration was examined in both the patients' plasma and in the control (normal plasma spiked with heparin).

Results: The median aPTT was 65.0 s (range 35.4–88.6 s; normal 24.1–30.9 s). Mixing study showed uncorrectable aPTT in four samples. Two samples from a patient with severe hepatic injury showed prolonged prothrombin time. Fibrinogen activity assays revealed decreased fibrinogen activity in five out of six patients. Interestingly, we found that the fibrinogen activity gradually increased with serial testing at different time interval at room temperature, which was similar to the pattern seen in plasma contaminated with heparin. The median level of fibrinogen activity at baseline, 60 and 90 min were 160.6 mg/dL (range 20.94–243.6), 352.6 mg/dL (range 220.4–588.1) and 446.1 mg/dL (range 256.4–455.4), respectively (normal 200–400 mg/dL). The protamine titration demonstrated the normalization of aPTT prolongation in all samples. The median concentration of protamine used to correct the aPTT was 0.05 mg/mL (range 0.02–0.055). Therefore, the results of mixing study, fibrinogen activity and protamine titration suggested the presence of heparin-like substance in the patients' plasma. Hence, protamine sulfate was given to two patients who sustained severe gastrointestinal bleeding and prolonged aPTT, which failed to improve after fresh frozen plasma and cryoprecipitate transfusions but responded to protamine sulfate with shortening of aPTT.

Conclusion: Heparin-like substance was demonstrated in plasma samples obtaining from patients with dengue hemorrhagic fever. Further study is required to validate current findings, which could be useful for the future management of hemorrhagic complication in patients diagnosed with dengue hemorrhagic fever.

PA3.14 – Coagulation Factor XI – II

PA 3.14-1

Enhanced factor XI-dependent thrombin generation by nanoparticle-bound polyphosphate

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Background: Inorganic polyphosphates (polyP) are negatively charged, linear phosphate polymers that are secreted from platelet dense granules. We previously reported (Choi et al, Blood 2011;118:6963–6970) that polyP supports both autoactivation of factor XI (FXI) and back-activation of FXI by thrombin. Thus, platelet polyP is likely a physiologically relevant cofactor for FXI activation *in vivo*.
Aims: Evaluate the ability of nanoparticle-bound polyP to support FXI-dependent thrombin generation.
Methods: Silica nanoparticles (SNP) with covalently bound polyP (SNP-polyP) were prepared by a modified Stöber process, based on the hydrolysis and condensation of tetraethyl orthosilicate in ethanol, using ammonium hydroxide as catalyst. Product was approximately 85 nm diameter nanoparticles with covalently attached long-chain polyP (300–1300 phosphates long, mode approximately 700mer). Thrombin generation (CAT) assays were conducted with factor (FXII)- or FXI-deficient plasma containing 20 mM phospholipids, 20 nM β-thrombin, and either: soluble polyP (0–70 μM phosphate monomer), SNP-polyP (0–100 mg/mL particles with 0–7 μM polyP), or SNP without polyP (0–100 mg/mL particles).
Results: Minimal thrombin generation was observed in FXII-deficient plasma in the presence of SNP lacking polyP. Adding either soluble polyP or SNP-polyP to plasma shortened the time to peak thrombin and greatly increased both the peak thrombin and the endogenous thrombin potential (ETP). Over the range of polyP concentrations evaluated, SNP-polyP was about tenfold more potent than soluble polyP in enhancing thrombin generation. In contrast, insignificant levels of thrombin were generated when either polyP or SNP-polyP were added to FXI-deficient plasma.
Conclusions: PolyP attached to nanoparticles was tenfold more potent than polyP in solution at stimulating thrombin generation under our assay conditions (in which polyP-stimulated thrombin generation is completely dependent on FXI). Such particles may be useful in enhancing thrombin generation at sites of injury *in vivo*.

PA 3.14-2

Identification and characterization of a highly specific FXIa inhibitor from Bungarus fasciatus venom

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Background: The high risk of excessive bleeding associated with most anti-thrombotic drugs available in the market has made it necessary to discover new anticoagulants with minimal bleeding side effects. Inhibitors targeting the intrinsic coagulation pathway while leaving the extrinsic and common pathways unaffected are potential candidates of such new anticoagulants.
Aims: (i) Identification and purification of novel peptides from *Bungarus fasciatus* venom, capable of specifically targeting the intrinsic coagulation pathway. (ii) Functional characterization of identified peptides.
Methods: *Bungarus fasciatus* venom was fractionated and examined for effects on prothrombin time (PT) and activated partial thromboplastin time (APTT) of human plasma. Fractions that prolonged APTT and left PT unaffected were further analyzed by mass spectrometry, protein sequencing, and circular dichroism spectroscopy and screened for specificity against different coagulation enzymes. Isolated candidate peptide was further characterized by a series of functional assays including inhibition kinetics, western blotting, surface plasmon resonance and blood coagulation assays.
Results: A kunitz-type protease inhibitor (termed BF01) showing highly specific inhibition of FXIa was purified. The protein was recombinantly expressed in *Escherichia coli* system, and purified by Ni-NTA beads. BF01 prolonged APTT and was demonstrated to interact and inhibit FXIa in a dose-and time-dependent manner from 50 nM to 5 μM and marginally inhibited Activated Protein C at > 10 μM, while inhibition of other protease in the coagulation pathways (Kallikrein, FXIIa, FXIa, FXa, FIXa, FVIIa, Urokinase, alpha-Thrombin) were not observed at doses up to 100 μM. BF01's inhibitory effect on the cleavage of FIX by FXIa was observed using western blotting through probing for FIX and FIXa.
Conclusions: We have identified and characterized the first FXIa-specific inhibitor from exogenous sources, which differs significantly from FXIa's physicological inhibitor Protease Nexin 2. This peptide may potentially be used as template for designing novel inhibitors against FXIa.

PA 3.14-3

The type I mutation causing factor XI deficiency in Ashkenazi Jews is a founder mutation of recent Eastern European origin

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Background: Factor XI (FXI) deficiency is one of the most frequent inherited disorders in Ashkenazi Jews (AJ). Two predominant founder mutations termed type II (p.Glu117Stop) and type III (p.Phe283Leu) account for most cases. The different ethnic distribution and the estimated coalescence times suggest there is an ancient Middle Eastern origin for type II mutation and a more recent European origin for type III mutation.
Aims: To investigate clinical and genetic aspects of a less common type I mutation (c.1716 + 1G>A) in AJ.
Methods: Bleeding manifestations, FXI levels and geographic origin of members of 13 unrelated families harboring type I mutation were determined. Eight intragenic polymorphisms and five polymorphisms flanking the *F11* gene, together spanning 1.43 Mb, were analysed in families with type I mutation, 16 unrelated type II homozygotes, 23 unrelated type III homozygotes and 438 AJ controls. Haplotype analysis was used to define a founder effect. The age of type I mutation was estimated by DMLE+2.0 software program.
Results: Among 414 unrelated Jewish patients with severe FXI deficiency (FXI level < 15 U/dL), the allele frequency of type I mutation was 1.21% compared to 53.9% and 43.8% for type II and type III mutations, respectively. Four of 16 type I heterozygotes (25%) and six of 12 (50%) compound heterozygotes for type I mutation (I/II or I/III), and a type I homozygote had bleeding manifestations mostly at injury sites expressing high fibrinolytic activity. Haplotype analysis disclosed that type I is a founder mutation that occurred approximately 600 years ago. The origin of the families carrying the type I mutation was from an Eastern European region surrounding the Carpathian mountains.
Summary: The type I mutation is a third founder mutation causing factor XI deficiency in AJ. The estimated ages of the three Jewish founder mutations reflect major landmarks in the history of the Jews. While the type II mutation occurred in early antiquity during the formation of the Jewish nation in the Middle East, and the type III mutation occurred in the middle ages during the consolidation of the AJ in Central Europe, the type I mutation occurred more recently when the Eastern European AJ communities were founded.

PA 3.14-4

Interim results (3-year) of a French non-interventional study to assess the long-term safety and efficacy of BeneFIX

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Background: Clinical studies have demonstrated the efficacy and safety of BeneFIX for on-demand treatment and prophylaxis. However, these studies can present limitations concerning extrapolation of their results to routine clinical practice and a non-interventional study with high quality standards is an appropriate mean to assess the long-term safety and efficacy in an unselected population of patients.
Aim: To evaluate the safety and efficacy of long-term BeneFIX therapy in the usual care settings in French patients with haemophilia B.
Methods: All patients receiving BeneFIX can be included in this non-interventional study. Data are collected on standardized case-report forms and monitoring visits are performed regularly to ensure high data quality.
Results: The study started in 2009. By January 2013, 56 patients from 17 sites were enrolled and evaluable for safety (median age: 18.06 years; range: 0.2–66.9). Among the 37 patients contributing to the efficacy analysis (FIX: C $\leq$ 1%, no inhibitor, $\geq$ 1 follow-up visit, sufficient diary information), 22 had at least a period of prophylactic treatment (median duration: 662 days) and 21 had at least a period of on demand treatment (median duration: 722 days). The mean dose per infusion for prophylactic treatment was 46.3 $\pm$ 12.38 IU/kg (median 45.7; range: 25–68). The annualized mean number of bleeding episodes was respectively 4.4 $\pm$ 3.7 (median 4.4) and 12.8 $\pm$ 11.8 (median 8.6) during prophylaxis and on-demand treatment. A total of 627 bleeding episodes occurred, mostly in a joint (42.6%). Overall, 84.2% of bleeding episodes were resolved with 1–2 injections. The mean dose per infusion for all bleeding episodes was 48.4 $\pm$ 12.8 IU/kg (median 49.7; range: 29–74). All subjective evaluations of efficacy were rated by the physician/patient as 'excellent' or 'good'. One previously untreated child with severe haemophilia B after 14 ED (11 in prophylactic and four on demand) with 83 UI/kg/twice a week BeneFIX consumption, developed inhibitor antibody at 1.7 Bethesda units (BU) titers. FIX gene analysis demonstrated complete deletion of the FIX gene. Development of inhibitory antibodies was not associated with severe allergic or even anaphylactic reactions following infusion of factor IX concentrates. Prophylactic treatment with rFactor VIIa (Eptacog alfa) was administrated until inhibitor disappeared.
Conclusions: This three-year interim analysis of this non interventional study supports the safety and efficacy of treatment with BeneFIX under usual care settings. Inhibitors against factor IX (FIX) are not very common, being reported in < 2% of patients with haemophilia B with a strong link between complete deletions and inhibitor development as described in literature.

PA 3.14-5

FXI deficiency and Gaucher disease: a potentiated bleeding risk?

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Background: Gaucher disease (GD) is an autosomal recessive deficiency of the enzyme glucocerebrosidase, required for the degradation of glycosphingolipids. Clinical features, consequent primarily on the effects of storage of un-degraded glycosphingolipid within the reticuloendothelial system include bone pain, hepatosplenomegaly, peripheral blood cytopenias and a poorly defined bleeding diathesis. Symptoms are highly variable and onset is often insidious and non-specific resulting in a prolonged time course from symptom onset to diagnosis. It is a pan-ethnic disorder, with an overall prevalence rate of one in 75,000 live birth; however, it is far more common in the Ashkenazi Jewish population with a carrier rate in 1 in 12. FXI deficiency similarly has an increased carrier rate of 1 in 8 in the Jewish population and has a highly variable bleeding phenotype. FXI deficiency may have an increased prevalence amongst patients with GD and there may be a synergistic effect that increases the prevalence of bleeding symptoms.
Aims: To ascertain the prevalence and nature of bleeding symptoms and prevalence of defined bleeding disorders/factor deficiencies with a cohort of GD patients.
Methods: A single centre cohort of patients with GD was evaluated. A retrospective review of clinical notes and laboratory data at diagnosis was undertaken. Prospective data regarding bleeding scores was obtained. Factor assays were performed using a standard 1 stage APTT-based assay. The study was approved by the local ethics committee.
Results: Amongst the cohort of 98 GD patients, 54% had bleeding symptoms at presentation: bruising (45%), epistaxis (11%), post-operative bleeding (8%), menorrhagia (2%). Bleeding was the primary presenting symptom in 19%. Sixty-five percent of patients were diagnosed by haematologists, including five patients who presented initially to haemophilia centres. Seventeen percent of patients were found to have reduced FXI levels within the heterozygous range (mean 56 IU/dL, range 39–68), 2% reduced von Willebrand antigen and activity and one a platelet storage pool defect. 6/17 (35%) of the patients with FXI deficiency were of known Jewish ethnicity, compared to 29/98 (28%) of the overall cohort. 14/17 (83%) of those with FXI deficiency had bleeding symptoms, with six suffering post-operative bleeding. This is in marked contrast to published cohorts of patients with partial/heterozygous FXI deficiency, of whom only 35% exhibit bleeding symptoms (Gomez & Bolton-Maggs, 2008). In GD, mild-moderate thrombocytopenia (mean 71 $\times$ 10^9/L, range 35–121) and cellular dysfunction secondary to lipid storage may act as modifiers of the bleeding phenotype.
Conclusions: GD commonly presents with bleeding symptoms and thus may initially present to haemostasis specialists. Patients with GD commonly have low FXI levels, within the heterozygous deficiency range, at presentation; bleeding symptoms are more prevalent in these patients than expected for patients with partial FXI deficiency. The diagnosis of GD should be considered in any patient with unexplained bleeding or bleeding out of keeping with the degree of a coagulation factor deficiency.

PA 3.14-6

Structure of coagulation FXI bound to a peptide derived from laminin

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Background: Coagulation factor XI (FXI) is the zymogen of the enzyme FXIa that contributes to haemostasis by activating factor IX (FXI). Laminin is an extracellular matrix protein which is an essential component of the basement membrane structure and becomes exposed when the vasculature is damaged. Recent work has shown that laminin can contribute to coagulation via activation of the contact system as well as through interaction with platelets [1–3]. The best characterised interaction of a coagulation factor with a matrix protein is the FIX (a FXIa substrate) interaction with collagen IV which is also a critical component of the basement membrane. The interaction of the FIX with collagen IV was shown to contribute to haemostasis *in vivo* and loss of this interaction has been characterised as a haemophilia [4].
Aims: To investigate the molecular basis of FXI ligand binding we crystallised a peptide derived from laminin with FXI.

Methods: We have determined the structure of a short peptide from laminin bound to the apple domain of the FXI zymogen using protein X-ray crystallography and molecular replacement techniques.
Results: The laminin peptide binds along a hydrophobic groove on the underside of the apple domains of FXI. A hydrogen bond network between the laminin peptide and residues on the apple domain surface is important in mediating this interaction.
Conclusion: *The FXI-laminin complex crystal structure is the first complex structure reported between a coagulation factor and an extracellular matrix protein.* The structure reveals a key binding site for laminin and also proposes a similar interaction between laminin and the FXI homolog prekallikrein. The interaction of FXI with laminin may represent another means to localise coagulation factors onto a surface formed by the basement membrane together with FIX.
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PA3.15 – Contact Activation – II

PA 3.15-1

DNA and RNA promote kallikrein-mediated activation of fXII and are cofactors for fXI feedback activation by thrombin

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Background: Polyanionic surfaces, such as dextran sulfate and kaolin, mediate contact activation in plasma by binding factor (f) XII and high molecular weight kininogen (HK). Interaction of fXII with a surface promotes its autoactivation. HK bridges fXI and prekallikrein (PK) to the surface. Generation of kallikrein activates additional fXII and augments production of fXIa, leading to enhanced thrombin generation. Thrombin also activates fXI, in a positive feedback step that also is surface-dependent. Recent studies have identified physiological activators of the contact system. Extracellular DNA and RNA have been shown to activate the contact pathway, but the mechanisms by which they promote activation are unclear.
Objective: To identify the mechanisms by which DNA and RNA promote contact activation.
Methods: DNA and RNA were isolated from mammalian cells. Binding of fXII, HK, fXI, Phe-Pro-Arg-chloromethyl ketone (FPRck)-fXIa and FPRck-thrombin to biotinylated-DNA and -RNA was quantified using surface plasmon resonance (SPR). The effects of DNA and RNA on contact activation were evaluated with chromogenic assays.
Results: Binding studies using SPR reveal that DNA and RNA bind fXII with K_d values of 30–200 nM, whereas HK binds with a K_d of 35–40 nM. Although DNA and RNA do not significantly promote fXII autoactivation on their own, they enhance fXIIa generation by fivefold in the presence of PK alone and 30-fold in the presence of both PK and HK. In the individual reactions, DNA augments both kallikrein activation of fXII and fXIIa activation of PK by 2-3-fold. In the feedback pathway, fXI (K_d of 0.6–4 nM), FPRck-fXIa (K_d of 11–14 nM) bind DNA and RNA with greater affinity than FPRck-thrombin (K_d of 600–1100 nM). DNA and RNA accelerate thrombin activation of fXI by 8- and 11-fold, respectively. However, DNA does not stimulate thrombin chromogenic activity.
Conclusion: Contact pathway factors (fXII, HK and fXI/fXIa) bind DNA and RNA with high affinity. Although they have little effect on fXII autoactivation on their own, DNA and RNA promote fXIIa generation in the presence of PK and/or HK, indicating that fXII activation by DNA and RNA is dependent on the plasma kallikrein system. DNA and RNA bind fXI and fXIa in an HK-independent manner and serve as cofactors for feedback activation of fXI by thrombin. Collectively, these results suggest that in addition to localizing and concentrating the contact proteins onto the surface to initiate contact activation, DNA and RNA also amplify the procoagulant response by promoting feedback activation of fXI by thrombin.

PA 3.15-2

Ongoing contact activation in patients with hereditary angioedema

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Background: Hereditary angioedema (HAE) is caused by a deficiency in C1-esterase inhibitor (C1INH). C1INH is the main inhibitor of the contact system and inhibits activated FXII (FXIIa), FXIa and kallikrein. Attacks of angioedema are caused by the release of bradykinin from high-molecular-weight kininogen, mainly mediated by kallikrein. Activation of FXII leads to the formation of α-FXIIa and β-FXIIa. Both enzymes can convert prekallikrein into kallikrein, but only α-FXIIa can initiate the intrinsic pathway of coagulation.
Hypothesis: In HAE patients the thrombotic risk is not increased during attacks. We hypothesized that contact activation preferentially leads to kallikrein and bradykinin formation and less to activation of the coagulation cascade via FXI activation in these patients.
Methods: In plasma samples of healthy controls ($N = 10$) and HAE patients ($N = 30$, 17 during remission and 13 during acute attack), we measured activation of the contact system as (i) the levels of C1INH-complexed with these enzymes (ii) the *in vitro* potential of the plasma to form inhibitory complexes when contact system is completely activated with a FXII trigger. Furthermore, the capacity of the plasma to inhibit kallikrein and the spontaneous kallikrein activity was measured.
Results: The basal levels of enzyme-inhibitor complexes were comparable between controls and patients during attack as well as during remission. There was no uninhibited FXIIa, FXIa or kallikrein present in the sample, since addition of C1INH did not change the complex levels. After *in vitro* activation of the plasma samples the levels of kallikrein-C1INH correlated positively with the capacity of the plasma to inhibit kallikrein ($r = 0.74$, $P < 0.001$), and negatively with the spontaneous kallikrein activity ($r = -0.54$, $P = 0.029$). Furthermore, the levels of FXIIa-C1INH, FXIa-C1INH and kallikrein-C1INH were at least 52% lower in samples taken during remission and 70% lower in samples taken during attack compared to samples from controls. The levels of FXIa-α_1-antitrypsin were more than 30% higher in patients compared to controls. Reconstitution of C1-esterase inhibitor after activation led to an increase in levels of FXIIa-C1INH and FXIa-C1INH, which, however, were 33% and 43% lower than in controls, while the levels of kallikrein-C1INH did not change.
Summary/Conclusion: In the basal samples we did not detect contact activation, even after correction of C1INH to normal levels for optimal enzyme inhibitor complex formation. After activation of the plasma samples *in vitro*, we found lower levels of all enzyme-C1INH

complexes in samples from HAE patients compared to controls, also in the presence of additional C1INH. The lowest levels were found in samples from patients during acute attack. In concert, this could indicate an ambient activation of the contact system and minor depletion of contact factors. Our results do not allow to demonstrate a preferential generation of kallikrein over FXIa. Regulation of the activity of FXIa and other enzymes of the coagulation cascade after a low degree of contact activation by other inhibitors is probably sufficient to prevent thrombosis.

PA 3.15-3

Binding and activation of coagulation factor XII by activated platelet subpopulations and microparticles

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Factor XII (fXII) has recently been implicated as having important roles in thrombosis, but the mechanisms of its physiological activation remain obscure. Stimulated platelets or platelet-derived material are the main candidate suspects for this.

In this study, fXII was activated by platelets stimulated with thrombin, collagen-related peptide, or calcium ionophore A23187; but not with 2-MeS-ADP, or non-stimulated. Activation proceeded in citrated plasma and in buffer with purified fXII, with an apparent K_m of 52 ± 14.4 nM. Inhibition of platelet-dependent fXII activation by annexin V and preferential fXII binding to phosphatidylserine-positive platelets (630 ± 90 molecules/platelet under physiological conditions) and procoagulant platelet-released microparticles implied essential role of the phosphatidylserine-expressing platelet subpopulation. Calpain inhibitors also significantly decreased fXII-activating activity of platelets stimulated with A23187 and thrombin that was coincident with the inhibition of microparticle production. Unexpectedly, platelet granule-secreted material did not activate fXII, but was found to inhibit fXIIa.

These results demonstrate that stimulated platelets regulate fXII activation in a complicated manner very similar to their regulation of thrombin generation: they activate fXII on their surface but produce soluble fXIIa inhibitor(s) thus creating a balancing system able to shift both ways. This can either serve to confine the clotting processes to the platelet surface, or as a flow-sensitive trigger that allows platelet-dependent coagulation onset when platelet-secreted inhibitors are removed by flow. This could explain why fXII is important in thrombosis, but not hemostasis.

PA 3.15-4

Neutrophil extracellular traps (NETs) and factor XII activation; a possible mechanism for increasing thrombotic risk during extracorporeal membrane oxygenation (ECMO)

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Background: Bleeding and thrombosis are risks associated with ECMO and may be linked to extracellular material released from neutrophils. Neutrophil extracellular traps (NETs) are a self-defence phenomenon and part of the innate immune response against microbial invasion. This novel activation pathway induces neutrophils to release decondensed nuclear DNA (polyphosphate PolyP) laced with histones that have antimicrobial properties. Links between NETs and autoimmune diseases and thrombosis have been established. Platelets also produce PolyP, which have been shown to induce NETosis in a dose response manner and can activate factor XII. The evidence suggests that increased NET activation in thrombosis may in fact be an aberrant over-shooting of the immune defence.

Aims: Our hypothesis was that NET activation as shown by increased extracellular DNA causes increase activation of factor XII which in turn increases the thrombotic risk in respiratory patients subjected to ECMO.

Methods: We measured extracellular DNA (plasQuant IT[TRADEMARK] picogreen kit), factor XIIa (UnitestTM FXIIa assay) and thrombin generation microparticle assay using the Tecan fluorescent plate reader with i-control software (Technothrombin® TGA assay). We studied 26 H1N1 patients with acute respiratory failure on ECMO. There were 12 males and 14 females, age range 19–55 years. Of those 26 patients 18/26 survived and 8/26 did not survive. Ethical approval had been given by the Biomedical Research Unit at the hospital.

Results: Extracellular DNA at admission was 1099 and 203 ng/mL at end of stay (normal plasma 136 mg/mL). Factor XIIa at admission was median 62.4 U/L (IQR 38.2–86.0 U/L) and at the end of the ECMO run was 77.5 U/L (IQR 32.1–181.8 U/L). This data shows no significance overall, Kruskal-Wallis $P = 0.831$. If however the XIIa data is split between survivors and non survivors the XIIa data for survivors shows a decreasing trend in activation with duration of extracorporeal support. In non-survivors an opposing trend of increasing activation from admission to the end of stay was seen. Thrombin generation at admission was 62.9 and 105.9 nM at the end of stay. A negative correlation was found between thrombin generation and extracellular DNA ($P = 0.0274$). There was a positive correlation between DNA and neutrophil count ($P < 0.0001$). Extracellular DNA levels were greater in non-survivors than survivors ($P = 0.0001$). Factor XIIa was associated with mortality ($P = 0.048$).

Conclusions: Increasing levels of factor XIIa and extracellular DNA appear to be poor prognostic indicators in patients on ECMO for H1N1. Post-modification of extracellular DNA appears to play an important role in modulating the coagulation response in this clinical setting. It is tempting to suggest factor XII could provide a therapeutic target to prevent or reduce thrombotic risk during ECMO without the additional bleeding side-effects seen with Heparin anticoagulation.

PA 3.15-5

Activation of coagulation factor XII is on the dextran sulfate surface is predominantly an autoactivation via a free substrate mechanism

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Background: Surface-induced activation of factor XII (fXII) is a crucial event of the internal pathway of blood plasma coagulation. Binding of fXII to the surface causes conformational change which leads to the fXII activation. Although presence of negatively charged surface is known to be necessary for this, the mechanism of this process remains unclear.

Aim: We used mathematical modelling and computer simulations to analyze possible mechanisms of fXII interactions with the surface and identify the correct activation pathway.

Methods: We assumed that there are two steps: spontaneous surface-induced activation of fXII, and consequent autoactovation of fXII by fXIIa. So surface is considered both as a trigger of contact activation and as an immobilization substrate for active form of f XII. Our model is notable for explicit account taken of role of surface concentration and molecular interactions with fXII. The models were solved using MATLAB (the MathWorks, Inc.).

Results: We developed two mathematical models for autoactivation of fXII. The first model required fXII first to bind the surface before it may interact with the surface-bound fXIIa via two-dimensional diffusion and become activated ('bound-substrate model'). According to the second model, surface-bound fXIIa activated a fluid-delivered form of factor XII ('free-substrate model').

We described these events in buffer system which contained pure fXII, trace amounts of factor XIIa (from 0% to 0.01% of total amount of fXII), activation surface (dextran sulfate) and albumin. We evaluated both these models and established that both of them fit with the majority of experimental data. Still, only the 'free-substrate model' was able to describe system's behavior in wide range of surface concentration and to obtain distinctive bell-shaped curve 'rate of activation vs. concentration of surface'.
Conclusion: Analysis of the present study suggests that 'free-substrate model' is the main mechanism of fXII activation during the initial stages of contact activation.

PA 3.15-6

Randomized trial of a quantitative, computerized pretest probability of acute coronary syndrome and pulmonary embolism in emergency department patients: safety, radiation exposure, and cost of care

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Background: Overtesting for acute coronary syndrome (ACS) and pulmonary embolism (PE) can harm patients.
Objective: Test the hypothesis that Pretest Consult, a validated, computerized method to estimate the pretest probability of both ACS and PE could safely reduce unnecessary exposure to medical radiation and wasted resource use.
Methods: Four center, prospective, randomized trial of medical device efficacy. Inclusions: age > 18, charted evidence of chest pain and dyspnea with a non-diagnostic electrocardiogram. Exclusions: known current diagnosis of ACS or PE, pre-arrival plan for admission from another physician, cocaine use, pregnancy and social situation precluding follow-up. Clinicians entered patient predictor data into Pretest Consult (the device) which randomly assigned to device output or sham. Device output showed estimated probabilities of ACS and PE, linked to specific diagnostic recommendations to minimize radiation exposure and to produce a posterior probability < 1%. Shams received nothing. Protocol-defined 90-day outcomes: (i) Dose (mSv) of chest radiation exposure with attention to the proportion with > 5 mSv radiation to the chest; (ii) Serious adverse events (SAEs) including delayed diagnoses; (iii) Same day admission rate with no significant cardiopulmonary diagnosis and length of stay (LOS); (iv) Readmission rate; (v) Direct costs and charges; (vi) Patient satisfaction. Sample size of 550 estimated to find a 10% difference in proportion with > 5 mSv chest radiation with $\alpha = 0.05$ and $\beta = 0.20$. Two-sided Ps from Mann–Whitney *U* or Exact test.
Results: Data complete for 541 patients. Means of ages and pretest probabilities were well matched between groups. Within 90 days, 16 (3%) patients had ACS and 11 (2%) had PE. No ACS, PE, or any other significant CP diagnosis was found in 236/277 (89%) vs. 242/264 (87%). The following compares the device ($n = 264$) vs. sham ($n = 277$) groups for the five predefined important outcomes: (i) The frequency of diagnosis of ACS or PE tended to be lower with use of the device vs. the sham 9/264 (3.3%) vs. 18/277 (6.5%), $P = 0.07$. (ii) The frequency of all-cause serious adverse events within 90 days tended to be lower with the device: 29/264 (11%) vs. 45/277 (16%) $P = 0.06$ and rate of delayed diagnosis was not different between groups (one had delayed ACS with device and one had delayed PE with sham). (iii) The same-day admission rate of patients who ultimately had no significant cardiopulmonary diagnosis was 104/264 (39%) vs. 119/277 (43%), $P = 0.38$. (iv) The 90 day readmission rate within 90 days was 8% with device vs. 10.5% with sham ($P = 0.3$). (v) The median chest radiation dose was 0.12 vs. 0.62 mSv ($P = 0.04$) and 30% vs. 37% of patients with no significant CP diagnosis had > 5 mSv ($P = 0.1$). (vi) Median costs were \$954 vs. \$1259 ($P = 0.03$) and charges were \$6299 vs. \$7565 ($P = 0.006$). (vii) Patients answered all questions 'very satisfied' in 20 vs. 22% ($P = 0.4$).
Conclusions: During a single encounter, the integration of Pretest Consult into the emergency care of patients with chest pain and dyspnea was safe and resulted in lowered median per patient radiation exposure with significantly lower costs and charges over the next 90 days.

PA3.16 – Cancer and Thrombosis – IX

PA 3.16-1

Thrombin and fibrinogen support prostate tumor growth in mice

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Background: Prostate cancer is the most common form of cancer in men and is second only to lung cancer as a leading cause of cancer related death in men. A role for hemostatic system components in this disease is suggested by previous studies where pharmacological anticoagulants limited prostate cancer growth in mice. Moreover, several clinical studies have shown that men on chronic anticoagulation therapy as well as men with hemophilia have a decreased incidence of prostate cancer, suggesting that hemostatic factors plays an important role in the development and/or progression of this malignancy.
Aim: Test the hypothesis that thrombin-mediated procoagulant function drives prostate cancer growth.
Methods: A transplantable prostate tumor cell line derived from a TRAMP (transgenic adenocarcinoma of the mouse prostate) mouse was inoculated into the dorsal subcutis of congenic C57Bl/6-derived mice with genetically-imposed alterations/deletions in prothrombin, thrombomodulin or fibrinogen.
Results: To determine the role of prothrombin in prostate cancer growth, cohorts of mice with genetically imposed low levels of prothrombin expression (approximately 10%) and wildtype mice were inoculated with TRAMP cells in parallel. Tumor growth was significantly diminished in mice with low prothrombin levels, resulting in an approximately 3-fold genotype-dependent difference in tumor mass by the end of the 3 week study period. Detailed histological analyses of tumor tissue showed a significant diminution in the mitotic index of tumors harvested from mice with low prothrombin levels, suggesting that thrombin function supports the proliferation of prostate cancer cells *in vivo*. Complementary studies of prostate tumor growth were performed in mice carrying a mutation in the native thrombomodulin gene that limits thrombin binding and protein C activation (TM^{Pro}). Here, the shift toward thrombin-mediated procoagulant function imposed by the TM^{Pro} mutation resulted in a significantly increased tumor growth rate relative to mice with normal thrombomodulin. Paralleling results in mice with a quantitative defect in prothrombin, comparisons of prostate tumor growth in fibrinogen-deficient and -sufficient mice revealed that the genetic elimination of this provisional matrix protein significantly limited prostate tumor growth.
Summary: These findings suggest that thrombin-mediated procoagulant function supports prostate tumor growth through at least one mechanism dependent on fibrinogen. These studies also suggest that therapies targeting thrombin or thrombin targets could limit prostate cancer progression.

PA 3.16-2

Effect of heaprins on the progression of tumor growth in mouse lewis lung carcinoma model

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Background: Heparin and low molecular weight heparin interact with growth factors and cellular receptors. Preclinical evidence suggests that heparins have an effect on tumor progression independent of anticoagulant activity. This study investigated whether heparin derivatives are able to inhibit tumor growth in a Lewis Lung carcinoma model.
Methods: C57BL/6 mice (6–8 weeks of age) were implanted with 5×10^5 LN7 tumor cells by dorsal subcutaneous injection in the upper back. Once tumors were palpable (7–10 days of growth), mice were treated with subcutaneous injections of heparin, enoxaparin, semuloparin or saline, daily for 2 weeks at approximately 1 cm away from the tumor site, in a dose range of 0.25–1.0 mg/kg. After the treatment period, animals were sacrificed and tumor weight, tumor volume, spleen weight and spleen size were measured. Plasma VEGF levels were measured by ELISA.
Results: At the 1.0 and 0.5 mg/kg dosages, enoxaparin and semuloparin decreased tumor volume compared to the saline control ($P < 0.01$). At the 1.0 mg/kg dosage heparin reduced the tumor size but mortality was higher due to bleeding. At a dosage of 0.25 mg/kg, semuloparin reduced tumor size and weight in comparison to saline control ($P < 0.01$). No significant differences among the agents were noted in spleen weight or size. Mortality rates of enoxaparin and semuloparin treated mice were low (< 10%) and were comparable between the two groups. A significant reduction in VEGF levels in the semuloparin group was observed.
Conclusions: Enoxaparin and semuloparin are more effective at reducing tumor growth compared to heparin. Enoxaparin and semuloparin were safer than heparin at the 1.0 and 0.5 mg dosages in terms of bleeding. Semuloparin downregulated VEGF levels in comparison to the other groups suggesting that this mechanism potentially plays a role in the control of the tumor growth. Therefore semuloparin may be a better alternate for the management of cancer associated thrombosis.

PA 3.16-3

TFPIa and TFPIβ inhibit tumor growth and associate with invasive tumor phenotypes

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Background: It is now recognized that tissue factor (TF) is not only expressed in normal tissue cells and trigger blood coagulation, but is expressed by many cancer cells and promotes tumor progression. Targeting TF in cancer may therefore be of dual benefit: (i) prevention of thrombosis, which is common in cancer, and (ii) suppression of tumor progression. TF pathway inhibitor (TFPI) modulates TF induced coagulation. TFPI is mainly expressed by the vascular endothelium, but is also found in cancer cells in two isoforms, TFPIα and TFPIβ. Both isoforms exert antitumor effects, such as increased apoptosis, and reduced proliferation and invasion in cell models.
Aims: To substantiate the role of TFPI in cancer biology, through studying TFPIα and TFPIβ expression- and cell surface association in various breast cancer cells, in relation to invasiveness, TF status and anticoagulant activity. Moreover, we attempt to replicate the antitumor effects of TFPI previously shown in cell models, by *in vivo* tumor growth, in nude mice.
Methods: TFPIα, TFPIβ and TF mRNA expression was measured by qRT-PCR, whereas protein levels were determined by ELISA. TFPI cell association was assessed by flow cytometry, Western blotting and immunofluorescence after phosphatidylinositol-phospholipase C (PI-PLC) treatment, and siRNA knock down of syndecan 1–4. Procoagulant activity was determined in a FXa activity assay. Nude mice were subcutaneously injected with MDA-MB-231 epithelial breast cancer cells with stable TFPI knock down, and tumor growth (mm^3) was monitored for 50 days after injection.
Results: Both TFPIα and TFPIβ mRNA were expressed in breast cancer cells, and expression of the two isoforms were highly correlated ($r = 0.94$, $P < 0.001$). Consistently, TFPIα represented the major isoform expressed, and correlated positively with TF both at mRNA and protein level. High TFPI expression was found in cells from basal-like, invasive tumor subtypes, whereas cells from luminal, non-invasive subtypes showed low TFPI expression.
Approximately 80% of the cell surface attached TFPI was bound through glycosylphosphatidylinositol (GPI), and although such anchoring was evident for both isoforms, TFPIβ represented the main GPI-bound isoform. The GPI-anchored TFPI demonstrated TF-FVIIa inhibitory activity. Additionally, we found that TFPIα was associated with the heparan sulphate proteoglycan syndecan 3, revealing GPI-independent cell association of this isoform in breast cancer cells.
In vivo, significantly smaller tumor sizes were detected in mice injected with breast cancer cells with high endogenous TFPI expression, compared to TFPI knock down cells. This effect was evident for both isoforms. Knock down of TFPI was confirmed in tumor xenograft samples.
Conclusion: From both cell- and animal models, we hereby reveal a biological importance of both TFPI isoforms in cancer. We show that anticoagulant active TFPIα and TFPIβ are expressed in breast cancer cells, and that TFPI is cell associated both through GPI-anchors, and GPI-independent binding to syndecan 3. Tumor growth suppressing effects of both isoforms of TFPI were seen *in vivo* in mice models. Our findings demonstrate a potential dual effect of TFPI in cancer development, both as a tumor suppressor, but also as a counterbalance to the procoagulant activity of TF positive malignant cells.

PA 3.16-4

Endothelial cell protein C receptor attenuates tissue factor-promoted tumor growth of malignant pleural mesothelioma

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Background: Tumor cell-associated tissue factor (TF) is known to contribute to tumor growth and metastasis. Recent studies showed that TF contributes to tumor growth in breast cancer through activation of TF-FVIIa-induced PAR2-mediated signaling. However, it is unclear at present whether TF-FVIIa-PAR2 signaling is responsible for TF-driven tumor growth in other types of cancers. Further, it is not entirely clear whether and how other hemostatic receptors present on tumor cells influence TF-dependent tumor growth.
Aim: The aim of the present study is to investigate the role of TF, endothelial cell protein C receptor (EPCR) and protease activated receptor-1 (PAR1) on tumor growth of malignant pleural mesothelioma (MPM) by utilizing MPM cells that lack or express TF, EPCR or PAR1, and a murine orthotopic model of MPM.
Methods: REN, MS-1 and M9K MPM cells were characterized for the expression of TF, EPCR, tissue factor pathway inhibitor (TFPI), thrombomodulin (TM), PAR1 and PAR2 by western blot analysis and/or in functional assays. TF, PAR1 or EPCR was either selectively

knocked-in or knocked-out in aggressive REN MPM cells and non-aggressive MS-1 and M9K MPM cells by stable transfection of plasmid DNA containing TF or EPCR cDNA, or specific shRNA constructs. REN, MS-1 or M9K MPM cells, or their variants (10^6 cells) were injected into the pleural cavity of nude mice (BALB/c, NU/J). Mice were sacrificed between 28 and 30 days following tumor cell implantation, the chest cavities were photographed, tumors were counted, weighed, and their volumes were calculated by measuring their dimensions. Tumor tissue samples were fixed and processed for immunohistochemistry.

Results: REN MPM cells express TF but not TFPI, EPCR or TM. MS-1 cells express TFPI, EPCR but not TF or TM. M9K MPM cells express TFPI, EPCR, TM but not TF. All three MPM cell types express PAR1 but not PAR2. Intrapleural administration of REN MPM cells in nude mice generated large tumors in the pleural cavity, some of tumors reaching the size of the heart. In contrast, intrapleural administration of MS-1 or M9K MPM cells resulted in relatively few tumors, and they barely reached the size of 2 mm. Suppression of TF or PAR1 expression in REN MPM cells markedly reduced the tumor growth. However, overexpression of TF in MS-1 or M9K MPM cells failed to promote the tumorigenicity of these cells. More importantly, introduction of EPCR expression in aggressive REN MPM cells attenuated the tumor growth completely. Conversely, EPCR silencing in MS-1 and M9K MPM cells overexpressing TF dramatically increased the tumorigenicity of these non-aggressive MPM cells. EPCR silencing in native MS-1 or M9K MPM cells did not significantly alter the low growth potential of these cells. Immunohistochemistry of tumor sections revealed that EPCR expression in MPM cells reduced tumor cell proliferation and increased tumor cell apoptosis.

Summary/Conclusions: Overall, our data indicate that TF promotes tumor growth of MPM in a PAR1-dependent mechanism, but expression of EPCR in TF-bearing MPM cells suppresses tumor growth. Our data suggest that EPCR acts as a tumor growth suppressor, at least in MPM.

PA 3.16-5

Role of thrombomodulin in melanoma progression

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Background: Thrombomodulin (TM) is a thrombin receptor that plays critical modulatory roles in inflammation, thrombosis, and carcinogenesis. The expression of thrombomodulin (TM) has been reported to negatively correlate with the metastatic potential of tumor cells. Thus, low TM expression has been found to be associated with high-malignancy phenotypes in oesophageal, lung and liver cancers. Melanoma, the most common fatal form of skin cancer, is an aggressive, therapy-resistant malignancy of melanocytes. The prognosis of patients diagnosed with metastatic melanoma is very poor and treatment alternatives are limited.

Aim: We propose that down-regulation of TM plays an important role in melanocyte transformation and melanoma progression.

Methods/Results: In this study, we monitored the expression of TM in murine and human melanoma cell lines in different stages of aggressiveness. The murine melanoma cell lines TM1 and B16F10, showed higher TM mRNA levels than melan-a (murine melanocyte), as analyzed by real-time PCR. We further analyzed two human melanoma cell lines displaying low (WM35) or high (A375) aggressive phenotypes. The mRNA expression level of TM was modestly (WM35) or dramatically (A375) down-regulated in melanoma cells as compared to human primary melanocytes, The lower expression of TM correlated with their degree of aggressiveness as determined by a migration assay which monitors the transmigration of human melanoma cells across an endothelial cell monolayer in a two chamber transwell plate. Interestingly, the over-expression of TM in A375 cell line reverted its aggressive phenotype as determined by the *in vitro* migration assay. Migration properties of A375 melanoma cells across endothelial monolayer stimulated with either thrombin or activated protein C (APC) were also investigated. The pretreatment of endothelial cells with thrombin significantly improved the migratory properties of A375 cells. By contrast, APC exhibited a protective activity by significantly inhibiting the migration of melanoma cells across the APC-pretreated endothelial cell monolayer.

Conclusion: These results suggest that TM plays a protective role in preventing melanocyte progression to melanoma and that its expression level may be used as an indicator in the prognosis of different stages of metastatic melanoma.

PA 3.16-6

Cisplatin increases tissue factor (TF) procoagulant activity on NT2 germ-cell tumor cells by a mechanism independent of apoptosis induction

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Background: Patients with germ-cell tumors (GCTs) are at increased risk of venous thromboembolism (VTE). Consequently, testicular cancer is categorized as a high-risk tumor entity in the Khorana score for the prediction of VTE risk in ambulatory cancer patients. Furthermore, cisplatin-based chemotherapy has been repeatedly shown to be independently associated with adverse cardiovascular events, suggesting that this cytotoxic drug elicits thrombogenic signals in leukocytes, endothelial and/or tumor cells. However, the molecular mechanisms underlying cisplatin-induced hypercoagulability remain obscure.

Aims: The aim of this study were to characterize constitutive TF expression by four different GCT cell lines and to study the effects of exposure to cisplatin on TF expression by NT2 cells.

Methods: Cell-associated TF procoagulant activity (PCA) was measured by single-stage clotting and fluorogenic thrombin generation assay in the presence or absence of inhibitory TF monoclonal antibody. Surface and total cellular TF antigen were analyzed by single-color flow cytometry and ELISA, respectively. Flow cytometric analysis of annexin V-FITC binding was used to assess phosphatidylserine (PS) membrane exposure on apoptotic cells.

Results: We found that three nonseminoma (NT2, 2102Ep, NCCIT) and one seminoma cell line (TCam-2) displayed significant TF-specific PCA by clotting and thrombin generation assay. Furthermore, all four cell lines stained positive for TF antigen by flow cytometry. Incubation of NT2 cells with 0.4 μM cisplatin (corresponding to the IC_{50} according to MTT cytotoxicity assay) for 48 h enhanced PS membrane exposure from 24 ± 11% to 38 ± 15% positive cells and increased cellular TF PCA 4.5-fold compared to vehicle-treated controls ($P < 0.01$). Interestingly, flow cytometry revealed enhanced TF antigen expression on both apoptotic and non-apoptotic cells, suggesting a mechanism of cisplatin-induced TF PCA expression independent of TF de-encryption during cell death. In line with this hypothesis, incubation of cisplatin-resistant NT2-R cells with 0.4 μM cisplatin had no effect on PS exposure (positive cells, 24 ± 5% vs. 25 ± 7%, $P = 0.4$), but dramatically increased cell-associated thrombin generation in a TF-dependent manner and induced TF-specific PCA 3.7-fold by single-stage clotting assay ($P < 0.01$), an effect accompanied by enhanced surface TF antigen staining (TF-positive cells, 15 ± 5% vs. 7 ± 3%; $P < 0.01$). Quantification of total TF antigen in cell lysates by ELISA revealed a 1.9- and 2.2-fold increase in cisplatin-treated NT2 and NT2-R cells, respectively, a finding that was also confirmed by Western blot analysis.

Conclusion: Constitutive TF expression may be a characteristic feature of GCT cells, possibly contributing to the elevated VTE risk in affected patients. Moreover, cisplatin-based chemotherapy may further potentiate this risk by increasing tumor cellular TF PCA indepen-

dently of apoptosis induction, e.g. via de-novo protein synthesis and/or inhibition of TF recycling.

PA3.17 – Hormones, Pregnancy and VT

PA 3.17-1

Thrombophilia and outcomes of ART procedures: a prospective Italian cohort study

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Background: Clinical pregnancy rate after Assisted reproductive technologies ART (ART) is low (20–35% per cycle started). Venous thromboembolism (VTE) and arterial thrombosis can occur after administration of drugs for ovulation induction. It is still controversial whether the presence of a thrombophilia could predispose women to infertility or to an adverse obstetric outcome following an ART cycle. However, many Infertility Clinics prescribe thrombophilia tests to all the women approaching this type of procedure, independently of the presence of additional risk factors. Low Molecular Weight Heparin (LMWH)and aspirin have been hypothesised to be effective in increasing clinical pregnancies rate in patients undergoing ART.
Aims: (i) To evaluate the prevalence of inherited and acquired thrombophilias in a large cohort of women suffering from infertility; (ii) to compare obstetric outcomes *before* and *after* performing screening and according to the presence and type of thrombophilia observed; (iii) to prospectively evaluate obstetric outcomes and thrombotic risk in women undergone thrombophilia screening according to the use of antithrombotic treatments.
Methods: Participants were 1107 women with fertility problems and a valid indication for ART treatment consecutively referred to our Atherosclerosis and Thrombosis Unit, between March 1998 and July 2011. All the participants were investigated for inherited and acquired thrombophilias. Before the thrombophilia screening data regarding maternal age, previous pregnancies and indication for treatment were collected. For each woman following information was collected for analysis: number of cycles and type of treatment (IUI/IVF/ICSI), antithrombotic prophylaxis (yes/no), antithrombotic drugs (low-dose aspirin, LMWH or both), period of prophylaxis and treatment outcomes.
The study was approved by the local ethics committee and complies with Declaration of Helsinki. Participants gave their written informed consent for present and future use of the clinical data.
Results: Median follow-up was 34.53 months (range 2–143 months). At the enrolment, 327 women (29.5%) have experienced 946 ART cycles. One hundred and twenty-two (37.3%) women obtained a clinical pregnancy (176 pregnancies), with 21(16%) live- born children. Overall, 115 (10.4%) women carried al least one thrombophilia (45 the FVL, 57 the PTm, 13 severe thrombophilias).
After screening, 595 (53.8%) underwent at least one attempt (1268 cycles, 27 lost to follow-up). In 424 (33.4%) cycles an antithrombotic prophylaxis (342 treated with aspirin, 66 with LMWH, 16 with aspirin + LMWH) was used. Pregnancy rate was 21.4% (265/1241 cycles) with 171 (13.8%) live-born children. A clinical pregnancy was obtained in 96 out of 424 (22.6%) treated cycles (*P*: 0.42, OR: 1.1, 95%CI: 0.8–1.5 vs. pregnancy rate in untreated cycles). The rate of live-born was not significantly different in treated vs. untreated cycles (*P*: 0.73, OR: 0.9, 95% CI: 0.6–1.3) prophylaxis. Overall, seven thrombotic events (three PE, three DVT, one arterial thrombosis; six of them in untreated women) were observed. Among them, 4 (0.3%, 2 DVT, 2 PE) occurred after screening, only one during LMWH prophylaxis.
Summary/Conclusions: The use of antithrombotic prophylaxis is not associated with an improved pregnancy rate or live-born children after ART. Thrombotic complications are less frequent in women receiving an antithrombotic prophylaxis.

PA 3.17-2

Unfractionated heparin, not enoxaparin, prevents down-regulation of endothelial protein C receptor by a pro-inflammatory cytokine on first trimester trophoblasts

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Background: Human first trimester trophoblasts must differentiate and invade the maternal endometrium, as well as give rise to placental villi. They come into contact with the maternal blood, contribute to changes in the maternal spiral arteries, and possibly participate in immune tolerance. Though anticoagulation and control of inflammation were shown to be strongly interconnected in many cell systems, to our knowledge no studies addressing directly the function and modulation of the anticoagulant pathway in human trophoblasts have been published. The importance of gaining basic knowledge on the anticoagulant functions of human trophoblasts has important clinical implications, because heparin prophylaxis is often given, albeit with no clear evidence-based benefit, to women with placenta-mediated obstetric complications (recurrent abortion, abruptio placentae, intrauterine growth restriction, late fetal death, pre-eclampsia) that may involve, at some point, a thrombotic event in the placental vessels.
Aims: To identify components of the protein C pathway on human first trimester trophoblasts, to evaluate protein C activating capacity on the trophoblast cell surface, and to test the effect of inflammatory mediators in the absence or in the presence of heparin on the expression of the anticoagulant pathway components on these cells.
Methods: Trophoblasts were isolated from first trimester placental tissue by sequential purification steps (99% purity). They were cultured in the presence or absence of various agents (TNF alpha, unfractionated heparin UFH, enoxaparin, alone and in combination) for maximum 24 h. Expression of thrombomodulin (TM) and endothelial protein C receptor (EPCR) was evaluated by flow citometry, RT-PCR and Western blot. Protein C (50 nM) activation was measured by a colorimetric assay, after thrombin addition (2 nM) to cultured trophoblasts.
Results: Human first trimester trophoblasts (cytokeratin seven positive, vimentin negative, HLA-G positive or negative) express EPCR and TM with variable intensity as mRNA, at the protein level and on the cell surface. Cultured, freshly isolated human trophoblasts activate protein C (1.1×10^{-7} nM/min/cell) at least to the same extent as HUVECs (1.56×10^{-14} nM/min/cell). When stimulated with the pro-inflammatory cytokine TNF-alpha (1 nM), EPCR but not TM expression was down-regulated by approximately 20%. EPCR downregulation was completely prevented by addition of UFH (1 IU/mL), but not by enoxaparin.
Summary/Conclusion: Human first trimester trophoblasts express a functional protein C anticoagulant pathway that can be modulated by inflammatory mediators such as TNF alpha, as in other tissues. Unfractionated heparin, but not enoxaparin, protects the trophoblast from TNF alpha-mediated down-regulation. If confirmed by further studies, these results may have important clinical implications.

PA 3.17-3

Prospective crossover trial of oral tranexamic acid and combined oral contraceptives in adolescent menorrhagia – interim report of a pilot study

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Background: Oral tranexamic acid (TA), an anti-fibrinolytic agent, has been shown to be more efficacious than progesterone-only hormonal therapy for menorrhagia in adult women. In adolescents with menorrhagia, TA has not been formally compared to the commonly prescribed combined oral contraceptive pills (COCP).

Aim: Among adolescents with menorrhagia, we hypothesized that oral TA would have equal or greater efficacy in reducing menstrual blood loss and improving quality of life when compared to COCP.

Method: Among adolescents with menorrhagia without an underlying bleeding disorder, organic pelvic pathology or thyroid disorder, a cross-over trial was undertaken as approved by the Institutional Review Board, to study the efficacy of TA (given according to FDA label for adult women at 1300 mg tid for 5 days, off-label use for adolescents < 18 years of age) vs. COCP (30 μg ethinyl estradiol/0.3 mg norgestrel daily, 21 days on/7 days off per cycle). This study was funded by Texas Children's Hospital's Pediatric Pilot Award. Informed consent was obtained from study participants/parents. Each medication was prescribed for three menstrual cycles with a 1 month wash out in-between medications. Menstrual cycle length, Periodic Blood Assessment Chart (PBAC) score for menstrual blood loss estimation, and quality of life assessment with PedsQL questionnaire was assessed at baseline and at the end of each medication arm. Data was analyzed using the mixed models repeated measure design in SAS.

Results: Seventeen patients were enrolled after obtaining informed consent; the mean age was 14.2 years (range 11.7–16.7). Five completed both arms of the study; two each are completing the first and second arm, resulting in nine currently evaluable patients. Eight patients were withdrawn from the study (2- due to adverse events, six for other reasons related primarily to study compliance).

When compared to baseline, significant improvement ($P < 0.05$) was demonstrated by both TA and COCP, in the length of menstrual cycle (mean reduction of 8.4 and 6.7 days), PBAC scores (mean score decrease of 578 and 552), and quality of life (mean score decrease of 17 and 14), though there were no statistically significant differences between TA and COCP ($P > 0.05$). All patients on TA reported 100% compliance while 57% on COCP reported periods of non-compliance. Seven patients (41%) experienced adverse events that were possibly drug-related – two on TA (28%; all mild – breakthrough bleeding/vomiting, lack of sleep) and five on COCP (62%; three mild – breakthrough bleeding, headache/weight gain, mood swings, two severe – seizure, generalized rash). None developed thrombosis.

Summary/Conclusion: This study is ongoing with four actively enrolled patients. Preliminary analysis of this ongoing pilot study suggests that TA may have efficacy comparable to COCP and holds promise for better control of menstrual bleeding, better quality of life, less toxicity and higher compliance likely due to the shorter duration of medication administration. Larger studies are needed to confirm these benefits of oral TA in menorrhagic adolescents as observed in our pilot study.

PA 3.17-4

Low-molecular-weight heparin added to aspirin in the prevention of recurrent early-onset preeclampsia in women with antiphospholipid antibodies

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Background: Early-onset hypertensive disorders (HD) and small-for-gestational age infants (SGA) are associated with placental vascular thrombosis, inheritable thrombophilia and antiphospholipid antibodies. Aspirin reduces the recurrence risk. Recently we reported the results of the FRUIT-RCT (ISRCTN 87325378) in women with a previous delivery for HD/SGA < 34 weeks and shown to have an inheritable thrombophilia without antiphospholipid antibodies: adding low-molecular-weight heparin (LMWH) to aspirin before 12 weeks gestation reduces recurrent HD onset < 34 weeks gestation. Here we present the results on women with antiphospholipid antibodies and no inheritable thrombophilia.

Aims: Adding LMWH to aspirin to reduce HD in women with previous early-onset HD (preeclampsia, HELLP syndrome, eclampsia) and/or SGA in the context of antiphospholipid antibodies.

Methods: In a multicentre RCT, 30 women were included before 12 weeks gestation. *Inclusion criteria*: previous delivery < 34 weeks gestation with HD and/or SGA; antiphospholipid antibodies (anti-cardiolipin antibodies > 10 GPL and/or MPL and/or lupus anticoagulant). *Intervention*: either daily LMWH (dalteparin, 5000 IU weight adjusted dosage) with aspirin 80 mg or aspirin 80 mg alone. *Primary outcomes*: recurrent HD onset (i) < 34 weeks and (ii) irrespective of gestational age. *Secondary outcomes*: recurrent SGA, preterm birth, maternal/neonatal hospitalization, spontaneous abortion and individual HD. *Analysis* by intention-to-treat. *Power calculations*: based on recurrence rate of 60% in Dutch population, 84 women needed to be randomized to detect a 50% reduction (two-tailed, an alpha of 0.05 and a power of 80%) and 66 inclusions for one-tailed, expecting no worsening of the outcome by LMWH.

Results: After the conclusion of the inheritable thrombophilia arm of the trial and given the low inclusion rate of women with antiphospholipid antibodies, an interim analysis was performed after delivery of 29 women, resulting in the advice to cease recruitment since accrual was low and the incidence of early onset HD was far lower than expected. The final analysis, performed on 30 women, did not reveal a reduction in the primary outcomes in the LMWH-and-aspirin arm, although there were no cases of HD. In the aspirin-only arm, there were two cases of HD, one before 34 weeks. Secondary outcomes did not differ between arms. No woman withdrew as result of adverse effects. Fourteen eligible women refused enrolment. Per-protocol analysis on 32 women did not alter the results.

The mean gestational age at delivery of the index pregnancies was 29 weeks, with 18 children born alive. The trial pregnancies had a mean gestational age at delivery of 37 weeks with all infants alive at birth. A single case of placental abruption occurred in the aspirin-only arm at 27^6 weeks in a woman hospitalized for fetal growth restriction and no HD; her index pregnancy had been terminated at 26^2 weeks for placental abruption and HD.

Conclusion: Adding LMWH to aspirin before 12 weeks gestation in women with antiphospholipid antibodies and prior delivery for HD/SGA < 34 weeks did not reduce recurrent HD either < 34 weeks or irrespective of gestational age.

PA 3.17-5

The minimal effective dose of tranexamic acid in women with menorrhagia

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Background: Menorrhagia is a common problem, affecting up to 15% of premenopausal women, that negatively impacts health and quality of life. Ten-30% of these women will have underlying bleeding disorders that are often not diagnosed until adulthood. Antifibrinolytic therapy with tranexamic acid (TA) represents a non-hormonal treatment option that reduces menstrual blood loss by up to 50%. However, its use is limited by dose dependent side effects and cost. The recommended dose is 3000–6000 mg/day, but in our experience a lower dose, with less side effects, is efficacious for the majority of women.

Aims: To determine if the minimal effective and tolerated dose (MED) of tranexamic acid for women with menorrhagia who have bleeding disorders is lower than the recommended dose in Canada of 4000 mg/day. A secondary objective is to determine if there is a difference in the MED of women with and without a bleeding disorder.

Methods: An open-label, uncontrolled, prospective dose escalation study is underway. Women 15–50 years with objective menorrhagia as defined by a and Pictorial Blood Assessment Chart (PBAC) score > 100, referred to a Hematology or Gynecology clinic are eligible for inclusion. The starting dose of TA is 1000 mg/day and may be escalated twice per cycle for a maximum of three cycles to a maximum dose of 6000 mg/day. Criteria for escalation are based on a quality of life assessment PBAC score. Any side effects are monitored. The MED is defined as the dose at which the PBAC score is < 100 or a percentage decrease in the PBAC score that is associated with the ability to continue normal daily activities. All women who had menorrhagia from menarche, and/or iron deficiency and/or any other bleeding history are investigated for an underlying bleeding disorder.

Results: We report the analysis of 71 women who have participated in the study to date. The mean age is 37.7 years (range 15.6–50.2 years). There are 16/71 (22.5%) with bleeding disorders, including 6 -Von Willebrand Disease (1 type 3, 5 type 1), 1-Hemophilia A carrier, 1-Immune thrombocytopenia 1-Plasminogen activator inhibitor-1 deficiency and 7-platelet dysfunction. The mean PBAC score at study entry for the entire group is 320 (range 104–1340), and for those with bleeding disorders, 353 (157–954). The mean end of study PBAC score for the entire cohort is 163 (range 15–664).The mean MED for the entire group was 2000 mg/day (1000 mg BID) and there was no difference between those with and without bleeding disorders. Iron deficiency was common with 69/71 women having s-ferritin < 30 μg/L. Tranexmaic acid was well tolerated and none of the participants stopped the medication prior to completion of the study.

Conclusion: Tranexamic Acid is an effective medication for women with menorrhagia and appears to be equally effective in women with underlying bleeding disorders. The mean MED was 50% less than the recommended dose in Canada for women with and without bleeding disorders. These preliminary findings may support the use of tranexamic acid at a lower than recommended dose and therefore be of benefit to a greater proportion of women with menorrhagia.

PA 3.17-6

Oral contraceptive use and tamoxifen therapy are not associated with increased levels of thrombin or impaired endogenous APC formation

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Background: Acquired resistance to activated protein C (APC) is a common phenotype in women receiving estrogen containing oral contraceptives (OC) or a selective estrogen receptor modulator (SERM).

Aims: To study if acquired APC resistance is associated with increased levels of thrombin formation we measured plasma levels of free thrombin and thrombin generation markers before and during OC use or SERM treatment. Simultaneously, plasma levels of APC were measured to identify a hormone associated endothelial cell dysfunction leading to impaired APC generation rates.

Methods: Blood samples were taken from women before starting with estrogen containing OC use or therapy with the SERM tamoxifen and in the first 3 months of application on a monthly basis. Thrombin and APC plasma levels were determined using recently developed oligonucleotide-enzyme capture assays (OECA) showing limits of detection of thrombin and of APC of 0.47 pM (17 pg/mL) and 0.39 pM (22 pg/mL), respectively. APC sensitivity was evaluated on basis of the effect of APC on the endogenous thrombin generation potential (ETP). In addition, plasma levels of coagulation factors fibrinogen, II, V, VII, VIII, IX, X, XI, XIII and of antithrombin (AT), protein C, d-dimer, prothrombin fragment 1 + 2 (F1 + 2), thrombin-AT-(TAT)-complexes, plasmin-α2-antiplasmin-(PAP)-complexes, and tissue-type plasminogen activator (t-PA) were measured using routinely established assays.

Results: Twenty-one women of the OC group (mean age, range: 21, 15–40 years) and 25 patients of the tamoxifen group (51, 28–71 years) were available for analysis. In both cohorts similar numbers of blood samples showed thrombin or APC levels above the respective limits of detection. OC use and SERM treatment were not associated with increased thrombin levels or a decrease in APC levels. APC sensitivity was significantly reduced in blood samples taken after 1 month of OC use ($P = 9 \times 10^{-6}$) or tamoxifen therapy ($P = 0.002$) in comparison to baseline results. The levels of indirect markers of thrombin formation F1 + 2, and TAT-complexes did not change significantly under OC or tamoxifen application. Observed changes of other parameters were consistent with previous reports. Coagulation factors, protein C, and PAP-complexes increased after start of OC use while AT and t-PA decreased. Under tamoxifen therapy coagulation factors, inhibitors, PAP-complexes, and d-dimer decreased over time while t-PA remained unchanged.

Summary/Conclusion: This study demonstrates that the hormone-induced APC-resistance phenotype is not regularly associated with increased rates of thrombin formation. As there was no dysbalance between plasma levels of thrombin and of APC, it seems unlikely that a hormone-induced endothelial cell dysfunction leading to impaired APC formation is involved in the pathogenesis of hormone related thrombosis.

PA3.18 – Inflammation

PA 3.18-1

Alterations of neutrophils production and functions at early stage of metabolic syndrome in a high fructose rat model

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1 In recent years, it became clear that the inflammatory response is intrinsically linked to metabolic pathways in vertebrates. Thus, a chronic and systemic low-grade inflammatory state is observed in metabolic syndrome (MetS) defined by a cluster of metabolic abnomalies including intra-abdominal adiposity, dyslipidemia, high blood pressure and altered glucose tolerance. This common disorder represents a major risk factor for atherosclerotic cardiovascular disease. The prevalence of MetS has dramatically increased worldwide due to a modern lifestyle and an increased of consumption of high-sugar diets especially fructose. By contrast to macrophage, neutrophils have classically received only little attention in MetS because these inflammatory cells were rarely detected in adipose tissue (AT).

2 We hypothesized that neutrophils' infiltration into AT may occur at early stage of MetS, in association with modulation of their major functions (activation, reactive oxygen species production, phagocytosis, apoptosis) and of their bone marrow production.

3 To test these hypotheses, we analyzed circulating neutrophil functions, their AT infiltration levels and the bone marrow efficiency in producing neutrophils at early stage in a high-fructose-fed Sprague-Dawley rat model. Male Sprague–Dawley rats were on regular (control rats [CR]) or fructose-enriched (60%) (fructose-fed rats [FFR]) diet during 6 weeks.

4 Compared to the CR group, the FFR group developed MetS i.e, hypertension, hypertriglyceridemia, fasting hyperglycemia and greater intra-abdominal AT volume and presented higher neutrophils infiltration in intra-abdominal AT ($+1.9 \pm 0.04$ fold increase, $P = 0.03$).

At resting state, no significant difference for circulating neutrophils functions was observed between two rats groups. In contrast, stimulated-neutrophils from the FFR group exhibited higher responses for all studied functions, compared to the CR group. Regarding PMA (1 μM) -activated neutrophils, CD18 (1.5 ± 0.04 fold increase, $P = 0.01$) and CD11b (1.3 ± 0.03 fold increase, $P = 0.02$) surface expression, and ROS production (1.5 ± 0.01 fold increase, $P = 0.001$) were significantly higher in the FFR group compared to the CR group. After activation with opsonized E. coli, significantly increased neutrophil phagocytic activity was observed in the FFR compared to the CR group (1.3 ± 0.02 fold increase, $P = 0.002$). These results suggest that early MetS induces circulating neutrophils priming.

In parallel, in bone marrow from FFR group, we observed a diminished clonal capacity of neutrophils progenitors (154.6 ± 12.77 vs. 206.5 ± 14.83, $P = 0.02$) and a higher proportion of apoptotic cells in granulocytic population (2.1 ± 0.03 fold increase, $P = 0.01$).

1. In conclusion, the results of this study provide evidence that at early stage of MetS, production, phenotype and function of neutrophils are modified that may explain the higher neutrophils infiltration in intra-abdominal AT infiltration. Understanding the contribution of neutrophils at early stage of MetS would help in development of novel therapies strategies against detrimental effects of this syndrome.

PA 3.18-2

The role of plasmin(ogen) in the pathogenesis of inflammatory bowel diseases

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Background and Aim: Inflammatory bowel diseases (IBD) are characterized by a chronic inflammation of the gastrointestinal tract, and are associated with defects in innate and adaptive immune pathways. However, the primary cause of IBD remains unclear. Plasmin(ogen) is an extracellular serine protease (zymogen), resulting in the activation of latent growth factors and matrix metalloproteases, degradation of extracellular matrix components, and fibrin clearance. It has reported that plasminogen (Plg) deficiency in mice results in spontaneous gastrointestinal ulceration. Plasmin(ogen) may play an important role in the pathogenesis of IBD. Therefore, we investigated the role of plasmin(ogen) in the pathogenesis of IBD.

Methods: We evaluated the parameters of intestinal inflammation in the wild type (Plg $^{+/+}$) and Plg deficient (Plg $^{-/-}$) mice. In addition, we examined that the effect of plasmin in intestinal epithelial Caco-2 cells.

Results: Plg deficiency in mice caused the reduction of colon lengths, and the increase of fecal IgA, proinflammatory cytokines expression, macrophages and T cells infiltration in colon. In addition, we found that Plg deficiency in mice promoted the increase of microfold cells (M cells) which acts as gatekeepers to the mucosal immune system. We also found that plasmin attenuated interleukin-1β (IL-1β)-induced the expression of receptor activator NF-κB ligand (RANKL) which regulates M cells differentiation in Caco-2 cells. Moreover, plasmin attenuated RANKL-induced M cells differentiation.

Conclusion: Plasmin(ogen) is associated with M cells differentiation by regulating the RANKL expression and RANKL-stimulated signal pathways. The excessive production of M cells by the defect of plasmin(ogen) might play an important role in the pathogenesis of IBD, and the regulation of plasmin(ogen) expression might provide a novel therapeutic approach to IBD.

PA 3.18-3

SMTP-7, a novel anti-inflammatory thrombolytic, treats serious cardioembolic stroke in a monkey model

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Background: Stroke is one of the major causes of death in industrialized countries. From WHO statistics, more than 6 million peoples die from stroke every year, and 80% of which is due to ischemic stroke. Moreover, it is also a problem of long-term hospitalization and sequelae. For the treatment of the acute cerebral infarction, rt-PA has been used as the standard drug. However, since rt-PA may cause serious hemorrhage side effect, only a few percent of the stroke patients benefits from rt-PA therapy. SMTP-7, a novel small molecule produced by a fungus, *Stachybotrys microspore*, promotes clot clearance and suppression of inflammatory and oxidative responses *in vivo*.

Aims: We attempted to obtain insights into therapeutic potential of SMTP-7 in comparison with rt-PA in a serious cardioembolic stroke model in a primate (cynomolgus monkey).

Methods: The monkey model of cerebral infarction was produced by injecting a blood clot that was generated from autologous blood into the middle cerebral artery. Three hours after injection of the clot, SMTP-7 (10 mg/kg, iv), rt-PA (3 mg/kg, iv) or saline were infused for 30 or 60 min.

Results: Cerebral infarct volume, neurological deficit score, and rate of cerebral edema were examined at 24 h after the thrombus injection.

The mean cerebral infarction volume was 10.12% in the SMTP-7 treated monkeys, 29.2% in the saline group, and 17.4% in the rt-PA group. The total of the neurological deficit score was 43 points in the SMTP-7 group, while 47 points in both the saline and rt-PA groups. The rate of cerebral edema in the SMTP-7 group was lower than those of in the saline and rt-PA group. Further, in the SMTP-7 treated monkeys, bleeding time was comparable to the saline group.
Conclusions: Our results showed that SMTP-7 is more effective and less hemorrhagic than rt-PA in a serious cardioembolic stroke model in monkeys. Taking that SMTP-7 has dual mode of action, thrombolytic and anti-inflammatory, this drug is clearly distinct from rt-PA in its molecular mechanism of action. This notion is supported by the present experiments using a primate model. Thus SMTP-7 can be the first, innovative one in a new category of drugs that treat acute ischemic stroke.

PA 3.18-4
Platelet-mediated innate immunity via toll-like receptor 2 stimulation and subsequent serotonin release

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Introduction: The innate immunity-mediating toll-like receptors (TLRs) influence the course of atherosclerosis. Platelets are involved in early and late stages of atherosclerosis and the stimulation of platelet TLR2 at high agonist concentrations induces platelet activation. It was previously shown that platelet serotonin induces activation of platelet tumor necrosis factor alpha converting enzyme (TACE), resulting in shedding of the adhesion molecule glycoprotein (GP)Ibalpha. We hypothesized that immunological platelet stimulation via TLR2 regulates serotonin release.
Methods: Platelet TLR2 was stimulated by incubation of human platelets with the TLR2 agonist Pam3CSK4 and compared to platelet activation by adenosine diphosphate (ADP, 20 μM), thrombin receptor activating peptide (TRAP, 30 μM), and phenylmaleinine phenylmaleic anhydride (PMA, 75 nM). Expression of surface markers was quantified by flow cytometry. Serotonin release was measured by high-sensitivity ELISA and dense granule release by electron microscopy. Leukocyte-endothelial interactions were analyzed by intravital microscopy.
Results: Incubation of platelets in the presence but not absence of plasma with Pam3CSK4 resulted in a dose-dependent increase of the surface expression of P-selectin and activated GPIIb/IIIa (up to 10-fold increase after incubation with 10–100 μg/mL Pam3CSK4, $P < 0.01$, $n = 8$). High-dose Pam3CSK4 also induced shedding of the TACE substrate GPIbalpha (50% decrease with 50 μg/mL Pam3CSK4, $P < 0.01$, $n = 8$) and exposure of phosphatidylserine (annexin V binding with > 10 μg/mL Pam3CSK4). Serotonin release was triggered by TLR2 stimulation with much higher potency: doses of Pam3CSK4 (0.1 μg/mL) that did not change P-selectin, GPIIb/IIIa activation, or GPIbalpha induced strong serotonin release ($P < 0.01$, $n = 8$). Perseverance of intact dense granules that did not undergo exocytosis suggested active serotonin release via membrane transporters.
Conclusion: Platelet TLR2 stimulation induces serotonin release much more potently than regular, 'hemostatic' platelet activation. This mechanism may constitute a novel innate immune function of platelets via selective release of the immuno-modulator serotonin.

PA 3.18-5
The role of a2AP in the development of renal interstitial fibrosis

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Background and Aim: Renal interstitial fibrosis is characterized by the excessive production, deposition and contraction of the extracellular matrix (ECM) protein, and is associated with macrophages infiltration and myofibroblats accumulation. In the process, the macrophages induce myofibroblats differentiation through the production of profibrotic factors, such as transforming growth factor-β (TGF-β) and connective tissue growth factor (CTGF), and then myofibroblasts promote the excessive production of the extracellular matrix (ECM) protein. However, the detailed mechanism remains unclear. We herein investigated the role of alpha 2-antiplasmin (α2AP) in the development of renal interstitial fibrosis in the obstructed kidney.
Methods: Renal interstitial fibrosis was induced by using unilateral ureteral obstruction (UUO) model in the wild type (α2AP+/+) and α2AP deficient (α2AP−/−) mice, and then we examined collagen deposition (Srius Red staining), myofibroblast differentiation (α-smooth muscle actin staining), macrophages infiltration (F4/80 staining) and the expression of α2AP. In addition, primary cultured macrophages from the α2AP+/+ or α2AP−/− mice were co-cultured with fibroblasts, and then we examined the degree of myofibroblast differentiation in fibroblasts.
Results: We found that the accumulation of α2AP was induced in the obstructed kidney, and α2AP deficiency in mice attenuated UUO-induced renal interstitial fibrosis. In addition, although α2AP deficiency did not affect UUO-induced macrophages infiltration, the degree of the myofibroblats differentiation in the co-culture of fibroblasts and α2AP−/− macrophages was significantly less than that in α2AP+/+ mice. We also found that CTGF induced the production of α2AP through integrin β3, ERK1/2, and JNK pathways in macrophages.
Conclusion: CTGF induced the expression of α2AP in macrophages, and CTGF-induced α2AP is associated with the development of renal interstitial fibrosis. The inhibition of α2AP-initiated pathways might provide a novel therapeutic approach to fibrotic diseases.

PA3.19 – Non-Inherited Risk Factors VT – IV

PA 3.19-1
Prediction of risk of venous thrombosis after cast immobilization of the lower extremity

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Background: Guidelines and clinical practice vary considerably with respect to antithrombotic treatment during plaster cast immobilisation of the lower extremity. This is partly due to the wide range of incidence rates that have been reported (asymptomatic venous thrombosis (VT): 4.3–36%; symptomatic VT: 0–5.5%). Knowledge on factors associated with an increased risk would provide a better basis to decide on individual thromboprophylaxis use.
Aims: To investigate the predictive value of genetic and acquired risk factors, levels of coagulation factors and other biomarkers on the occurrence of VT after cast immobilisation of the lower extremity.

Methods: We used data from a large population based case-control study (MEGA study, 4468 cases, 6156 controls, consent and ethical approval obtained) into risk factors for a first VT. Identification of predictor variables to be included in the model was mainly based on reported associations in the literature or on a relative risk (RR) > 1.2 or $P \leq 0.25$ in the univariate analysis. Using multivariate logistic regression several prediction models were created to determine the additional predictive value of genetic factors and biomarkers. In addition to the complete model (all variables), a reduced model (minimum number of predictors with a maximum predictive value) and a clinical model (acquired risk factors only, no blood draw and assays required) were created. To determine the discriminatory power, the area under the curve (AUC) was calculated by means of a receiver operating characteristic. Validation was performed in another case control study into the aetiology of VT (THE VTE study, 784 cases, 523 controls with valid information on all acquired risk factors, Leiden/Cambridge) and is about to be repeated in a third (the Milan study 1765 cases, 2088 controls).
Results: The complete prediction model consisted of 30 predictors including three genetic factors and six biomarkers. For this model, an AUC of 0.89 (95% CI; 0.85–0.94) was found in subjects with plaster cast of the lower extremity compared with an AUC of 0.84 (95% CI; 0.78–0.90) for the clinical model (consisting of only 13 acquired predictors). The addition of biomarkers to the clinical model resulted in a higher AUC than addition of genetic risk factors, with an AUC of 0.88 (95% CI; 0.84–0.93) vs. an AUC of 0.870 (95% CI; 0.82–0.92). The reduced model (containing 10 predictors including one genetic and three biomarkers) resulted in an AUC of 0.88 (95% CI; 0.84–0.92). Validation in THE VTE data showed good performance with an AUC of 0.83 (95% CI; 0.66–0.99) for the clinical model. The complete model and the reduced model will be validated in the Milan study because THE VTE study currently lacks data on biomarkers.
Conclusion: These results show that information on acquired risk factors, coagulation factors and genetic determinants in patients with plaster casts leads to an improved accuracy in the prediction of VT risk. In daily practice, the clinical model may be the preferred model as its factors are most easy to determine, while the model still has good predictive quality. When sufficiently validated, this model may be helpful in clinical decisions on thromboprophylaxis.

PA 3.19-2

Chronic VTE treatment VTE with rivaroxaban. Updated results of the prospective Dresden NOAC registry (NCT01588119)

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Background: In the EINSTEIN extension trial, rivaroxaban was superior over placebo in long-term prevention of recurrent venous thromboembolism (VTE) with low rates of bleeding complications. However, patients in RCT's present a selected population treated under a strict protocol and followed for a short period of time. Consequently, efficacy and safety of new oral anticoagulants (NOAC) need to be confirmed in cohorts of unselected patients in daily care.
Aims: To evaluate the efficacy, safety and management issues of rivaroxaban anticoagulation in chronic VTE treatment in daily care.
Patients and Methods: The Dresden NOAC registry is a prospective, non-interventional registry. A network of more than 230 physicians from private practice and hospitals enrol eligible patients, who are centrally followed by the registry office. Inclusion criteria are: (i) indication for therapeutic NOAC anticoagulation > 3 month; (ii) age > 18 years; (iii) written informed consent; (iv) availability for follow-up. No Exclusion criteria apply. In the registry, up to 2000 patients will receive prospective follow up (FU) by phone visits at day 30 day and quarterly thereafter to collect efficacy and safety data. All events are centrally adjudicated based on copies of reports, patient charts, autopsy reports and death certificates and using standard definitions.
Results: Until December 31th 2012, 1665 patients were registered. Of these, 168 patients were registered to receive rivaroxaban for chronic VTE treatment (index event > 6 month previous). Mean age is 62.5 years and, therefore, older than the EINSTEIN extension population (58.2 years). 43.5% of patients receive prolonged treatment for their first VTE event and 56.5% for recurrent VTE.
During follow-up, one recurrent VTE event occurred (phlebitis; 0.8 per 100 patient years). Four patients (3.2 per 100 patient years) had major cardiovascular events (1 ACS, 1 TIA, 1 stroke, 1 retinal artery thrombosis). Furthermore, 45 patients (35.7 per 100 patient years) had bleeding complications, in one case major bleeding according to ISTH definition (0.8 per 100 patient years, non-fatal hematemesis). One patient (0.8 per 100 patient years) died of terminal malignant disease.
At 6 month after enrolment, 88.5% of patients were still taking rivaroxaban. The remaining patients had planned end of treatment (2.5%) or were switched to other anticoagulants (9.0%, mostly for side effects or cost issues).
Conclusions: In unselected patients in daily care, chronic VTE treatment with rivaroxaban is effective and safe with low rates of cardiovascular or bleeding events. Overall, adherence to rivaroxaban therapy is over 90% and unplanned discontinuations occur in < 10%, mainly because of side effects, cost issues or concomitant diseases.
For presentation at the ISTH meeting, updated results on demographic and clinical characteristics at baseline, cardiovascular and bleeding event rates will be provided.

PA 3.19-3

Physicians' compliance with the Padua Prediction Score for preventing venous thromboembolism among hospitalized medical patients

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Background: Hospitalization for acute medical illnesses confers an eight-fold increased risk of venous thromboembolic (VTE) disorders that persists for up to 3 months. We have recently identified and validated a suitable and effective risk assessment model, the Padua Prediction Score (PPS), for optimal stratification of the thrombotic risk in hospitalized medical patients (Barbar et al, J Thromb 2010;8:2450–7). For the purpose of that investigation, attending physicians were not informed about the thrombotic risk of their patients and, as a result, < 40% managed their patients correctly.
Aims: In an attempt to assess whether awareness of the thrombotic risk – as assessed with the PPS – has the potential to increase the rate of appropriate thromboprophylaxis in high-risk medical patients, we performed a second prospective investigation within the same framework as the first.
Methods: Attending physicians were alerted about the thrombotic risk of their patients, and the orders for the related drugs were recorded. In addition, the thrombotic and haemorrhagic events occurring up to 90 days after patients' recruitment were registered.
Results: Out of 1600 consecutive patients admitted to the Second Division of Internal Medicine of the University Hospital of Padua (Italy) between January 2010 and December 2011, 803 patients met the inclusion criteria, of whom 296 (39.6%) at high risk and 507 at low risk of VTE based on the PPS. Of the 296 high-risk patients, 262 (88.5%) received adequate pharmacological prophylaxis during hospitalization, this proportion being more than twice as high as that (186/469, 39.6%; $P < 0.00001$) recorded in the previous study. VTE complications developed in seven of the 296 high risk patients (2.3%): four in the group of 262 (1.5%) who received prophylaxis and three in the group of 34 (8.8%) who did not, leading to an absolute difference of 7.3 (95% CI, 2.3–16.9), which is fully consistent with that (8.8; 95%

CI, 4.8–13.1) recorded in the previous study. Non-fatal bleeding complications were observed in three out of the 262 patients (1.1%) who received prophylaxis, and in none of those who did not.
Conclusions: Awareness of the thrombotic risk, as conferred by a self-explanatory, easy and suitable risk assessment model has the potential to remarkably increase the rate of appropriate VTE prophylaxis among hospitalized medical patients. The adoption of adequate prophylaxis strategies in high-risk patients eventually improves their outcome.

PA 3.19-4
Exposure to air pollution increases the risk of isolated pulmonary embolism relative to deep vein thrombosis

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Background: Exposure to inhaled particulates has been associated with increasing morbidity and mortality. Recently, there has been a growing interest in the effect of air pollution as a possible risk factor for venous thromboembolism (VTE) and links between air pollution and arterial and venous thrombosis are currently under investigation.
Aims: This study investigated the role of elevated levels of fine particulate matter (PM) in the development of isolated (without deep vein thrombosis) pulmonary embolism (PE).
Methods: The cases included 105 patients (M/F 35/70, age range 22–95 years) consecutively admitted to our Thrombosis Unit, between February 2008 and October 2012, with a diagnosis of first episode of PE without deep vein thrombosis (DVT). The control group included 161 patients (M/F 72/89, age range 20–89 years) consecutively referred to our Thrombosis Unit with objectively proven DVT without PE. Mean concentration of PM10 and PM2.5 were computed using data from monitors located at two different sites of Padua City.
Results: Using the 95th percentile of PM10 and PM2.5 measured in patients with DVT without PE in the month (73 mg/m^3 in both cases) and trimester (64 and 56 mg/m^3, respectively) before the event we found a significant increased risk (OR 2.23, 95% confidence interval [CI]: 1.02–4.93, for month; OR 4.48, 95% CI: 1.97–10.17, for trimester) of PE for PM10 and a significant increased risk (OR 2.32, 95% confidence interval [CI]: 0.99–5.45, for month; OR 4.22, 95% CI: 1.85–9.64, for trimester) of PE for PM2.5. In the multivariate logistic regression analysis (considered as possible confounders: age, gender, type of presentation, thrombophilia, heart diseases, smoking status, distance from the monitor sites, social-economic status, presence of COPD or asthma) for the variable PM10 an OR of 2.45 (95% CI: 1.04–5.79) in the month before admission and 5.24 (95% CI: 2.21–12.43) in the trimester before admission was found. For the variable PM2.5 were obtained an OR of 2.69 (95% CI: 1.06–6.83) in the month before admission and an OR of 6.19 (95% CI: 2.55–10.05) in the trimester.
Conclusions: A significant association was found between PM10 and PM2.5 exposure and isolated PE. At present, it is only possible to hypothesize that air pollution may be involved in the generation of *in situ* thrombosis in the pulmonary artery. Larger prospective multicentre investigations are needed to confirm this finding and clarify the potential underlying mechanisms.

PA 3.19-5
Cerebral vein thrombosis: long-term recanalization rate and clinical outcome

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Background: Only few studies with very small sample sizes have investigated on the long-term recanalization rates of cerebral venous thrombosis (CVT) and on the role of recanalization on clinical outcomes in these patients.
Aim of the Study: To assess the recanalization rate and potential risk factors for residual thrombosis in patients with a first episode of CVT. To evaluate the role of a complete recanalization on long term clinical outcome of these patients.
Methods: CVT patients with follow-up imaging tests performed at 3 and(?) 12 months after the index event were included. Vessel status at follow-up was categorized as complete, partial, or no recanalization. A good clinical recovery was defined as a score of 0–1 on the modified Rankin Scale (mRS). The following potential predictors of absence of recanalization and of clinical outcome were evaluated: age, gender, previous venous thromboembolic event, hormone therapy, unprovoked nature of CVT.
Results: Three hundred and nine CVT patients (71.5% female, mean age 40.2 years) were included; complete and partial recanalization were detected in 143 patients at 3–6 months and in 166 patients 7–12 months (71.8% vs. 769%; $P > 0.2$). At univariate analysis, only age > 45 years was associated with a higher risk of no recanalization ($P < 0.05$). This result was confirmed at multivariate analysis with a per year Odds Ratio [OR] of 1.02 (95% confidence interval [CI] 1.00–1.04; P 0.02). mRS at the time of follow-up imaging was available for 248 patients (80.3%); 223 of these patients (89.9%) had a good recovery. Complete or partial recanalization and hormone therapy were associated with a better outcome at the univariate analysis and these results were confirmed at the multivariate analysis (OR 2.63, 95% CI 1.05–6.61, P 0.039 and OR 3.88, 95% CI 0.99–15.19, P 0.05 respectively).
Conclusions: About three forth of CVT patients have complete or partial recanalization in the first months after the index event. Age was the only independent predictor of absence of recanalization. Complete or partial recanalization, together with hormone therapy, is associated with a better outcome in these patients.

PA 3.19-6
The contribution of immobility risk factors to the risk of venous thrombosis in the older population

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Background: Venous thrombosis is common in the older population. Assessment of risk factors is necessary in order to implement optimal preventive measures within this population.
Aims: In a population aged 70 and older, we determined associations between immobility-related risk factors and venous thrombosis, specifically, hospitalization, surgery, fracture, plaster cast (or splint) use, minor injuries, and transient immobility at home. Moreover, the contribution of these risk factors to the relative risk of thrombosis was assessed.

Methods: The Age and Thrombosis, Acquired and Genetic risk factors in the Elderly (AT-AGE) study is a two-center population-based case–control study performed between 2008 and 2011 in the Netherlands and the USA. Consecutive cases aged 70 years and older with a venous thrombosis and control subjects aged 70 years and older were included (consent en ethical approval obtained). Exclusion criteria were active malignancy and severe cognitive disorders. Participation in the cases was 476 (69%) and in the control subjects 461 (73%). For the current analyses, we only included cases with a first-time deep venous thrombosis in the leg or pulmonary embolism (n = 406) and control subjects without a history of thrombosis (n = 433). Odds ratios (OR) and population attributable risks (PAR) were calculated for in-hospital and out-of-hospital immobility. All ORs were adjusted for age, sex, body mass index and study center.
Results: There was a 15-fold (OR 14.8; CI 95 4.4–50.1) increased risk of thrombosis within 2 weeks of a hospital discharge and this risk extended for 3 months after discharge (OR 3.0; CI 95 1.7–5.3). Recent surgery yielded a 6.6 fold (OR 6.6; CI 95 3.7–11.7) fold increased risk. Moreover, out-hospital immobility defined as fractures (OR 12.6; CI 95 3.7–43.6), plaster cast (or splint) (OR 6.2; CI 95 2.0–18.8), minor leg injuries (OR 1.9; CI 95 1.1–3.3), and transient immobility at home (OR 5.0; CI 95 2.3–11.2) were all associated with thrombosis. A PAR of 27% for in-hospital immobility and 15% for out-of-hospital immobility was estimated.
Summary/Conclusions: In those over 70 years of age, immobility-related risk factors, including hospitalization, surgery, fracture, plaster cast (or splint), minor injury of the leg, and transient immobility at home were strong risk factors for venous thrombosis. Preventative measures should not focus solely on hospital settings.
Funding: Netherlands Heart Foundation (grant NHS 2009B50).

PA3.20 – Post-Thrombotic Syndrome

PA 3.20-1

Predictive value of the HAS-BLED score for major bleeding in patients with venous thromboembolism during anticoagulant treatment

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Background: The HAS-BLED score enables a risk estimate of major bleeding in patients with atrial fibrillation on vitamin K-antagonists (VKA) treatment, but has never been validated in patients with venous thromboembolism (VTE).
Aim: We analyzed the predictive value of the HAS-BLED score in VTE patients on VKA treatment.
Methods: Medical records of 416 VTE (165/416 pulmonary embolism and 251/416 deep vein thrombosis) patients starting VKA treatment for VTE in 2006 were searched for items on the HAS-BLED score (hypertension; renal or liver disease; stroke; major bleeding or anemia; age >65 years; use of NSAIDs, anti-platelet therapy or alcohol) and major bleeding events. Data on labile INR (time within therapeutic range <60%) was not available. Area under the curve (AUC) of the receiver operating characteristic (ROC) was calculated for the endpoint of major bleeding during VKA therapy, according to the ISTH criteria. Follow-up was defined as time between initiation of VKA treatment and major bleeding, or discontinuation of VKA treatment, or death, or June 2012, which ever came first.
Results: Median follow-up time for the endpoint of major bleeding was 175 days (2.5–97.5 percentiles 9–2304 days). Major bleeding occurred in 18/416 (4.3%, 95%CI 2.7–6.8%) patients. The AUC for the HAS-BLED score was 0.73 (0.62–0.84). In a multivariate Cox regression analysis, age >65 years, renal and liver impairment were predictive of major bleeding, with hazard ratios of 6.5 (95%CI1.6–26.5), 9.0 (95%CI1.4–56.2) and 15.6 (95%CI 1.0–245.3), respectively.
Conclusion The HAS-BLED score offers useful prognostic capacity in predicting major bleeding in VTE patients on VKA therapy. These results should be confirmed by other larger cohorts of VTE patients.

PA 3.20-2

Post-thrombotic syndrome does not confer a hypercoagulable state as measured by thrombin generation

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Background: Post-thrombotic syndrome (PTS) is a common complication of deep vein thrombosis (DVT). Its development has been associated with an increased risk of recurrent thrombosis.
Aims: We sought to establish whether the development of post-thrombotic syndrome conferred a hypercoagulable state following completion of anticoagulation for a first DVT.
Methods: This sub-study was undertaken in participants with a first DVT prospectively recruited to the Camberwell DVT outcomes study. DVT treatment comprised of 3 or 6 months anticoagulation for distal and proximal DVT respectively. Patients were clinically evaluated for PTS utilising the Villalta scale at 6 weeks and 6 months post end of anticoagulation. Plasma was collected for thrombin generation at the time of first clinical assessment. Thrombin generation was measured in platelet poor plasma using calibrated automated thrombography with a final tissue factor concentration of 5 pM and phospholipids 4 μM.
Results: One hundred and thirty-three patients were recruited, of which 107 completed anticoagulation and provided plasma for thrombin generation. Mean age of this cohort was 46.8 (± 15) years with males accounting for 51.4%. Fifty-seven percent were Caucasian, 33.6% African-Caribbean and the remainder mixed or other ethnicity. DVT involved the proximal vasculature in 51.4% with 57.0% having a provoking factor for DVT. Heritable thrombophilia was detected in 22.4%, with 51.4% having FVIII > 150 IU/L. During follow up, 29.0% developed mild PTS and 19.6% moderate or severe PTS. There was no significant difference in age, gender, BMI, presence of provoking factor or involvement of proximal vasculature between those with mild and those with moderate/severe PTS. There was no significant difference in thrombin generation parameters between those with and without PTS. On stratification by severity, those with moderate/severe PTS had significantly higher peak thrombin (389.5 nM) compared to those with mild PTS (345.7 nM; P = 0.023). This was no longer significant after adjustment for age, gender, BMI, ethnicity, presence of provoking factor and involvement of proximal vasculature; ethnicity demonstrated the strongest independent relationship to peak thrombin (P = 0.001). This led us to investigate ethnic differences in PTS; there was no significant difference in PTS development between Caucasians and African-Caribbeans. However, African-Caribbean ethnicity was significantly associated with increased moderate/severe PTS (33.3%) compared to Caucasians (13.1%; P = 0.023). On univariable analysis, African-Caribbean ethnicity was associated with moderate/severe PTS with OR 1.6 (95% CI 0.94–2.74, P = 0.081 [using no PTS as the reference]). After adjustment for age and involvement of the proximal vasculature, African-Caribbean ethnicity was significantly associated with moderate/severe PTS with OR 2.0 (95% CI 1.04–3.89, P = 0.036).
Summary/Conclusion: PTS does not confer a global hypercoagulable state as measured by thrombin generation. Its association with an increased risk of recurrence may be mediated by local hypercoagulability at the site of previous thrombus or by alternative mechanisms. African-Caribbean ethnicity was unexpectedly associated with an increased risk of moderate/severe PTS.

PA 3.20-3

Cost-effectiveness of additional catheter-directed thrombolysis for deep vein thrombosis

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Background: Following a proximal deep vein thrombosis (DVT) treated with standard anticoagulation and compression therapy at least 25% develop post-thrombotic syndrome (PTS). The CaVenT study was the first randomized controlled trial to evaluate additional catheter-directed thrombolysis (CDT) with alteplase and showed an absolute reduction in PTS of 15% after 2 years. However, in addition to this benefit CDT involves a small bleeding risk, lengthens inpatient stay, and consumes more resources.

Aims: Consequently, we aimed at performing a health economic evaluation using a decision model to assess the cost-effectiveness of additional CDT compared with standard treatment alone.

Methods: A Markov model with a third-party payer perspective and a lifetime horizon compared standard treatment with CDT in addition to standard treatment in a hypothetical cohort with high proximal DVT and low risk of bleeding. All patients received anticoagulation in the first 6 months' cycle of the model. In the subsequent cycles patients could transition to the health states (i) post DVT, (ii) PTS, (iii) severe PTS, (iv) permanently disabled from an intracranial bleeding, or v) dead. Model input data obtained from the CaVenT study included PTS development, risk of major bleeding from CDT, and utilities for post DVT states with and without PTS development. The remaining clinical inputs were obtained from the literature. Costs were obtained from the CaVenT study, hospital accounts and the literature.

We applied utilities and costs to each event and outcome over their expected durations. Results were expressed in monetary costs (April 2012 US $), quality adjusted life years (QALYs); both discounted at 3.0% annually, and incremental cost-effectiveness ratio (ICER). Model uncertainty was assessed with one-way and probabilistic sensitivity analyses.

Results: Base case analyses demonstrated that additional CDT accumulated 32.31 QALYs compared to 31.68 QALYs following standard treatment alone. Direct medical costs were $64,709 for additional CDT and $51,866 for standard treatment. The incremental cost-effectiveness ratio (ICER) was $20,429/QALY gained. Projected life expectancy was 30.9 years following standard treatment and 30.8 years in the CDT strategy, corresponding to a loss of 1.1 months or 32 days from the additional risks associated with CDT. One-way sensitivity analysis showed that the model was sensitive to the clinical efficacy of both treatment strategies, but the ICER remained < $55,000/QALY gained over the full range of all parameters. The probability that additional CDT is cost-effective was 82% at a willingness to pay threshold of $50,000/QALY gained.

Summary/Conclusion: Additional CDT is likely to be a cost-effective alternative to standard treatment for patients with high proximal DVT and low risk of bleeding.

PA 3.20-4

Role of inflammation, tissue remodeling, and vascular function in the development of post thrombotic syndrome: a case–control study

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Background: Post thrombotic syndrome (PTS) is a prevalent sequel of deep vein thrombosis (DVT) of the leg. PTS develops in 20–50% of patients and manifests itself with symptoms of heaviness, pain, cramps, itching, and tingling of the affected leg. In severe cases ulceration can occur. There is restricted knowledge on PTS aetiology and therapeutic options are limited. Insight in pathogenic processes involved in PTS could be gained by studying the relationship between biomarkers and PTS potentially leading to improved treatment options.

Aims: We aimed to investigate the role of inflammation, tissue remodelling, and vascular function in the aetiology of PTS using a panel of potential plasma biomarkers.

Methods: Patients with a history of DVT with PTS (Villalta ≥ 5) defined as cases and patients with a history of DVT without PTS defined as controls were selected from the outpatient clinic of the Maastricht University Medical Centre and the Flevohospital Almere, theNetherlands. To be included the required minimum time since DVT was 24 months. In addition, healthy individuals (HI) without a history of venous thromboembolism were invited to participate. Cases, controls, and HI were matched for gender, age, and BMI. Blood was collected and a panel of predefined markers was determined using enzyme-linked immunosorbent assays. Median levels of biomarkers were compared between the three categories with the Kruskal-Wallis test, and post hoc Bonferroni correction was applied (significance level < 0.0167).

Results: Ninety subjects were included. Blood collection was performed at a median follow up after first DVT of 83 months (range 53–102). Mean Villalta score was 8 (Standard deviation 4) for cases and 2 (1) for controls.

CRP showed a significant trend of increasing CRP in controls (1.4 μg/mL [0.7–3.3]), cases (2.5 μg/mL [1.3–4.8]), and consequently healthy individuals (3.1 μg/mL [1.1–9.1)] $P = 0.045$. Median levels of Thrombomodulin were found to be higher in controls (4.4 ng/mL [3.9–5.3]) compared to cases (4.2 ng/mL [3.2–4.7]) $P = 0.046$ and HI (3.8 ng/mL [3.4–4.2]) $P = 0.006$. Levels of von Willebrand Factor (vWF) were higher in patients compared to HI. This difference was significant comparing controls (205% [165–235]) and HI (153% [103–200]) $P = 0.005$, but not comparing cases (179% [150–201]) and HI $P = 0.115$.

No differences were found for: Il-6, Il-8, TNF-α, SAA, E-selectin, P-selectin, MMP-9, TIMP-1, sICAM-1, and sICAM-3.

Conclusions: There is marked absence of inflammatory activity in cases at this point in time. Higher levels of CRP as well as lower levels of Thrombomodulin in cases may be a reflection of compensatory anti-inflammatory mechanisms in the chronic phase after DVT. The higher levels of Thrombomodulin in controls, compared to cases and HI may be explained by the anti-inflammatory and anti-coagulant properties of Thrombomodulin resulting in a vasculoprotective effect in controls. High vWF in cases and controls reflects endothelial activation in patients vs. HI, but does not appear to be a marker for PTS.

PA 3.20-5

Venous thromboembolism and subsequent receipt of disability pension – a population-based prospective cohort study

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Background: Venous thromboembolism (VTE) is an important cause of morbidity and mortality. However, the burden of VTE in terms of permanent work disability has never been assessed at the population level.

Aims: To estimate the incidence of disability pension (DP) after a first event of VTE compared to subjects without VTE, and among provoked/unprovoked cases of VTE.
Methods: After passing all formal regulations (Institutional regulatory boards, the Norwegian Data Protection Authority), data from the Tromsø Study (baseline 1994–95) and the North-Trøndelag Health Study (HUNT) (baseline 1995–97) were merged by the 11-digit personal identification number with National Insurance Administration DP data from Statistics Norway. All participants, aged 25–64, in the two studies with no previous VTE and no prior DP or DP within 12 months from baseline were included. Study participants were followed from baseline through the end of 2007 for objectively verified diagnoses of VTE and the end of 2008 for DP, defined as a disability pension of 50% or more. Study subjects without DP were censored at age 65, at date of emigration or death, whichever came first. In total 60,580 subjects were included. Cumulative proportions of DP per 100 participants were analyzed in survival analyses by VTE exposure. Cox-regression models were used to calculate hazard ratios (HRs) for DP by VTE as main exposure with 95% confidence interval (CI). Age, sex, education, body mass index, cardiovascular diseases, cancer, muscle-skeletal diseases, psychiatric diseases, and self-perceived health assessed at study start were included in the regression models for potential confounding effects.
Results: At study end 75 of 357 (21%) incident VTE cases received DP relative to 9052 of 60,223 (15%) non-VTE subjects. The study subjects were observed over 8,240,118 study months, mean 136 (1–174 months). Within 10 years from baseline, the cumulative proportion of DP among VTE subjects was 16.3% (95% CI: 12.4–20.2) compared with 11.9% (95% CI: 11.6–12.3) among non-VTE subjects. The HR for DP among VTE to non-VTE cases changed from 1.45 (95% CI: 1.16–1.82) to 0.96 (95% CI: 0.76–1.20) when adjusted for age and self-perceived health. No other factors at baseline had a confounding effect on the association of VTE with DP. Within 3 and 6 years after VTE, the cumulative proportion of DP per 100 VTE subjects were 15.4 (95% CI: 11.4–19.4) and 21.3 (95% CI: 16.6–26.0). The HR for DP among VTE subjects aged 50–64 at time of VTE was 4.6 (95% CI: 2.3–8.9) relative to VTE subjects aged 25–49 years. There was no difference in DP for provoked and unprovoked VTE (HR 1.00; 95% CI: 0.94–1.07).
Conclusions: Within 6 years of first diagnosis of VTE more than 20% of Norwegians received DP, and the probability of receiving DP was higher among those aged 50 or more at time of diagnosis. After controlling for age and baseline self-reported health, DP was not received more often by VTE subjects. This study does not indicate that VTE is associated with permanent work disability.

PA 3.20-6

Prospective study to identify risk factors for post thrombotic syndrome and evaluate role of compression ultrasound sonography to decide discontinuation of anticoagulant

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Background/Aim: To identify patients with unprovoked Deep vein thrombosis (DVT) where anticoagulation could be discontinued and identify possible risk factors for post thrombotic syndrome (PTS).
Methods: This was designed as a prospective randomized study which included patients of 12–75 years age presenting to our clinic with unprovoked lower limb proximal DVT. Patients with active malignancy, pulmonary embolism and sites of thrombus other than the lower limbs, DVT post surgery or bed ridden medical patients, other provoked DVT were excluded. Patients were anticoagulated and monitored as per standard procedures. Patients were advised to wear compression elastic stocking below knee from early morning to bed time for prevention of PTS. PTS was analysed and graded as per the Villalta PTS scale. Compression ultrasound sonography (CUS) of affected limb was done after 6 months of therapy and results of CUS were used to determine further treatment. CUS was done at two sites: Common femoral vein (CFV) just above the junction of saphenous vein to femoral vein and the popliteal vein (PV). Ratio of compressed to uncompressed diameter > 40% identified patients with significant residual thrombus whereas patients with < 40% were defined as patients with resolution of thrombus. Patients with significant residual thrombus were continued on anticoagulants in group A. Patients with resolution of thrombus were randomized to continue the drug in group B or assigned to drug discontinuation in group C. Adequacy of anticoagulation was assessed by INR of 2–3 on atleast 50% of visits. Events were recorded as a fresh episode of DVT in same limb or at other site confirmed on compression ultrasound, bleeding episodes, symptoms and signs related to PTS.
Results: Forty-four patients were enrolled. Of 44 patients (34 males, 10 females), median age 35 years (range 16–65 years), 25 patients (56.8%) showed significant resolution of thrombus at 6 months and were randomized into groups B (n = 13) and C (n = 12). Nineteen patients with residual thrombus were treated in arm A where anticoagulation was continued. None of the patients in any arm suffered a recurrence after a median followup of 12 months (range 11–17.8 months). However the post thrombotic syndrome was seen in a total of 17 patients (38.6%), 11 in arm A and six in arm B+C (P = 0.03). The grade of PTS was mild as per the Villalta PTS scale in all patients. Two of 13 patients in arm B developed PTS as compared to 4/12 patients in arm C. Inadequate anticoagulation in patients with or without PTS was 47% and 14.8% (P = 0.035, odds ratio 4.9, 95% CI 1.01–28.57) respectively. Mean duration of symptoms prior to starting anticoagulation was 2.52, 3.84 and 5.4 weeks in groups A, B and C respectively with no significant difference.
Conclusion: Absence of thrombus or a significant resolution of thrombus on followup CUS identifies group of patients in whom oral anticoagulation could be withdrawn. Inadequate anticoagulation after diagnosis of DVT identifies a group of patients who would be at risk for PTS. Risk of PTS is not dependent upon the duration of symptoms prior to diagnosis.

PA4.01 – New Antiplatelet Agents – I

PA 4.01-1

Tumor vascular disrupting agent DMXAA inhibits platelet activation and thrombosis via inhibition of TXA2 – TP signaling and PDE

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Background: 5, 6-dimethylxanthenone-4-acetic acid (DMXAA) is a tumor vascular disrupting agent under clinical trials as an adjacent antitumor agent. DMXAA is structurally similar to flavone-8-acetic acid (FAA), an old tumor vascular disrupting agent with antiplatelet and antithrombotic effects. In contrast to FAA, which causes bleeding in tumor patients, no bleeding has been reported in patients receiving DMXAA. Whether DMXAA also affects platelet function is not clear.
Aims: We sought to investigate whether DMXAA also inhibits platelet activation and thrombus formation.
Methods: The antiplatelet effects of DMXAA were assessed using human washed platelets or platelet-rich plasma induced by multiple agonists. The TXB_2 levels were measured using an ELISA kit. The phosphorylation of Erk1/2 and Akt were measured using western blot. The assay for PDE activity was performed using HPLC. The antithrombotic effects were evaluated using both $FeCl_3$-injured mouse

mesenteric arterial thrombus model and laser-injured mouse cremaster arteriole thrombus model.

Results: Here we demonstrated that DMXAA concentration-dependently inhibited human platelet aggregation and ATP release induced by U46619, arachidonic acid, ADP, collagen or ristocetin. Moreover, DMXAA inhibited phosphorylation of Erk1/2 and Akt downstream of thromboxane synthase inhibition and TXA_2 receptor (TP) antagonism. DMXAA also inhibited phosphodiesterase (PDE). The antiplatelet effects were further confirmed using mice intravenously given DMXAA. DMXAA dramatically inhibited thrombus formation in $FeCl_3$-injured mouse mesenteric arterial thrombus model and laser-injured mouse cremaster arteriole thrombus model. Notably, DMXAA did not significantly increase bleeding at a dose achieving antithrombotic effects similar to clopidogrel in mice.

Summary/Conclusions: For the first time, we found that tumor vascular disrupting agent DMXAA has potent antiplatelet and antithrombotic effects without increased bleeding. As a well-evaluated antitumor agent with safe profile, DMXAA may be used as an efficacious and safe antiplatelet drug.

PA 4.01-2

Antiplatelet activity of N,N'-substituted piperazines

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Background: Antiaggregant drugs play an important role in the prophylaxis and therapy of ischemic stroke and cardio-vascular deceases. Nevertheless the development of novel drugs possessing high therapeutical activity and safety with no pronounced resistance remains an actual topic.

Aims: The study investigates *in vitro* and *in vivo* pharmacobiological activity in a series of novel N,N'–substituted piperazines with different hydrophobicy and acid-base properties; the correlation between their phisycochemical properties and antiaggregant and vasodilatory activity.

Methods: Antiaggregant properties of N,N'–substituted piperazines were evaluated in donor blood based on their ability to reduce platelet aggregation induced by ADP, collagen, ristocetin, thrombin, epinephrine and arachidonic acid (CHRONO-LOG 490-4D); their influence on thromboelastogram of blood clot (thromboelastograph TEG 5000) and functional activity of regular blood elements; and by experimental rodent model of laser-induced arterial thrombosis coupled with intravenous or oral administration of the compounds. The dose – effect dependence was studied and the semi-inhibition constants of thrombocyte aggregation (ED50) were calculated. The results were compared with the ones from parallel studies with aspirin.

Results: It was experimentally determined that N,N'–substituted piperazines have antiaggregant properties when platelet aggregation is induced by ADP, collagen, ristocetin, thrombin, epinephrine and arachidonic acid. The study of N,N'–substituted piperazines chemical structure relation to their activity showed higher activity in the compounds having guanidine, sulfamide and tetramethoxyalkyl substituents (compounds VRV-0411, VRV-0511). The semi-inhibition constants of thrombocyte aggregation (ED50) in the tests of ADP, epinephrine, thrombin, and collagen induced aggregation for most active compounds VRV-0411, VRV-0511 were statistically significantly lower than the constants for model compound (aspirin). In the tests of arachidonic acid induced platelet aggregation the compounds VRV-0411, VRV-0511 had the constants comparable to the ones of aspirin. The compounds VRV-0411, VRV-0511 in the doses up to 2 mM/kg do not influence the functional activity of regular blood elements. Experiments according to rodent model of laser-induced arterial thrombosis showed that during the course of oral administration of compounds VRV-0411, VRV-0511, aspirin (7 days, once per day) ED50 equals to 0.0045, 0.0035, 0.0035 mM/kg, respectively. After the intravenous injection of compounds VRV-0411, VRV-0511, aspirin ED50 equals to 0.002, 0.009, 0.003 mM/kg, respectively.

Conclusion: The studies of antiaggregant properties of N,N'–substituted piperazines in donor blood and on experimental rodent model of laser-induced arterial thrombosis allowed to determine the relation of pharmacological activity of the compounds on their chemical structure and to define the compounds having wide spectrum of antiaggregant activities. Preclinical studies of the most effective N,N'–substituted piperazines are under way to introduce novel drugs to the market.

PA 4.01-3

PAR-1 antagonists inhibit thrombin-induced platelet activation whilst leaving the PAR4-mediated response intact

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Background: Thrombin-induced platelet activation is initiated by PAR1 and PAR4 receptors. Vorapaxar, a PAR1 antagonist, has been assessed in patients with acute coronary syndromes (ACS) and stable atherosclerotic disease in addition to standard-of-care treatment. Significant benefit was seen in those with a prior history of myocardial infarction and vorapaxar was not associated with significantly increased CABG-related major bleeding or differences in reoperation for bleeding or fatal bleeding in patients undergoing CABG surgery, which is associated with excess thrombin generation.

Aims: Investigate the effects of selective PAR1 antagonism on thrombin-induced platelet activation.

Methods: All studies were conducted with informed consent and medical ethics approval. *In vitro* studies were performed using healthy volunteers. Samples were pre-incubated with the PAR-1 antagonist, SCH79797 (1 μM) or pre-exposed to PAR-4 agonist peptide (2 mM) to induce receptor desensitization. *Ex vivo* studies were also performed on ACS patients randomised in the TRACER study to receive vorapaxar (40 mg loading dose, 2.5 mg daily) (n = 13) or placebo (n = 17). Blood samples were taken from the patient group at baseline and 4 h, 1 and 4 months during drug administration. Platelet-rich plasma was prepared and the platelets labelled with fluo-4 AM. Thrombin-induced calcium mobilisation (0.1–1 U/mL) was assessed by flow cytometry in the presence of extracellular calcium (1 mM).

Results: Vorapaxar treatment significantly reduced the peak calcium response of 2608 ± 1962 and 4835 ± 3702–251 ± 407 and 613 ± 1070 (mean ± SD, $P < 0.001$) using 0.1 and 0.3 U/mL thrombin, respectively (4 h post vorapaxar), and extended the time to reach the peak calcium response (5.6 ± 2.4 and 9.7 ± 14.3–110.0 ± 23.4 and 61.3 ± 31.6 s ($P < 0.001$). There was residual, delayed calcium mobilisation in response to thrombin which was unaffected by vorapaxar treatment. These findings are consistent with calcium mobilisation mediated via the PAR-4 receptor and were reproduced *in vitro* using SCH79797. PAR-4 receptor desensitization, in combination with SCH79797, completely inhibited thrombin-induced calcium mobilisation confirming that the residual, delayed calcium mobilisation was mediated via PAR-4.

Conclusion: Vorapaxar selectively antagonises the PAR1-mediated component of thrombin-induced platelet activation, leaving the PAR4-mediated response intact. This result may explain why vorapaxar is well tolerated in patients undergoing CABG surgery since higher thrombin levels in this setting may override the effects of PAR1 antagonism through PAR4 activation and thus preserve haemostasis. Further assessment in this setting may be warranted.

PA 4.01-4

The role of glaucocalyxin A in inhibiting platelet activation and protecting against hypoxic ischemic brain injury

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Background: Platelets play a pivotal role in atherosthrombosis and antiplatelet agents prove to be useful in preventing acute clinical events, including myocardial ischemia and stroke.
Aims: In this study we investigate the antiplatelet and neuroprotection effect of glaucocalyxin A (GLA), a diterpenoid isolated from Rabdosia japonica (Burm F) Hara var glaucocalyx (Maxim) Hara (Labiatae).
Methods: GLA was purified and tested for its effect on platelet activation using washed platelets freshly isolated from peripheral blood of healthy donors. Experiments for platelet function used in this report include platelet aggregation, flow cytometry, microfluidic chamber, and clot retraction. Hypoxic-ischemic brain damage (HIBD) was induced by unilateral carotid ligation and 2.5 h of hypoxia in postnatal day 7 (P7) rat pups. GLA was administered immediately before hypoxia. The effect of GLA on infarct volume was evaluated at 48 h after HIBD by TTC staining.
Results: We showed that pretreatment of human platelets with GLA (0.01 and 0.1 μg/mL) selectively inhibited platelet aggregation induced by collagen ($P < 0.001$ and $P < 0.001$, respectively). This inhibition also occurs in other agonist-induced platelet aggregation when platelets were preincubated with higher doses of GLA (5, 50 μg/mL). Platelet inside-out signaling was inhibited by GLA as indicated by p-selectin secretion and Pac-1 binding assay. GLA reduced platelet adhesion on collagen surfaces and clot retraction, a process mediated by outside-in signaling. Further study showed that GLA inhibited collagen-stimulated tyrosine phosphorylation of Src, Syk and PLCγ2, the signaling events in collagen receptor GPVI pathway. We next examine whether GLA protected against hypoxic-ischemic brain damage (HIBD) in newborn rats. The infarct volume in the GLA (500 and 1 mg/kg)-treated animals ($29 \pm 0.37\%$, $n = 10$ and $27 \pm 0.69\%$, $n = 10$) was significant reduced compared with the vehicle group ($39 \pm 0.83\%$, $n = 10$) ($P < 0.05$). Underlying mechanistic study showed that GLA treatment significantly decreased cleaved caspase-3 levels ($P < 0.05$), a marker of apoptosis.
Summary: The present results provide the molecular basis for the inhibition by GLA of collagen-stimulated platelet activation and the evidence that GLA protects against hypoxic-ischemic brain injury in neonatal rats. Our data suggest that GLA could potentially be developed as a novel agent for the prevention of thrombosis and ischemia.
This work was supported in part by grants from the National Science Foundation of China (NSFC81071410) and Jiangsu Province's Key Discipline of Medicine (XK201118).

PA 4.01-5

Plain pegylated liposomes inhibit platelet activation by HOCl modified albumin, thrombospondin peptide RFYVVMWK and high shear modified von Willebrand factor

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Background: Although several anti-platelet drugs exist to prevent thrombosis, there still is a need for new strategies to protect against platelet-mediated atherothrombosis, as bleeding risk is a substantial limitation of current antiplatelet therapy. Proteins or peptides with amyloid-like structure activate platelets as shown by Herczenik et al. Modification of proteins like LDL, albumin, fibrinogen by HOCl, or of von Willebrand factor by shear conditions induce self-association, a hallmark of misfolded proteins. The amyloid-like carboxyterminal peptide RFYVVMWK that is conserved among the products of all five thrombospondin genes is known to activate platelets. All these proteins/peptides expose hydrophobic surface areas. Pegylated liposomes are in use for many years as a vehicle for drug delivery and targeting, without inducing bleeding problems. The presence of PEG on the surface of such liposomal carrier has been shown to extend blood-circulation time.
Aims: The effect of plain pegylated liposomes, with no drugs encapsulated into them and no molecules attached to their surface, on platelet adhesion to and activation by HOCl modified proteins (LDL, albumin, fibrinogen), thrombospondin peptide RFYVVMWK and high shear modified von Willebrand factor was studied.
Methods: Plain pegylated liposomes were synthesized using 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC) and distearoyl phosphatidyl-ethanolamine methyl polyethylene glycol 2000 (DSPE-PEG 2000). The liposomes were filtered in an extruder apparatus through polycarbonate filters to form liposomes of 120–140 nm in size, according to Yatuv et al. The effect of plain pegylated liposomes on platelet adhesion, activation, secretion, aggregation, and thrombus formation was investigated by aggregometry, flow cytometry, thrombus formation in *in vitro* perfusion chambers, confocal laser scanning microscopy and plasmon resonance.
Results: Plain pegylated liposomes inhibited in a dose dependent manner platelet adhesion and aggregation on von Willebrand factor in a plate(let) and cone analyser at 1800/s. In addition the liposomes inhibited platelet adhesion, GPIIb/IIIa activation as tested by fibrinogen and PAC-1 binding, granule secretion (CD62P and CD63 surface exposure), aggregation, microparticle formation and association between platelets and monocytes induced by HOCl modified proteins (LDL, albumin, fibrinogen) and by thrombospondin peptide RFYVVMWK and inhibited thrombus formation on HOCl modified proteins in the BioFlux *in vitro* perfusion system.
Summary/Conclusion: As such pegylated liposomes have been already used in humans, for example together with recombinant factor VIII or G-CSF without adverse effects, they might be a promising new approach to be tested as anti-thrombotic therapy for acute intervention.

PA 4.01-6

Sildenafil reduces platelet activity via both NO synthase and NO synthase-independent pathways

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Introduction: Originally used primarily for the treatment of erectile dysfunction, sildenafil is now more widely applied in the treatment of a range of conditions that result in regional blood supply deficiencies. Sildenafil is a selective phosphodiesterase 5 (PDE5) inhibitor which enhances the nitric oxide (NO) pathway by preventing the breakdown of cyclic guanosine monophosphate (cGMP). NO signalling is an important inhibitory regulator of platelet function and it is known that platelets highly express PDE5. Platelet hyperactivity can lead to arterial thrombosis causing fatal ischaemic diseases. Platelets are conventionally thought to obtain most of their NO from endothelial nitric oxide synthase (eNOS) however more recently, reduced nitrate and nitrite from dietary sources (i.e green leafy vegetables) or NO metabolism have been suggested as alternative sources of bioactive NO.
Aims: The aim of this study were to investigate the effects of sildenafil on platelet function *in vitro* and *in vivo* and to identify the upstream sources of NO.
Methods: Collagen-induced human platelet aggregation responses to sildenafil citrate (phosphodiesterase 5 inhibitor), 1H-[1,2,4]oxadiazolo [4,3-a]quinoxalin-1-one (ODQ – soluble guanylyl cyclase (sGC) inhibitor), Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME, NOS inhibitor), $NaNO_2$ (nitrite) and ascorbic acid (reducing agent) were obtained via *in vitro* optical aggregometry. The effects of sildenafil and $NaNO_3$ (nitrate) on collagen-induced platelet aggregation were investigated *in vivo* in wild-type C57bl/6 and $eNOS^{-/-}$ mice by mea-

suring radiolabelled platelet thromboembolism in real-time via external scintillation probes connected to a spectrometer.
Results: *In vitro*, sildenafil caused significant, concentration-dependent inhibition of platelet aggregation which also occurred in the presence of L-NAME but was antagonised by ODQ. Ascorbic acid induced a concentration-dependent inhibition of platelet aggregation which was enhanced by $NaNO_2$ and blocked by ODQ. $NaNO_2$ caused a concentration-dependent inhibition of aggregation in the presence of a low concentration of sildenafil. *In vivo*, sildenafil caused a significant inhibition in platelet aggregation in wild-type but not $eNOS^{-/-}$ mice. $NaNO_3$ was associated with a non-significant trend towards inhibition of platelet aggregation in W.T mice and significantly inhibited aggregation in $eNOS^{-/-}$ mice.
Summary/Conclusions: Sildenafil induced sGC-dependent inhibition of platelet function *in vivo* and *in vitro* suggesting the presence of an upstream source of NO in both preparations. *In vitro*, sildenafil acted independently of NOS but was able to act downstream of reduced nitrite. The ability of nitrite to exert an inhibitory effect in the absence of exogenous reducing agents suggests an endogenous mechanism for reduction of nitrite to NO in platelets. In contrast, the effects of sildenafil *in vivo* were mediated predominantly via eNOS. During vascular dysfunction characterised by loss of eNOS, nitrate exerts an inhibitory effect upon platelet function *in vivo*, presumably due to reduction to NO. In conclusion, sildenafil and nitrite/nitrate may be beneficial in reducing the risk of platelet-driven cardiovascular diseases by enhancing NO-mediated inhibition of platelet function.

PA 4.02-1
Retinoic acids control translational events in platelets

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Background: Recently we were able to demonstrate that human platelets reproducibly form progeny, and continue to produce daughter cells in the circulation. This process occurs in freshly-isolated platelets as well as in stored platelets. In addition, the ability to produce progeny appears to depend on protein synthetic events. Retinoids are a group of naturally-occurring vitamin A derivatives that regulate cellular differentiation, growth, and therefore protein synthetic events. Some retinoid X receptor family members have previously been reported in platelets. All-trans retinoic acid (atRA) signals through the retinoic acid receptor (RAR), which is expressed by most cell types. The stereoisomer of atRA is 9-cis retinoic acid (9-cisRA), which is a high-affinity ligand for the retinoid X receptor family (RXR).
Aims: We wanted to examine if RAR and RXR family members are expressed and control translational events in platelets during progeny formation.
Methods: Freshly isolated human platelets were used for all experiments. Expression of RAR and RXR were examined on RNA and protein levels. Protein-protein-interactions and RNA binding capacities were studied by combining immune-precipitation and RNA binding assays.
Results: RNA coding for RAR and RXR family members were detected by next generation sequencing as well as by PCR approaches. In immunocytochemical studies we were able to detect expression of RAR and RXR family members in platelets that form new cell bodies. First, RARs showed co-localization with cytoskeletal proteins indicating regulatory functions in regards of cytoskeletal rearrangement during platelet progeny formation. Second, we demonstrated that RARs bind to mRNAs. The binding capacity was influenced by retinoid treatment. In addition, we were able to demonstrate translational regulation of target proteins due to retinoid treatment, providing mechanistic insights into regulatory processes involved in platelet progeny formation.
Conclusions: These data demonstrate that platelets utilize nuclear receptors for new functions like translational regulation. In addition, we show that progeny formation is a regulated process by internal or external cues.

PA 4.02-2
Platelet Rap1 signaling, mediated by CalDAG-GEFI and P2Y12, contributes to atherosclerotic lesion development in mice

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Background: The development and progression of atherosclerotic lesions is mediated by various blood cell types, including platelets and neutrophils. We recently identified CalDAG-GEFI (CDGI), a guanine nucleotide exchange factor for Rap GTPases, as a critical component of integrin-mediated adhesion of platelets and neutrophils at sites of vascular injury. In platelets, signaling by CDGI mediates the early activation of Rap1, while the Gi-coupled receptor for ADP, P2Y12, the target of the clinically used drug Plavix, is required for sustained Rap1 activation and thrombus stability.
Aim: In this study, we evaluated lesion formation in atherosclerosis-prone low-density lipoprotein receptor deficient $Ldlr^{-/-}$ mice lacking CDGI and/or P2Y12 in hematopoietic cells only.
Methods: $Ldlr^{-/-}$ mice were irradiated and reconstituted with bone-marrow from wild-type (WT), CalDAG-$GEFI^{-/-}$ (CdgI), $P2y12^{-/-}$ (P2y12) or $CdgI^{-/-}$ $P2y12^{-/-}$ (DKO) mice. Reconstituted mice were fed a high-fat diet (21% fat, 0.2% cholesterol) for 12 weeks, ad libitum, and hearts and aortas were harvested. Oil Red O staining was performed on cross sections of the aortic sinus and the lesion size was quantified. After 8 weeks of high fat diet, blood was drawn from the retro-orbital plexus and blood cell counts as well as platelet activation were determined. Blood was also perfused over collagen in a microfluidics chamber system and integrin-mediated adhesion of platelets and neutrophils was studied.
Results: Atherosclerotic lesions in the aortic sinus of $Ldlr^{-/-}$;$CdgI^{-/-}$ chimeras were approximately 42% smaller than those in $Ldlr^{-/-}$;WT controls (0.18 ± 0.02 vs. 0.31 ± 0.05 mm^2,respectively) ($n = 13$, $P < 0.001$). Lesions in $Ldlr^{-/-}$;$P2y12^{-/-}$ chimeras were also significantly smaller than those in controls (0.22 ± 0.10 mm^2; $n = 13$, $P < 0.05$), confirming recent findings in $Apoe^{-/-}$; $P2y12^{-/-}$ mice. $Ldlr^{-/-}$;DKO chimeras had the smallest lesions, reduced by 48% compared with WT controls, (0.16 ± 0.02 vs. 0.31 ± 0.05 mm^2,respectively) ($n = 13$, $P < 0.001$), but they were not statistically different from $Ldlr^{-/-}$; $CdgI^{-/-}$ chimeras. Platelet counts were similar in blood from hypercholesterolemic $Ldlr^{-/-}$; $CdgI^{-/-}$, $Ldlr^{-/-}$; $P2y12^{-/-}$, and $Ldlr^{-/-}$; DKO chimeras. Consistent with previous findings, the fraction of neutrophils in relation to all white blood cells was elevated in $Ldlr^{-/-}$; $CdgI^{-/-}$ but not in $Ldlr^{-/-}$; $P2y12^{-/-}$ chimeras. Platelet adhesion and activation on collagen under flow was markedly impaired in $Ldlr^{-/-}$; $CdgI^{-/-}$ and $Ldlr^{-/-}$;DKO blood. In contrast, a partial defect in platelet adhesion and activation was seen in $Ldlr^{-/-}$; $P2y12^{-/-}$ blood. Consistently, firm neutrophil adhesion to collagen-bound platelets was reduced by > 90% in $Ldlr^{-/-}$;$CdgI^{-/-}$ and $Ldlr^{-/-}$; DKO blood, while no significant reduction was observed in $Ldlr^{-/-}$; $P2y12^{-/-}$ blood. Total cholesterol and triglyceride levels were similar among groups.
Summary/Conclusion: Our findings reveal a critical role for CDGI and P2Y12, and Rap1-dependent platelet activation, in promoting atherosclerotic lesion development in hypercholesterolemic mice. Further studies are required to determine if the observed protection in $Ldlr^{-/-}$; $CdgI^{-/-}$ chimeras is in part due to impaired neutrophil function.

PA 4.02-3

Antithrombotic actions of statins involve PECAM-1 signalling

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Background: Statins are widely prescribed cholesterol-lowering drugs that are a first-line treatment for coronary artery disease and atherosclerosis, reducing the incidence of thrombotic events such as myocardial infarction and stroke. Statins have been shown to reduce platelet activation, although the mechanism(s) through which this occurs is unclear.

Aims: Since several of the characteristic effects of statins on platelets are shared with those elicited by the inhibitory platelet adhesion receptor PECAM-1, we investigated a potential connection between the influence of statins on platelet function and PECAM-1 signalling.

Methods: Washed human platelets were treated for 5 min with increasing concentration of simvastatin or fluvastatin, prior stimulation for 90s with collagen-related peptide CRP-XL (0.5 mg/mL) and aggregation measured at 37 °C under constant stirring conditions. Changes in ATP concentration were used as a measure of dense-granule secretion and monitored simultaneously with aggregation in an optical lumi-aggregometer using a luciferase detection system. Flow cytometry was used to examine fibrinogen binding to integrin $a_{II}bb_3$ and a-granule secretion. Washed human platelets were incubated with simvastatin or control for 5 min prior to CRP-XL (1 mg/mL) stimulation in the presence of EGTA (10 mM), indomethacin (10 mM) and apyrase (2 U/mL). PECAM-1 was immunoprecipitated from lysates using anti-PECAM-1 antibody (WM59) and immunoblotted with an antiphosphotyrosine antibody (4G10). Analysis of thrombus formation under arterial flow conditions were performed using DIOC6 labelled human citrated blood and perfused over a collagen coated Vena8 BioChip (Cellix Ltd, Dublin, Ireland) with and without prior incubation with statins, at a shear rate of 20 dynes/cm^2. Z-stack images of thrombi were obtained every 30 s for up to 10 min using a Nikon eclipse (TE2000-U) microscope (Nikon, Surrey, UK). *In vivo* Thrombus formation was performed using C57BL/6 and PECAM-1 knockout mice, where platelets were fluorescently labeled by injection of Alexa488-conjugated anti-GPIb antibody. After laser injury of the cremaster muscle arterioles, accumulation of platelets was assessed. Images were captured prior to and after the injury by a Hamamatsu charged-coupled device camera C9300 in 640 × 480 format (Hamamatsu Photonics, UK) and analysed using slidebook5 software (Intelligent Imaging Innovations, Denver, USA).

Results: Statins were found to inhibit a range of platelet functional responses and thrombus formation *in vitro* and *in vivo*. Notably, these effects of statins on platelet function *in vitro* and *in vivo* were diminished in PECAM-1$^{-/-}$ platelets. Activation of PECAM-1 signalling results in its tyrosine phosphorylation, the recruitment and activation of tyrosine phosphatase SHP-2, the subsequent binding of phosphoinositol 3-kinase (PI3-K) and diminished PI3-K signalling. Statins resulted in the stimulation of these events, leading to the inhibition of Akt activation.

Conclusions: Together, these data provides evidence for a fundamental role of PECAM-1 in the inhibitory effects of statins on platelet activation, which may explain some of the pleiotropic actions of these drugs.

PA 4.02-4

Hydrophobic regions on protein surfaces, an ancient damage-associated molecular pattern, induce platelet activation

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Background: Immune responses are initiated by pathogen-associated molecular patterns or by tissue-derived danger/alarm signals. Hydrophobic portions of proteins act, when exposed, as universal damage-associated molecular patterns, as shown by Matzinger. Herczenik et al. demonstrated that misfolded/partly unfolded proteins were able to activate platelets. Exposure of *hydrophobic* regions on its surface, the thermodynamic *hallmark* of a misfolded/partly *unfolded* protein, is recognized by the chaperone BiP/GRP78.

Aims: As platelet activation can be regarded as a response to injury, we studied the role of exposed hydrophobic areas in platelet activation by misfolded/partly unfolded proteins.

Methods: The effect of the completely different misfolded proteins on platelet adhesion, activation, secretion, aggregation, and thrombus formation was investigated by aggregometry, flow cytometry, thrombus formation in *in vitro* perfusion chambers, confocal laser scanning microscopy and plasmon resonance.

Results: The chaperones GRP78, and protein disulfide isomerase showed prominent, punctuate staining, with striking co-localization at the platelet surface. All tested misfolded proteins, HOCl oxidised Albumin, Eap from S. aureus, human neutrophil alpha defensin modified plasma proteins, thrombospondin-1 in its amyloid like state and von Willebrand factor under high shear conditions, induced platelet adhesion, GPIIb/IIIa activation, granule secretion, platelet aggregation and *in vitro* thrombus formation. All used agonistic proteins exposed aggregation-prone hydrophobic regions. Soluble BIP/GRP78 as well as soluble PDI, inhibitors of the PDI and the hydrophobic probe 4,4′-bis(1- anilinonaphthalene 8-sulfonate) (bis-ANS) inhibited platelet activation in a dose dependent manner.

Summary/Conclusion: Human platelets present the chaperone BIP/GRP78 on their surface. Platelet activation by misfolded proteins is inhibited by soluble chaperones that recognize exposure of hydrophobic regions on their surface, as well as by the hydrophobic probe bis-ANS. As hydrophobic regions on protein surfaces, an ancient damage-associated molecular pattern, induce platelet activation, platelet activation in thrombosis might be regarded as a mechanism to initiate repair, remodeling and defense against microorganisms, rather than inappropriate activation of haemostatic mechanisms. Future work in this filed might lead to an antithrombotic agent that will inhibit thrombosis, but spare haemostasis

PA 4.02-5

Amplification of platelet activation by surface pannexin-1 hemichannels

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Background: Pannexin 1 (Panx1) forms an ion channel permeable to molecules up to 1000 Da and represents a non-lytic, non-vesicular ATP release pathway in erythrocytes, leukocytes and neurons. A role for related connexin gap junction proteins in haemostasis and thrombosis has recently been reported (Vaiyapuri et al. 2012, Circulation 125, 2479; Angelillo-Scherrer et al. 2012 Circulation 124, 930) whereas the expression and role of platelet pannexins remains unknown.

Aims: To determine the expression and functional role of pannexins in human platelets using a variety of molecular, cellular and functional techniques.
Methods: Washed suspensions of human platelets were isolated from informed consenting healthy donors with local ethical committee permission. Platelets were lysed in RIPA buffer for protein studies or fixed with paraformaldehyde for immunohistochemstry (IHC). mRNA from ultra-purified platelets was analysed by quantitative PCR. Aggregometry, ATP release, and calcium measurements were performed under conditions where P2X1 function was preserved (0.32 U/mL apyrase). Block of Panx1 was achieved with the two structurally unrelated and widely used inhibitors at concentrations that do not affect connexin channels; probenecid (Prb, 100 μM) and carbenoxolone (Cbx, 10 μM).
Results: Panx1, but not Panx2 or 3, was detected in platelet mRNA samples at a similar level to P2X1. Western blotting showed that Panx1 ran as a PNGaseF-sensitive glycosylated protein at 48 kDa. From biotinylation and IHC studies, Panx1 was found to be predominantly located on the surface membrane. Prb or Cbx inhibited Ca^{2+} influx to a range of agonists by up to 30%. Prb and Cbx also inhibited ATP secretion and aggregation, with the greatest effect (80% reduction in aggregation and 75% reduction in secretion) observed at low concentrations of collagen (0.5 μg/mL) where desensitisation of P2X1 receptors with α,β me-ATP had an equivalent effect to Panx1 blockade. Co-immunoprecipitation experiments with Panx1 and P2X1 demonstrated a weak association between these proteins.
Summary/Conclusions: Panx1 protein is present on the surface of platelets as the fully glycosylated species, which supports formation of ATP-permeable hemichannels but not gap junctions. The data suggest that Panx1 contributes to ATP release and secondary activation of platelets, particularly at low levels of collagen where aggregation depends upon secondary activation of P2X1 receptors. Panx1 may therefore have an amplifying role in arterial thrombosis.

PA 4.02-6
PKCd negatively regulates platelet function in response to thrombin via GPIba

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Background and Aims: The protein kinase C (PKC) family is essential for platelet function in response to various platelet agonists including thrombin. Thrombin signaling in platelets is mediated by a specialized family of G-protein-coupled receptors known as protease-activated receptors (PARs) and by GPIbα. Indeed, GPIbα, PAR1, and PAR4 are considered the high-, medium-, and low-affinity thrombin receptors in human platelets, respectively. It has been shown that PKC delta (PKCδ) negatively and positively influences platelet function in response to collagen and PAR stimulation, respectively. However, its importance in response to thrombin stimulation via the high-affinity GPIbα binding site is not known. This study was therefore designed to examine the role of GPIbα in platelet PKCδ signaling and function.
Methods and Results: We found that in human platelets, pre-treatment with a specific PKCδ membrane translocation inhibitor δ(V1-1)TAT significantly potentiated platelet aggregation and activation, as well as PKCδ phosphorylation on Tyr^{311}, in response to a priming concentration of thrombin. However, the responses to high concentrations of thrombin or to the PAR agonist peptides, TRAP1 and TRAP4, were not affected. This potentiation process is GPIbα-dependent, as specific inhibition of the high affinity thrombin-binding site on GPIbα reverses this effect. These results were reproduced using platelets from $PKC\delta^{-/-}$ mice and in platelets from WT mice pre-treated with δ(V1-1)TAT. Notably, tail bleeding times and blood platelet counts in $PKC\delta^{-/-}$ mice were significantly decreased following low thrombin injection as compared to WT mice, which is indicative of enhanced platelet activation. In addition, lung histological sections from $PKC\delta^{-/-}$ mice reveal exacerbated pulmonary thromboembolisms in response to a low-dose thrombin challenge. Importantly, $PKC\delta^{-/-}$ mice treated with a blocking anti-mouse GPIbα antibody were protected from these effects.
Conclusion: This study adds new insights into the role of PKCδ in platelet function downstream of GPIbα, where it negatively regulates platelet aggregation and activation in response to a priming concentration of thrombin. Thus, PKCδ may constitute a target in the management of thrombotic events.

PA4.03 – Platelets and Leukocytes

PA 4.03-1
Membrane fragments from dying platelets promote leukocyte aggregation and vascular obstruction during ischemia-reperfusion injury

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Background: Ischemia-reperfusion (I/R) injury is a prominent feature of a wide range of human diseases, characterised by tissue damage initiated by vessel obstruction and exacerbated by blood reperfusion. I/R injury to the intestines can result in remote organ injury to the lung, leading to the acute respiratory distress syndrome, a condition associated with considerable morbidity and mortality. Both leukocytes and platelets are known to be important in this process however it is unclear how they promote remote organ injury.
Aim: We have examined the mechanism by which platelets in the ischemic mesenteric circulation promote leukocyte adhesion and infiltration into the lung parenchyma during mesenteric I/R injury.
Methods: A mouse mesenteric I/R injury model was utilised and the injury was induced by clamping the mouse superior mesenteric artery for 30, 60 or 90 min followed by blood flow restoration. The adhesive behaviour of leukocytes and platelets in the mesenteric circulation and in the lung was monitored by intravital microscopy and histology, respectively.
Results: Utilising a confocal imaging technique that enables detailed analysis of the entire mesenteric microvasculature during I/R injury we confirmed extensive leukocyte adhesion and rolling onto the surface of ischemic endothelial cells. Unexpectantly, we demonstrated that up to 50% of rolling leukocytes formed aggregates. These aggregates developed in the mesenteric venous circulation within minutes of blood reperfusion which increased as a function of ischemia time (30–90′). Anti-Gr1Ab staining revealed that neutrophils were the dominant leukocyte population forming aggregates, with aggregate size varying from 2 to > 10 cells. Co-staining platelets and neutrophils revealed the presence of both cell types in the aggregates and depletion of platelets from the circulation completely eliminated aggregate formation. Similarly, WT mice transplanted with bone marrow from P-selectin deficient mice revealed that platelet P-selectin ($P\text{-}sel^{PL}$) was critical for neutrophil aggregation. Greater than 80% of the platelets within the leukocyte aggregates expressed phosphatidylserine (PS^{+ve}) on the cell surface and analysis of leukocyte aggregate formation under shear conditions *in vitro* confirmed that PS^{+ve} platelets were both necessary and sufficient to induce leukocyte aggregate formation. PS^{+ve} platelets lose their cytoskeletal network and the membranes became fragile under shear, leading to the extrusion of long membrane strings and large cellular fragments. These strings/fragments were pulled from the surface of platelets by rolling leukocytes, forming a physical bridge between adjacent leukocytes. Injection of PS^{+ve} platelet fragments into the portal circulation during intestinal I/R injury greatly enhanced leukocyte aggregation in the portal vein and lung vasculature, leading to increased leukocyte infiltration and interstitial oedema.
Conclusion: These studies define a new proinflammatory function for dying platelets that involves the shear-dependent formation of membrane strings/fragments, capable of inducing leukocyte aggregation and vascular obstruction. PS^{+ve} fragments from dying platelets can

circulate from the portal circulation to the lung thereby promoting pulmonary leukocyte aggregation and remote organ injury.

PA 4.03-2

Platelets limit the histotoxic activities of infiltrating neutrophils and act as vascular healing patches in inflamed tissues

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Platelets are now recognized as major actors of innate and adaptive immune responses. In particular, it is well established that platelets support leukocyte infiltration into diseased tissues in various pathophysiological situations such as cancer, atherosclerosis, dermatitis, stroke, and acute lung injury. In addition to this leukocyte supporting action, platelets exert protective effects on vessels at sites of leukocyte infiltration, resulting in prevention of tissue hemorrhage. Thus, although platelets promote leukocyte infiltration through the activated endothelium, notably by loosening endothelial junctions and supporting endothelial activation, they also play a highly positive, vascular protective role in inflamed tissues. Here, using models of acute dermatitis, peritonitis, and pneumonitis, and *in vitro* experiments using isolated human platelets and neutrophils, antibody-induced profound thrombocytopenia and neutropenia in mice, we investigated the role of platelets in regulating the activation of infiltrating neutrophils. We show that GPVI-dependent early recruitment of platelets to the activated endothelium is required for prevention of neutrophil-induced bleeding in both immune-complexes-induced dermatitis and LPS-induced pneumonitis. We further show that although thrombocytopenic mice display reduced neutrophil infiltration in inflamed skin, lungs and peritoneal cavity, they are also characterized by an increased activation of infiltrating neutrophils, as indicated by the increased release of myeloperoxidase and elastase by their neutrophils, as compared to that of control mice. The ability of platelets to counter neutrophil-derived histotoxic activities was confirmed by co-incubating isolated human neutrophils and platelets, in the presence or absence of the pro-inflammatory cytokine TNF-alpha. Using pharmacological inhibitors of GPVI and ADP signalling, we found that this anti-neutrophil action of platelets was independent of GPVI but requires platelet activation. Together, our results indicate that although platelets favour neutrophil infiltration, they also limit the histotoxic activities of infiltrating neutrophils and act as vascular healing patches in inflamed tissues, thus limiting vascular injuries and bleeding.

PA 4.03-3

Platelet thrombi utilize co-operative biochemical and biophysical mechanisms to induce excessive leukocyte accumulation to sites of endothelial injury

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Background: Ischemia-reperfusion injury is associated with an intense inflammatory infiltrate that exacerbates tissue injury. It is generally considered that ischemic injury to the endothelium, leading to upregulated expression of adhesion molecules and proinflammatory cytokines, is the principal mechanism promoting leukocyte recruitment and inflammation. A growing body of evidence has suggested a potentially important role for the proinflammatory function of platelets in exacerbating leukocyte recruitment, inflammation and tissue damage following I/R injury, however the mechanisms responsible for this remain poorly defined.

Aim: To investigate the mechanisms by which platelets induce an exaggerated leukocyte recruitment response at sites of endothelial perturbation.

Methods: We established a murine model of intestinal I/R injury and used confocal intravital microscopy to analyse platelet and leukocyte adhesive responses in ischemic tissue. We subsequently developed a model of mechanical injury using micromanipulator needles in the mesenteric vasculature of mice to enable investigation of the mechanisms regulating platelet-leukocyte interactions. Particle image velocimtery (PIV) and computational fluid dynamics (CFD) were used to measure rheological parameters.

Results: Intravital confocal imaging of the murine intestinal microvasculature following I/R injury identified distinct patterns of leukocyte recruitment in the ischemic tissue, ranging from minimal stable adhesion to regions of extensive leukocyte accumulation and tissue infiltration. Significantly, the regions of exaggerated leukocyte adhesion correlated with the presence of platelet-rich thrombi, with leukocytes preferentially adhering to larger thrombi (surface area $> 0.5 \times 10^3$ mm^2). Leukocytes adherent to thrombi had a polarised, elongated morphology with enhanced motility, suggesting a potentially important role for thrombi in promoting localized infiltration of leukocytes into ischemic tissue. To identify the mechanisms by which platelet thrombi mediate efficient leukocyte recruitment *in vivo*, we developed a localised model of microvascular injury using microinjector needles. In this model we controlled the level of platelet activation by microinjecting platelet agonists and identified thrombin as the most potent inducer of leukocyte-thrombus interactions. There was a direct correlation between thrombus size and the efficiency of leukocyte recruitment and computational fluid dynamic analysis (CFD) revealed that the three-dimensional shape of the thrombus resulted in the formation of low shear pockets (< 100/s) where leukocyte accumulation preferentially occurred. Notably, large thrombi and potent platelet stimulation were not sufficient to induce efficient leukocyte recruitment in arteries. However, controlled manipulation of arterial flow rates demonstrated that the bulk flow rate had a profound impact on leukocyte-thrombus interactions, with an approximately 80% reduction in arterial flow leading to a > 50 fold increase in leukocyte recruitment.

Conclusion: These studies identify the key biochemical and biophysical factors which modulate leukocyte-thrombus interactions *in vivo*. Our results define the cooperative influences of both biochemical (platelet activation status and thrombin generation) and biophysical factors (loco-regional rheological effects imparted by thrombus size and prevailing bulk flow) in regulating leukocyte recruitment by microvascular thrombi. These findings may help explain the potent thromboinflammatory response that is characteristic of I/R injury, where excess thrombin generation and platelet activation, in combination with disturbed blood flow amplifies the thromboinflammatory response.

PA 4.03-4

Platelet FLow-Induced PRotrusions (FLIPRs): a landing strip for circulating monocytes

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Background: Platelets are the most important cells in the primary prevention of blood loss after injury. In addition, platelets provide a negatively charged phospholipid surface to support coagulation and are also involved in inflammatory responses through their interaction with leukocytes.

Methods: We perfused platelets over a fibrinogen surface and activated them with the GPVI agonist collagen related peptide (CRP). We

describe the formation of extremely long, negatively charged membrane strands that emerge from platelets adhered under flow conditions.

Results: FLow-Induced PRotrusions (FLIPRs) are formed downstream of adherent and activated platelets, and reach lengths up to 250 mm, which is 80-fold more than the size of and individual platelet. FLIPR formation occurs on 19.5 ± 4.1% of the platelets in a process that depends on the activation of calpain and is accompanied by a disassembly of the actin and microtubule organization. Due to shearing forces, FLIPRs severe/disintegrate leading to the generation of procoagulant microparticles. Furthermore our data reveal that FLIPRs may serve as a surface extension for tethering of monocytes required for monocyte accumulation at the vessel wall and subsequent recruitment of platelet-derived FLIPRs/microparticles from the surface. Monocyte binding to the membrane strands was dependent on the interaction between P-selectin -and PSGL-1.

Conclusion: Our study demonstrates that platelets have the capacity to create extremely long membrane extensions that may serve as capturing devices for circulating monocytes.

PA 4.03-5

Zebrafish as a model to investigate the effect of P2Y12 knockdown on leukocyte migration

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Background: Activation of the platelet $P2Y_{12}$ receptor by ADP induces degranulation of α and dense granules from the platelet. α-granules release a myriad of different proteins including pro-inflammatory mediators that are involved in vascular inflammation as well as antimicrobial peptides. Clopidogrel and ticagrelor are $P2Y_{12}$ inhibitors that are used in the treatment and prevention of arterial thrombosis. Ticagrelor yields greater and more consistent $P2Y_{12}$ inhibition compared to clopidogrel. In the PLATO study, ticagrelor reduced mortality compared to clopidogrel in patients with acute coronary syndromes and post hoc analysis has suggested that partly this may be due to differential effects of the drugs on susceptibility to pulmonary infection and its complications. Zebrafish represent an ideal model to study inflammatory processes as transgenic zebrafish lines with fluorescently tagged macrophages and neutrophils enable excellent *in vivo* visualisation of migration. Temporary knockdown of gene function is achieved in the zebrafish by injection of morpholino antisense oligonucleotide.

Aims: To investigate the effect of $P2Y_{12}$ receptor knockdown on macrophage and neutrophil migration to injury.

Methods: The zebrafish transgenic *fmsgal4;UNM;mpoGFP* was utilised, in which mcherry red fluorescent protein is driven by the macrophage *fms* promoter, and Green Fluorescent Protein (GFP) is driven by the neutrophil specific myeloperoxidase (*mpo*) promoter. These double transgenics were injected at the 1 cell stage with 2.1 μg of either $P2Y_{12}$ or control morpholino antisense oligonucleotide. The effect of $P2Y_{12}$ knockdown was assessed by quantification of thrombus formation after laser-induced injury of the dorsal aorta. At 3 days post fertilisation (dpf) the ventral wall of the dorsal aorta was injured by laser, inducing thrombosis. The inflammatory response was induced by injury to the tail fin of 3 dpf larvae. Subsequent migration of macrophages and neutrophils were ascertained by counting number of macrophages or neutrophils present within the region between the tail injury site and loop of circulation 1, 4 and 8 h post injury.

Results: $P2Y_{12}$ receptor knockdown significantly reduced thrombus area in response to vessel injury ($2.26 \pm 0.4 \times 10^6$ pixels^2 control $0.12 \pm 0.01 \times 10^6$ pixels^2 $P2Y_{12}$ $P < 0.001$). The numbers of macrophages and neutrophils at the site of tail injury were determined for both the control and $P2Y_{12}$ knockdown groups. There was no significant difference between the groups in the numbers of macrophages or neutrophils at the site of injury.

Summary and Conclusion: $P2Y_{12}$ receptor knockdown significantly reduced thrombus area in response to vessel injury. However there was no significant effect on migration of macrophages and neutrophils to sites of injury. We therefore found no evidence that $P2Y_{12}$ plays a role in leukocyte migration in this model. Further work is required to explore potential mechanisms for differential effects of ticagrelor and clopidogrel on innate immunity.

PA 4.03-6

Cell-specific role of junctional adhesion molecule-A in flow-dependent atherosclerosis

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Background: Leukocyte transendothelial migration is a crucial event in atherosclerotic lesion formation and is controlled by a multi modal cascade comprising adhesion molecules, integrins and cytokines. Junctional Adhesion Molecule- (JAM-) A, as a transmembrane protein mainly located in endothelial cell (EC) junctions and on leukocytes, displays a wide variety of functions in cell polarity, barrier function, leukocyte adhesion as well as stem cell adhesion and differentiation.

Aims: Little is known about the involvement of JAM-A from differing cellular origins to atherogenesis. As JAM-A from differing cellular origins display different or even contrary effects in a number of inflammatory disease models, we wanted to delineate the role of leukocytic, endothelial and somatic JAM-A on atherogenesis.

Methods: Besides common (immune-) histology for plaque assessment, molecular biological approaches for protein expression pattern recognition and functional assays for *in vitro* transendothelial migration, multi-photon laser scanning microscopy (MPLSM) was used to obtain crucial data e.g. *ex vivo* protein localization, *in vivo* transendothelial migration and *ex vivo* vessel permeability. Transgenic mice deficient for endothelial or leukocyte or both JAM-A origins with or without apolipoprotein ($Apoe^{-/-}$) deficiency were used.

Results: Deficiency of JAM-A expression in endothelial cells reduced atherosclerotic lesion and necrotic core formation and T- cell and macrophage content in $Apoe^{-/-}$ mice. In contrast, JAM-A deficiency in leukocytes increased lesion size, necrotic core and infiltration in chimeric $Apoe^{-/-}$ mice, amounting to indifferent effects in $Apoe^{-/-}$ mice with a somatic JAM-A deletion. In line with decreased macrophage transendothelial migration in plaques of mice lacking endothelial JAM-A, a reduced monocyte transendothelial migration could be demonstrated in *in vitro* assays and *in vivo* in mice deficient for endothelial JAM-A by high-resonance scanning MPLSM. Further, MPLSM of atherosclerotic carotid arteries *ex vivo* revealed a focal concentration and redistribution of endothelial JAM-A to the luminal surface in lesion-proneareas, which also could be validated in appropriate *in vitro* assays. Deletion of endothelial JAM-A had no direct influence on barrier function in contrast to deletion of leukocytic JAM-A, which showed an increase in vessel permeability.

Conclusion: Our data identify specific and targetable functions of endothelial JAM-A in atherosclerosis, which are mediated by its upregulation and redistribution under atherogenic conditions. Moreover, we were able to demonstrate contrary effects for JAM-A from differing origins in atherogenesis, emphasizing the importance of a comprehensive assessment of targets.

PA4.04 – Thrombocytopenia Models

PA 4.04-1

Only severe thrombocytopenia results in bleeding and defective thrombus formation in mice

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Background: Platelets are essential players in thrombosis and haemostasis and their counts in peripheral blood average at 250 platelets/nL in humans. Thrombocytopenia is diagnosed if the platelet count is below 100 platelets/nL blood, but patients only have to undergo medical treatment to avoid excessive bleeding, if the platelet count is below 30 platelets/nL. On the other hand, it is not entirely clear how platelet counts affect the occurrence of acute ischaemic disease states, such as myocardial infarction or stroke.

Aims: Mice, which display a platelet count of 1000 platelets/nL blood, are an important model system to study haemostasis as well as the pathomechanisms underlying ischaemic diseases. To date, no systematic study has been reported that assessed the significance of platelet counts for normal haemostasis and experimental ischaemic diseases in mice.

Methods: To address the question to which extent the platelet count affects normal haemostasis and the occurrence of thrombotic events, we reduced platelet counts in mice to defined ranges between 0 and 1000 platelets/nL by platelet-depleting antibodies. Mice were then subjected to five different *in vivo* models to assess haemostatic and thrombotic function: tail bleeding time assay, thrombosis models in aorta, carotid artery and mesenteric arterioles as well as the transient middle cerebral artery occlusion (tMCAO) model of ischaemic stroke.

Results: We show that thrombotic occlusion of injured aortas and carotid arteries (large arteries) were partially impaired when the platelet count was reduced by 70% or 80%, respectively. In contrast, tail bleeding times and thrombus formation in small arterioles were largely unaffected by reductions of the platelet count up to 97.5%. Similarly, infarct growth and neurological deficits following tMCAO were unaffected by reductions of the platelet count up to 90% whereas a further reduction was protective.

Conclusion: These results reveal that arterial thrombosis, cerebral infarction and haemostasis in mice efficiently occur at unexpectedly low platelet counts which may have implications for humans at risk of thrombotic or hemorrhagic disease. Furthermore, our experimental thrombosis models indicate that occlusive thrombus formation in large vessels may be more sensitive to a reduced platelet count compared to small vessels.

PA 4.04-2

A novel non-antibody mediated model of thrombocytopenia allows efficient adoptive transfer of platelets and assessment of platelet function

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Background: Thrombocytopenic murine models have provided numerous insights into the role of platelets in normal physiology and disease. However, current methods of platelet depletion rely on either chemotherapeuticor antibody-based approaches. Off-target effects on the vasculature make interpretation of any results obtained using the chemotherapeutic approach problematic, while persistence of circulating antibody and rapid recovery of native platelets make adoptive transfer approaches technically challenging. Furthermore, antibody-mediated platelet opsonization and splenic clearance can induce immunoregulatory cytokine expression.

Aim: Develop a novel non-antibody mediated model of thrombocytopenia that will allow efficient adoptive transfer of platelets and assessment of platelet function

Methods and Results: Mice expressing simian diphtheria toxin receptor (DTR) on the megakaryocyte/platelet lineage (DTR^{PF4Cre}) were generated by crossing B6-iDTR mice (containing a simian DTR allele whose expression is blocked by an upstream *loxP*-flanked STOP sequence) with PF4-Cre mice. Since the cells of non-primate species lack the cell-surface receptor recognized by DT, mice are unaffected by injection of DT. Injection of 10 ng/g DT every third to DTR^{PF4Cre} mice resulted in complete platelet ablation (< 5%) of platelets by day 6 after injection, which was maintained through day 10. Depletion of megakaryocytes was confirmed by evaluation of bone marrow histology. Administration of an identical DT dose to control B6-iDTR mice (no DT expression) was without effect. Platelet-ablated mice were viable, and abnormal purpura was noted only with trauma.

To test the feasibility of adoptive transfer, donor platelets (DTR^{PF4Cre} negative) were transfused on day 1 after treatment with DT. 8×10^8 platelets injected by tail-vein or retro-orbital injection raised the circulating platelet count to approximately 3×10^5/mL in a 10–13 week old mouse. The function of the transferred platelets was assessed in a tail bleeding assay. Bleeding time in normal mice was 182 ± 73 s and platelet count was approximately 1000 K/μL. In platelet-depleted mice, bleeding did not cease prior to 600 s. Platelet repletion to 100–200 K/μL resulted in correction of the tail bleeding time to 340 ± 191 s. Bleeding time was normalized by platelet repletion to > 300 K/μL.

Conclusions: We have developed a novel non-antibody mediated method of platelet depletion that readily allows adoptive platelet transfer and assessment of platelet function. We expect that utilization of this model will allow the rapid assessment of platelet-specific roles in both genetic and acquired murine models of disease.

PA 4.04-3

Fucoidan improves adenovirus mediated thrombocytopenia and enhances viral liver transduction

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Background: Fucoidin is a known potent P-selectin antagonist and an inhibitor of platelet leukocyte- endothelial- interactions. Platelet P-selectin and Von Willebrand factor play a critical role in inducing thrombocytopenia following IV administration of adenovirus in mice. Adenovirus induces and potentiates ADP-induced platelet aggregation and results in rapid expression of P-selectin and the virus has been observed inside platelets. Adenovirus-platelet aggregates are taken up by macrophage and reduce liver transduction in mice; hindering the efficiency of adenovirus applications. Despite the documentation of CAR; the primary viral attachment receptor on human platelets, there are no reports proving this receptor on mice platelets. The direct effect of fucoidan on platelet functions is not fully understood and its effect on adenovirus mediated thrombocytopenia has not been investigated.

Aim: To study the effect of fucoidan on platelet- adenovirus interaction and platelet activation as well as the virus induced thrombocytopenia and viral liver transduction following intravenous administration of virus into mice.

Methods: Platelet adenovirus interaction was tested *in vitro* under the effect of fucoidan using Cy3 conjugated adenovirus by fluorescent microscopy. The presence of CAR on mice platelets was tested alongside with human platelets as positive control as well as CHO cells as negative control, by flow cytometry using mouse anti CAR and FITC-rabbit anti mouse as well as by RT-PCR. Mice were injected intrave-

nously with single dose of fucoidan 10 mg/kg 10 min before injection of Ad5 1 × 10 11 vp/mL and compared them to other mice injected with adenovirus only. Platelet count was examined 1 and 48 h following injection. Viral hepatic transduction was tested in mice receiving Ad5CTL (CAR binding) and Ad5KO1 (mutated to abate CAR interactions). *B*-gal expression was analysed visually and quantitatively using a *B*-gal ELISA.

Results: Fucoidan resulted in 3 -fold decrease in platelet P-selectin expression at 1 h after Ad administration to mice. Thrombocytopenia at 48 h was observed in both groups however; the reduction of platelet count was 46% in the group receiving fucoidan compared to 65% in the Ad only group. CAR was detected on mice platelets using flow cytometry (23 ± 0.04% $n = 3$). Fucoidan did not decrease Ad-platelet binding.

However, it increased liver transduction in mice receiving Ad5CTL (CAR binding) and Ad5KO1 (CAR mutated) compared to those not receiving fucoidan with significant increase in *B*-gal expression in mice injected with Ad5KO1.

Conclusions: Fucoidan inhibits P-selectin, improves thrombocytopenia following Ad administration, enhances liver transduction but does not abolish platelet Ad binding. Ablation of viral CAR interactions may have potential in improving safety and efficacy of adenovirus administration.

PA 4.04-4

Suspected clopidogrel induced thrombocytopenia – identifying the real culprit

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Background: Drug induced thrombocytopenia (DIT) is an idiosyncratic immune-mediated reaction which most typically occurs 1–2 weeks after commencement of a new drug. A high index of suspicion is required when considering a drug-induced thrombocytopenia since testing for drug-dependent antibodies (apart from those due to heparin and quinine) is generally unavailable. The main differential diagnosis is primary immune thrombocytopenia.

Aims: A case of suspected clopidogrel induced thrombocytopenia is detailed in order to highlight the challenges in making such a diagnosis.

Methods: Serum samples were collected from the index case and referred to the BloodCenter of Wisconsin for testing. A NOD-SCID mouse which lacks xenoantibodies and therefore allows infused human platelets to circulate, was used to study drug-dependent clearance of platelets by drug-dependent antibodies *in vivo*. (methods as described previously in Bougie DW, et al Blood. 2010;116(16): 3033–3038)

Results: The index case was a female who developed a lacunar stroke. The patient was taking Aspirin, Rosuvastatin and Enalapril long term. Platelet count was normal at 171 × 10^9/L. Over the ensuing 3–4 weeks, a significant number of medication changes were made. Aspirin was ceased and the patient was placed on Clopidogrel. Enoxaparin was prescribed as venous thromboembolism prophylaxis. The platelet count remained normal during this time. Enoxaparin was ceased and the patient was commenced on Dantrolene, Duloxetine, Ciclesonide and then Escitalopram. Some 5 days later, the patient complained of bleeding gums and was found to have a platelet count of 4 × 10^9/L with no other abnormality on full blood count or blood film. All medications (apart from Rosuvastatin and Enalapril) were ceased and oral prednisone was commenced. A diagnosis of heparin induced thrombocytopenia was considered clinically unlikely and heparin antibody testing was negative. The platelet count began to increase within a few days of cessation of the suspected drugs and returned to normal within 1 week. Of the numerous medications the patient had been recently prescribed, Clopidogrel was considered the likely culprit. Patient serum was sent to the BloodCenter for further testing and was found to cause clearance of human platelets in mice injected (IP) with Clopidogrel. No effect was seen with patient serum alone or with normal serum given to mice challenged with Clopidogrel.

Summary/Conclusion: This case illustrates the potential difficulty in determining the cause of an acute, severe thrombocytopenia in a patient who has been commenced on several new medications. Clopidogrel was considered the most likely cause and specific testing confirmed this. As a result, the patient's ongoing anti-platelet therapy had to be altered accordingly.

PA 4.04-5

Megakaryocytes, not only victims, but also participants in ITP

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Background: Immune thrombocytopenia (ITP) is an autoimmune disease characterized by platelet destruction accelerated. Many abnormal immune responses are involved. Autoantibody secreted long-lived plasma cells in bone marrow may be a reason for incurable of ITP. The bone marrow microenvironmental niches are important for the generation and maintenance of these cells. Some studies had revealed that megakaryocytes can secret several cytokines, some of which are very important to plasma cell survival and antibodies release.

Aims: Based on the potency of megakaryocytes in the immune modulation, we studied the interactions of megakaryocytes and plasma cells in bone marrow from ITP patients *in vitro*. To clarify that whether megakaryocytes modulate the function and survival of platelets reactive plasma cells within bone marrow in ITP patients.

Methods: It was detected that megakaryocytic cell surface expression of the protein levels of APRIL and CD154 in ITP patients by flow cytometry. Megakaryocytes was co-cultured with isolated plasma cells and lymphocytes in the bone marrow of ITP patients *in vitro* and these were analysed that the survivion and activation of several T cells subtype and plasma cells.

Results: The surface APRIL ($P = 0.035$) and of CD154 ($P = 0.039$) expression levels of megakaryocytes in ITP patients were significantly increased. The megakaryocytes in the bone marrow of ITP patients plays a role in survivion of other immune cells. The megakaryocytes can promote surviving of CD4+ T cells, CD8+ T cells, CD27+ B cells and activation of CD4+, CD8+ T cells. HLA-DR expression was elevated in these immune cells when co-cultured with megakaryocytes. Moreover, we studied the role of APRIL and CD154 in the interreaction between megakaryocyte and immune cells in ITP. When the co-culture system was processed with anti-APRIL ($P = 0.038$), anti-CD154 ($P = 0.047$) antibody, the proliferation of plasma cells was weakened and platelet-specific autoantibody secretion was reduction. However, when TPO was added to the co-culture system, the number of CD138+ plasma cells was increase in the co-cultured system.

Conclusion: Our study for the first time suggested that megakaryocytes play an important active role in the modulation of activation and survival of bone marrow platelets-reactive plasma cells in ITP. Megakaryocytes are not only victims, but also participants of the pathologic immune reaction in ITP.

PA 4.04-6

Tissue plasminogen activator induced fibrinolyis on rotational thromboelastometry in chemotherapy-induced thrombocytopenia

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Background: Dropping platelet counts are a significant problem in patients with haematological malignancies treated with high dose chemotherapy. There is general consensus that platelet counts below 10×10^9/L are managed with prophylactic platelet transfusions. Nonetheless clinical significant bleedings may occur above this threshold and also, indeed, despite the prophylactic transfusion policy (Stanworth et al, 2012, ASH). Assessing the individual patient risk of serious bleeding complications is challenging and does not seem to depend solely on platelet counts. In absence of reliable platelet function tests in thrombocytopenic patients there is a clinical need for developing new haemostatic assays as predictors of bleeding.

Aims: In this study, the susceptibility to fibrinolysis, in thrombocytopenic individuals, was assessed by inducing tissue plasminogen activator (tPA)-mediated lysis in an adapted ROTEM test. The aim was to investigate whether any heterogeneity between healthy volunteers and thrombocytopenic patients might exist.

Methods: Ten healthy volunteers, with normal platelet counts and without any use of medication, were selected. Samples with different platelet counts (range 15–248 $\times 10^9$/L) were obtained by replacing platelet rich plasma (PRP) with platelet poor plasma (PPP) without changing the haematocrit or the fibrinogen concentration. Blood samples from 21 thrombocytopenic patients were not manipulated. Lysis speed (mm/minute) was calculated during three periods: maximal clot formation time (MCF-t) – lysis onset time (LOT) (100%-85%), MCF-t – lysis time (LT) (100%-10%) and LOT – LT (85%-10%).

Results: Fibrinolysis speed did not show any significant differences among different, manually diluted platelet counts (15–248 $\times 10^9$/L) with constant fibrinogen levels in healthy volunteers. Supraphysiological fibrinogen levels were found in a concentration-dependent manner when platelet counts decreased in chemotherapy induced thrombocytopenic patients. Fibrinogen levels in the lowest group of platelets (0–10×10^9/L) were significantly higher compared to the group just above the transfusion threshold (10–20 $\times 10^9$/L) (5.0 g/L vs. 4.1; $P = 0.005$). A decrease in platelet counts and subsequent higher fibrinogen levels resulted in a significantly lower lysis speed. This effect was observed both in physiologic conditions (i.e. without addition of tPA) as in tests where fibrinolysis was induced with a high concentration of tPA. One major bleeding (WHO grade 3) was reported in a patient with a platelet count of 16×10^9/L and a relatively low fibrinogen level (2.2 g/L) compared to other patients with platelet counts between 10 and 20 $\times 10^9$/L (median 4.1 g/L, interquartile range 3.4–4.3).

Conclusion: Supraphysiological fibrinogen levels were found, and might be associated with lower lysis speed, in patients with severe chemotherapy-induced thrombocytopenia. The preventative effect of higher fibrinogen levels might play a role in assessing the bleeding risks in these patients. Adjusted reference values for fibrinogen and other haemostatic proteins might be applicable during severe thrombocytopenia for establishing a more correct probability of bleeding tendencies. Our future investigations will focus on fibrinogen and other markers as possible predictors of bleeding in these patients.

PA4.05 – Genetic Platelet Disorders – II

PA 4.05-1

Proteomic analysis of platelets from patients with X-linked thrombocytopenia with thalassemia (XLTT)

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Background: X-linked thrombocytopenia with thalassemia (XLTT) is a rare inherited disorder resulting in problems of bleeding mainly in males. It is caused by the mutation 216R>Q in exon 4 of the *GATA-1* gene. The GATA-1 protein is a transcription factor involved in hematopoiesis. Clinical manifestations of XLTT include thrombocytopenia, altered platelet function and morphology, slight hemolysis and splenomegaly. We have recently reported that males also develop slight to moderate bone marrow fibrosis. In similarity to the disorder Gray platelet syndrome, large platelets deficient in alfa-granule are typical. Proteomics is a method used for analysis of the proteome, i.e. the translated set of proteins in a defined tissue, cell type or fluid. Extracted proteins can be separated by isoelectric point and molecular mass to produce high-resolution maps by two-dimensional electrophoresis (2-DE). This enables individual quantification and identification of proteins that are altered by disease.

Aims: The aim of this study is to increase the knowledge on XLTT pathogenesis by identifying specific protein alterations in platelets. Such a detection of protein alterations in XLTT platelets is also valuable for the general understanding of platelet function in health and disease.

Methods: Blood samples from four male XLTT patients from two recently reported Swedish families (one patient from family A, three patients from family B) were collected after informed consent and approval by the regional ethical review board in Uppsala, Sweden. Their platelet counts were 35, 50, 42 and 76 $\times 10^9$/L respectively. Whole blood was also collected from four age and sex-matched healthy volunteers. Platelets were isolated by differential centrifugations, using optimized conditions to keep them in an inactive state. The platelet pellet was gently washed to avoid plasma protein contamination. Platelet proteins were resolved in a highly denaturing solution containing urea and detergent and separated by 2-DE. By software evaluation, individual platelet proteins were quantified and compared between controls and XLTT. For statistical calculations, student's t-test and multivariate analysis (partial least squares) were used.

Results: On average, 850 individual protein spots were detected in each gel. With a chosen significance level of $P < 0.05$, about 60 protein spots were found altered in XLTT platelets. From quantitative 2-DE gels, 25 spots were chosen for initial identification. Preliminary mass-spectrometry analysis has so far identified nine of the differentially expressed proteins. Only one of these proteins, gamma-actin, was up-regulated (fold change 1.98). Of the eight downregulated proteins (fold changes 0.14–0–56), the majority are involved in modeling of actin and the cytoskeleton. Two downregulated proteins, regulator of G-protein signaling 10 (RGS10) and Acyl-protein thioesterase 1, both inhibit protease-activated receptor-1 (PAR-1) signaling dependent functions.

Conclusions: There is limited knowledge available on the pathogenic events caused by *GATA-1* mutations. XLTT is a non-progressive inherited disorder but shows similarities to the malignant disease primary myelofibrosis. XLTT might thus be a model for *GATA-1* related thrombopoietic changes contributing to myelofibrosis. By platelet proteomics, we have found altered protein expressions regarding the cytoskeleton and regulators of PAR-1 signaling pathways. Additional proteins with altered expressions are soon to be identified, and the consequences evaluated.

PA 4.05-2

Inherited mild bleeding disorders (MBD) of undefined cause (BUC): platelets express decreased tissue factor-dependent FXa generation and low thrombin generation in platelet-rich plasma

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Background: Hereditary mild bleeding disorders (MBD), usually manifested with skin and mucous bleeding are of heterogeneous nature, involving different pathogenic mechanisms, including platelet function defects, decreased amount and function of plasma VWF, mild defects of clotting factors and also, increased fibrinolysis. However, up to 60% of all the patients with MBDs have abnormal bleeding of undefined cause (BUC). We previously showed that platelets contribute to the clotting system providing not only an anionic phospholipid-rich membrane, but also functional Tissue Factor (TF).

Aims: To revisit the old concept of Platelet Factor 3 (platelet procoagulant activity) now focusing our attention on platelet TF activity in patients with BUC.

Methods: TF-induced FXa generation was measured as described (Panes et al. Blood 2007;109:5242) in washed, resting and VWF-Ristocetin-stimulated platelets. Thrombin generation (TG) in PRP was performed by modified CAT method, with no TF and phospholipids added to the assay and assessed by the Velocity index (Vi, nM thrombin/min). Clot lysis time in PRP was measured as described (Panes et al. Platelets 2012;23:36). These measurements were compared in 72 Controls, 81 patients with BUC and 20 with type 1 VWD, of similar ages (21, 19 and 21, respectively). Anionic phospholipids exposure was assessed by annexin V binding (FC).

Results: Controls and patients with BUC had normal VWF:Ag, RCo and CBA, normal platelet aggregation-secretion and clotting screening tests. Clot lysis time, measured in PRP was similar in the three groups. Medians (ranges) of FXa generating potential (nM 10^{-7} platelets), expressed as the ratio of FXa generated by VWF-Ristocetin-stimulated platelets and resting platelets was lower in Controls than in BUC and VWD patients: 8.3 (3.4–30.1), 4.4 (1.2–10.9) and 9.8 (4.5–17.4), respectively ($P = 0.0042$). In TG assay, patients with BUC had lower Vi than Controls, either in non-stimulated (5.0 vs. 6.2) and VWF-Ristocetin (21.5 vs. 28) or TRAP-stimulated platelets (33.3 vs. 44) ($P < 0.05$–$P < 0.01$, Kruskall–Wallis with Dunn's multiple comparison test). Patients with VWD also had significantly decreased Vi in stimulated platelets. Annexin V binding was similar in Controls and BUC patients after stimulation with VWF-R (12% vs. 10.4% of labeled platelets) and TRAP (22.3% vs. 15%), respectively.

Conclusions: (i) FXa generation dependent on platelet Tissue Factor showed that platelets of patients with BUC generate less FXa than normal platelets. (ii) The velocity to generate thrombin in PRP in an assay with no added TF and phospholipids was significantly lower in platelets from patients with BUC than Controls. (iii) These results were unrelated to the ability of BUC platelets to expose anionic phospholipids, as shown by their normal annexin V binding. Up to 60% of patients with MBDs remain undiagnosed after a comprehensive laboratory work up. The decreased procoagulant activity of platelets observed in these patients probably constitutes a bleeding risk factor that may be pathogenically related to the symptoms in a proportion of these patients. This finding would represent a renewed version of the old-fashioned platelet factor 3. (Fondecyt 1110404 and 1130853).

PA 4.05-3

A novel mutation in the dry motif of the P2Y12 receptor combined with a function-reducing polymorphism in PAR1 in a patient with a bleeding disorder

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Background: The study of patients with bleeding problems is a powerful approach in determining the function and regulation of important proteins in human platelets. As part of the Genotyping and Phenotyping of Platelets (GAPP) consortium we have identified a number of mutations in receptor genes that could contribute to bleeding tendency in patients, including mutations in the P2Y12 receptor (P2Y12R (1,2)). We have identified a patient, with a chronic bleeding disorder, expressing a homozygous mutation, predicting an arginine to a cysteine (R122C) substitution in their P2Y12R. Importantly this mutation is found within the DRY motif of this receptor which in other G protein-coupled receptors (GPCRs) plays a critical role in regulating conformational states.

Aims: To examine the consequences of this mutation upon P2Y12R function in the index and affected family members patient's platelets and cell lines.

Methods: Platelet function was assessed by measuring platelet aggregation responses in platelet rich plasma (PRP) whilst functional responses to the P2Y12R were assessed by measuring VASP phosphorylation. HA-tagged wild type (WT) or R122C-P2Y12R were expressed in 1321N1 astrocytoma cells and receptor function assessed as previously described (1).

Results: ADP-stimulated aggregation was significantly reduced as a result of a significant impairment of P2Y12R activity in the patient and family members. There was a significant reduction in R122C variant expression at the cell surface in both cell lines and in platelets as a consequence of increased agonist-independent internalization followed by subsequent receptor traffic to lysosomes. Strikingly, members of this family also showed a significant reduction in thrombin-induced platelet activation due to an intronic polymorphism in the PAR-1 gene shown to be associated with reduced PAR1 receptor expression (3).

Conclusions: We have identified a novel P2Y12R defect associated with patient bleeding and demonstrated that the DRY motif of the P2Y12R is critical in maintaining the receptor in a basal non-activated state. In addition our study is the first to demonstrate a deficit in two stimulatory GPCR pathways that regulate platelet activity further indicating that patient bleeding is a complex trait.

References:

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PA 4.05-4

PTGS1 compound heterozygosity impairs gene expression and platelet aggregation and is associated with severe bleeding complications

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Background: The prostaglandin endoperoxide synthase (PTGS1, COX1) catalyze the conversion of arachidonic acid (AA) into

prostaglandin H_2 intermediates and thromboxane A_2 (TxA_2). Polymorphisms of genes involved in AA metabolism are potential modifiers in platelet function. The Aspirin-like defect (ALD) is an inherited platelet function disorder that involves the AA pathway, however, the molecular basis remains unknown.

Aim: A patient without bleeding history prior surgical interventions but with postoperative recurrent disseminated bleeding showed strongly deminished platelet aggregation response on AA indicating an ALD. Our study describes the molecular genetic investigation of the AA metabolism genes in this patient.

Methods: Platelet function was analyzed by light transmission and whole blood impedance aggregometry using different agonists including ADP, AA and U46619. Molecular genetic investigation included exon re-sequencing of the *PTGS1, TBXAS1* and *TBXA2R* genes. In addition, gene transcripts were quantified in platelet RNA using real-time-PCR (qRT-PCR).

Results: AA-induced platelet aggregation response was significantly decreased, whereas platelet aggregation induced by U46619 as indicator for TxA_2-receptor function was unaffected. The *TBXAS1* and *TBXA2R* genes revealed regular DNA sequences, whereas, the coding region of the *PTGS1* gene of the patient was compound heterozygous (c.22C>T; c.50C>T). We found that *PTGS1* mRNA expression in the patient's platelets was significantly decreased compared to unaffected normal controls (5-fold) or single heterozygotes (2-fold).

Conclusions: Compound heterozygosity in the 5′ region of the *PTGS1* gene impairs transcription and is associated with a phenotype of severe bleeding complication. Therefore, surgeons and anaesthesiologists should be aware of risks which go with *PTGS1* polymorphisms prior elective surgery.

PA 4.05-5

Platelet defects in congenital variant of Rett syndrome patients with FOXG1 mutations or reduced expression due to a position effect at 14q12

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Background: The Forkhead box G1 (*FOXG1*) gene encodes a transcriptional repressor essential for early development of the telencephalon. Intragenic mutations and gene deletions leading to haploinsufficiency cause the congenital variant of Rett syndrome, a neurodevelopmental disorder characterized by microcephaly, hand stereotypies, severe intellectual disability, absent language, seizures, and corpus callosum hypoplasia. Recently, the presence of long-range regulatory elements for *FOXG1* expression in the region distal to *FOXG1* was hypothesized, based on descriptive studies in patients with a chromosome translocation breakpoint located distally to *FOXG1* or with 14q12 deletions that do not harbor the *FOXG1* gene.

Aims: Given that abnormal platelet morphology and function has been described in several other neuronal disorders, we have used platelets from Rett syndrome-like patients to study FOXG1 expression and platelet ultrastructure.

Methods: Platelet studies were performed in six patients, three of them carrying a balanced translocation with breakpoint in the chromosome 14q12 region, one patient having a 14q12 microdeletion excluding the *FOXG1* gene, and two previously described *FOXG1* mutation positive patients.

Results: The hypothesis of putative long-range FOXG1-regulatory elements was supported by our finding of reduced FOXG1 mRNA and protein levels in platelets and skin fibroblasts from these cases compared to normal controls. Electron microscopy of their platelets showed some enlarged, rounder platelets with often abnormal alpha and fewer dense granules. Platelet function studies were possible in one 14q12 translocation patient with a prolonged Ivy bleeding time and a patient with a heterozygous FOXG1 p.Tyr416X mutation. Both had a prolonged PFA100 occlusion time with collagen and epinephrine, and aggregations with low dose of ADP and epinephrine were also reduced while ATP secretion was normal.

Summary/Conclusions: Our study shows for the first time by using platelets functional evidence of *cis*-regulatory elements in the 14q12 region that result in reduced FOXG1 expression in patients having translocations or deletions in that region. The platelet functional abnormalities deserve further investigation regarding a non-transcriptional regulatory role for FOXG1 in these enucleated cells.

PA 4.05-6

Demonstration of novel gain-of-function mutations of aIIbβ3: association with macrothrombocytopenia and Glanzmann thrombasthenia-like phenotype

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Background: Integrin αIIbβ3 is indispensable for normal hemostasis, but its role for thrombopoiesis is still controversial. Although platelet counts and morphology in Glanzmann thrombasthenia (GT), which is a congenital bleeding disorder due to qualitative or quantitative defects of αIIbβ3, are usually normal, several αIIb and β3 mutations have been identified in patients with congenital macrothrombocytopenia very recently.

Aims: To investigate genetic background and molecular mechanism of congenital macrothrombocytopenia associated with αIIbβ3 abnormalities.

Patients/Methods: We analyzed three unrelated Japanese families with congenital macrothrombocytopenia. Expression and activation state of αIIbβ3 in platelets was examined by flow cytometry and immunoblotting. Sequence of whole coding region and exon-intron boundaries of *ITGA2B* and *ITGB3* genes was performed. The effects of mutations on αIIbβ3 activation state and phosphorylation of FAK were analyzed in transfected cells.

Results: We newly identified three mutations; two mutations in highly conserved G^{991}FFKR sequence in juxtamembrane region of αIIb, p.G991C and p.F993del, and one donor site mutation of intron 13 of *ITGB3* leading to 40 amino acids deletion, p.D621_E660del, in the membrane proximal β-tail domain of β3. Case 1, whose platelets showed GT-like marked reduction in surface αIIbβ3 expression (3–11% of normal control) and macrothrombocytopenia with around $40 \times 10^3/\mu L$ of platelet counts, was a compound heterozygote with *ITGA2B* p.G991C and a novel nonsense mutation, *ITGA2B* p.R422*. Case 2 and Case 3 were heterozygotes of *ITGA2B* p.F993del and *ITGB3* p.D621_E660del, respectively, and their platelets showed moderate reduction of surface αIIbβ3 expression (60–70%) with 29–$113 \times 10^3/\mu L$ of platelet counts and increased platelet size. All three mutations, *ITGA2B* p.G991C, *ITGA2B* p.F993del and *ITGB3* p.D621_E660del, led to highly activated conformation of αIIbβ3 and spontaneous tyrosine phosphorylation of FAK in transfected cells.

Conclusion: These results suggest that spontaneous activation and aberrant outside-in signaling caused by mutations around membrane region of αIIbβ3 may lead to abnormal platelet number and morphology with impaired surface αIIbβ3 expression.

PA4.06 – ADAMTS13: Basic – II

PA 4.06-1

Anti-idiotypic DARPin molecules – potential new treatment tools for acquired thromobotic thrombocytopenic purpura (aTTP)?

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Background: The hallmark of aTTP is a severe ADAMTS13 deficiency resulting from autoantibodies (Abs) neutralizing and/or accelerating ADAMTS13 clearance. Despite the success of plasma exchange the risk of relapse is approximately 40%. A therapy using small molecules capable to neutralize inhibitory anti-ADAMTS13 Abs and to eliminate anti-ADAMTS13 specific memory B- and plasma cells would be highly desirable.

Aim: In order to develop such a therapy we used previously generated spleen-derived inhibitory monoclonal anti-ADAMTS13 Abs, having in common one of four CDR3 motifs shared by two aTTP patients, to search for small anti-idiotypic molecules mimicking the conformational ADAMTS13 epitope and consequently thus specifically bind and neutralize inhibitory anti-ADAMTS13 Abs in aTTP patients when administered.

Method: As source of anti-idiotypic small molecules we choose a large combinatorial small protein library of Designed-Ankryin-Repeat-Proteins (DARPins, Molecular Partners AG, Switzerland) that are characterized by: (i) a high diversity through a library size of 10^{15-23}, allowing to find molecules to any target including conformational epitopes, by (ii) high stability once expressed, and by (iii) being immune tolerant when administered. Two DARPin libraries coding for either 2 (N2C-library) or 3 (N3C) randomized ankyrin repeat modules were screened against an equimolar pool of three inhibitory anti-ADAMTS13 antibodies using Ribosomal display. Selected anti-idiotypic single DARPin clones from the fourth panning round were purified, their DNA sequence analyzed and their expressed proteins tested for specificity and neutralization potential towards all inhibitory Abs ($n = 5$) holding the same CDR3 motif as the selecting Abs, by ELISA or FRETS assay. Furthermore the binding capacity of an equimolar pool of the selected DARPins towards anti-ADAMTS13 Abs in plasma of 37 different aTTP patients was tested by ELISA.

Results: Nine, as revealed by their DNA sequence, unique anti-idiotypic DARPins were highly specific for their targets. Preincubation with spleen-derived inhibitory anti-ADAMTS13 Abs (equimolar mAbs pool) with a 10-fold molar excess of four 4 anti-idiotypic DARPins restored ADAMTS13 activity in plasma in a dose-dependent manner as assessed by FRETS assay. Moreover, the four anti-idiotypic DARPins bound anti-ADAMTS13 antibodies from plasma of 27/37 (73%) randomly picked aTTP patients and reduced binding to immobilized recombinant ADAMTS13 by 70–95% when pre-incubated with five additional patient plasma.

Conclusions: Using spleen-derived inhibitory anti-ADAMTS13 Abs of one patient we were able to select four different highly specific anti-idiotypic DARPins that not only bind to anti-ADAMTS13 Abs being composed of a similar CDR3 motif than the selecting Abs, but also to a substantial proportion of anti-ADAMTS13 Abs in plasma of randomly selected aTTP patients. Our results are promising and hint at a limited number of different anti-idiotypic molecules necessary to neutralize inhibitory anti-ADAMTS13 Abs. Affinity and neutralizing capacities of all nine selected anti-idiotypic DARPins are underway.

PA 4.06-2

Identification of glycosylation sites in plasma derived ADAMTS13 employing tandem mass spectrometry

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Background: ADAMTS13 is a plasma metalloproteinase that regulates platelet adhesion and aggregation by its ability to process ultra-large von Willebrand factor (VWF) multimers on the surface of endothelial cells. Acquired deficiency of ADAMTS13 causes a rare and life-threatening disorder called thrombotic thrombocytopenic purpura (TTP). Several studies have shown that aberrant glycosylation can play an important role in the pathogenesis of autoimmune diseases. Aberrant glycosylation of antigens can not only create new ligands for different B cell epitopes but might also play a role in presentation of peptides to T cells. Several potential N or O-linked glycosylation sites have been predicted or identified in recombinant ADAMTS13. However, it is not known which of these sites are indeed glycosylated in plasma ADAMTS13.

Aim: In the study we analyzed the presence of N-linked or O-linked sugars on plasma derived ADAMTS13.

Methods: Plasma ADAMTS13 was purified using a monoclonal antibody directed against the disintegrin like domain. Sites of N-linked glycosylation were determined by the use of N-glycosidase-F, which removes the entire carbohydrate from the side chain of asparagines. In this process the asparagine residues are deaminated to aspartic acid, resulting in an increase of the peptide mass of 1 Da. Following trypsin or chymotrypsin digestion, deglycosylated peptides were identified by tandem MS.

Results: Nine of the predicted N-linked sites were identified in or near the metalloproteinase, spacer, thrombospondin type 1 repeat (TSR1) and theCUB domain of plasma ADAMTS13. Moreover, all seven predicted O-fucosylated sites were identified in the TSR1 domains of plasma ADMTS13 by performing searches of the MS/MS data for loss of glucose (hexose, 162 Da), fucose (deoxyhexose, 146 Da), or glucose-fucose (308 Da). Sequential loss of the glucose-fucose disaccharide gives a characteristic fragmentation pattern, allowing easy identification of modified peptides. With the use of electron transfer dissociation (ETD) we confirmed unambiguously the modified sites. ETD fragmentation produces ions that retain the post translation modification allowing a precise identification of the modified site.

In addition to N- and O-linked modifications, two C-mannosylation sites were identified within the TSR domains of ADAMTS13. Peptides with an increase of mass by 162 Da from the predicted tryptic fragments that contained a WXX_nW motif were subjected to MS/MS. C-mannosylation of Trp can be identified through the addition of 162 Da, but also by the loss of 120 Da in MS/MS spectra, a characteristic cross-ring fragmentation product of aromatic C-glycosides.

Summary/Conclusion: Taken together our data clearly identify different glycosylation sites on plasma ADAMTS13. Analysis of the glycosylation pattern gives novel information on the biochemical properties of plasma derived ADAMTS13 and might provide new insight in the initiation of autoimmune reactivity against ADAMTS13 in patients affected by acquired TTP.

PA 4.06-3

Prophylactic and therapeutic efficacy of a recombinant ADAMTS13 in a mouse model of thrombotic thrombocytopenic purpura

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Deficiency of ADAMTS13, a von Willebrand factor (VWF)-cleaving protease, is the key factor in the pathogenesis of thrombotic thrombocytopenic purpura (TTP), a life-threatening thrombotic microangiopathy. Baxter is developing a recombinant ADAMTS13 (rADAMTS13, a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13) product for the potential prophylaxis and treatment of TTP. A recently established disease model in ADAMTS13 ko mice (B6.129-ADAMTS13^{tm1Dgi}) was used to test the effectiveness of this drug candidate. In brief, animals are challenged with a high dose of human recombinant von Willebrand factor (rVWF) containing ultra-large VWF multimers to induce TTP-like symptoms.

In two studies, 10 ADAMTS13 ko mice per group received a single dose of 200 FRETS-U/kg rADAMTS13 5 min–120 h before or 15–180 min after challenge with rVWF. Buffer, administered either 5 min before or 15 min after challenge, was used as negative control. Efficacy was defined as the degree of prevention of platelet drop and increase in LDH. Schistocytosis and organ damage were also assessed.

All buffer-treated animals that received rVWF were severely thrombocytopenic and showed increased LDH levels, schistocytosis and organ damage. Efficacy of rADAMTS13 was treatment interval-dependent in both studies. Platelet count at termination of all rADAMTS13-treated animals was statistically superior to that of buffer-treated controls ($P \leq 0.0001$). However, clinically relevant protection was seen only for treatment intervals ≤ 72 h; animals that received prophylactic treatment 120 h before administration of rVWF showed severe thrombocytopenia. Treatment with rADAMTS13 stabilized the platelet count and prevented further development of thrombocytopenia. Serum LDH levels in rADAMTS13-treated animals were statistically significantly lower than in buffer-treated controls ($P \leq 0.0001$) in both the prophylactic and therapeutic setting. Other endpoints, such as schistocytosis and organ damage, confirmed the treatment interval-dependent efficacy for platelet count.

In conclusion, Baxter's rADAMTS13 was shown to be effective in an rVWF-induced animal model closely mimicking the situation in patients with hereditary TTP, and is thus a promising drug candidate for future clinical trials.

PA 4.06-4

Treatment of reperfusion injury with recombinant ADAMTS13 in a porcine model of acute myocardial infarction

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Background: No reflow and decreased microvascular perfusion after percutaneous coronary intervention increase morbidity and mortality in ST-elevation myocardial infarction (STEMI) patients. No reflow may be mediated by platelet vessel wall interaction that is governed by von Willebrand factor. ADAMTS13 is a metalloprotease that cleaves von Willebrand factor, thereby reducing its prohemostatic properties. There is considerable evidence that ADAMTS13 levels decrease and von Willebrand factor levels increase in STEMI patients. Recombinant ADAMTS13 has been effective in reducing cerebral infarct size in a murine model of stroke.

Aims: In this study recombinant ADAMTS13 was tested as a potential treatment of no reflow in a porcine model of cardiac ischemia and reperfusion.

Methods: In 23 female swine (median age 83 days, median weight 30 kg) a balloon was inflated in the circumflex coronary artery for 75 min. Fifteen minutes after reperfusion, an intracoronary bolus of either recombinant ADAMTS13 (400 U/kg, Baxter Innovations Vienna, Austria) or vehicle was given.

Results: ADAMTS13 activity significantly increased in treated pigs (from median 18%, IQR 14.5–24.0 to median 324%, IQR 117.0–384.0, $P = 0.003$) whereas no change was observed in the control group. Animals were sacrificed 7 days later for histopathology. There was no difference in the size of myocardial necrosis as assessed with plasma Troponin T measurements, continuous 12 leads ECG, macroscopical infarct analysis, and histopathology using phosphotungstic acid-hematoxylin staining. Microvascular obstruction as estimated by staining with anti-CD31/Hematoxylin and counting of vessels and microthrombi was similar for both groups.

Conclusions: Intracoronary treatment with recombinant ADAMTS13 did not prevent formation of microthrombi and did not decrease infarct size in this porcine coronary model of ischemia and reperfusion.

PA 4.06-5

A rat model reveals feasibility of rADAMTS13 therapy in the presence of inhibitory antibodies

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Anti-ADAMTS13 autoantibodies that either neutralise the activity of ADAMTS13 or enhance protein clearance are the major cause of severe ADAMTS13 deficiency in patients suffering from acquired TTP. Standard care treatment of these patients involves frequent plasma exchange with fresh frozen plasma. Although substitution therapy with recombinant (*r*) ADAMTS13 is considered preferable, the therapeutic efficacy is likely complicated by free circulating anti-ADAMTS13 autoantibodies that bind and neutralise infused rADAMTS13.

We addressed the feasibility of substitution therapy with rADAMTS13 in acquired TTP by simulating the dynamic *in vivo* situation in a rat model. Development of this model was based on our previous *in vitro* studies which demonstrated a clear linear correlation between inhibitor titer and the amount of rADAMTS13 required to overwhelm the antibodies and to normalise ADAMTS13 activity.

Defined quantities of a purified polyclonal goat anti-ADAMTS13 antibody were administered to normal rats, resulting in animals with a defined inhibitor titer of 12 BU/mL. These animals were then dosed with a single bolus of various concentrations of rADAMTS13. Citrated plasma was obtained at different time points (up to 336 h post-infusion) and levels of ADAMTS13 acitivity, free goat anti-ADAMTS13 inhibitor and anti-ADAMTS13 immune complexes were determined. Pharmacokinetic parameters including *in vivo* recovery and terminal half-life of rADAMTS13 were calculated based on the measured ADAMTS13 FRETS-VWF73 activities. Animals were monitored for clinical signs throughout the observation period. Kidneys were removed at the end of the observation period for histopathological analysis.

At a sufficient dose, rADAMTS13 was capable of building up ADAMTS13 activity in the presence of free inhibitors which became complexed and neutralised immediately upon infusion of rADAMTS13. The terminal half life of rADAMTS13 was calculated to be about 18 h, similar to that in the absence of inhibitors. The inhibitory antibodies remained bound in anti-ADAMTS13 immune complexes which

decreased over time. Even at the highest dose of rADAMTS13 tested, no clinical signs were observed and kidneys showed no abnormalities. The combined data therefore suggest that the immune complexes formed upon administration of rADAMTS13 were cleared over 48 h without causing any obvious adverse events.
Our findings support the concept of achieving therapeutic ADAMTS13 levels in the presence of neutralising anti-ADAMTS13 antibodies, and provide a rationale for rADAMTS13 dosing in patients with acquired TTP.

PA 4.06-6
ADAMTS13 accelerates the cell engraftment efficacy in mouse model of bone marrow transplantation

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Background: von Willebrand factor (VWF) plays a pivotal role on physiologic hemostasis, mediating platelet aggregation under high shear stress conditions. However excessive functions of VWF could cause thrombotic occlusion of microvasculature such as arterial capillaries, where blood flow creates a typical high shear stress. The VWF-cleaving protease ADAMTS13 is therefore thought to down-regulate precisely the VWF function to maintain enriched microcirculation.
Aims: In this context, we hypothesized that this ADAMTS13 role might contribute to better donor cell homing and engraftment in various cell therapy approaches, in which fluent blood flow could be critical in the microcirculation system. To test this hypothesis, we studied the donor cell engraftment in the bone marrow transplantation (BMT) model in *Adamts13*−/− mice.
Methods: Irradiated recipient mice were received 2×10^6 GFP positive cells from the sex-matched GFP donor mice. All of irradiated recipient mice without receiving BMT died within 21 days. Although there is no difference between *Adamts13*−/− and wild-type mice in survival rate after 7 days of BMT, Kaplan-Meier analysis revealed that the percent ratio of survival rate starts significantly declining after 14 days of BMT in the group of *Adamts13*−/− mice. The successful cell engraftment in BMT was assessed by the number of GFP-positive neutrophils in peripheral blood at the several time points from BMT.
Results: The duration achieving the number of GFP-positive neutrophils over 500/μL was found to significantly delay in the *Adamts13*−/− mice, as compared with the wild-type mice (20.2 ± 3.8 vs. 14.4 ± 3.3 days). The delayed cell engraftment observed in the *Adamts13*−/− mice became normalized by the bolus administration of recombinant ADAMTS13 (10 μg/mouse) at the day 0 of BMT. Bone marrow analysis at the day 1 of BMT revealed that the number of GFP-positive blood cells in bone marrow was significantly reduced in the *Adamts13*−/− mice as compared with the wild-type mice, which could result in the delayed cell expansion at the day 7 and day14 of BMT in *Adamts13*−/− mice. The single bolus injection of recombinant ADAMTS13 was found to fully correct the delayed cell expansion in bone marrow in the *Adamts13*−/− mice.
Summary/Conclusion: Our results indicate that the regulation of VWF-mediated thrombotic or inflammatory responses by ADAMTS13 could contribute to better microcirculation which could be critical for efficient donor cell homing and engraftment in BMT, suggesting a therapeutic potential of ADAMTS13 in cell therapy approaches.

PA4.07 – Haemophilia A: Clinical – XIII

PA 4.07-1
Relation between cut-off value of the Bethesda assay and the detection of low titre inhibitors in previously untreated children with severe haemophilia A

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Background: About one third of patients with severe haemophilia A (sHA) develop inhibitors towards infused FVIII. The inhibitor risk depends on genetic and non genetic factors. After the introduction of recombinant FVIII products the reported inhibitor incidences have increased. It is still unclear whether this is caused by higher intrinsic immunogenicity, by more frequent testing, higher awareness in reporting low titre inhibitors or higher sensitivity of the performed tests with a lower cut-off.
Aim: To describe the impact of the cut-off value of the Bethesda assay on the overall inhibitor outcome in previously untreated (PUPs) children with sHA.
Patients/Methods: The RODIN Study (Research of Determinants of Inhibitor development) included PUPs with sHA for the first 75 exposure days (E.D). Uniform prospective data and well-defined outcomes were collected from this patient cohort regularly tested for inhibitors in 29 haemophilia centres in Europe, Israel and Canada. The investigators are members of the European Paediatric Network for Haemophilia Management (PedNet) and/or the RODIN Study group. The centres, which all used the Nijmegen modification of the Bethesda assay, were asked to report on the cut-off value that was used during the study period (2000–2010) and also the frequency of testing during the first 20 E.D and between 20 and 75 E.D.
Results: Inhibitory antibodies were detected in 188 (30%) out of 621 eligible patients. Of all inhibitor patients, 124 (66%) developed a high-titre (> 5 BU) and 64 (34%) a low-titre. Patients developed inhibitors after a median of 15 E.D (inter-quartile range (IQR) 10–20 days), at a median age of 15.5 months (IQR 10.7–19.6 months). During the first 20 E.D, patients were tested every 3–5 E.D in 27 centres. After 20 E.D, the frequency of testing was unchanged in 6 centres and reduced (5–10 E.D to 3 months) in 15. There were 10 centres that used low cut-off values (0.3 and 0.4 BU/mL) and 19 using high cut-off values (0.5 and 0.6 BU/mL, the latter being the highest cut-off value for positivity). Centres that used the lowest cut-off values followed 203 PUPs (32.7%); centres that used the highest followed 418 (67.3%). The inhibitor incidence was 27.5% and 31.6%, respectively. In both groups, 66% of the inhibitors were diagnosed with a high and 34% with a low-titre.
Conclusion: A lower cut-off value did not influence the detection rate of inhibitors. Also the frequency of low vs. high titre inhibitor patients was similar for both cut-off values.
This abstract is submitted on behalf of the PedNet and RODIN Study Group.

PA 4.07-2

Long-lasting recombinant factor VIII Fc fusion (rFVIIIFc) for perioperative management of subjects with haemophilia A in the phase 3 A-LONG study

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Background: To improve the pharmacokinetics (PK) of factor VIII (FVIII), Fc technology was used to develop a monomeric recombinant FVIII Fc fusion protein (rFVIIIFc), with an extended half-life compared with currently available recombinant factor VIII (rFVIII) products. rFVIIIFc comprises a rFVIII molecule genetically linked to the Fc domain of immunoglobulin G_1, with no intervening sequence. The recently completed A-LONG phase 3 study evaluated safety, efficacy, and PK of rFVIIIFc for prophylaxis, treatment of acute bleeds, and perioperative control of bleeding in previously treated subjects with severe haemophilia A and demonstrated a 1.5-fold increase in half-life for rFVIIIFc vs. rFVIII (Advate®).

Aims: To evaluate the efficacy of rFVIIIFc for haemostatic control in the setting of major surgery.

Methods: Eligible male subjects ≥ 12 years old, with severe haemophilia A (< 1 IU/dL [1%] endogenous FVIII), a history of ≥ 150 prior exposure days (ED) to FVIII, and no current/prior FVIII inhibitors, received either individualised prophylaxis (25–65 IU/kg every 3–5 days; Arm 1), weekly prophylaxis (65 IU/kg; Arm 2), or episodic (on-demand; Arm 3) treatment. Subjects from each arm were eligible to enter the surgery subgroup for assessment of rFVIIIFc in perioperative management if they required major surgery, had ≥ 12 EDs to rFVIIIFc and negative inhibitor titres following this period and within 4 weeks prior to surgery. Dosing for subjects in this subgroup was determined by the investigator based on the subject's rFVIIIFc PK profile, dose regimen of FVIII generally required for the planned surgery, and bleeding status.

Results: Overall, nine major surgeries were performed in nine subjects (eight subjects from Arm; 1 subject from Arm 2), including knee arthroplasty ($n = 5$), laparoscopic inguinal hernia repair ($n = 2$), appendectomy ($n = 1$), and arthroscopy ($n = 1$). Haemostatic response with rFVIIIFc was rated by investigators/surgeons as excellent (8/9) or good (1/9) for all nine surgeries. Median (range) estimated blood loss, available for 7/9 surgeries, was 15.0 (0, 600) mL during surgery and 0.0 (0, 1100) mL post-operatively (post-surgical drainage). A single injection of rFVIIIFc was sufficient to maintain haemostasis to the end date/time of all major surgeries, at a median (range) dose of 51.4 (50, 77) IU/kg. Median (range) rFVIIIFc consumption (summarized over all injections during each referenced time period) was 80.6 (65.8, 115.4) IU/kg on the day of major surgery, 161.3 (45.8, 237.3) IU/kg for Days 1–3 days following surgery, and 387.1 (28.1, 728.8) IU/kg for Days 4–14 following surgery. No subjects reported a bleeding episode during the postoperative or rehabilitation periods. Overall, seven adverse events (AEs) were reported in 4 (44.4%) subjects in the surgery subgroup, of which six AEs were of mild or moderate severity, and one AE was considered severe. Two serious AEs (inguinal hernia and appendicitis) were reported in two subjects. All AEs during the perioperative period were assessed by the investigators as unrelated to rFVIIIFc treatment.

Summary/Conclusions: The results from this surgery study showed that rFVIIIFc effectively maintained haemostasis in all major surgeries, and suggest that perioperative haemostasis achieved after infusion of rFVIIIFc was comparable to that expected for similar surgeries in subjects without haemophilia.

PA 4.07-3

The importance of biomarkers of joint damage in monitoring the efficacy of different prophylaxis regimens for severe haemophilia A

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Background: Haemophilic arthropathy, with characteristic joint damage, is the main cause of morbidity in individuals with severe haemophilia A. The most important clinical strategy for management of these patients is treatment by continous prophylaxis with intravenously applied factor (F) VIII. Recently, it was shown that serum and/or urine biomarkers of cartilage turnover in joints reflected the degree of total joint degradation in haemophilia patients.

Aims: The aims of this study were to detect correlations between serum and urine concentrations of biomarkers of joint cartilage degradation and the radiological score for haemophilic arthropathy, as well as to estimate whether measurement of these biomarkers could be useful in monitoring the efficacy of different (secondary) prophylaxis regimens for severe haemophilia A.

Methods: This single-center study included 20 adult males with severe haemophilia A manifested by plasma FVIII < 1% of normal, without inhibitor. The first group involved five patients treated with full-dose prophylaxis: 20 U/kg three times per week. The second group included five patients given intermediate-dose prophylaxis: 10–15 U/kg three times per week. The third group consisted of 10 patients treated on demand (i.e. only in acute bleeding episodes). The following joint cartilage degradation products were measured: serum cartilage oligomeric matrix protein (COMP) and urinary C-terminal telopeptide of type II collagen (CTX-II). Blood and urine samples were collected initially, before the start of treatment (marked as COMP-1 and CTX-II-1) and after 3 months follow-up (marked as COMP-2 and CTX-II-2). Radiological evaluation of haemophilic arthropathy was estimated initially according to the Pettersson score. Approval from the local Ethics Committee and informed written consent were obtained from each subject.

Results: The mean age of the patients was 32 years (range 19–55). The results showed significant positive correlations between the number of points in the Pettersson score and both COMP level ($r = 0.602$, $P = 0.006$) and CTX-II level ($r = 0.580$, $P = 0.009$). In the group of patients given full-dose prophylaxis, the mean value for COMP-2 was significantly lower than that for COMP-1 ($P = 0.043$), while in the group of patients receiving intermediate-dose prophylaxis and in those treated on demand the mean values of COMP-2 were not significantly changed when compared to those for COMP-1. Likewise, in the group of patients treated with full-dose prophylaxis, the mean value for CTX-II-2 was significantly lower than that for CTX-II-1 ($P = 0.014$). Moreover, the mean value of CTX-II-2 was also significantly decreased compared to that for CTX-II-1 ($P = 0.028$) in the group

receiving intermediate-dose prophylaxis. The mean values of CTX-II in the group of patients treated on demand showed no change.
Conclusions: Joint cartilage degradation products, such as the biomarkers: serum COMP and urinary CTX-II, can provide an estimation of the amount of joint damage in patients with haemophilia A. Measurement of serum/urinary biomarker levels is useful for monitoring the efficacy of the applied doses of FVIII in different treatment approaches towards these patients.

PA 4.07-4

Factor VIII genotype and correlation with the hemophilia severity score

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Background: Hemophilia A is classified into mild (> 5–40%), moderate (2–5%) and severe (≤ 1%) disease based upon plasma factor activity levels. Severity of bleeding is commensurate with the baseline factor levels in general; however heterogeneity of bleeding patterns in patients with severe disease is well described. The Hemophilia Severity Score (HSS) is a validated measure of phenotypic severity that takes into account the annual incidence of joint bleeds, the World Federation of Hemophilia Orthopedic joint score and annual factor consumption. The joint score and factor consumption are adjusted for age at the start of prophylaxis and body weight. Multiple factor VIII (F8) mutations have been described.
Aim: We examined the relationship between genotype and phenotypic severity in a cohort of patients with severe hemophilia A.
Methods: After informed consent was obtained, patients with severe hemophilia A (≤ 1%) were recruited from The Emory Hemophilia Treatment Center during routine clinic visits. Patients with concomitant bleeding disorders or thrombocytopenia (< 100,000/μL) were excluded. Each patient received a HSS score. In addition, genotype data was recorded if known or performed if unknown. Mutation analysis was performed by sequencing, multiplex ligation-dependent probe amplification, and polymerase chain reaction for inversions in F8 introns 22 and 1. The F8 genotype was classified as large deletions (single exon or multiple exons), nonsense mutations, intron 1 and 22 inversions, small deletions/insertions/combined deletions and insertions, missense mutations, frameshifts and splice sites. We considered severe molecular defects to include intron 1 and 22 inversions, nonsense mutations and large deletions, while less severe molecular gene defects included missense, splice site, small deletions and frameshift mutations. Associations between genotype and HSS phenotype were examined via one-way ANOVA. Association between severity of mutation and HSS phenotype was determined via t-test.
Results: To date, 71 patients with severe hemophilia A enrolled on the study. The patients ranged in age from 3 to 61 years. Full HSS and genetic data were available on 48 patients. Four (8%) patients manifested large deletions, 18 (38%) had inversion 22, 4 (8%) exhibited intron 1 inversion, 3 (6%) had nonsense mutations, 7 (15%) demonstrated frameshifts, 10 (21%) with missense mutations and 2 (4%) had splice site changes. The mean HSS differed significantly among the mutation types $P = 0.032$. The patients that manifested severe mutations had a significantly higher mean HSS (1.05 vs. 0.54) than those with less severe mutations, $P = 0.0045$.
Summary/Conclusion: We have demonstrated that bleeding severity as scored by the HSS correlates with the type and severity of genetic mutations. We surmise that patients with less severe mutations may produce small amounts of F8 and this may correlate with a less severe bleeding phenotype. These results suggest that assessment of F8 genotype, above and beyond F8 activity, may provide useful information regarding phenotypic severity in patients with severe hemophilia.

PA 4.07-5

A new treatment concept for haemophilia: safety, pharmacokinetics and pharmacodynamics of single i.v. and s.c. doses of a monoclonal anti-TFPI antibody in healthy males and haemophilia subjects

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Background: Regular prophylaxis with either factor VIII or IX is the current gold standard of care for patients with severe haemophilia to prevent joint damage. Frequency of the injections, poor venous access, cost, compliance and time commitment continue to be barriers for widespread use across the world. Novo Nordisk has developed a monoclonal antibody (mAb 2021) targeting tissue factor pathway inhibitor (TFPI). If successful, mAb 2021 has the potential to alter current concepts of prophylaxis in all types of haemophilia, including convenient subcutaneous (s.c.) administration with potential for improved compliance.
Aims: Safety was the primary objective. Secondary objectives were pharmacokinetics (PK) and pharmacodynamics (PD) of mAb 2021 after single i.v. and s.c. doses in healthy subjects (HS) and subjects with haemophilia A or B (patients).
Methods: This was a phase 1, multi-centre, placebo-controlled, double-blind trial. Escalating single i.v. and s.c. doses were administered to healthy subjects ($N = 28$) and patients with haemophilia ($N = 24$). Informed consent was obtained from all participating trial subjects. The trial was approved by the relevant ethical committees. I.v. dose cohorts for HS: 0.5, 5, 50 and 250 μg/kg; and for patients: 250, 1000, 3000 and 9000 μg/kg. S.c. dose cohorts for HS: 50, 250 and 1000 μg/kg; and for patients: 1000 and 3000 μg/kg. Four subjects were included in each dose cohort of which one received placebo.
We registered all Adverse Events (AEs), including Serious Adverse Events (SAEs), local tolerability, laboratory assessments, anti-drug antibodies, vital signs and ECG. A mAb 2021 ELISA was used for PK, and a residual TFPI functionality assay based on FXa generation for PD. An ELISA measured plasma TFPI.
Results: There were no SAEs and no anti-drug antibodies. Fifty-seven of 76 AEs were mild, 17 were moderate and two were graded as severe (one endodontic procedure, and one sciatica, both unlikely related to mAB 2021). Nineteen AEs occurred after placebo. Five AEs were judged by investigators as related, of which three occurred after administration of mAb 2021. Two of these were graded as mild and one as moderate severity, the latter a small superficial thrombophlebitis in a HS in the 1000 μg/kg s.c cohort, manifesting only as local skin tenderness and diagnosed with ultrasound. The symptom disappeared spontaneously, without treatment, the day after diagnosis. Injection site reactions were few, all mild except for one moderate.
There were no clinically relevant changes in Platelets, AT, APTT and PT. As expected a dose dependent procoagulant effect of mAb 2021 was seen as increased levels of D-dimers and prothrombin fragments 1 + 2.
Non-linear PK of mAb 2021 was observed due to target mediated clearance. A maximum AUC of 33.960.278 h*ng/mL and a maximum concentration of 247.104 ng/mL was measured at the highest dose, 9000 μg/kg i.v. Residual TFPI functionality and Total free plasma TFPI levels decreased in a mAb 2021 concentration dependent manner.
Conclusions: mAb 2021 was found to be safe after i.v. and s.c. administration. PK was influenced by target mediated clearance. A mAb 2021 concentration dependent effect was observed on plasma TFPI functionality and levels.

PA 4.07-6

Validation of the colorado adult joint assessment scale in patients with severe hemophilia A

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Background: Valid rating scales for the physical examination of joints are required for controlled studies of hemarthropathy in patients with hemophilia A. The Hemophilia Joint Health Score (HJHS) was previously validated in a pediatric population; a new joint rating scale for adults with hemophilia is also needed.

Aim: To demonstrate the validity of the Colorado Adult Joint Assessment Scale (CAJAS) for measuring joint status in adults with severe hemophilia A

Methods: Data for this validation study were obtained from a prospective, randomized, parallel-group study (SPINART; ClinicalTrials.gov identifier: NCT00623480), which compared routine prophylaxis vs. on-demand treatment with sucrose-formulated recombinant factor VIII in 84 male patients aged 15–50 years. Baseline CAJAS scores were evaluated for linearity (ie, distribution properties of baseline scores, range of observed values, floor and ceiling effects, correlation with bleeding episodes) and reproducibility (ie, variability among different raters, sites, and countries). Item analysis was conducted using multivariate analysis to evaluate item uniqueness, scoring difficulty, and discriminating properties (ie, ability to discriminate between healthy and unhealthy joints). Factor analysis was conducted using the maximum likelihood method to identify domains specific to the adult patient population.

Results: CAJAS scores moderately correlated with age ($r = 0.52$), similar to the expected correlation of joint health with total lifetime bleeding ($r = 0.43$–0.50) reported for older joint assessment scales and consistent with the expectation that all patients with hemophilia will experience joint bleeding episodes and that older patients will have accumulated more bleeding episodes than younger patients. The CAJAS demonstrated excellent reproducibility between countries despite widely varying degrees of joint damage and evaluator training. Few patients (3%) had CAJAS scores of 0, and no patient had a maximum score, demonstrating no adverse floor or ceiling effects. The CAJAS scores had excellent correlation with radiologic evaluations of joint damage ($r = 0.71$); this correlation is higher than the correlation of the physician joint health assessment to HJHS ($r = 0.42$) reported by Feldman et al. Items clustered by two important International Classification of Functioning, Disability, and Health factors that were likely related specifically to adult functions: Activity Functional Impairment and Structural Functional Impairment. Internal reliability was good, with an overall Cronbach's alpha of 0.68; this was lower than the Cronbach's alpha reported in the literature for the HJHS (alpha, 0.86). This result may have been due to the increased heterogeneity of disease severity in the SPINART population.

Conclusions: The CAJAS demonstrated good to excellent content validity and reliability for use in adults. Although the CAJAS is similar to previous scales, such as the HJHS, it also incorporates items consistent with adult joint health that were deleted from later versions of the HJHS. The items removed from HJHS were shown to be important and reliable items that increase the overall discriminant ability of the CAJAS.

PA4.08 – Heparin and Heparinoids – II

PA 4.08-1

Procoagulant red blood cells diminish the protective effect of prothrombinase on factor Xa inhibition by fondaparinux and low molecular weight heparin

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Background: Numerous red blood cell (RBC) disorders have been linked with having procoagulant RBCs that over-express phosphatidylserine on their outer leaflets. We have previously demonstrated that prothrombinase (IIase) formed on procoagulant RBCs protects Xa from inhibition by antithrombin (AT) + unfractionated heparin, consistent with vesicles and platelet IIase systems. Others have also shown that IIase on vesicles and platelets also protects Xa from inhibition by fondaparinux and low molecular weight heparin (LMWH). However, inhibition of the RBC-IIase system by fondaparinux and LMWH has never been studied.

Aim: This study was performed to determine the inhibition of Xa within the IIase complex by AT+fondaparinux or AT + LMWH when the IIase forms on a procoagulant RBC surface.

Methods: Discontinuous second order rate constant assays were carried out to obtain k_2-values for inhibition of free or RBC-IIase-bound Xa by AT + fondaparinux or AT + LMWH. Freshly isolated RBCs were activated for 15 min with phosphatidic acid and calcium at room temperature prior to addition of Va, Xa, Ca^{2+} and pefabloc-TH in buffer. After an additional 3 min incubation, IIase was reacted with prothrombin (II), followed by addition of inhibitors (AT + fondaparinux or AT + LMWH) at specific time intervals. The reactions were neutralized by simultaneous addition of polybrene, Na_2EDTA and S-2222 in buffer. The remaining enzyme activity was obtained at 405 nm, and the k_2-values calculated from plots of lnVt/Vo vs. time.

Results: The k_2-values for inhibition of free Xa by AT+fondaparinux and AT+LMWH were $6.15 \pm 0.87 \times 10^6$/M/min and $1.35 \pm 0.36 \times 10^7$/M/min, respectively. However, incorporation of Xa in the RBC-IIase (without II) gave k_2-values that were slightly increased to $7.57 \pm 2.58 \times 10^6$/M/min for AT + fondaparinux and $1.73 \pm 0.43 \times 10^7$/M/min for AT+LMWH. Thus, protection of Xa by IIase appeared to be diminished on the surface of RBCs. When experiments were repeated with addition of II to the RBC-IIase system, there was a further slight increase in k_2-values to $8.38 \pm 2.58 \times 10^6$/M/min for AT+fondaparinux and $1.93 \pm 0.82 \times 10^7$/M/min for AT + LMWH. Since protection of Xa is not observed on RBCs, we performed additional experiments with synthetic phospholipid vesicles to confirm whether the protective effect was retained in that lipid system. We observed that, relative to free Xa, the k_2-values of the vesicle-IIase system (without II) were reduced to $2.33 \pm 0.83 \times 10^6$/M/min for AT + fondaparinux ($P < 0.001$) and $1.18 \pm 0.07 \times 10^7$/M/min for AT + LMWH ($P$ = ns), thus re-establishing the protective effect.

Summary/Conclusion: Inhibition rates from this study are one and two orders of magnitude lower for LMWH and fondaparinux, respectively, compared to previously reported rates for heparin. Moreover, IIase protection of Xa inhibition by AT + fondaparinux and AT + LMWH was not observed on procoagulant RBCs. In fact, incorporation of Xa within the RBC-IIase even trends slightly towards increased rate of inhibition, which is opposite to results previously reported for other cell surfaces. To ensure the functionality of our system, we repeated the experiments utilizing vesicles instead of RBCs, and demonstrated that Xa protection was restored to previously reported values for both inhibitors. Therefore, surface complexation of IIase on RBCs may provide suitable conditions for relative enhancement of Xa inhibition by fondaparinux and LMWH.

PA 4.08-2

Binding and inhibition of drug transport proteins by heparin – a significant role in modulation of multidrug resistance in human breast cancer cells

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Background: A number of transmembrane drug transporter proteins, including the ATP-binding cassette (ABC) active transporters which require the energy of ATP hydrolysis as well as non-ABC transporters, are involved in multidrug resistance (MDR) to chemotherapeutic agents. In cancer patients undergoing chemotherapy, treatment with heparin has been shown to prolong survival compared to patients who have not received heparin treatment, yet the fundamental mechanisms underlying this survival benefit are largely unknown. We hypothesised that one of the possible mechanisms is that heparin treatment enhances the efficacy of chemotherapy.
Aims: To clarify whether heparin could act as aMDR modulator and thereby increase the potency of chemotherapeutic drugs in cancer patients.
Method: Four cancer cell lines, including a human enriched breast cancer stem cell (CSC) line, two of human breast cancer cell lines, MCF-7 and MDA-MB-231, and a human lung cancer cell line A549 were used. The ability of heparin to bind to individual drug transporter proteins was investigated by extraction on Heparin-agarose beads. The effect of heparin on the function of drug transporter proteins was investigated by measuring the rate of efflux of different drug transporter substrates from cancer cells in the presence of heparin using flow cytometry; Measurement of the effect of heparin on the ATPase activity of each of the ABC transporter proteins was carried out using individual ATPase kits (Solvo Biotechnology, Hungary). shRNA for *lung-resistance-protein* (LRP) – one of major non ABC transporters, was used to generate stable LRP knockdown cell lines. The sensitivity of chemotherapeutic drugs in treated cells was measured by cytotoxicity assay.
Results: Heparin was shown to bind to several of the drug transport proteins from ABC and non ABC transporter system. Among the ABC system, heparin binding caused a significant inhibition of the ATPase activity of ABCG2, and to a lesser extent of ABCC, and this was associated with significant inhibition of the efflux function observed as enhanced intracellular accumulation of substrates of the ABC transporter system in tumour cells. The cytotoxicity towards breast CSCs of each of a number of chemotherapeutic substrates of the ABC transporter system was increased when used in combination with heparin. As one of the major non-ABC transporter proteins, LRP was also confirmed to be a heparin binding protein. The expression of LRP was up or down regulated by 17β-estradiol treatment or shLRP transfection, respectively results which corresponded with the rate of intracellular accumulation and cytotoxicity of doxorubicin in MCF-7 cells.
Conclusion: Heparin acts as an inhibitor of a range of ABC and non-ABC transporter proteins, by blocking transporter protein activity and modulating drug metabolism and toxicity. It is proposed that these interactions are responsible, at least in part, for the increased survival of heparin treated cancer patients.

PA 4.08-3

Heparin induced apoptosis of breast cancer cells is associated with modulation of endogenous Protein Kinase C (PKC) activities

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Background: Heparin exerts a pro-apoptotic effect on a variety of cells, but the fundamental basis of this action is little understood. It may well be due to its effect upon the primary signalling pathways regulating the cell cycle, such as the PKC system since previously heparin has been shown to antagonise serum or phorbol 12-myristate 13-acetate (PMA) induced PKC isoenzyme gene expression. These effects may arise from the ability of heparin to bind to and modulate the function of many of the growth factors and other agonists found in serum. Whether heparin is also able to affect PKC activity directly remains unclear.
Aims: The investigation of the principal interactions contributing to the pro-apoptotic effect of heparin on breast cancers cells.
Methods: Two human breast cancer cell lines (MCF-7 and MDA-MB-231) were cultured under serum-free conditions (in which cells are maintained by the activity of autocrine growth factors) in the presence of either unfractioned heparin (UFH) or a low molecular weight heparin (LWMH, Fragmin). Cell cycle and apoptosis assays were performed using flow cytometry. Based upon observations obtained by gene microarray analysis, the expression of a selection of pro-apoptotic proteins was analysed by Western blot. The level of phosphorylated protein kinases in signalling pathways involved in apoptosis was investigated in treated cells by Western blot and immunofluorescence assay.
Results: Cell cycle profiling of cell derivedDNA showed that MCF-7 cells were arrested in the G0-G1 phase when treated with each of the heparins. The percentage of apoptotic cells was also significantly increased by heparin treatment. Moreover, the percentage of apoptotic cells induced by treatment with heparin in combination with chemotherapeutic drug(s) was higher than in cells treated with drug or heparin alone. The level of phosphorylated protein kinases of signalling pathways involved in apoptosis was also modulated by heparin in both cell lines, including the reduction in the level of phosphorylation of members of the Raf/MEK/ERK pathway (p-ERK, p-Ref) and PI-3K/Akt pathway (p-Akt, p-PTEN)., The endogenous activity of each of a number of PKC isoenzymes, which are capable of triggering an apoptotic response, was also reduced by heparin treatment, including p-PKCα/βII, p-PKCδ, p-PKCθ, and p -PKD/PKCμ. In addition, the activity of p-PKCε, a transforming oncogene and possible tumour biomarker was reduced. The expression of a numbers of pro-apoptotic proteins was also regulated by heparin in both cell lines and was confirmed by RT-PCR and Western blot – these included TNFα, p-GSK3α/βII and active-caspase-3, and regulator proteins p21, p53 and Hsp90.
Conclusion: The pro-apoptotic action of heparin is associated with the modulation of the activity of a number of key signalling pathways, including endogenous PKC activity as well as increased expression of a number of pro-apoptotic proteins. This ability of heparin to promote apoptosis may contribute to the favourable effect of heparin treatment on cancer therapy.

PA 4.08-4

Protamine sulfate neutralization of anticoagulation by a potent antithrombin-heparin covalent complex

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Background: Heparin is a well-established anticoagulant for treatment of thromboembolic disease. Heparin catalyzes inhibition of several coagulation factors, including thrombin (IIa) and factor Xa (Xa), by enhancing the activity of antithrombin (AT). Despite development of low molecular weight heparins (LMWHs) and factor-specific direct anticoagulants, unfractionated heparin (UFH) remains the anticoagulant of choice in some clinical situations. Limitations of UFH include a short intravenous half-life, AT-dependence, and only one in three molecules have anticoagulant activity. We have developed a potent anticoagulant consisting of covalently linked AT and UFH (ATH). ATH has increased half-life, both AT-dependent and independent activities, faster reaction rates with coagulation factors, and 100% of its heparin chains are anticoagulant. Thus, ATH may be a more favourable anticoagulant than UFH for specific indications. When a patient receiving

heparin is experiencing acute bleeding or requires emergency surgery, anticoagulation is neutralized by administration of protamine sulfate. Protamine is also used to reverse heparin following cardiopulmonary bypass surgery (CPB). Protamine is a small charged protein which binds electrostatically to heparin forming an inactive complex. Protamine is less effective at inhibiting LMWHs, and factor-specific inhibitors currently do not have any known direct antidote. Thus far, ATH neutralization with protamine has not been studied.

Aim: To determine if protamine can neutralize the anticoagulant activity of ATH.

Methods: ATH or AT + UFH were exposed to various concentrations of protamine, before incubating with purified IIa or Xa. Residual enzyme activity against chromogenic substrates was monitored spectrophotometrically and expressed as a percentage of uninhibited enzyme activity in the presence of an equal amount of protamine. Additionally, normal human pooled plasma mixed with 0.2 U/mL ATH or UFH was treated with protamine, followed by initiation of clotting with calcium and dilute tissue factor. Time to clot was monitored turbidimetrically and defined as the time to reach half maximal turbidity.

Results: Inhibition of IIa by ATH or AT + UFH was fully reversed with protamine, both requiring the same molar ratio of protamine: anticoagulant. A 4:1 molar ratio of protamine:UFH recovered 88.7 ± 2.2% of uninhibited Xa activity, whereas the maximum recovery of Xa activity in the presence of ATH was 78.4 ± 1.6%. Increasing amounts of protamine gave no further reversal. In plasma, 0.2 U/mL ATH and UFH extended the time to clot from 3.2 ± 0.1 to 13.6 ± 0.6 and 18.7 ± 1.3 min, respectively. Protamine, at 1 μg/mL, reduced the time to clot for both ATH and UFH to 3.8 ± 0.1 min, although it had a slight anticoagulant effect (4.6 ± 0.1 min) in plasma on its own.

Conclusions: IIa inhibition by ATH or AT + UFH was completely abolished by protamine in a purified system. Interestingly, in similar experiments with Xa, protamine did not entirely reverse the inhibitory effects of ATH, or even those of AT + UFH. However, protamine fully neutralized ATH and UFH anticoagulation when determined by clot formation in plasma. Further experiments studying protamine efficacy against ATH *in vivo* are warranted. These results contribute significantly to the development of ATH as a clinical therapeutic option.

PA 4.08-5

In vitro immunogenicity assessment of branded enoxaparin and a US generic version of enoxaparin

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Background: With the approval of generic enoxaparin for clinical use in the US, and the fact that that low molecular weight heparins (LMWHs) are not immunogenically inert, it is of interest to understand if the immunogenic response of the generic enoxaparin is equivalent to that of branded enoxaparin (Lovenox®).

Aims: The focus of these *in vitro* studies was on heparin-induced thrombocytopenia (HIT) where cross-reactivity of generic enoxaparin to HIT antibodies was compared to the reactivity of branded enoxaparin.

Methods: Five batches each of branded (Sanofi-aventis; Bridgewater, NJ) and generic (Sandoz US; Princeton, NJ) enoxaparin were studied. Drugs were purchased through hospital pharmacies as pre-filled syringes containing 40 mg drug. HIT antibodies from multiple patients (minimum $n = 9$ sera strongly ELISA and SRA positive) and platelets (pre-screened for platelet assay reactivity) from different donors were employed to assure the robustness of the data. Platelet activation by HIT antibodies was determined by the clinical ^{14}C-serotonin release assay (SRA; using washed platelets) and the heparin-induced platelet aggregation assay (using platelet rich plasma). The metabolic capacity of platelets after incubation with HIT antibodies and LMWH was determined by our MPA colorimetric assay (*Platelets* 2012;23:69) using the AQ_{ueous} One Solution (Promega; Madison, WI) indicator dye to detect mitochondrial activity. PF4 displacement by LMWH was determined using the PF4-Enhanced ELISA (GTI; Waukesha, WI) and detecting HIT antibody binding.

Results: Over multiple runs mean values did not reveal significant differences between the generic in comparison to the branded enoxaparin for PF4 affinity or effect on platelet function (activation, aggregation, metabolic potential) due to cross-reactivity with various HIT antibodies. However, what was revealed were variable responses between the tested batches of generic enoxaparin. This batch-to-batch variation was not observed with the branded enoxaparin that gave a more consistent response. This signal would have been masked if individual data were not evaluated and the data was only analyzed as an average.

Conclusion: This was a comprehensive evaluation of the immunogenic potential of generic enoxaparin compared to branded enoxaparin using multiple sources of HIT antibodies as well as several different assay systems. While no obvious difference between drugs in these *in vitro* assays was observed in a general sense, the significant finding was a higher batch-to-batch variation for the generic enoxaparin. This finding is of importance since an enhanced immunogenic response of even some batches of generic enoxaparin make the branded and generic materials non-equivalent. It also would raise concern for clinicians of true interchangeability between branded and generic products. It is thus of clinical interest to include investigations into the immunogenic response of complex biological drugs, such as generic LMWHs, for regulatory recommendations.

PA 4.08-6

Non-anticoagulant heparin analogue protects against ConA-induced acute hepatitis and sepsis

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Sepsis is a worldwide healthcare problem for which adequate therapy is currently not available and severe sepsis is associated with a mortality rate of up to 50%. Recent findings have identified extracellular histones as major mediators of hyperinflammation in sepsis. Histones are major protein components of neutrophil extracellular traps that are formed in response to infection. Consequently histones were identified as interesting therapeutic targets for the treatment of hyperinflammation and sepsis.

Unfractionated heparin (UFH) has the highest negative charge density of all known biological molecules and is a good candidate to bind positively charged extracellular histones to potentially neutralize them. The disadvantage of heparins for clinical use is the associated risk for bleeding complications. To this end, we prepared a heparin-analogue, HA, that has only marginal anticoagulant effects.

The *in vitro* and *in vivo* effects of UFH and HA were tested and we demonstrate that both UFH and HA dose-dependently inhibit the cytotoxic effects of histones on endothelial cells. In a ConA-triggered mouse model for fatal liver injury and in a CLP model, HA administration could increase survival from 40% in the untreated group till 90–100% in the treated groups, with no signs of bleeding in the treated groups.

We show that HA can be used as a safe and effective means of protection against histone-mediated death *in vivo* and conclude that HA has a strong therapeutic potential to treat hyperinflammatory responses in humans, such as occur during sepsis.

Disclosure of Interest: The author declares no conflict of interest.

PA4.09 – Von Willebrand Factor – VI

PA 4.09-1

Acquired von willebrand factor deficiency and bleeding risk in a rabbit model of aortic stenosis

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Background: Acquired deficiency of von Willebrand factor (VWF) is characterized by the loss of high molecular weight (HMW) multimers. HMW multimers have been reported in association with high shear-cardiovascular disorders, mainly in aortic valve stenosis. Although it has been shown that acquired deficiency of VWF is improved after correction of the pathological condition, the causal evidence of a pathological shear condition on HMW multimers changes has not been established in an experimental model *in vivo*. Moreover the involvement of these selective HMW multimers in the bleeding risk is not demonstrated.

Aims: Our aim was to provide an *in vivo* causal evidence between loss of HMW multimers and aortic stenosis in an animal model and to evaluate the bleeding risk induced by the stenosis.

Methods: We developed a rabbit model of calibrated supra aortic stenosis. All experiments were conducted in syngeneic male New Zealand rabbits (2.7–3 kg) in accordance to the declaration of Helsinki. The ascending aorta was surgically exposed after a median sternotomy under general sedation. A severe stenosis > 75% was performed using a vascular silicone occluder placed on the ascending aorta. The occluder was inflated with a device allowing a reproducible stenosis. Blood was sampled at baseline (T0) and at 30 min (T30) after stenosis for VWF antigen and multimeric profile assessment in 17 animals. The multimeric structure of plasma VWF was analyzed as previously described in humans and the revelation was adapted for rabbits. The percentage of the highest molecular weight multimers (more than 15 mers) was determined using densitometric scanning. Results were expressed as a ratio vs. baseline value.

In this model, a hepatosplenic surgical cut was performed in 20 rabbits randomly allocated to two groups (stenosis/sham, $n = 10$ each) to evaluate blood loss. Blood was collected through four gauze compresses previously disposed around the liver and the spleen. Hepatosplenic blood loss was considered as the weight of compresses after procedure. Potential confounding variables (pH, platelet count, HCO3, PCO2, body temperature) were monitored throughout procedure, body temperature was maintained using a heating blanket.

Results: A significant decrease of HMW multimers was observed (ratio 0.77 ± 0.07; $P < 0.0001$) after the induction of the stenosis when compared to baseline values. HMW multimers remained unchanged in sham group (1.03 ± 0.07;ns). VWF:Ag remained unchanged in the both groups during the whole procedure.

A trend for an increase in blood loss was observed in the aortic stenosis group ($11.8\ g \pm 5.6$) when compared to the sham group ($7.1\ g \pm 2.1$; $P = 0.06$). No significant changes were observed in confounding variables.

Summary/Conclusions: We provide the first *in vivo* causal evidence of HMW multimers loss associated with aortic stenosis. This selective loss of HMW multimers was associated with a moderate increase in bleeding risk.

PA 4.09-2

Loss of either Fut1 or Fut2 fucosyltransferase is associated with significantly elevated von Willebrand Factor (Vwf) levels in mice

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Background: von Willebrand Factor (VWF) is a critical coagulation glycoprotein which stabilizes Factor VIII, adheres at sites of vascular injury, and binds platelets. VWF is synthesized in megakaryocytes and vascular endothelium, where it undergoes complex post-translational processing, including extensive glycosylation, prior to secretion. VWF levels vary widely in humans, and the carbohydrate blood group, ABO, is a well-known genetic modifier of VWF. 'H' glycan is the precursor structure for ABO, and thus is also a candidate to influence VWF. H glycan is generated by two alpha-1,2-fucosyltransferase genes, *FUT1* and *FUT2*. In humans (but not mice), *FUT1* is expressed in erythrocytes and vascular endothelium. In both humans and mice, *FUT2* (and to a lesser extent, *FUT1*) is expressed in mucosa. Human homozygotes for *FUT1* nonsense mutations ('Bombay' blood type) are very rare and exhibit low VWF levels, likely due to the absence of H (and therefore ABO) on endothelial-cell derived VWF. On the other hand, individuals homozygous for *FUT2* nonsense mutations ('non-Secretor') are common and have been reported to have slightly higher VWF levels, but the literature is conflicted. To investigate the hypothesis that mucosal H glycan expression influences VWF, we examined Vwf levels in *Fut1* and *Fut2* knock-out mice.

Methods: *Fut1* and *Fut2* knock-out animals (backcrossed > 20 generations to C57BL6/J prior to analysis) were obtained from the Jackson Laboratory. Heterozygous F1 animals were intercrossed to generate F2 offspring. Littermate controls were generated for each knock-out mouse line separately. Male mice aged 8–12 weeks were studied to avoid the effects of estrous and aging. Twenty-six individuals were analyzed per group except for *Fut1*−/− ($n = 25$). Platelet-poor plasma was assayed for Vwf antigen (Vwf:Ag) in duplicate by sandwich ELISA [rabbit anti-human VWF (Dako); sheep anti-rabbit VWF (Abcam)] using a pooled C57BL6/J reference standard (100 arbitrary Units/uL).

Results: *Fut2*−/− animals exhibited significantly higher (> 2-fold) Vwf:Ag levels compared to their wild-type littermates (*Fut2*−/− = 217.9 ± 75.3 U/uL; *Fut2*+/+ = 106.7 ± 31.7 U/uL; $P = 5.7 \times 10^{-8}$). Interestingly, *Fut2*+/− animals (heterozygotes) had an intermediate elevation in Vwf:Ag levels (*Fut2*+/− = 155.4 ± 59.7 U/uL; $P = 5.9 \times 10^{-4}$), consistent with an allele dose effect. *Fut1*−/−animals also exhibited Vwf:Ag levels nearly double that of their wild-type littermates (*Fut1*−/− = 186.1 ± 70.8 U/uL; *Fut1*+//+ = 97.9 ± 28.9 U/uL; $P = 2.2 \times 10^{-6}$).

Conclusions: Loss of either *Fut1* or *Fut2* expression is associated with significantly higher Vwf:Ag in mice. This supports previous human studies which found modestly increased VWF levels in non-Secretors. We speculate that the large effect size of *Fut1* and *Fut2* on Vwf levels observed in mice is due to the absence of the confounding effects of endothelial H expression which occurs in humans. Although the mechanism by which mucosal H expression influences circulating Vwf is not yet known, it must be distinct from the classical model of VWF glycosylation in the Golgi of endothelial cells as the tissue-specific expression patterns of *Vwf* and the *Fut* genes do not overlap in mice. We conclude glycosylation variants may influence VWF by more complex mechanisms than previously suspected.

PA 4.09-3

Incorporation of von Willebrand Factor into a fibrin network induces activation of von Willebrand Factor enabling binding of GPIb

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Background: Attachments of platelets from the circulation onto a growing thrombus is a complicated process involving several receptors on the platelet and components in the thrombus. Platelet binding to a thrombus under arterial flow indicates a role for Von Willebrand Factor (VWF) and Glycoprotein Ib (GPIb).

Aim: We investigated a possible interaction between Von Willebrand Factor and fibrin(ogen).

Methods: We measured the interactions of von Willebrand Factor with fibrin(ogen) using different enzymes in a purified system and plasma based system. The following enzymes were used: (i) Plasma purified thrombin cleaves fibrinopepties A (FpA) and B (FpB) and interacts with fibrinogen; (ii) Plasma purified γ-thrombin cleaves FpA and FpB but does not interact with fibrinogen due to not active exosite I; (iii) Arvin only cleaves FpA but not FpB; (iv) The venoms from snakes Agkistrodon contortix contortix and Contralux atrox cleave FpB and the first 42 amino acids (where FpB is located), respectively. To study binding kinetics between fibrin, VWF and Glycoprotein Ib we used ellipsometry and surface plasmon resonance. To visualise the effect of VWF on fibrin structures Scanning Electron Microscopy was introduced.

Results: Applying ellipsometry and surface plasmon resonance we observed that VWF does not bind to either fibrinogen nor to polymerized fibrin. Interestingly, we did establish binding of VWF to fibrin monomers during the process of fibrinogen-to-fibrin conversion in the presence of thrombin. This indicates that VWF is incorporated into the fibrin mesh and that the incorporation more probable occurs in the E domain where FpA and/or FpB are situated. Furthermore, using either γ-thrombin, Arvin, Agkistrodon contortix contortix or Contralux atrox we were able show that VWF was incorporated into the fibrin mesh indicating that cleaving of either FpA or FpB is sufficient. To identify the binding place of VWF to fibrin monomers we tested mutant RGGS-VWF in our system. The lack of incorporation of mutant RGGS-VWF into fibrin indicates that VWF most probably binds to fibrin via its RGD sequence located in the C-terminal part of VWF. Moreover, using truncated GPIb proteins we demonstrated that the incorporated VWF into the thrombus is able to bind GPIb. Furthermore, we have accomplished incorporation of VWF into a developing fibrin mesh under conditions of flow in a purified system. Using Scanning Electron Microscopy we have observed that increasing concentrations of VWF in plasma result in a denser fibrin mesh with significantly thinner fibres.

Summary/Conclusion: We have shown that the C-terminal part of VWF and the E-domain of fibrin monomers are involved in the incorporation of VWF into a fibrin mesh. This interaction explains the incorporation of platelets into a growing thrombus under conditions of arterial shear.

PA 4.09-4

Von willebrand factor acts as a novel antagonist for nitric oxide synthase to promote insulin resistance in hypoxia

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Background: Insulin resistance (IR) and impaired nitric oxide (NO) availability is reported in respiratory diseases such as chronic obstructive pulmonary disease (COPD), obstructive sleep apnoea (OSA) and high altitude (HA) induced cardio respiratory diseases (CRDs). NO plays an important role in preventing vascular diseases through regulation of vascular tone and endothelial functions. High plama level of vWF has been reported in all above mentioned diseases due to endothelial dysfunction. However, the molecular mechanism responsible for impaired NO production and IR in hypoxia remain unknown.

Aim: In this study, we investigated the possible mechanism of impaired NO production and IR during hypoxia in a mice model.

Methods: Mice (Swiss albino, 25–30 g, male, $n = 6$/group) were subjected to HAH (in an environmental chamber) equivalent to the prevailing atmospheric conditions at an altitude of 7628 m (282 mm Hg barometric pressure) having O_2 content approximately 8.5%, humidity $55 \pm 5\%$ and temperature 25 ± 2 °C for 0–24 h. Age and sex matched control mice (not exposed to HAH) were maintained in 21% O_2 with same environmental conditions. Blood was drawn though retro-orbital method and different assays were performed. The expression of vWF was analyzed by ELISA and SDS-immunobloting. The NO assay was done by oxyhaemoglobin methods. The IR resistance parameters were evaluated by glucose tolerance test (GTT), insulin tolerance test, D-[U-^{14}C] – glucose uptake and HOMA-IR. The interaction between vWF and nitric oxide synthase were studied by far-western blotting, coimmunoprecipitation and plasma surface resonance spectroscopy. *In vivo* inhibition of vWF was performed by tail vein injection of anti-vWF neutralizing antibody 2 h before HAH exposure.

Results: Exposure to hypoxia showed a time dependent increase of IR (GTT, ITT & HOMA-IR) as well as multimeric form of vWF and subsequent decrease of NO production. Pre-incubation of time dependent hypoxia exposed animal plasma or recombinant human-vWF dose dependently inhibited insulin (0.24 μM) induced NO production and higher dose of insulin (1.2 μM) reverses the effect. In contrary, pre-incubation of immunodepleted (vWF) plasma failed to inhibit insulin induced NO production, whereas vWF immunoneutralization attenuated hypoxia induced IR and D-[U-^{14}C] – glucose uptake. Furthermore, the interaction between vWF and nitric oxide synthase (NOS) were studied by Far-western blotting, coimmunoprecipitation, surface plasma resonance spectroscopy. The kinetic analysis showed that the dissociation constant (K_D), inhibitory constant (K_i) and IC_{50} are 1.79×10^{-8} M, 250 and 18.31 pM respectively suggesting vWF binds to NOS with higher affinity having greater efficacy for the activator (insulin) inhibition.

Conclusion: These results demonstrated that vWF acts an antagonist for NOS, inhibits insulin induced NO production and exerts IR. These data may have important implications in understanding the pathophysiology of hypoxia induced cardiopulmonary and other metabolic diseases.

PA 4.09-5

Vimentin is a ligand for von Willebrand factor: the role of this interaction in platelet adhesion

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Background: Platelet adhesion to ruptured atherosclerotic plaque or exposed subendothelial matrix is critical to both pathological thrombosis and normal hemostasis, respectively. In this context, the participation of Von Willebrand factor (VWF) under high hydrodynamic force is well documented. The binding of the GPIba from the glycoprotein (GP)Ib/IX/V complex to the A1 domain of VWF is considered as a key step to recruit the rapidly flowing platelets. This interaction enables the platelets to 'roll' over the VWF surface and allows them to firmly adhere to the exposed plaque or extracellular matrix through other platelet receptors. While studying the platelet adhesive/activation properties of two recombinant VWF fragments, the isolated A1 domain and a fragment encompassing the A1A2A3 domains that lacks the binding site for integrin aIIbb3, we obtained intriguing results. First, platelets rolled more slowly on a surface coated with A1A2A3 protein than on an A1-coated surface under arterial shear stress. Second, in sharp contrast to the A1 protein, the A1A2A3 protein activated platelets in a manner similar to the activation induced by full-length VWF under high shear stress.

Aims: This result suggested that the triple A domain protein provides an additional adhesive contact point on platelets. We proposed the existence of another receptor on platelets that binds to the A1A2A3 domains. We considered vimentin since it was identified as a binding protein to the isolated A2 domain of VWF in another ongoing project from our laboratory and vimentin can be detected on the surface of platelets.

Methods: We examined the effect of anti-vimentin antibodies on blocking the interaction of platelets with VWF using parallel flow chamber and flow cytometry. Purified plasma VWF and recombinant proteins were utilized to perform protein-protein binding analyses. Finally, transgenic mice models were used to investigate vimentin as a potential receptor for VWF.

Results: Purified plasma VWF and wild type (WT) A1A2A3 protein bound to vimentin in a ristocetin-dependent manner, whereas an A1A2A3 mutant expressing increased GPIba-binding activity bound without the need of ristocetin. Vimentin was detected on the surface of platelets by flow cytometry. The binding of the A1A2A3 mutant to vimentin on platelets, and the binding of isolated A2 domain to purified vimentin were inhibited with anti-vimentin antibody, V9. Platelet adhesion to WT A1A2A3 protein, and collagen fibrils were inhibited (40–75%) by anti-vimentin antibody under high shear stress. To test whether the interaction of GPIba with A1 domain directly regulates the exposure of vimentin on platelets, we used V9 as a probe of vimentin expression. We perfused blood over a surface coated with a mixture of A1 domain and V9 or A1 and mouse IgG. Platelets rolled and did not attach stably on the A1/IgG surface, but many of them firmly bound to the A1/V9 surface. Lastly, vimentin-deficient mice had an increased tail bleeding times, and the isolated platelets from these mice exhibited reduced adhesion to VWF at high shear stress compared to wild type mice.

Conclusion: We have demonstrated a previously unknown function of vimentin in platelet adhesion to VWF under high shear stress.

PA 4.09-6

Conformation and N-linked glycan determinants within the A1A2A3 domains play critical roles in modulating human von Willebrand factor interaction with macrophages

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Background: Recent studies have shown that hepatic Kupffer cells play a key role in modulating *in vivo* clearance of von Willebrand Factor (VWF). However the molecular mechanisms through which macrophages interact with VWF remain poorly understood.

Aims: In this study, we have utilised a novel Hi-Content-Analysis methodology to characterize the role of VWF domains and glycans in regulating macrophage binding.

Methods: Plasma-derived human VWF was purified using cryoprecipitation and 2BCl gel filtration. Recombinant full length VWF and VWF fragment VWFA1A2A3 were expressed in HEK23T cells, and purified by Nickel-affinity chromatography. VWF point mutations of interest (N1515Q; N1574Q; N1515Q/R1450E and R1450E) were introduced using site-directed mutagenesis. Finally, macrophage-VWF binding was analysed using PMA-differentiated THP-1 cells. VWF binding was quantified using FITC-labelled polyclonal VWF and Hi-Content-Analysis.

Results: In the presence of divalent cations, plasma-derived VWF (pdVWF) and full length recombinant VWF (rVWF) bound to differentiated THP-1 cells in a time- and dose-dependent manner. Binding of pdVWF was markedly attenuated ($P = 0.003$) following pre-treatment with PNGase F, suggesting a critical role for VWF N-linked glycans in modulating macrophage binding. Moreover, pdVWF binding to THP-1 was also significantly inhibited in the presence of either 10 mM mannose or 10 mM galactose respectively. In contrast, pdVWF-macrophage interaction was markedly enhanced in the presence of ristocetin. Similarly, introduction of the VWD Type 2B mutation R1450E also resulted in a striking increase (13-fold; $P < 0.001$) in macrophage-binding. In the presence of ristocetin, VWF R1450E macrophage binding was also increased, but to a lesser degree than the effect observed with wild-type rVWF. Cumulatively these data suggest that VWF confirmation also plays a critical role in regulating macrophage interaction. In view of the major effects of ristocetin and R1450E in regulating the interaction of full length VWF with macrophages, we further investigated the specific contribution of the VWF A1A2A3 domains. Recombinant VWF A1A2A3 also bound macrophages in a dose-dependent manner. Moreover, A1A2A3 binding was significantly enhanced in the presence of ristocetin, and/or following introduction of the R1450E substitution. PNGase-digestion significantly attenuated A1A2A3 binding, suggesting that local N-linked glycans may be specifically important. In keeping with this hypothesis, macrophage-binding was reduced by 50% following introduction of the point mutation N1515Q. In contrast however, VWF A1A2A3 N1574Q binding was unaffected. Loss of the N1515 sugar chain could inhibit macrophage binding by reducing a specific lectin-glycan interaction, or through a secondary local conformational effect. In support of the latter hypothesis, we observed that the inhibitory effect of N1515Q was lost in the double mutant VWF N1515Q/R1450E, which displayed enhanced macrophage-binding in keeping with that of the single mutant VWF R1450E.

Summary/Conclusions: These novel data clearly demonstrate that the conformation and N-linked glycan determinants within the A1A2A3 domains play critical roles in modulating human VWF interaction with macrophages. Further studies will be required in order to further localize VWF binding motifs, and to identify the specific macrophage receptors involved in the physiological clearance of human VWF.

PA4.10 – Anticoagulant Agents – XVII

PA 4.10-1

Real-world management, clinical outcome and predictors of thromboembolism during a 1-year follow-up in patients with atrial fibrillation: results from the ATA-AF study

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Background: Prevention of thromboembolism (TE) is a cornerstone in the management of atrial fibrillation (AF). Oral anticoagulants (OAC) offer the best prevention; however, vitamin K antagonists (VKA) are cumbersome to use, their prescription is generally far from evidence-based recommendations, and some grey zones exist even in recent guidelines as for the optimal treatment of AF patients. Finally, real-world data on long-term management of AF are limited and therefore needed, since they could provide interesting information on the present attitudes and related outcomes, and be an useful benchmarking for the future scenario of treatment with new OAC.

Aims: Aim of the present study was to evaluate real-world management, clinical outcome and predictors of TE in patients with AF and followed-up for 1 year.

Methods: Data were collected from May 2010 to July 2011 in the context of the observational ATA-AF study endorsed by the Italian Scientific Societies of Hospital Internists (FADOI) and Cardiologists (ANMCO). The survey prospectively enrolled, in Internal Medicine and Cardiology settings, patients with current or previous diagnosis of AF, who were followed-up for 12 months. The association with TE was evaluated for a number of potential predictors: age ≥ 75, $CHADS_2$ score ≥ 2, modified HASBLED (without 'labile INR') ≥ 3, previous TE, recent major bleeding, paroxysmal AF, valvular AF, cognitive/functional dysfunction. A P value < 0.05 was considered statistically significant.

Results: A total of 1368 patients were evaluated, with median age 76 years. At baseline, 21.4% of patients had diagnosis of paroxysmal AF, and 27.7% valvular disease. $CHADS_2$ score was ≥ 2 in 62.1% of patients, while 23.0% of patients could be considered at high hemorrhagic risk (modified HASBLED ≥ 3). Percentages of use of VKA or other antithrombotic therapy were 65.7% and 27.1% at study enrollment, and 65.4% and 27.2% among patients who completed the one-year follow-up. Discontinuation of VKA occurred in 116 patients, mainly due to physician's decision; 31 patients started OAC treatment during the follow-up. One-year all-cause mortality was 10.3%, and 22.2% of patients had hospitalization within this time period. Thromboembolism or major bleeding occurred in 2.9% and 1.6% of patients, respectively. $CHADS_2$ score ≥ 2 ($P < 0.05$), modified HASBLED ≥ 3 ($P < 0.05$), previous TE ($P < 0.01$) and cognitive/functional dysfunction ($P < 0.01$) appeared significantly related to the occurrence of thromboembolic events.

Summary/Conclusions: Long-term prevention of TE is challenging in patients with AF, since they frequently have concomitant high thrombotic and hemorrhagic risk. In our survey, the percentage of use of VKA was high if compared to previous studies; in the next years we will evaluate if new OAC might overcome restrictions and disadvantages of VKA and extend OAC therapy. According to our study, occurrence of TE is more frequent than major hemorrhages, and this could suggest the opportunity of further efforts to optimize prevention of TE. This attitude could be particularly stringent in patients who appeared at increased risk of TE in our analysis (those with positive history or present high thrombotic risk, and patients generally receiving suboptimal prevention due to hemorrhagic risk or poor compliance).

PA 4.10-2

Warfarin monitoring with the Fiix-prothrombin time (Fiix-INR) increases time within target range and reduces dose adjustment need compared to standard monitoring using INR

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Background: The quality of vitamin K antagonist (VKA) management has major impact on the efficacy and safety of the anticoagulation, ie the thromboembolic and major bleeding rate. Thus, poor clinical outcome is more frequent among poorly managed patients with a low time within target range (TTR). A low TTR is commonly due to a fluctuating PT-INR. Since PT-INR fluctuation is partly caused by rapid changes in factor VII concentrations that experiments suggest have only minor influence on thrombin generation *in vivo*, we invented a new prothrombin time, Fiix-PT, that is sensitive to the more stable factors II and X only.

Aims: We hypothesized that dosing VKA based on the Fiix-PT (Fiix-INR) could lead to a more stable anticoagulant effect and subsequently better anticoagulation outcome than dosing based on PT-INR monitoring.

Methods: In the single center clinical Fiix study, patients on warfarin with INR target range 2–3 are randomized into two arms, ie. dosing based on Fiix-INR or based on PT-INR (standard control) aiming for at least 1200 patient year observation time. The study is patient, dose-manager and assessor blinded. Fiix-INR and PT-INR values are reported by the laboratory in a blinded manner as 'INR'. Software assisted dosing is managed by specially trained staff using the DAWN® anticoagulation software. Clinical events and surrogate parameters are continously monitored. Surrogate parameter results based on the first nine study months are presented here as per protocol analysis, ie periods of initiation and temporary discontinuation of warfarin are excluded. Numerical and categorical data were analyzed with the Mann-Whitney and Chi square tests respectively.

Results: After 9 months the observation time was on average 5.5 months for each patient, i.e. 224 patient years in 484 patients in the Fiix group and 222 years in 488 patients in the PT group. There were 6.3% fewer monitoring tests in the Fiix group. The median INR was 2.5 in the Fiix group vs. 2.4 ($P = 0.0003$) in controls. Extreme high and low INR′s were less common in the Fiix group. The proportion of tests within target range was 5.2% higher in the Fiix INR group, 71.7% vs. 68.1% ($P < 0.001$). The center TTR was also higher (82.7% vs. 79.4% respectively, $P < 0.0001$). The median individual TTR was 87.2% vs. 81.7% ($P = 0.0007$). The mean (median) interval between monitoring tests was longer or 23.2(21) in the Fiix group vs. 21.9(20) days in controls, $P < 0.01$. The mean dose adjustment frequency was reduced by 11.2% in the Fiix group, from 44.6% to 39.6% ($P < 0.0001$). When only patients observed for 7–9 months were analyzed the dose adjustment frequency was reduced further (13.4%, $P < 0.0001$).

Conclusions: These first surrogate parameter results of the Fiix study show that within 6 months of randomization, the Fiix-INR has led to increased anticoagulation stability and reduced dose adjustment frequency. The improvement has occurred despite a high TTR in the standard monitoring control group. Although preliminary, the results suggest that monitoring VKA therapy with the Fiix-INR instead of

the PT-INR may hold a potential to increase both safety and efficacy of VKA therapy.

PA 4.10-3

Efficacy and safety of weight-adjusted heparin prophylaxis for the prevention of acute venous thromboembolism among obese patients undergoing bariatric surgery: a systematic review and meta-analysis

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Background: Obesity is a growing global problem putting people at risk for VTE. Obese patients are often excluded from clinical trials or are not recruited in sufficient number to assess safety and efficacy of LMWH in this population. The bariatric surgical population is a particularly high risk population for VTE. It is unclear if standard (i.e. non-adjusted) thromboprophylaxis doses of low-molecular weight heparin (LMWH) provide adequate protection for obese patients undergoing bariatric surgery, or if higher doses are required.
Aims: To determine whether a weight based thromboprophylactic dosing regimen is safe and effective in the post-operative period for obese patients undergoing bariatric surgery.
Methods: A systematic literature search was performed using MEDLINE and EMBASE. The primary outcome measures were VTE and major bleeding events. Venous thromboembolism was defined as symptomatic proximal lower limbs (popliteal vein or more proximal) deep vein thrombosis or pulmonary embolism. Weight-adjusted thromboprophylactic LMWH dosing was defined as the use of a higher than standard recommended dose. Major bleeding was defined as per the ISTH definition. Pooled proportions for the different outcomes during hospitalization were calculated.
Results: A total of seven studies (one randomized controlled trial and six cohort studies) containing 2396 patients met the inclusion criteria and were included in the analysis. Post bariatric surgery patients receiving weight-adjusted prophylactic doses of LMWH, had an in hospital rate of VTE of 0.54% (95% CI: 0.2–1.0%) compared to 2.0% (95% CI: 0.1–6.4%) for those that did not weight adjust doses. Rates of major bleeding were for both groups: 1.6% (95% CI: 0.6–3.0%) for patients receiving weight-adjusted dosing compared to 2.3% (95% CI: 1.1–3.9%) for those receiving standard doses of LMWH.
Summary/Conclusions: Adjusting the dose of LMWH for thromboprophylaxis post-bariatric surgery seems to be associated with a lower rate of VTE compared to a strategy of not adjusting the dose. This practice does not lead to an increase in adverse major bleeding events. Future studies assessing the efficacy and safety of weight adjusted dosing of bariatric surgical patients are needed to confirm these findings.

PA 4.10-4

A 7-year outcome study on the effect of warfarin and co-morbidities in critically injured patients. Has the outcome improved?

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Aim: To establish whether warfarinised trauma patient with injury score of ISS 9 and above and in comparison to controlled group of similar characteristics who non-warfarinsed patients in the UK.
Patients and Methods: We conducted first multi-centred retrospective study in UK, Europe and Australia by analysing all patients who were entered onto a large national database, (Trauma Audit and Research Network) TARN, for data collected on both groups simultaneously between 2005 and 2012.
Results: A total of 101,794 adult trauma patients dating from November 2005 to July 2012 were retrospectively analysed. Out of these, 445 patients were taking warfarin at the time of trauma. These cohorts were compared with a controlled group. Significant differences were found in age, ISS, GCS, (median age 74.8 vs. 52.6), ISS (median 16 vs. 9), and had significantly increased mortality rate at 30 days post injury (76/445 = 17.1% vs. 6816/101,794 = 6.7%; $P < 0.0024$; odds ratio (OR) 2.45; 95% confidence interval (CI) 71.7–84.2) (Table 1).
Further analysis of sub-grouping to modulated variability of age, ISS and GCS also further revealed that the warfarinised trauma patients had a statistically significant higher mortality rate than age and ISS matched control group (54/155 = 34.8% vs. 2782/11,561 = 24.1%; $P \geq 0.001$; OR 1.72; 95% CI [1.3–2.3] Table 2).
Table 1. Baseline clinical characteristics of study patients.
PMC, per existing medical condition.
Table 2. Baseline and clinical characteristics of patients study.
Conclusion: This largest UK study showed the trend of mortality rate has not changed since 1994[1]. The outcome has not altered despite trauma guidelines and trauma centred[2]. Within the trauma study group warfarinised patients had the highest mortality rate compared with controlled group. There are no specific guidelines in the management of these subgroup trauma patients, which is further complicated with co-morbidities.
References:
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2. NCEPOD. Trauma: who cares? A report of the National Confident Enquiry into Patient Outcome and Death, 2007. http://www.ncepod.org.uk/2007t.htm

PA 4.10-5

The effect of rivaroxaban and dabigatran on the surface architecture of clots formed from plasma enriched with different levels of autologous platelets

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Background: Current guidelines for treating patients with thromboembolism and concomitant thrombocytopenia are based on anecdotal data. We have developed an *in vitro* assay using thromboelastography (TEG) to describe the effects of several anticoagulants on plasma clot formation in the presence of autologous platelets at levels below 150×10^9/L. We observed that clotting of plasma containing predefined platelet counts is less compromised by the presence of factor-specific oral anticoagulants than with heparinoids. As fibrin and platelets are two key determinants for those TEG parameters showing pronounced differences, we further characterized the fibrin clot structure to investigate how the structures of theses clots were altered by the presence of (a) platelets at varying concentrations, and (b) different anticoagulants.
Aim: To determine the effects of various anticoagulants on the surface architecture of clots formed from plasmas enriched with different levels of autologous platelets.
Methods: Fresh human platelet-rich plasma and platelet-poor plasma (PPP) were obtained from each donor and subsequently mixed to produce plasma samples with varying platelet counts. Plasma containing platelets < 10 (PPP), 30, or 150×10^9/L, was mixed with 30 μg/mL corn trypsin inhibitor, and one of the following anticoagulants at therapeutic concentration: 0.3 IU/mL UFH, 1.0 IU/mL dalteparin, 1.0 IU/mL fondaparinux, 150 ng/mL rivaroxaban and 180 ng/mL dabigatran. Clotting was initiated by addition of 10 mM $CaCl_2$ and monitored with TEG for a maximum of 180 min. After completion of clot formation, the clots were fixed with 2% v/v glutaraldehyde in

0.1 M phosphate buffer (pH = 7.4), washed, and then stained with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer. The clots were dehydrated with gradient ethanol series, dried, mounted onto stubs, and gold sputter-coated for scanning electronic microscopy examination at 20,000× magnification. The diameter and instances of fibers and the porosity of the fibrin clots were quantitated. The fibrin fibers composed of single strand were defined as minor fibers whereas those with ≥ 2 visible strands in a bundle formation were defined as major fibers.

Results: Without anticoagulant, clots formed in low-platelet-count plasma had less minor fibers and increased porosity. For clots that contained the same platelet count, dabigatran or rivaroxaban reduced the number of minor fibers compared with control clots without anticoagulants ($P < 0.05$). Although there was no significant change in the number of major fibers, these fibers were thicker in the presence of dabigatran or rivaroxaban ($P < 0.05$). Clots containing dabigatran were more porous than controls and those with rivaroxaban ($P < 0.05$). None of the samples containing heparinoids clotted, thus their structures could not be studied.

Summary/Conclusion: Our study demonstrates the differences in the plasma clot structure affected by the presence of various anticoagulants and/or platelets levels. Heparinoids have more prominent anticoagulant effects on platelet-enriched plasma as compared with rivaroxaban and dabigatran. As the platelet counts decrease, plasma clots contain less minor fibers, which consequently lead to thicker major fibers and increase porosity. These changes are exacerbated by the presence of new factor-specific oral anticoagulants, in particular for dabigatran.

PA 4.10-6

Determinants for anticoagulant use in patients with first-time venous thromboembolism: VTE Epidemiology Group (VEG) Study

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Background: The benefits and limitations of vitamin K antagonists and low molecular weight heparins in the management of venous thromboembolism (VTE) are well documented. Initiation of anticoagulant therapy is necessary to avoid recurrent events and other potentially fatal complications. Therefore, the factors resulting in the decision to initiate anticoagulation therapy need to be studied.

Aims: To assess determinants of the initiation of anticoagulation use in a cohort of patients without active cancer and with first-time VTE.

Methods: Patient data were retrieved from the subset of general practices in England contributing to the Clinical Practice Research Database (CPRD) linked to data from the Hospital Episodes Statistics (HES) and the Office for National Statistics (ONS). From January 2001 to October 2011, all VTE cases in the CPRD were verified with an algorithm based on a review of clinical notes, hospital diagnoses, cause of death and anticoagulation therapy. Patients were excluded if they survived < 7 days, had a history of active cancer or had previous use of anticoagulants. We conducted a nested case–control study defining all patients who received anticoagulant treatment in the following 90 days as cases, and those without anticoagulant treatment as controls. Patients who developed recurrent VTE during the observational period were censored. Adjusted odds ratios (OR) with 95% confidence intervals (CI) were derived from multivariate logistic regression models to estimate the association between the determinant of interest and anticoagulant use.

Results: The cohort comprised 20,995 anticoagulant-naïve, non-cancer VTE patients. The mean age was 62.7 years; 45.5% were male. There were 17,503 cases (83.4% of the VTE cohort), of whom 6196 (35.4%) had provoked VTE and 11,307 (64.6%) had unprovoked VTE. There were 3492 controls (16.6% of the VTE cohort).

Type of VTE was the strongest predictor for anticoagulant use: OR = 2.06 (95% CI 1.89–2.25) for pulmonary embolism (PE) and OR = 2.93 (95% CI 2.37–3.63) for PE with deep vein thrombosis (DVT) compared with DVT alone. Male patients and those aged 60–79 years at the time of first VTE were most likely to receive anticoagulants. Other determinants for anticoagulant use were unprovoked VTE (OR = 1.53; 95% CI 1.41–1.66), body mass index ≥ 30 kg/m^2 (OR = 1.12; 95% CI 1.02–1.24), rheumatologic conditions (OR = 1.71; 95% CI 1.36–2.14) and history of asthma (OR = 1.63; 95% CI 1.16–2.29). The following factors were determinants of non-initiation of anticoagulant treatment: age < 50 and > 80 years, current and ex-smokers, lowest socioeconomic status (OR = 0.80; 95% CI 0.68–0.94), diabetes (OR = 0.79; 95% CI 0.69–0.91), history of varicose veins (OR = 0.54; 95% CI 0.43–0.67), peripheral vascular disease (OR = 0.79; 95% CI 0.66–0.93) and major bleeding in the previous year (OR = 0.52; 95% CI 0.35–0.77). Previous antiplatelet therapy was not significantly associated with anticoagulation initiation.

Summary/Conclusions: Real-life data from the UK setting show that at least 83.4% of patients with a first VTE receive anticoagulation treatment. Further studies confirming our findings, and strategies to increase anticoagulation initiation where it may be beneficial should be considered.

PA4.11 – Blood Coagulation Tests – XIV

PA 4.11-1

Increase of INR above 2–2.5 and within therapeutic range does not change the thrombin generation capacity of patients on warfarin

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Background: In clinical practice conventional monitoring of vitamin K antagonists with INR does not always predict the bleeding or thrombotic risk. Thrombin generation test (TGT) in plasma rich (PRP) or poor (PPP) in platelets is a promising tool for assessing haemostasis.

Aim: We investigated all thrombin generation parameters in patients on warfarin and their relation to INR.

Methods: Fifty six patients (27 male/29 female) with a median age of 66 (18–89) years were enrolled in the study. They were anticoagulated with warfarin for a median period of 3 (0.5–16) years with an INR of 2.5(1.5–4.0).Twenty nine received warfarin as primary prophylaxis (22 atrial fibrillation-7 valve replacement) and 27 for secondary prevention (thrombotic events). All patients were in steady state at the time of sampling and on no other medication affecting haemostasis. Thrombin generation was measured in CAT (Calibrated Automated Thrombinoscope).Citrated PRP with CTI was triggered with 0.5 pM TF whereas PPP analysis was run in non-CTI samples triggered with phospholipids and TF (5 pM). We assessed all time (lagtime and ttpeak) and amount parameters (ETP, Peak) from the TG curve. Plasma levels of clotting factors (F) FII, FVII, FX, FIX were measured. Patients were analysed in five groups based on INR (1.5–1.9/2–2.4/2.5–2.9/3–3.4/3.5–4).

Results: We found strong positive correlation between INR and time parameters (r 0.9) and negative correlation between INR and amount parameters (r 0.85) and factors levels (r 0.8) in both PRP and PPP TGT ($P < 0.001$).However when we analyse patients by INR group we find significant differences in all TG parameters only between group 1 and 2. Patients in group 2 and 3 differed significantly only when PPP was used. Interestingly despite the strong negative correlation between INR and all factors measured, FIX showed the modest decrease keeping almost haemostatic levels (37%) even in the group with the highest INR.

Conclusions: Based on our results, TGT identifies as critical point an INR value of 2(PRP) or 2.5(PPP).It also highlights that further increase within the usual therapeutic range (2.5–4.0) does not offer significant reduction in thrombin generation which might be due to the relatively high levels of factor FIX. Our findings in clinical terms could raise dual interpretation. First that patients are deeper anticoagulated only when they reach an INR > 2(PRP) or 2.5(PPP) and secondly that further INR increase within the therapeutic range (2.5–4) does not necessarily add significantly either in anticoagulation or in bleeding risk. Prospective trials with clinical endpoints concerning antithrombotic efficacy and bleeding risk are needed before implementing TGT information into clinical practice.

PA 4.11-2

The SAW-CT assay: a point-of-care clotting assay based on surface acoustic waves (SAWs) to monitor anticoagulant pharmacotherapy

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Aim: A microfluidic and scaleable μ-fluidic coagulation assay that can be used bed-side.

Methods: We describe a new microfluidic coagulation assay in that SAWs rapidly mix and recaclify citrated blood. Coagulation kinetics were quantified by automatic image processing methods and compared with standard methods: used in clinical routine laboratories.

Results: Human whole blood (citrated) was spiked with a range anticoagulants in clinical use, such as the direct thrombin inhibitor argatroban (0–4000 ng/mL), unfractionated heparin (0–1.0 IU/mL), the platelet integrin inhibitor abciximab (0 vs. 4 μg/mL) and the new anticoagulants dabigatran (0–1000 ng/mL) and rivaroxaban (0–500 ng/mL). The SAW-CT was prolonged in dose-linear fashion and this was highly correlated with custom methods e.g. aPTT, PT or Ecarin Time. These results prove the suitability of our SAW-CT method to monitor anticoagulant therapy of clinically relevant doses of currently used anticoagulants. A series of clinical samples of patients under dabigatran also showed excellent correlation with their aPTT. Pretreatment *in vivo* with aspirin did not affect alter SAW-CT in 125 ng/mL dabigatran treated blood (351 ± 13 s before vs. 373 ± 49 s after aspirin), in 250 ng/mL rivaroxaban treated blood (521 ± 58 s before vs. 528 ± 28 s after aspirin) and untreated blood (204 ± 25 s before vs. 240 ± 25 s after aspirin). Also, treatment with clopidogrel alone or with aspirin showed no influence on SAW-CT.

Conclusion: Our method can be downscaled to a fully automatic, small hand held device and did not require trained personnel to operate. This might be an advantage in situations where the coagulation status needs a quick answer, e.g. in emergency settings and unconscious patients.

PA 4.11-3

Protein C and factor VII concentrations can predict outcomes of surgical intensive care unit patients

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Background: In surgical intensive care unit (SICU), most of the patients suffered major surgery or serious trauma, coagulopathy always happened and correlated with outcomes of these patients.

Aims: Characterizing the evolution of coagulation/anticoagulation markers' concentrations in SICU patients, and finding out the valuable markers which may help in predicting mortality risk and identifying potential therapeutic targets.

Methods: One hundred and fifty-two patients entering our hospital's surgical ICU were investigated, the soluble P -selection, factor VII, protein C, antithrombin, fibrinogen and d-dimer levels on the first week of admission were detected daily, and deaths during the first month were registered.

Results: Among all the coagulation tests, protein C and factor VII levels of the first day were lower ($P < 0.05$) in non-survivors ($n = 31$, 20.4%), irrespective of the presence of sepsis, type of admission and type of surgery. In a multivariable analysis with SICU mortality as the dependent variable, the initial protein C concentration < 49.5% and factor VII concentration < 51.0% were independent risk factor for SICU death. In survivors, both of protein C and factor VII levels increased gradually and got higher concentrations in the seventh day than those in the first day ($P < 0.05$); in non-survivors, comparing with the first day, only factor VII levels decreased to a lower concentrations in the seventh day ($P < 0.05$).

Conclusion: In SICU patients, initial protein C and factor VII concentrations were generally low and independently associated with a higher risk of mortality; the factor VII level seemed to be a better initial therapeutic target than protein C for patients.

PA 4.11-4

Changes in hemostasis during the perioperative period of orthopedic surgery

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Background: Thrombosis and major bleeding are significant, albeit poorly understood, concerns during the perioperative period. Several hemostatic parameters are known to influence the hemostatic system and may contribute to the delicate balance between thrombosis and bleeding.

Aim: We sought to better understand the mechanism of perioperative hemostasis by investigating the changes in coagulation factors during the perioperative period.

Methods: We performed a prospective cohort analysis of 70 subjects undergoing non-emergent orthopedic surgery of the knee ($n = 28$), hip ($n = 35$), or spine ($n = 7$) between August 2011 and November 2011. Plasma was collected preoperatively (T1), 1-h into the operation (T2), 1-h (T3), 24-h (T4) and 48-h postoperatively (T5). Coagulation assays were performed for factor VII, factor VIII, von Willebrand Factor (vWF), and fibrinogen. Mean curve profiles were compared using linear mixed models with time and main effect terms of interest.

Results: Of the 70 subjects, mean age was 64.1 + 9.8 years, 61% were female, and 74% were Caucasian. Coagulation assays changed significantly throughout the perioperative period. Factor VIII (T1 – 148.3% [standard error, 8.0], T2 – 129.1% [8.1], T3 – 130% [8.9], T4 – 140% [8.3], T5 – 175.8% [9.6], $P < 0.0001$), vWF (T1 – 171.3% [8.9], T2 – 139.8% [7.9], T3 – 149% [8.0], T4 – 216.9% [15.4], T5 – 298.9% [14.8], $P < 0.0001$) and fibrinogen (T1 – 488.7 mg/dL [15.7], T2 – 428.4 mg/dL [14.7], T3 – 421.4 mg/dL [15.8], T4 – 560.9 mg/dL [18.5], T5 – 885.1 mg/dL [28.1], $P < 0.0001$) significantly increased, while factor VII (T1 – 131.9% [9.6], T2 – 115.2% [10.3], T3 – 112.1% [9.1], T4 – 65.1% [4.3], T5 – 52.7% [2.2], $P < 0.0001$) significantly decreased during the perioperative period. Overall, factor VIII increased by 18.5%, vWF by 74.5%, and fibrinogen by 81.1%, while factor VII decreased by 60.0%. The change in hemostasis was independent of age, sex and race.

Conclusions: Among subjects undergoing non-emergent orthopedic surgery, factor VIII, vWF and fibrinogen increased, while factor VII decreased during the perioperative period. Understanding differences in hemostatic markers may better explain the pathophysiology of the heightened risk of thrombotic and bleeding events during the perioperative period.

PA 4.11-5

External quality assessment of platelet function by the PFA-100: an update from the RCPAQAP Haematology

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Background: The PFA-100 is commonly used in Haemostasis laboratories for screening and monitoring of both von Willebrand disease (VWD) and platelet function. Testing by PFA-100 requires fresh whole blood, so External Quality Assessment (EQA) of PFA-100 test practice requires an innovative approach.
Aims: The RCPAQAP Haematology, an international EQA provider, established a formal PFA-100 program in 2010 with 50 participants currently enrolled. Our objective is to provide test challenges encompassing 'normal' and 'variable case-dysfunctional' samples for participants.
Methods: A novel approach was developed whereby a range of formulated test tubes are distributed to EQA participants to which citrated normal whole blood collected on site is added, thereby creating test material that can be locally evaluated. Several surveys have been conducted over the past few years (total of 12 challenges), with most designed to mimic a mild or severe primary haemostasis defect, or an aspirin-like effect.
Results: Numerical results for PFA-100 closure times (CTs) and interpretive comments provided by participants analysed over the past few years have consistently yielded results within expectations, with good reproducibility evidenced by repeated challenges. For example, coefficients of variation (CVs) generated for two cartridge types (C/ADP and C/Epi) for 2012 challenges [median (range): 14.8 (0.0–29.5) and 10.9 (4.4–17.3)] were similar to those for native whole blood [15.0 (14.2–15.7) and 14.7 (14.3–15.0)]. The median (range) CTs for each challenge, as obtained by participants, was within the regions of expectations according to sample challenge details. For example, PF12-08b yielded maximally prolonged (i.e. > 250s) median (range) CTs for both C/ADP 301s (147–301s) and C/Epi 301s (211–301s) for over 90% of the results returned. Interpretations were, in general, also consistent with expectations and test data provided by laboratories.
Conclusions: An EQA process for the PFA-100 has been successfully introduced that includes very reproducible data, thus providing a valuable mechanism for monitoring and improving laboratory test performance. Future goals include an expansion in challenge material to help assess performance with newer anti-platelet agents.

PA 4.11-6

Thrombin generation assay (TGA) is not able to predict hemostatic efficacy of by-passing agents in patients with hemophilia and inhibitors: results from *in vivo* studies

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Background: Therapy with by-passing agents (BPA) in patients with hemophilia and inhibitors still lacks a laboratory test able to assess the hemostatic response that may help clinicians in therapeutic choices. Thrombin generation assay (TGA) has been used at this aim by spiking *in vitro* haemophilic plasma with BPA, however *in vivo* evidence remains limited.
Aim: The aim of this study was to evaluate whether or not TGA is able to predict the response to the administration of either rFVIIa or aPCC at different doses in patients with hemophilia and inhibitors.
Methods: In this study, thrombin generation was assessed *in vivo* in platelet-rich (PRP) and platelet-poor (PPP) plasma with the addition of corn trypsin inhibitor in 15 patients with hemophilia A and high-responding inhibitors (median age: 34 years). Four parameters of the TGA curve were evaluated: lagtime, endogenous thrombin potential (ETP), peak and time-to-peak. TGA was assessed in 11 patients prior, 30 min and 3 (rFVIIa 120 and/or 270 μg/kg) or 6 (aPCC 80 IU/kg) h after drug injection in a non-bleeding state and a minimum wash-out period of 48 h from the last injection. It was also assessed once daily prior and 30 min after BPA administration in nine patients undergoing orthopaedic surgery. Haemostatic treatment to cover surgical procedures and post-operative period was established irrespective of TGA measurements.
Results: In the group of 11 patients who received BPA in a non-bleeding state, the baseline values of the TGA curve were highly variable between patients as well as in the same patient both in PRP and in PPP (ANOVA $P = 0.01$). Overall, ETP increased after drug administration in all cases, with similar results obtained in PRP (median ETP increase: 585 nM × min, IQR: 226–997) and PPP (median increase: 552 nM × min, IQR: 415–1013) and no difference observed with respect to product type and/or doses used. In all patients who underwent surgery, the TGA curve was evaluated over a 7-days post-operative period and a lack of response was observed over time in all patients irrespective of type and/or intensity of treatment with BPA. Five post-operative bleeding complications were observed (four major and one minor), however the TGA curve was not able to discriminate between patients who bled and those who did not bleed. For patients who underwent TGA measurement both in a non-bleeding state and during surgery, there was no correlation between the values obtained in the two different settings.
Conclusions: Our data show that *in vivo* TGA values are highly variable between different subjects as well as in the same subject; TGA is not able to discriminate the type and the dose of drug used and during post-operative period a 'lack of response' was observed irrespective of the therapeutic regimen adopted.

PA4.12 – Coagulation Factor V

PA 4.12-1

Isolated decrease of factor v in children treated by 6-mercaptopurine for acute lymphoblastic leukemia

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Background: Maintenance therapy for acute lymphoblastic leukemia (ALL) aims to eradicate leukemic cells persisting after remission and after intensive chemotherapy. It is based on oral daily 6-mercaptopurine (6-MP) and oral weekly methotrexate (MTX). Side effects of 6-MP (hepatotoxicity, myelotoxicity) are correlated with concentrations of its various metabolites, and depend on genetic polymorphisms of enzymes involved in its metabolism.
Aims: Among 62 children treated for ALL in the Pediatric Hematology and Oncology Department of the University Hospital of Rouen, between 1 January 2007 and 31 December 2011, four patients developed an unexplained decrease of Prothrombin Time (PT) during maintenance therapy despite the absence of any hepatic injury.
Methods: Factor V activity (FV:Act) was quantified by a one-stage clotting assay, FV antigen (FV:Ag) was measured by enzyme-linked immunosorbent assay (ELISA)
Results: The decrease in prothrombin time seen in the four patients was observed after a mean time of 6 months (range: 1–17 months) of maintenance therapy. Of all the prothrombin complex factors, FV:Act was the lowest (mean: 30%, range: 20–54), whereas vitamin K dependent factors were in the normal range. FV:Act decrease was unrelated to hepatic cytolysis. Disseminated intravascular coagulation and acquired inhibitors to FV were ruled out, as well as toxicity to MTX. High concentrations of methylated metabolites of 6-MP (6-MMPN, mean 17,620 pmol/8.10^8 erythrocytes, range: 10,456–27,184 pmol/

8.10^8 erythrocytes) were observed at the same time (levels of 6-MMPN superior to 5000 pmol/8.10^8 erythrocytes are considered hepatotoxic). Drug imputability (Naranjo probability scale) in FV decrease shows a definite reaction to 6-MP in three cases and a probable reaction in one case. In two patients, FV:Ag was in the same range as the FV:Act. Patients were asymptomatic. The deficiency was reversible upon discontinuation or dose reduction of 6-MP.

Summary/Conclusion: Seven cases of a FV decrease have previously been described in the literature during 6-MP or azathioprine treatment for ALL or inflammatory bowel disease. These case reports have not described either a correlation with high levels of 6-MMPN or an association with FV levels (activity and antigen). The mechanism of action of 6-MP toxicity on FV levels remains unknown. The correlation between FV:Ag and FV:Act suggest either a direct effect on FV transcription or a lack of post-translational modifications of FV (phosphorylation, sulfatation or glycosylation) induced by a metabolite of 6-MP, which could prevent FV production or secretion. As patients were asymptomatic, the interest to maintain or reduce 6-MP treatment remains to be defined in such circumstances.

PA 4.12-2
Cleavage at Arg1018 is not required for factor V activation

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Background: The coagulation cascade is initiated following vascular injury which results in the exposure of endothelial tissue factor to the blood flow and that in turn results in robust thrombin generation. The proteolytic conversion of prothrombin (Pro) to thrombin is catalyzed by the prothrombinase complex. This enzymatic complex is composed of the cofactor factor Va (fVa), the enzyme factor Xa (fXa) associated on a procoagulant membrane surface in the presence of divalent metal ions. Factor V (fV) circulates as a single chain inactive precursor consisting of three domains A1-A2-B-A3-C1-C2. The molecule must be activated by thrombin following three sequential cleavages at Arg^{709}, Arg^{1018}, and Arg^{1545} to generate the active cofactor (fVa) composed of heavy and light chain. It was previously reported that cleavage at Arg^{1545} by thrombin requires prior cleavage at Arg^{1018}.

Aim: To determine the role of cleavage at Arg^{1018} during activation of fV.

Methods: We used site directed mutagenesis to create several recombinant fV molecules with the activation sites mutated to Glutamine (R to Q). We have also used a recombinant mutant factor V molecule with the region 1000–1008 from the B region deleted ($fV^{\Delta B9}$). We have created recombinant fV molecules as follows: fV^{WT} (wild type), fV^{QQR} (only cleavage at Arg^{1545} is available), fV^{RQQ} (only cleavage at Arg^{709} available), fV^{QRQ}, (only cleavage at Arg1018 available), fV^{RQR} (cleavages at Arg^{709} and Arg^{1545} available), fV^{QQQ} (no cleavage available), $fV^{\Delta B9/RQR}$ and $fV^{\Delta B9/QRQ}$. The recombinant molecules were expressed in COS-7 cells, purified to homogeneity and assayed for clotting activity as well as in prothrombinase assays using purified reagents. Western blotting followed by staining with specific monoclonal antibodies to the heavy and light chain of the cofactor was used to evaluate the integrity of the recombinant fV/fVa molecules.

Results: Two-stage clotting assays revealed that the clotting activities of fVa^{QQR}, fVa^{RQQ}, and fVa^{QRQ} were reduced while fV^{QQQ} was devoid of clotting activity. In addition, fVa^{RQR} and $fVa^{\Delta B9/RQR}$ have similar clotting activities as fVa^{WT}. In contrast, fVa^{QRQ}, and $fVa^{\Delta B9/QRQ}$ are impaired in their clotting activities and have clotting activities similar to the activity expressed by fV^{QQQ}. Kinetic analyses demonstrated that fVa^{RQR} and $fVa^{\Delta B9/RQR}$ have similar affinities for fXa, while fVa^{QRQ}, and $fVa^{\Delta B9/QRQ}$ were impaired in their interaction with fXa. The k_{cat} values for prothrombinase assembled with fVa^{RQR} and $fVa^{\Delta B9/RQR}$ were similar to the k_{cat} obtained with prothrombinase assembled with fVa^{WT}, while prothrombinase assembled with fVa^{QRQ} and $fVa^{\Delta B9/QRQ}$ had 2- and 7-fold reduced catalytic efficiency respectively, when compared to the k_{cat} values obtained with prothrombinase assembled with fVa^{WT}. Finally, the k_{cat} value for prothrombinase assembled with fVa^{QQR} was approximately 50% lower than the k_{cat} obtained with prothrombinase assembled with fVa^{WT}.

Conclusion: Our data demonstrates that cleavage at Arg^{1018} is neither essential nor required for activation of factor V. These data further promote our understanding of the mechanisms regulating blood coagulation and the assembly of prothrombinase.

PA 4.12-3
Galectin-8 does not function as a factor V receptor on the surface of CMK cells and *ex vivo*-derived megakaryocytes

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Background: Plasma-derived factor V is endocytosed by megakaryocytes and is subsequently modified to form the unique platelet-derived cofactor molecule. Factor V endocytosis is mediated by a two receptor system including an uncharacterized, specific factor V receptor and low density lipoprotein receptor related protein-1 (LRP-1), a ubiquitous endocytic receptor. Studies are currently underway to identify the unknown factor V receptor. A recent study by Zappelli and colleagues suggested that galectin-8, an intracellular galactoside-binding protein, is expressed on the cell surface of DAMI cells, and may function as the unknown, specific factor V receptor [J Biol Chem. 2012; 287(11): 8327–35].

Aims: In the current study, a role for galectin-8 in factor V endocytosis was further characterized using the megakaryocyte-like cell line, CMK, a well-characterized model of factor V endocytosis, as well as *ex vivo*- derived megakaryocytes.

Methods: CD34+ cells were isolated from umbilical cord blood and induced to differentiate *ex vivo* into megakaryocytes for 10 days. Galectin-8 cell surface expression was determined by flow cytometry using a specific, inhibitory monoclonal antibody directed against the N-terminus of galectin-8. The role of galectin-8 in factor V endocytosis was also assessed using inhibitors of galectin-8 ligand binding.

Results: *Ex vivo*-derived megakaryocytes were identified by their forward and side scatter, as well as expression of the megakaryocyte/platelet-specific marker CD41. Flow cytometric analyses of these cells under non-permeabilizing and permeabilizing conditions demonstrated intracellular but not extracellular expression of galectin-8. This observation is consistent with data from Rowley et al. showing galectin-8 mRNA in human platelets [Blood. 2011; 118(14):e101–11] as well as its known intracellular roles in cellular differentiation, growth regulation, and apoptosis. Cell surface and intracellular galectin-8 expression were also assessed in CMK cells. Under conditions where endocytosis of fluorescently-labeled factor V (30 nM) was observed, galectin-8 expression was only detected following cell permeabilization. Endocytosis assays were performed in the presence of lactose, a β-galactoside known to block carbohydrate-dependent binding of galectins to their ligands, as well as an inhibitory antigalectin-8 antibody to assess the role of galectin-8 in factor V binding and endocytosis. Preincubation of the cells with lactose and mannose had no effect on endocytosis of fluorescently-labeled factor V or 125I-labeled factor V (30 nM) by *ex vivo*-derived megakaryocytes or CMK cells. Mannose was used as a negative control as its structure is similar to lactose, but it does not interfere with galectin ligand binding. Consistent with this observation, the presence of the inhibitory anti-galectin-8 antibody had no affect on factor V endocytosis by either of these cell types.

Conclusions: These combined observations suggest that, while galectin-8 may be involved in factor V endocytosis in DAMI cells, its role in factor V endocytosis by other megakaryocyte model systems including megakaryocytes derived *ex vivo* from human cord blood, remains unclear.

PA 4.12-4

Molecular characterization of FV deficiency in a large cohort of Italian patients

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Background: Factor V (FV) deficiency is a rare coagulation disorder characterized by low or unmeasurable plasma levels of functional and immunoreactive FV and associated with a hemorrhagic phenotype of variable severity. It is transmitted as an autosomal recessive trait (OMIM +227,400) with a prevalence of about one in 1 million and, among rare inherited coagulopathies, is the least characterized from the molecular point of view.
Aims: To investigate the molecular basis of the disease in 24 Italian FV-deficient patients, nine of whom affected by a severe form and 15 with mild deficiency.
Methods: Mutational screening was performed by direct sequencing of PCR products covering all FV gene (*F5*) exons and a region of *F5* promoter. Missense mutations were investigated by *in-vitro* expression of mutant FV in COS-1 cells, followed by SDS-PAGE of immunoprecipitated proteins, as well as by enzyme immuno assays on cell lysates and conditioned media.
Results: DNA sequencing disclosed 22 mutations, ten of which (three nonsense, one splicing, five missense, and one frameshift) were hitherto unknown. Moreover, five individuals were found to carry the HR2 haplotype, a *F5* allele responsible for a mild form of FV deficiency. Whilst the identified nonsense and frameshift mutations (W1797X, S1849X, L1908X, V381QfsX1) introduce premature stop codons, thus likely resulting in unstable transcripts and eventually in low protein synthesis, the pathogenic role of missense and splicing mutations was experimentally verified. All analyzed missense mutations (N409D, D1669G, G1867R, C2033Y, C2038Y) were shown to impair FV secretion, leading to a concomitant reduction of functional and immunologic FV levels. The role of the splicing mutation (c.5879-11_-12delinsAA) is currently being investigated by producing the mutant transcript in HeLa cells. Bioinformatics analyses suggest that the mutation, interrupting the polypyrimidine tract, might cause the skipping of exon 20. Interestingly, four mutations (R1133X, Y1702C, c.248+1G>A, and G1902D), already described in the literature, were each identified in two unrelated patients of our cohort, suggesting that these variants may be recurrent in the Italian population.
Conclusions: This work reports the identification and the functional characterization of 10 novel genetic defects responsible for FV deficiency in the Italian population.

PA 4.12-5

Factor V is endocytosed and trafficked to proplatelet extensions by megakaryocytes derived *ex vivo* from human umbilical cord blood

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Background: Platelet- and plasma-derived factor Va are absolutely essential for thrombin generation catalyzed by the prothrombinase complex. The platelet-derived form represents only approximately 20–25% of the total factor V pool; however, several observations suggest that it is physically and functionally distinct and plays a more relevant role in clot formation. Thus, any alterations in this pool may have a profound effect on the maintenance of normal hemostasis.
Aim: The mechanism by which platelets acquire, modify, and store factor V is being investigated. In the current study, megakaryocytes differentiated *ex vivo* from umbilical cord blood or bone marrow-derived $CD34^+$ cells were examined as potential models to study the formation of the distinct platelet-derived factor V pool.
Methods: $CD34^+$ cells were isolated from human umbilical cord blood or bone marrow and induced to differentiate into megakaryocytes by culture in the presence of stem cell factor, thrombopoietin, and/or interleukin-3. Endocytosis of AlexaFluor488-labeled factor V (30 nM) was assessed by flow cytometry or confocal microscopy and correlated with expression of megakaryocyte-specific markers. DNA content was assessed by propidium iodide staining followed by flow cytometry.
Results: *Ex vivo*-derived megakaryocytes were identified by their forward and side scatter and expression of megakaryocyte/platelet-specific markers by flow cyytometry. Expression of these markers by bone marrow-derived megakaryocytes increased over time of culture. Of the cells capable of factor V endocytosis, $19.1 \pm 6.3\%$ are positive for CD41 on day 3. This increases over time to $98.2 \pm 4.1\%$ by day 10. Similar observations were made for CD61, von willebrand factor, and P-selectin. Additionally, cells with up to 16N DNA content could be visualized. However, these cells do not appear to produce platelets *ex vivo* suggesting that they may not be suitable to study the entire factor V trafficking pathway. Umbilical cord blood is often used as an alternative source of $CD34^+$ cells for *ex vivo* differentiation into megakaryocytes. In addition, these cells exhibit higher proliferation and are able to generate proplatelets, possibly making them a more suitable model system. Thus, their use for this study was also assessed. Factor V endocytosis by cord-blood derived megakaryocytes was observed on days 5–13 of culture, consistent with what was observed in the bone marrow-derived cells. Expression of CD41 increased from 32.4% on day 5 to $55 \pm 8.1\%$ on day 12 of culture. Additionally, cells that endocytose factor V and are also CD41 positive follow a similar trend, increasing from 25.1% on day 5 to $60.3 \pm 0.35\%$ on day 12. Day 13 cells were incubated with fluorescently-labeled factor V for 8 h just prior to culture on fibrinogen-coated slides to induce proplatelet formation. Following its endocytosis, factor V was observed in the proplatelet extensions by confocal microscopy.
Conclusions: Cord blood-derived megakaryocytes endocytose factor V in a manner similar to bone marrow-derived cells and traffic it to proplatelet extensions. These observations suggest that cord blood-derived megakaryocytes may be a suitable model to study the formation of the distinct platelet-derived factor V pool.

PA 4.12.6

A new regulatory function of activated factor V: inhibition of the activation by tissue factor/factor VII (a) of factor X

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Background: We observed that minute amounts of thrombin or the enzyme Russell's viper venom activating factor V (RVV-V), added to plasma strongly diminish the potential of that plasma to generate thrombin after being triggered by tissue factor.
Aim: To find the mechanism behind this phenomenon.
Methods: Calibrated automated thrombin generation (CAT) was measured in platelet poor plasma using different triggers (tissue factor, kaolin, factor IXa or direct activation of factor X) in the absence and presence of factor V(a). The effect of FV(a) on the activation of factor X by tissue factor (TF) and factor VII(a) was determined in a purified system.
Results: Thrombin generation (TG) initiated by tissue factor is strongly and dose-dependently inhibited by addition of activated factor V (FVa) or by addition of a factor V activator (thrombin or RVV-V). The two forms of activated factor V (FVa_1 and FVa_2) diminished thrombin generation similarly while FV_1 and FV_2 showed no effect. No inhibition by FVa is seen when TG is triggered via the intrinsic pathway or by direct activation of factor X. The effect is independent of proteins C and S and tissue factor pathway inhibitor (TFPI). The inhibiting action is also observed in hemophilia A and B samples. In

factor VII -deficient plasma the effect is seen when it is spiked with recombinant factor VII (FVII) and to a much lesser extent when spiked with recombinant FVIIa. In a purified system, FVa also dose-dependently inhibits the activation of FX by TF/FVII(a). The inhibitory effect is neutralized by antibodies against the light chain of FVa but not by antibodies against the heavy chain.

Conclusions: We report a new regulatory function of factor Va where it inhibits the activation of factor X. This observation can be explained by assuming that factor Va, via its light chain, binds to the complex TF/FVII(a) and prevents it from activating factor X. We assume that this mechanism reduces the possibility that thrombin and factor Xa escaping from a wound area into the circulation, together with blood-borne tissue factor, would trigger intravascular coagulation.

PA4.13 – Coagulation Factor VIII – VI

PA 4.13-1

Assessment of structural comparability between rFVIIIFc and unmodified B domain-deleted FVIII by complementary biophysical methods

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Background: Recombinant Factor VIIIFc (rFVIIIFc) is a long-lasting FVIII molecule developed to reduce dosing frequency and increase protection for patients with severe hemophilia A. rFVIIIFc is a fully recombinant protein consisting of a single molecule of B domain-deleted (BDD) human coagulation factor VIII (FVIII) covalently linked to the dimeric Fc domain of human immunoglobulin G1 (IgG1) with no intervening sequence. The specific activity of rFVIIIFc, on molar basis, and its affinity for von Willebrand factor (VWF) are comparable to those of commercially available BDD rFVIII and full-length rFVIII products, indicating that the Fc moiety does not impair the association of rFVIIIFc with other elements of the Xase complex or with VWF.

Aims: This study was undertaken to assess the structural comparability between the BDD-FVIII and Fc constituents of rFVIIIFc and their respective free counterparts, and to evaluate the relative orientation and conformational dynamics of the FVIII and Fc elements within the rFVIIIFc molecule.

Methods: The following methods were employed to evaluate the structural comparability of rFVIIIFc and rFVIII, as well as the conformational dynamics of rFVIIIFc: (i) hydrogen-deuterium exchange (H/DX) mass spectrometry (MS), (ii) multiplexed surface plasmon resonance (SPR) affinity analysis with a panel of anti-FVIII antibodies, (iii) Small-angle X-ray scattering (SAXS), (iv) X-ray crystallography, and (v) negative stain electron microscopy (EM).

Results: H/DX-MS analysis was performed on rFVIIIFc and its recombinant FVIII and Fc constituents with a sequence coverage of 94% for FVIII (416 peptides) and 87% for Fc (50 peptides). Over a time course of 4 h, no statistically significant differences in deuterium exchange rates were observed for any peptide pairs, indicating a high degree of structural comparability between the BDD-FVIII and Fc constituents of rFVIIIFc and their respective free counterparts. Consistent with these findings, members of a panel of 23 anti-FVIII monoclonal antibodies, which recognize epitopes distributed among the different domains of FVIII, bound to both rFVIII and rFVIIIFc with comparable affinities as determined by SPR array analysis. SAXS analysis combined with molecular dynamics simulation enabled derivation of a minimal ensemble of structures for rFVIIIFc in which the Fc domain is extended away from, and does not interact with, the FVIII component. The X-ray crystallographic structure of rFVIIIFc, determined at low resolution (approximately 5 Å) was comparable to published FVIII atomic coordinates 2R7E and 3CDZ. Electron density for the Fc domain was not observed, indicating that the tethered Fc domain experiences conformational freedom in relation to the FVIII component. Negative stain EM analysis of 5853 particle pairs (60°/0°) showed that 3184 rFVIIIFc particles adopted the same conformation (six out of 10 classes). In the resulting 3D reconstruction at 27 Å resolution, the Fc domain projects maximally away from the FVIII component.

Conclusions: The results from these complementary biophysical approaches are in agreement with respect to the structure of rFVIIIFc. The linkage of Fc to FVIII does not alter the conformation of either component. The rFVIIIFc linkage permits conformational flexibility between the two components, with the appended Fc domain extending away from the FVIII component.

PA 4.13-2

Structure of membrane-bound porcine factor VIII

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Factor VIII (FVIII) is a multidomain blood plasma glycoprotein, when defective or deficient causes for hemophilia A, a severe hereditary bleeding disorder. Upon initiation of coagulation FVIII is cleaved by Thormbin and the active form FVIIIa, acts as co-factor to the serine protease Factor IXa (FIXa) within the membrane-bound tenase complex. Human FVIII (hFVIII) is a large glycoprotein of approximately 280 kDa Molecular weight, composed of six domains: A1-A2-B-A3-C1-C2. *In vitro*, FVIII exists as a mixture of heterodimers of a variable length heavy chain (HC) of the A1-A2 domains with different length of the B domain, and a constant length light chain (LC) of the A3-C1- C2 domains. The LC holds the FVIII membrane-binding sites. The HC holds the main FIXa interaction sites. The LC and HC are non-covalently linked via divalent metal cations. Recombinant FVIII concentrate is the most effective drug against hemophilia A and commercially available FVIII is expressed as full-length (FVIII-FL) or B-domain deleted (FVIII-BDD). Porcine (p) FVIII-BDD shares 84% amino acid sequence identity with hFVIII-BDD, has 10–14 fold higher expressions and is used as replacement therapy in patients who develop inhibitory antibodies against the human form, as it forms functional complexes with human FIXa.

The focus of this work is to achieve a high resolution structure of membrane-bound pFVIII-BDD close to physiological conditions, by combining cryo-EM and structure analysis. For this purpose we have expressed, purified and organized helically pFVIII-BDD onto single bilayer lipid nanotubes (LNT) containing phsophatidylserine in a 20 mM HEPES buffer, pH 7.4, containing 150 mM NaCl and 5 mM $CaCl_2$. The calculated cryo-EM structure for membrane-bound pFVIII-BDD at subnanometer resolution was further refined by fitting the existing hFVIII-BDD crystal structure within the density map. A pFVIII-BDD homology structure was further generated with the fitted hFVIII-BDD structure as a template.

The presented membrane-bound pFVIII-BDD structure further confirms the previously refined 3D structure for helically organized hFVIII-LC on LNT and that the FVIII membrane-bound organization differs from that of the FVIII organized in 3D crystals in solution. Fitting the FVIII domains from the hFVIII-BDD crystal structures within the density map resolved by cryo-EM shows that the C2 domain is the main FVIII membrane interaction site. The C1 does not interact directly with the membrane interface when the pFVIII-BDD molecules are helically organized onto the LNT surface. A new

interface is formed between the C1 and the A1 and A3 domains, creating a platform for the A2 domain holding the main FIXa interaction site. This organization of the FVIII-LC domains has been previously observed for plasma derived hFVIII in membrane-bound 2D crystals and hFVIII-LC helically organized onto LNT.
Comparing the membrane-bound organization for both human and porcine FVIII-BDD as resolved by cryo-EM will provide a structural basis for comparing the activity of mutations, complementing the existing homology models. Understanding the differences between the organization of porcine and human FVIII-BDD bound to LNT will provide a structural scaffold for mapping the functionally important FVIII interaction sites and improve drug design based on recombinant FVIII.

PA 4.13-3

Dose response relationship and duration of effect of a PEGylated recombinant FVIII conjugate, N8-GP, in a new tail vein transection bleeding model in anaesthetized FVIII k/o mice

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Background: Turoctocog alfa is a new serum-free B-domain truncated recombinant FVIII for treatment and prevention of bleeding in patients with haemophilia A. N8-GP is turoctocog alfa with a 40 kDa PEG conjugated to the O-glycan in the 21 amino acid B-domain. The potency of Advate®, turoctocog alfa and N8-GP are similar in the standard mouse tail transection model, while the half-life and duration of effect is longer for N8-GP. We now report on a sensitive tail vein transection bleeding model in anaesthetized FVIII k/o mice, where clinically relevant FVIII doses are effective. The new model was used to investigate the dose response relationship of N8-GP and the duration of effect of N8-GP and turoctocog alfa on bleeding time and blood loss.
Methods: FVIII k/o (exon 16 disrupted) male mice on a mixed background, C57Bl6/129, were dosed with N8-GP, turoctocog alfa, or vehicle in the right lateral tail vein 24 or 48 h before injury. The bleeding challenge comprised a template-guided transection of the left lateral tail vein at a tail diameter of 2.7 mm in isoflurane anaesthetized mice. The tail was immersed in saline (37 °C) allowing visual recording of the bleeding for 60 min, whereafter the blood loss was determined by measuring the haemoglobin concentration. Data are reported as means ± SEM. One-way ANOVA with post test for linear trend or Bonferroni's adjustment for multiple comparisons was applied for analysis of data.
Results: The haemophilic phenotype was verified in all animals as: (i) intact primary haemostasis with a primary bleeding lasting ≤ 3 min after injury and (ii) subsequent re-bleeding episodes depending on the actual treatment. Dose response relationships were demonstrated for total bleeding time and blood loss when tail vein transection was made 24 h after administration of N8-GP. Mice dosed at 20, 10, 5, 2.5, 1.25, or 0 U/kg had total bleeding times of 7.0 ± 1.0, 11.8 ± 2.2, 25.3 ± 4.7, 25.1 ± 4.5, 36.1 ± 3.5, and 46.0 ± 1.4 min, respectively ($P < 0.0001$). The corresponding blood losses were 1372 ± 359, 2286 ± 592, 4659 ± 993, 5679 ± 1108, 7120 ± 533, and 7382 ± 714 nmol haemoglobin, respectively ($P < 0.0001$). A study comparing N8-GP 10 U/kg, turoctocog alfa 10 IU/kg, and vehicle dosed 48 h before tail vein transection had total bleeding times of 18.6 ± 3.1, 34.3 ± 4.6, and 36.5 ± 3.0 min with corresponding blood losses of 2832 ± 829, 6325 ± 782, and 6117 ± 498 nmol haemoglobin, confirming the prolonged duration of effect of N8-GP when compared to turoctocog alfa ($P < 0.05$ for total bleeding time and $P < 0.01$ for blood loss).
Conclusion: Dose response relationship was demonstrated at 24 h for N8-GP when applied in clinically relevant doses using a new tail vein transection bleeding model in anaesthetized FVIII k/o mice. Furthermore, the prolonged duration of effect of N8-GP was confirmed.

PA 4.13-4

Impact of factor VIII A2 domain stabilization on activated factor X generation

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Background: An important negative regulator of activated factor VIII (FVIIIa) cofactor activity is subunit dissociation. Factor VIII (FVIII) molecules with stabilized activity have been generated by elimination of charged residues at the A1-A2 and A2-A3 interfaces. These molecules exhibited reduced decay rates as part of the factor Xase complex (FXase) and retained their activities under thermal and chemical denaturing conditions (Wakabayashi H, et al. *J Thromb Haemost*. 2009;7 (3):438–44).
Aim: The goal of this study was to determine the relationship between FVIII A2 subunit stabilization and its cofactor activity.
Methods: We established the A2 stabilization of two molecules 'D519VE665V and D519VE665VE1984A' and performed extended kinetic characterization of these mutants to select a candidate for further testing in animal models. These mutants differed by their FVIII and FVIIIa stability as assessed by FXase decay.
Results: Biacore studies with FVIII immobilized on an A2 subunit-specific antibody indicated comparable molecular integrity (90%) for both D519VE665V and D519VE665VE1984A after thrombin activation and 30 min of buffer wash. In contrast, only approximately 25% of B-domain' deleted FVIII (BDD) remained intact under the same conditions. Coatest assay (Chromogenix) results show D519VE665V had twice the specific activity of D519VE665VE1984A, which had comparable activity to BDD. The difference in chromogenic specific activity does not correlate with FXase decay data and raises the possibility that mutations conferring A2 subunit stability affect FVIIIa interactions with other components of the FXase complex, activated factor IX (FIXa) and factor X (FX). A kinetic assay indicated a 2-fold increase in apparent FIXa affinity for D519VE665V vs. BDD, regardless of whether FVIIIa or FIXa were varied in the assays. In contrast, D519VE665VE1984A affinity was comparable to BDD. Finally, an FXase kinetic assay with 100-pM FIXa and 10-nM FVIIIa concentrations indicated similar FX activation kinetics for all variants and BDD.
Conclusion: Taken together, our results indicate that mutations in FVIII that stabilize A2 subunit association can also affect FVIIIa interactions with its FXase partners, which raises the possibility that there may be an optimum balance between increasing A2 stability and cofactor activity.

PA 4.13-5

Increased mortality risk in US Hemophilia A inhibitor patients

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Published mortality studies are limited in hemophilia inhibitor populations but indicate that there is no increased risk of mortality in FVIII inhibitor patients. In an updated assessment of the mortality of Hemophilia A patients with inhibitors in the US, we analyzed retrospective data on 7605 males with severe hemophilia A over a 12 year period (1998–2011) using the CDC UDC database. Inhibitor cases were participants who had either an elevated inhibitor titer, were on immune tolerance or who had failed ITI according to data from their most recent UDC visit. There were a total of 641 cases and the remaining 6964 participants served as controls. Information from mortality data

forms was used to determine date and cause of death for all study subjects. During the study period, 48 of the 435 participants who died had an inhibitor. Demographic characteristics most strongly associated with death were increased age and Medicare health insurance. Among clinical characteristics increased number of reported bleeds, signs of liver disease and infection with either HIV or HCV were all strongly associated with increased odds of death. After adjustment for all other causes of death, the odds of death were 70% higher (odds ratio = 1.7; 95% confidence interval 1.2–2.4) among inhibitor cases than non-inhibitor subjects ($P < 0.01$). Over 40% of deaths in inhibitor cases were due to bleeding, especially intracranial hemorrhage, compared to 12% of deaths in non-inhibitor patients ($P < 0.001$). Of the 48 inhibitor cases who died, 31 (65%) had used one or more bypassing agents in the year prior to the last UDC visit. The data suggest that people with Hemophilia A and an inhibitor may be at higher risk of death.

PA 4.13-6

VWF affects the clearance and biodistribution of recombinant factor VIII Fc fusion (rFVIIIFc)

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Background: rFVIIIFc is a fully recombinant fusion protein consisting of a single B domain deleted human FVIII covalently attached to the dimeric Fc domain of human IgG1. rFVIIIFc was shown to have a 1.5–fold extended half-life and decreased clearance compared to rFVIII in patients with hemophilia A in clinical studies. The Fc region of rFVIIIFc binds to the neonatal Fc receptor (FcRn), which is part of a naturally occurring pathway that cycles IgG back into circulation, delaying lysosomal degradation. Clearance studies in FcRn knock-out mice indicated a similar role for FcRn in the prolonged half-life of rFVIIIFc and studies with FcRn–chimeric mice show that the decreased clearance of rFVIIIFc is mediated by FcRn expressed in somatic cells and not hematopoietic cells.

Biodistribution studies with ^{125}I–rFVIIIFc identify liver as the dominant clearance organ and primary liver cell co-culture indicated uptake of rFVIIIFc by somatic liver sinusoidal endothelial cells (LSEC), rather than Kupffer cells (KC) or hepatocytes (HC). However, in blood, 95% of FVIII circulates as a non-covalent complex bound to von Willebrand Factor (VWF) suggesting that VWF binding may also play an important role in the clearance of rFVIIIFc.

Aims: Investigate the contribution of VWF binding to the clearance and biodistribution of rFVIIIFc compared to rFVIII in order to understand the mechanism leading to the long-lasting half–life of rFVIIIFc.

Methods: Pharmacokinetic studies compared rFVIII and rFVIIIFc in VWF-KO and FVIII/VWF–KO double knockout (DKO) mice. Biodistribution studies compared ^{125}I–rFVIII and ^{125}I–rFVIIIFc in DKO and HemA mice by quantitative whole body autoradiography and quantitating the radioactivity remaining in perfused organs.

Results: VWF stabilizes rFVIIIFc in the blood and extends rFVIIIFc half-life in HemA mice ($t_{½}$ = 13.7 h) in contrast to VWF-KO ($t_{½}$ = 2.5 h) or DKO mice ($t_{½}$ = 1.6 h). Thus rFVIIIFc is cleared rapidly in VWF-KO mice, but even more rapidly in mice lacking both VWF and endogenous murine FVIII. Biodistribution studies show that the liver is the predominant clearance organ for both rFVIIIFc and rFVIII. For both molecules, over 10% of the initial dose is found in liver within the first 15 min after injection in HemA mice. In contrast, 30% of the initial dose of rFVIIIFc is found in the liver of DKO mice showing that VWF delays liver uptake and improves rFVIIIFc half–life in mice with circulating VWF. In addition, RT-PCR confirms that all three primary liver cell types (LSEC, KC and HC) express FcRn.

Summary/Conclusions: Together with our previous observation that the FcRn from LSEC is primarily responsible for extended half-life of rFVIIIFc, our results support the following conclusions:

1 Similar to FVIII, the majority of rFVIIIFc circulates as a complex with VWF and this interaction strongly influences the clearance of both FVIII and rFVIIIFc.
2 Free rFVIIIFc is taken up by LSEC, and cycled back into circulation by an FcRn–dependent mechanism.

Effects of VWF on the clearance of rFVIIIFc using both *in vitro* cell uptake and *in vivo* immunohistochemistry studies are ongoing.

PA4.14 – Extrinsic Pathway of Coagulation

PA 4.14-1

Mechanism by which platelet-bound Prothrombinase maximizes platelet procoagulant activity

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Background: The generation of physiologically relevant concentrations of thrombin, effected by Prothrombinase, a Ca^{2+}-dependent, stoichiometric (1:1) complex composed of factors Va and Xa assembled on an appropriate membrane, is absolutely essential for an appropriate hemostatic response. Physiologically, activated platelets provide this surface and in so doing promote prothrombin cleavage initially at Arg271 and subsequently at Arg320 to form thrombin. Cleavage at Arg271 generates the inactive intermediate prethrombin-2. In contrast, when Prothrombinase is assembled on the surface of phospholipid vesicles of defined content (PCPS vesicles), initial cleavage at Arg320 is preferred, generating the enzymatically-active intermediate meizothrombin, which expresses substantial anticoagulant activity.

Aim: Our aim is to define the unique functional and structural attributes of the platelet-bound complex that dictate initial cleavage at Arg271. Studies from other laboratories indicate that the prothrombinase complex is capable of binding two conformationally distinct forms of prothrombin, a 'proteinase-like' state optimally positioned for cleavage at Arg271, and a 'zymogen-like' state, optimally positioned for cleavage at Arg320. We hypothesized that platelet-bound Prothrombinase binds its substrate in a 'proteinase-like' state.

Method: Two experimental approaches were developed to address the following questions. Does platelet-bound Prothrombinase bind prothrombin in a 'proteinase-like' state that allows the irreversible incorporation of Phe-Pro-Arg-chloromethyl ketone (FPR-CK) into a pseudo-active site, whereas vesicle-bound Prothrombinase, which binds prothrombin in a 'zymogen-like' state, does not? Secondly, would increasing concentrations of dansylarginine N-(3-ethyl-1,5-pentanediyl)amide (DAPA), a reversible, active-site inhibitor of thrombin, force prothrombin into a 'proteinase-like' state and promote cleavage at Arg271 by vesicle-bound Prothrombinase?

Results: We observed that increasing the concentrations of DAPA in prothrombin activation reactions containing vesicle-bound Prothrombinase resulted in increased utilization of the prethrombin-2 pathway, suggesting that as the DAPA concentrations increased, so did the concentrations of prothrombin in a 'proteinase-like' state. When an identical experiment was performed with platelet-bound Prothrombinase as the enzyme, increasing the concentrations of DAPA increased the rate of prethrombin-2 formation, again suggesting that DAPA interactions with prothrombin forced the substrate into a more 'proteinase-like' state, making it a better substrate for platelet-bound Prothrombinase. The ability of platelet-bound Prothrombinase to bind and configure prothrombin in a 'proteinase-like' state was confirmed in experiments in which inactive platelet-bound Prothrombinase effected the incorporation of FPR-CK into prothrombin. The formation of FPR-prothrombin was verified by demonstration that the thrombin formed subsequent to its cleavage had no enzymatic

activity. In contrast, FPR-CK was not incorporated into prothrombin when inactive vesicle-bound Prothrombinase was used under similar conditions.

Summary/Conclusions: Our data support the concept that platelet-bound Prothrombinase orients prothrombin into a 'proteinase-like' state to promote the rapid generation of thrombin at a site of vascular injury, thus optimizing the procoagulant response by preventing the generation of the anticoagulant intermediate, meizothrombin. We speculate that the activated platelet membrane supports the binding of the Prothrombinase constituents in a manner that dictates prothrombin cleavage at Arg271 and the preferential use of the prethrombin-2 pathway of activation.

PA 4.14-2

Differential effects on TFPI levels upon exposure of human endothelial cells and cynomolgus monkey vascular beds to TFPI KPI-2 antibody mAb 2021 and TFPI KPI-3 antibody mAb 0001

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Background: Tissue factor pathway inhibitor (TFPI) is synthesized by endothelial cells as two isoforms. It is stored intracellularly in a full-length form (TFPI α) and present on the cell membrane mainly as the GPI-anchored TFPI β form. Heparin induces synthesis and secretion of TFPI from endothelial cells *in vitro* and increases the TFPI plasma level *in vivo* by an unknown mechanism. A recent publication describes the haemostatic effect of monoclonal antibody mAb 2021 that binds to the Kunitz proteinase inhibitor (KPI) 2 domain of TFPI. Here, we compare the *in vitro* and *in vivo* effects of mAb 2021 on TFPI secretion to the effects of heparin and an antibody binding to the KPI-3 of TFPI (mAb 0001).

Aims: To evaluate the effect of mAb 2021 and mAb 0001 on TFPI secretion from endothelial cell culture and on TFPI levels shortly (0.5–2 h) after administration to cynomolgus monkeys.

Methods: Endothelial-like cells ECV304 were treated with 5 U/mL heparin or 10 nM of mAb 2021 or mAb 0001 for 24 h in serum-free medium with 0.5% BSA. Cynomolgus monkeys were dosed intravenously with mAb 2021 or mAb 0001 (20 mg/kg) and TFPI levels were followed by measuring total-TFPI (the sum of free TFPI and TFPI in complex with antibody) and free-TFPI in plasma samples. The different TFPI levels were quantified by specific sandwich ELISAs.

Results: *In vitro*, treatment with heparin caused a 4-fold increase in TFPI levels in the supernatants from endothelial cell cultures compared to non-treated controls. Similarly, a 3-fold increase could be measured after treatment with 10 nM of mAb 0001, whereas 10 nM of mAb 2021 had no measurable effect on the TFPI levels. *In vivo,* dosing cynomolgus monkeys with mAb 0001 caused a steep increase in the total TFPI plasma level within 30 min. At 2 h the increase had continued, but at a much slower rate. The rate of accumulation of total-TFPI after mAb 2021 treatment was much slower and largely linear over the observation period. In both studies, the free TFPI levels were reduced after mAb administration.

Conclusions: TFPI KPI-3 mAb 0001 demonstrated a strong stimulatory effect on the secretion of TFPI from endothelial cells, comparable to the effect of heparin. In contrast, TFPI KPI-2 mAb 2021 had no effect on TFPI secretion. Data from cynomolgus monkeys demonstrated a rapid increase of total-TFPI in animals treated with mAb 0001. This increase is most probably due to both an accumulation of total-TFPI but also a secretion of TFPI as seen by endothelial cells *in vitro*. Animals treated with mAb 2021 showed a slow and linear increase in total-TFPI level, most likely caused only by an accumulation of total-TFPI and no secretion. The results demonstrate that the biological effects of the two antibodies are distinctly different. Only the KPI-3 antibody seems to affect the endothelial secretion of TFPI and may work by the same mechanism as heparin. However, further studies are needed to fully elucidate the mechanism of action.

PA 4.14-3

Increased expression of TFPI by NaBut is due to changes in the acetylation pattern of the TFPI promoter

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Background: Tissue factor pathway inhibitor (TFPI) is the physiological inhibitor of tissue factor (TF) induced coagulation. Increased levels of TFPI are correlated with antithrombotic and anticancer effects. Sodium butyrate (NaBut) is a histone deacetylase inhibitor that is used in treatment of cancer, and it is able to modulate gene transcription and apoptosis. In another study, we found that NaBut was able to increase the expression of TFPI in HEK293 cells, and we have previously reported that the transcriptional activity of the promoter was mediated by the −287T/C promoter SNP where the presence of the C allele resulted in higher promoter activity compared to the T allele.

Aim: The aim of the present study was to acquire information on whether the effect of NaBut on TFPI expression involved the TFPI promoter; specifically the potential role of the −287T/C SNP in this context, and also to identify nuclear factors that bind this SNP.

Methods: The TFPI promoter was studied in HEK293 cells using a reporter gene approach by measuring the luciferase activity after transient transfections with the various promoter constructs and subsequent treatment of the cells with ± 1 mM NaBut. Binding studies of nuclear proteins to the −287T/C SNP were performed using EMSA. Identification of binding factors was done with EMSA and immunoprecipitation, and the histone acetylation pattern of the promoter was studied using ChIP.

Results: Treatment of transfected cells with NaBut resulted in increased luciferase activity compared to the corresponding activity in untreated cells. This increase was dependent on the region surrounding the −287 SNP and was highest when the −287T wild type (wt) was present. EMSA studies with a probe containing either the −287T wt or the C variant allele revealed DNA binding activity of nuclear proteins from the HEK293 cells to both SNP variants; the activity was most prominent for the C allele. Treatment with the transfected cells with NaBut did not change the DNA binding activity to the SNP. The transcription factor FOXP3 was identified to bind to the T allele. Since NaBut is a histone deacetylase inhibitor, we analysed the effect on histone proteins H3 and H4. The global acetylation pattern revealed increased acetylation of both histones. Preliminary results indicate changes also in the acetylation pattern of the histone proteins H3 and H4 in the TFPI promoter after NaBut treatment. It is recognized that also non-histone proteins can be acetylated and studies are in progress to examine whether treatment with NaBut also affects the acetylation of FOXP3.

Conclusions: We conclude that NaBut affects the TFPI expression by a mechanism that involves the TFPI promoter region directly. A role of the −287T/C SNP is indicated together with changes in the acetylation pattern of the histone proteins H3 and H4 in the promoter region of TFPI. We have identified that the transcription factor FOXP3 binds specifically to the −287T allele and we hypothesize that NaBut affects the acetylation pattern of the factor. The findings may in part explain the anti-cancer effect of NaBut.

PA 4.14-4

Sodium butyrate induces TFPI-mediated apoptosis through the AKT/mTOR pathway

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Background: Sodium butyrate (NaBut) is a histone deacetylase inhibitor that has been shown to regulate gene expression and induce apoptosis in cancer cells. Tissue factor (TF) pathway inhibitor (TFPI), which regulates the TF-dependent pathway of blood coagulation, can also induce apoptosis and inhibit cell proliferation in cancer cells. In the present study we have found that NaBut elevates the endogenous expression of TFPI and induces apoptosis in HEK293 cells.
Aims: Our aim has been to further investigate the NaBut induced increase in TFPI expression and apoptosis and to explore the potential signaling pathways underlying the involvement of TFPI.
Methods: HEK293 cells were treated ± 1 mM NaBut, and The Cell Death Detection ELISA^{PLUS} kit and flow cytometry were used to assess the apoptotic activity of the cells. TFPI expression was determined using qRT-PCR and ELISA. TFPI and AKT knock down was performed with the siRNA approach. The PI3K/AKT/mTOR signaling pathway was examined by Western blot with antibodies against AKT, Phospho-Akt, mTOR and Phospho-mTOR. The activation of AKT was also determined using ELISA. Together with the knock down of AKT, the PI3K inhibitor LY294002 was used to study the involvement of AKT in NaBut induced TFPI expression and apoptosis.
Results: NaBut treatment of HEK293 cells resulted in an increased apoptotic activity accompanied by increased TFPI expression. TFPI expression increased in a time- and dose-dependent manner. When TFPI was knocked down, the apoptotic activity of the cells was impaired. NaBut treatment of the cells resulted in elevated phosphorylation of AKT and mTOR. Preliminary results indicate that inhibition of AKT phosphorylation or knock down of AKT by siRNA technology, results in an abolished effect of NaBut on TFPI expression. Studies are in progress to evaluate the effect of inactive AKT/downregulated AKT levels on the apoptotic activity of the cells after treatment with NaBut.
Conclusions: We conclude that TFPI participates in mediating the NaBut induced apoptosis in HEK293 cells since knockdown of TFPI impairs the increased apoptosis caused by NaBut treatment. Due to the activation of AKT and mTOR we hypothesize that the PI3K pathway is involved in this process. These results confirm previous findings that TFPI has anti-apoptotic properties in transformed cells and may partly explain the anti-cancer effect of NaBut.

PA 4.14-5

Factor VII antigen levels are differentially associated to etiological subtypes of ischemic stroke

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Background: Factor VII (FVII) plays a key role in the coagulation cascade and has thus been implicated in thrombotic diseases. Several polymorphisms in the FVII gene, *F7*, as well as environmental factors have been associated with plasma FVII antigen (FVIIag) levels. While higher levels of FVII have been reported in patients with cardiovascular disease, the relationship between FVII and ischemic stroke (IS) remains unclear. IS is a heterogeneous disorder comprised of four main etiologic-subtypes: large-vessel disease (LVD), small-vessel disease (SVD), cardioembolic (CE) stroke and cryptogenic stroke. The mechanism of disease and the frequency of established risk factors differ between subtype, eg atrial fibrillation (AF) is much more prevalent in CE stroke compared to the other subtypes. Analysis with respect to subtype is therefore of importance. No studies have yet looked at FVII plasma levels in relation to IS etiologic subtypes.
Aims: (i) To investigate SNPs in *F7* in relation to IS and the etiological subtypes; (ii) To determine whether AF affects FVIIag levels; (iii) To investigate whether elevated plasma levels of FVIIag are associated with an increased risk of IS, and more specifically whether there are subtype-specific differences in FVIIag.
Methods: Two SNPs were analyzed in the Sahlgrenska Academy Study on Ischemic Stroke (SAHLSIS), which comprises 844 patients with IS and 668 controls. Stroke subtypes were defined according to the TOAST criteria. Blood samples were collected from the first 600 patients in the acute phase and 3 months after index stroke, as well as the first 600 controls. These were used for subsequent FVIIag ELISA analysis.
Results: (i) The SNP rs510317 was associated to elevated FVIIag levels, while rs6046 was associated to lowered FVIIag levels. No SNP was associated with overall IS after correcting for traditional risk factors. rs510317 was independently associated to the subtype LVD (OR 1.53, 95% CI 1.02–2.30; $P = 0.04$), however this did not withstand correction for multipe testing. (ii) Stroke patients with AF had significantly lower FVIIag levels compared to those without AF, both when comparing patients with and without warfarin. (iii) Elevated FVIIag levels were independently associated with IS at both time-points. Significant differences in FVIIag levels were observed between subtypes. LVD and SVD were associated with increased FVIIag after adjusting for traditional risk factors. CE stroke was associated with decreased FVIIag levels. This was not only attributed to the high proportion of patients on anticoagulant therapy in this group, but also due to the fact that patients with AF have lower levels of FVII.
Conclusion: (i) No association between the two SNPs in *F7* and overall IS or the etiologic subtypes were detected after correction for traditional risk factors and multiple testing. (ii) AF was determined to be an important and not fully recognized confounding variable in analyses of FVIIag. (iii) FVIIag is independently associated to overall IS and significant differences exist in FVIIag levels between etiological subtypes. These findings highlight the importance of using etiological sub-classification of IS in future prospective and case-control studies on FVII.

PA 4.14-6

Endogenous tissue factor pathway inhibitor and tissue factor expression in rabbit bleeding and clotting models

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Background: Patients with haemophilia rely on prophylactic or on-demand replacement therapy that involves frequent intravenous injections of Factor VIII or Factor IX. Tissue factor pathway inhibitor (TFPI) is an endogenous inhibitor of coagulation initiation. TFPI is present in several forms; the majority is associated with the endothelium and a minor fraction is present in plasma and in platelets. Compounds that neutralise the function of TFPI present a novel strategy for future haemophilia therapy. The monoclonal antibody mAb 2021 that inhibits human TFPI is currently in clinical development with the aim of providing a subcutaneously administered prophylaxis option for patients with haemophilia.
Aims: We wanted to characterize experimental bleeding and clotting models with respect to endogenous expression of TFPI and tissue factor (TF).
Methods: We used immunohistochemistry to study the expression patterns of TFPI and TF in select rabbit tissues including the cuticle, ear,

and facial vein, which are sites commonly investigated in experimental bleeding and clotting models.
Results: TFPI was prominently present in the microvasculature of the cuticle and ear tissue, but only to a lesser extent in the endothelium of large vessels such as the facial vein. TF on the other hand was prominently expressed in the adventitia of medium-sized and large vessels, but not associated with the capillary network. The relative expression level of TFPI and TF thus varied greatly with TFPI dominating in the microvasculature and TF dominating in large vessels. In the rabbit cuticle, which contains a fine network of capillaries, the balance was shifted towards TFPI expression. In the facial vein, the balance was shifted towards TF expression, and there was an intermediate situation in the ear tissue.
Conclusions: The expression patterns of TFPI and TF show clear and consistent differences between the tissues that are studied in common experimental bleeding and clotting models. While these inherent differences may have implications for the interpretation of experimental results, the prominent expression of TFPI in the cuticle and ear suggest that these tissues provide relevant bleeding models, in which to study the effect of novel TFPI-neutralizing compounds.

PA4.15 – Factor XIII

PA 4.15-1
Factor XIII deficiency in elective hip or knee surgery

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Background: Factor XIII (FXIII) plays an essential role in fibrin stabilization and crosslinking of antifibrinolytic proteins to the fibrin clot. It is also known that FXIII has an impact on wound healing. Congenital FXIII deficiency is rare, whereas acquired FXIII deficiency is more common.
Aim: Evaluation of FXIII as useful predictor of total blood loss and in consequence of possible bleeding complications in surgery. Here, we report FXIII levels over time before and after elective hip and knee surgery correlated with blood loss.
Method: In this prospective study on 109 patients (m = 52/f = 57; mean age: 63.5 ± 10.9 years) 64 hip (58.7%) and 45 knee surgeries (41.3%) were performed. Blood samples were collected pre- and post-op 4–8 h and on Day 1, 2, 3, 4, 6 to analyse FXIII activity and antigen, VWF ristocetin cofactor activity and antigen among other coagulation parameters. Furthermore, volume of blood loss was assessed intra- and postoperatively. The analyses of data were done using descriptive statistics.
Result: A continuous decrease of FXIII activity was detected in the whole group from pre- (109.9 ± 21.9%) to D4 post-op (74.8 ± 18.3%; nadir) and in both subgroups depending on type of surgery. From D5 post-op, the FXIII-level increased in the total and all subgroups. Furthermore, highly significant differences of FXIII activity were found between patients with high amounts of blood loss in contrast to small blood loss (82.5 vs. 67.8%; $P < 0.000$). There was a significant correlation between intra-op and total blood loss, but not between post-op and total blood loss. This correlation was even more significant by cumulating the intra-op and D1-blood loss. Main blood loss occurred post-op (665 ± 621 mL in contrast to intra-op 458 ± 252). FXIII immediately post-op correlated with intra-op blood loss ($P < 0.01$) and total blood loss ($P < 0.05$). Furthermore no correlation was found between FXIII activity and VWF ristocetin cofactor and antigen.
Conclusion: FXIII activity strongly correlates with intra-op as well as post-op blood loss with a nadir on day 4 post-op. FXIII immediately post-op is an predictor of total blood loss and in consequence of possible bleeding complications. Avoiding prolonged hospital stay due to large blood loss might be of economic importance, e.g. costs of FXIII concentrate vs. prolonged hospitalization.

PA 4.15-2
Effects of free factor XIII activation peptide on factor XIII function and fibrin formation and structure

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Background: We have previously shown that the activation peptide of coagulation factor XIII (AP-FXIII) is released into plasma upon activation by thrombin and can be detected in plasma samples from patients with an acute thrombotic event.
Aim: The aim of this study was to investigate whether free AP-FXIII may affect FXIII function, fibrin clot formation and structure, and thrombin function in terms of a negative feedback regulation.
Methods: FXIII function was assessed with the FXIII biotin incorporation assay. A turbidimetric clot formation assay was used to study fibrin clot formation in plasma. Clot formation and viscoelastic properties of the clot were also analysed by rotation thrombelastometry (ROTEM) performed in plasma and whole blood. The structure of plasma clots was investigated by permeation analysis and scanning electron microscopy (SEM). Thrombin activity was determined using chromogenic substrates. All experiments were performed in the presence of different concentrations of AP-FXIII or a scrambled peptide of the same amino acid composition but in random order as a negative control.
Results: AP-FXIII reduced FXIII-dependent biotin incorporation by up to 50% in a dose-dependent manner. When FXIII was pre-activated, AP-FXIII had no effect suggesting that AP-FXIII interferes with FXIII activation but not its transglutaminase reaction. In the turbidimetric clotting assay, kinetics of fibrin clot formation were not altered but maximum absorbance was reduced by up to 46% by increasing AP-FXIII concentrations. Maximum clot firmness measured by ROTEM was reduced by up to 30%. Plasma clots made in the presence of AP-FXIII showed significantly reduced permeability with a 40% reduction in pore size. SEM data, however, showed no significant effect of AP-FXIII on plasma clot structure. Thrombin activity towards chromogenic substrates were not altered by AP-FXIII. The scrambled peptide had no effects in any of the experiments.
Conclusions: We have found first evidence that AP-FXIII inhibits FXIII activation and has an effect on plasma clot structure. We propose that AP-FXIII might act as a negative feedback regulator of FXIII activation, and might interact with fibrin fibres during polymerisation and/or crosslinking. Further studies are needed to elucidate the underlying mechanisms and potential relevance of our findings.

PA 4.15-3
Factor XIII and corneal wound healing

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Background: In addition to its role in hemostasis, coagulation factor XIII (FXIII) is involved in wound healing and also exerts several other cellular functions. Beside plasma, the dimer of its potentially active A subunit (FXIII-A) is also present in various cells, like platelets, monocytes/macrophages, osteoblasts and chondrocytes. We have previously reported that FXIII is present in normal tears at very low concentration (Orosz et al. J Immunol Methods 2010;353:87–92), but following corneal surgery, particularly following corneal transplantation, its concentration increases up-to 25–40-fold (Orosz et al. Clin Chim Acta 2011;412:271–6). Based on these results we presumed that FXIII plays a role in the wound healing of corneal tissue.
Aims: The aim of the study was to provide more direct proofs on the involvement of FXIII in corneal wound healing and to explore if the cellular form of FXIII (cFXIII) is expressed in cells of human cornea.

Methods: Scratch wound healing experiments were performed on virus transformed human corneal epithelial cell (HCET) culture in the presence and absence of recombinant human FXIII-A_2 (rFXIII). Wound closure was monitored by video-microscopy and the area uncovered by cells was recorded at various times. The effect of rFXIII on cell proliferation and migration was investigated by EZ4U and xCELLigence RTCA DP Instrument using CIM-Plate 16, respectively. Thrombospondin-1 (TSP-1) content of the cells and media was determined by ELISA technique and Western blotting. Frozen sections of normal human corneas obtained from enucleated bulbus with posterior segment tumor were immunostained for cFXIII, for transgultaminase (TG) 1 and 2. Detection of cFXIII was combined with labeling for CD11b, CD34, CD45, CD68 and CD163 in double immunofluorescent staining. Corneas were analyzed for FXIII-A by Western blotting and FXIII-A mRNA by RT-PCR technique. The study was approved by the Regional Medical Ethics Committee.
Results: Addition of rFXIII to HCET cell culture, even at low concentration, significantly accelerated the healing of scratch wound. The improved scratch wound healing was due to increased proliferation of the cells rather than to an effect on their migration. Treatment of HCET cells with rFXIII resulted in the down-regulation of TSP-1 expression.
A significant part of keratocytes showed intensive staining for cFXIII, but not for TG-1 or TG-2. Neither epithelial nor endothelial cells were labeled by anti-FXIII antibody. FXIII-A positive cells were unevenly distributed in the corneal stroma, abundant in the subepithelial tertile and sparse in the subendothelial tertile. FXIII-A+ cells showed co-staining for CD34, however, a significant number of CD34+ cells were negative for cFXIII. The presence of cFXIII in the cornea was also confirmed by Western blotting. RT-PCR experiments indicated that stromal keratocytes are capable of synthesizing cFXIII.
Conclusions: FXIII promotes the healing of scratch wound in HCET cell culture. cFXIII is abundant in subepithelial keratocytes. It is suggested that in the case of corneal injury cFXIII released from subepithelial keratocytes, plus FXIII present in tears, play a role in the repair of corneal injury. Keratocyte FXIII might also be involved in the structural organization of corneal stroma.

PA 4.15-4

Pre-clinical safety and prolonged pharmacokinetic/pharmacodynamic (PK/PD) properties of a recombinant fusion protein linking activated coagulation factor VII with albumin (rVIIa-FP)

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Background: Recombinant factor VIIa (rFVIIa) is approved to control bleeding in haemophilia patients who developed inhibitory antibodies to replacement therapy. rFVIIa is rapidly eliminated with a terminal half-life of approximately 2.5 h in humans. This short half life of rFVIIa necessitates frequent injections and considerably limits prophylactic use. A recombinant fusion protein linking activated factor VII with albumin (rVIIa-FP) was therefore developed to extend the half life of rFVIIa.
Aim: The present studies were conducted to support the clinical efficacy and tolerability evaluation of rVIIa-FP by gathering knowledge about its pharmacodynamic activity based on procoagulant effects in a preclinical venous stasis model and by assessing PK and safety aspects in animals.
Methods: During a GLP-compliant toxicity program in several animal species, the anaphylactic or allergic, immunogenic and prothrombotic potential of rVIIa-FP, its impact on safety pharmacology variables and systemic toxicity parameters as well as the local tolerability were assessed applying single or repeated dosing regimens. The PK profiles of both rVIIa-FP and NovoSeven®, a licensed rFVIIa, were recorded measuring selective FVIIa activity after a single treatment of cynomolgus monkeys at clinically relevant dose levels of 2700 and 270 μg/kg, respectively. When investigating PD responses and duration thereof, rVIIa-FP and NovoSeven® were administered intravenously to normal rabbits before induction of venous stasis followed by assessment of clot formation and systemic haemostasis parameters. In parallel, FVIIa activity was recorded in plasma samples.
Results: Overall, the safety program showed that administration of rVIIa-FP was well tolerated with no findings indicative of adverse systemic toxicity, anaphylactic or allergic reactions and without any safety pharmacology or local intolerability concerns. Intriguingly, in both animal species tested, the systemic bioavailability, clearance and in addition the terminal half-life ($t_{1/2\beta}$) of rVIIa-FP were significantly better in comparison to NovoSeven®. When recording FVIIa activity in plasma samples derived from monkeys, the 11-fold higher area under the curve (AUC) was accompanied by a fourfold longer $t_{1/2\beta}$, while *in vivo* recovery (IVR) of rVIIa-FP exceeded that of NovoSeven® by 70%. In rabbits, the 20-fold higher AUC was associated with a 5-fold extension in $t_{1/2\beta}$ and a fourfold increase in IVR when measuring selective plasmatic FVIIa activity. In this animal model, the procoagulant effect of rVIIa-FP was not different from NovoSeven® after treatment under acute conditions. However, at advanced time-points the prolonged systemic availability of rVIIa-FP translated into prolonged haemostatic activity.
Summary/Conclusion: Consequently, these animal studies confirmed that the recombinant albumin fusion technology was successfully applied to human recombinant FVIIa for improvement of pharmacokinetic parameters. The non-clinical pharmacology and safety program proved prolonged activity and a favourable tolerability profile of rVIIa-FP. The presented investigations did not reveal any safety concerns and support the evidence necessary for further clinical development in humans (PROLONG-7FP).

PA 4.15-5

Molecular interaction of factor XIII subunits

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Background: Plasma factor XIII (FXIII) consists of two catalytic A subunits (FXIII-A) produced by cells of bone marrow origin and two protective/inhibitory B subunits (FXIII-B) synthesized by hepatocytes. FXIII-B, a mosaic protein, consisting of 10 sushi domains, circulates in excess; approximately 50% of it is free in the plasma. The formation of FXIII-A_2B_2 complex takes place in the plasma. The exact binding constant between the two subunits is not known and the structures involved in the association of the two types of subunits are not well characterized. FXIII is also present in body fluids, cerebrospinal fluid, tears, bronchoalveolar lining fluid, although in much less concentration than in the plasma. However, in these body fluids the majority FXIII-A exists as free, non-complexed dimer.
Aims: To investigate the binding kinetics of the two subunits and to locate the binding site of FXIII-A on the B subunit. Using the Kd established for the association to calculate the ratios of free to total FXIII-A_2 in different body fluids and compare them with experimentally determined values.
Methods: Binding constant was measured by surface plasmon resonance and also by an ELISA type assay. Using this Kd and the total FXIII subunit concentrations in the body fluids the theoretical ratio of free to complexed FXIII-A_2 was calculated. The actual concentrations of free FXIII-A_2 was determined by ELISA technique after the complete removal of free FXIII-B and FXIII-A_2B_2 complex by immunoabsorption. To locate the epitopes responsible for the association of the two subunits a monoclonal anti-FXIII-B antibody preventing complex formation between the two types of subunits was produced. Epitope mapping was carried out by tryptic digestion of FXIII-B bound to our surface linked antibody. The tryptic fragments that remained associ-

ated with the antibody were identified by MALDI TOF analysis. Recombinant isolated sushi domains 1–2 of FXIII-B were produced in insect cells.
Results: The Kd was found to be in the range of 2.93–5.43 $\times$ 10^{-10} by both methods. Using this Kd it was calculated that approximately 1% of plasma FXIII-A_2 should be in free form. This value was experimentally confirmed using FXIII-B depleted plasma. In body fluids with much lower FXIII subunit concentrations, like cerebrospinal fluid or tears, a much higher percentage (80–90%) of FXIII-A_2 existed in free form. The calculated and measured values agreed in these cases, too. The antibody that prevented complex formation reacted with isolated sushi domains 1–2 produced in insect cells. The sequence of the tryptic fragments that remained associated with the antibody corresponded to the overlapping sequences between amino acids I90-K103 in the second sushi domain.
Conclusion: The two FXIII subunits are tightly associated, but depending on the concentration of the subunits certain amount of free FXIII-A_2 is also present in body fluids. The small percentage of free FXIII-A_2 in the plasma might contribute to the so-called innate plasma transglutaminase activity. Our results together with those of Suori et al. (Biochemistry 2005;47:8656–64) suggest that the N-terminal part of FXIII-B (sushi domains 1–2) are involved in the formation of FXIII-A_2B_2 complex.

PA 4.15-6
Common FXIII polymorphisms associate with abdominal aortic aneurysms and with overall survival rate

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Background: An abdominal aortic aneurysm (AAA) involves the dilatation of the abdominal aorta and if left untreated leads to high mortality due to rupture. Deposition of an intraluminal thrombus increases the rate of AAA expansion. It is thought blood coagulation FXIII may play a role in AAA due to its part in thrombus formation. Three common FXIII polymorphisms have been identified; FXIII-A Val34Leu, FXIII-B His95Arg and FXIII-B Splice Variant (intron K nt29576C-G). FXIII-A Val34Leu is a polymorphism that is known to affect FXIII activation rate and fibrin structure. FXIII-B His95Arg is a polymorphism associated with a moderately increased risk for venous thrombosis, and with increased dissociation of the FXIII subunits in plasma. The FXIII-B Splice variant leads to a novel splice acceptor site and a protein 15 amino acids longer at the C-terminus. The roles of these FXIII polymorphisms in AAA are currently unknown.
Aims: Our aims were to analyse FXIII-A Val34Leu, FXIII-B His95Arg and FXIII-B Splice Variant in patients with AAA compared with control subjects and assess the effect of on AAA survival.
Methods: DNA was extracted from 529 AAA patients and 469 controls. All subjects were enrolled as part of the Leeds Aneurysm Development Study (LEADS). All patients were Caucasian, aged $\geq$ 55 years and had an AAA $\geq$ 3 cm. These were compared to age and sex matched controls (also all Caucasian) who had an aortic diameter $\leq$ 2.9 cm. Samples were analysed by real-time PCR for the FXII-B His95Arg, FXIII-A Val34Leu and FXIII-B Splice Variant polymorphisms. Genotype distributions were tested by Chi-squared and Student *t* test, survival was analysed by Kaplan-Meier.
Results: Our data showed that FXIII-B Arg95 was more prevalent in AAA patients than controls. This difference was significant with 24% (n = 130) of the AAA patients possessing $\geq$ 1 Arg allele compared with 16.2% (n = 76) of the controls possessing $\geq$ 1 Arg allele (P = 0.001). There was no difference regarding FXIII-A Val34Leu or FXIII-B Splice Variant genotype distribution between AAA patients and controls (P = 0.666 and P = 0.733 respectively). Mean crude survival for AAA patients with > 1 Leu allele was 6.230 (CI 5.78–6.68) compared to 6.85 years (CI 6.48–7.22) for Val/Val, (P = 0.064). Although there was an increase in overall mortality for AAA, it was not significant. There was no significant difference in mean crude survival for FXIII-B His95Arg or FXIII-B Splice Variant polymorphisms.
Summary: This data suggests that (i) FXIII-B His95Arg may increase risk of rupture, complications or repair in AAA, (ii) FXIII-A Val34-Leu and FXIII-B Splice Variant polymorphisms alone are not a risk factor for AAA and (iii) FXIII-A Val34Leu influences overall mortality in this group of patients.

PA4.16 – Diagnosis of VTE – VI

PA 4.16-1
Conventional or age-adjusted D-dimer cut-off values to exclude venous thromboembolism in older patients: a systematic review and meta-analysis

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Background: Since D-dimer levels increase with age, the proportion of false-positive D-dimer tests for detecting venous thromboembolism using conventional cut-off values (500 μg/L) increases in the elderly, and the specificity decreases. Age-adjusted D-dimer cut-off values (age*10 μg/L in patients aged > 50 years) have therefore been introduced.
Aims: To review the diagnostic accuracy of D-dimer testing in elderly patients suspected of venous thromboembolism, using conventional or age-adjusted D-dimer cut-off values.
Methods: MEDLINE and Embase databases were searched for studies, published before June 21, 2012, and authors of primary studies were contacted. We selected all studies that enrolled older patients suspected of venous thromboembolism in whom D-dimer testing -using both conventional and age-adjusted cut-off values- and reference testing were performed. The quality of the studies was appraised using the QUADAS-2 tool. 2 $\times$ 2 tables were reconstructed and stratified by age-category and applied D-dimer cut-off value.
Results: Thirteen cohorts including 4875 patients aged over 60 years and a non-high clinical probability, were included in the meta-analysis. The specificity of the conventional cut-off value decreased with increasing age, from 39.6% (95% confidence interval 33.6–46.5%), to 24.7% (19.7–30.5%) and 14.7% (11.2–19.2%) in patients aged 61–70, 71–80 and > 80 years respectively. Age-adjusted cut-off values revealed higher specificities over all age categories: 49.9% (42.8–58.9%), 44.4% (37.6–51.5%) and 35.8% (29.4–42.9%) respectively. Sensitivities of the age-adjusted cut-off remained above 97% in all age categories.
Conclusions: The application of age-adjusted cut-off values nearly doubles the specificity without modifying the sensitivity, thereby largely improving the clinical utility of D-dimer testing in the elderly.

PA 4.16-2
Arterial disease in patients with symptomatic venous thromboembolism. Findings from the RIETE registry

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Background: The relationship between venous thromboembolism (VTE) and atherosclerosis has not been consistently explored.

Aims and Methods: We used the RIETE Registry data to compare the rate of arterial events and VTE recurrences during the first 3 months of therapy, and to identify risk factors for both events.
Results: As of October 2012, 17,485 consecutive patients in RIETE had information on risk factors for atherosclerosis and subsequent arterial events. Of these, 8272 initially presented with deep vein thrombosis (DVT) and 9213 with pulmonary embolism (PE). In patients presenting with DVT, the number of patients dying of arterial events (ischemic stroke 11, lower limb ischemia 4, myocardial infarction 3, mesenteric ischemia 3) exceeded the number of those dying of recurrent PE ($N = 11$). In patients initially presenting with PE, the rate of fatal arterial events after the second week of therapy (myocardial infarction 7, ischemic stroke 3, other 2) exceeded the rate of fatal recurrent PE ($N = 7$). On multivariate analysis, prior arterial disease (odds ratio [OR]: 1.4; 95% CI: 1.03–1.8), diabetes (OR: 1.4; 95% CI: 1.01–1.8), statin therapy (OR: 1.4; 95% CI: 1.07–1.8) and smoking habit (OR: 1.4; 95% CI: 1.1–1.9) independently predicted the risk for recurrent VTE, while unprovoked VTE (OR:0.6; 95% CI:0.4–0.96) and cancer (OR: 2.5; 95% CI: 1.6–3.9) independently predicted the risk for subsequent arterial events.
Conclusions: In patients with acute VTE the mortality due to subsequent arterial events may be worse than that due to VTE recurrences. Some risk factors for these complications have been identified.

PA 4.16-3
Incidence of superficial vein thrombosis: a community-based study

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Background: Superficial vein thrombosis (SVT) is perceived as a common and not entirely benign disease, managed by both general practitioners and vascular physicians.[1] Deep vein thrombosis (DVT) affects approximately 6–12 persons per 10,000 per year and Pulmonary embolism (PE) 3–6 per 10,000 per year, yet the incidence of SVT is still unknown.
Aims: To assess, in an unselected population and a well-defined geographic area, the annual incidence of symptomatic SVT of the lower limbs associated or not with concomitant symptomatic DVT and/or symptomatic PE.
Methods: A cross-sectional study was conducted for 1 year, involving the entire 265,686 residents of the urban area of Saint-Etienne, France, aged 18 years or older.
For each clinical suspicion of SVT, a standardized questionnaire was completed by the general practitioner and/or the vascular physician. Compression ultrasonography was systematically performed by the vascular physician in order to confirm SVT and to check for the existence of concomitant DVT. Each clinical suspicion of concomitant PE had to be confirmed by spiral computed tomography or high probability ventilation/perfusion lung scan. Based on these data, outcomes were validated by an independent adjudication committee. This protocol was approved by the ethical committee of the University Hospital of Saint-Etienne. All patients were informed of the study, however, no written consent was required.
All vascular physicians of the predefined area participated in this study. All general practitioners of this area were informed of the study and asked to refer any patient with clinically suspected SVT to a vascular physician in order to perform compression ultrasonography, as recommended in the latest ACCP guidelines.
Annual incidence rates (per ten thousand) were calculated as the number of cases of SVT occurring during the study period divided by the population of the predefined area aged 18 years or more. Ninety-five percent Confidence Intervals (CI) were also calculated. The frequencies of concomitant DVT and/or PE and their 95% CI were estimated.
Results: Between November 14, 2011 and November 13, 2012, a total of 175 patients had confirmed SVT. The incidence rate of symptomatic confirmed SVT was 6.6 per 10,000 per year (PY) (95% confidence interval [CI]: 5.6–7.6). Among them, 43 (24.6%; 95% CI: 18.4–31.6) were associated with concomitant DVT (of which 21 were proximal), and 8 (4.6%; 95% CI: 2.0–8.8) were associated with concomitant PE, all confirmed by spiral computed tomography.
Conclusion: Incidence of SVT is sizeable. It appears to be between the incidence of DVT and that of PE.
Reference:
1. Decousus H et al. The POST study. *Ann Intern Med* 2010;152 (4):218–224.

PA 4.16-4
Exploring decisions to withhold diagnostic investigations in Dutch nursing home patients with a clinical suspicion of venous thromboembolism: a mixed method study

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Background: Imaging investigations can contribute to improved outcomes in patients with suspected venous thromboembolism by increasing the probability of the appropriate diagnosis and subsequent guidance of appropriate treatment-decisions. However, attendance of a hospital and undergoing diagnostic investigations can be burdensome for frail older patients. Therefore, many physicians would prefer to confirm or refute diagnoses without referring their frail older patients for additional diagnostic investigations.
Aims: To explore decisions to withhold additional diagnostic investigations in nursing home patients with a clinical suspicion of venous thromboembolism.
Methods: A mixed-method study was performed. The frequency of decisions to forgo diagnostic investigations was established with a cross-sectional observational study in nursing homes in the Netherlands on diagnostic strategies for elderly patients with a suspicion of venous thromboembolism. Patient characteristics, bleeding-complications and mortality were related to the decision to withhold investigations. For a better understanding of the physicians' decisions, 21 individual face-to-face in-depth interviews with elderly care physicians were performed and analysed by two researchers using the grounded theory approach.
Results: Referal for additional diagnostic investigations was forgone in 104/268 (38.8%) patients with an indication for diagnostic work-up. 'Blind' anticoagulant treatment was initiated in 79 (76.0%) of these patients. Patients in whom investigations were withheld were older and mortality and major-bleedings occurred more often within 3 months, compared to the referred patients (respectively 83.8 vs. 80.9 years; 34.0% vs. 18.2% and 6.7% vs. 1.2%). In their decisions to forgo diagnostic investigations, elderly care physicians estimated the proportionality of investigations: Considering the impact of the potential disease given the patients' burden and prognosis of chronic diseases, physicians estimated the benefits of the diagnostic investigation for the patient. This was balanced against the considered burden and risks of the diagnostic investigation and whether performing investigations agreed with established management goals in advance care planning. As a result of their decisions to forgo diagnostic investigations ('non-diagnosis decisions'), physicians were left with diagnostic uncertainty which they managed by acceptation of the possible consequences of the potential disease.
Conclusions: Referral for additional diagnostic investigations is commonly withheld in Dutch nursing home patients with a clinical suspicion of venous thromboembolism. Patients in whom investigations were withheld had higher mortality- and bleeding rates than referred patients. Physicians mainly based their decisions on their judgment concerning the proportionality of the investigation. Given the worse

outcomes of the non-referred patients and complexity of the decisions, more attention for decisions concerning withholding diagnostic investigations in older patients with suspected venous thromboembolism is needed.

PA 4.16-5

Prevalence of pulmonary embolism in patients with syncope

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Background: The clinical features of pulmonary embolism (PE) are extremely variable. While fainting or syncope has been reported to occur in up to 25% of patients with PE, the prevalence of PE among patients with syncope is unknown.
Aims: The primary study outcome was to assess the prevalence of PE in a large series of consecutive patients presenting with the first episode of syncope.
Methods: All patients with the first episode of transient and short-lasting loss of consciousness, consecutively referred to our Department in a 20-month period, had a diagnostic workup for the assessment of the most common causes of syncope, and were evaluated for the presence of PE with the use of an internationally accepted algorithm including a pre-test clinical probability (PTP according to the method of Wells et al.) and a high-sensitivity quantitative D-dimer assay. If the PTP was low and D-dimer was negative, PE was excluded. All other patients underwent confirmatory diagnostic tests (either computerized tomography or ventilation/perfusion lung scanning). A written informed consent was obtained from all patients. The study was approved by the local ethics committee.
Results: Among 201 eligible patients, 177 met the inclusion criteria. The mean age was 75 ± 14 years: 78 (44.1%) were males. In 100 (56.5%) no clear explanation for the loss of consciousness could be identified. One hundred and five patients (52.2%) had low PTP and negative D-dimer. Among the remaining 72 patients with high PTP and/or positiveD-dimer, PE was detected in 23. They all belonged to the group of 100 (23.0%) patients presenting with apparently unexplained syncope. Hence, the prevalence of PE in our series of unselected patients with syncope was 13.0% (95% CI, 8.0–17.9). This proportion became 23.0% (95% CI, 15.2–32.5) when confined to patients with unexplained syncope, and increased up to 31.9% (95% CI, 21.4–44.0) in patients with high PTP and/or positive D-dimer. All patients with PE-related syncope received conventional anticoagulation and had a favourable outcome.
Conclusions: Pulmonary embolism can account for up to 18% of all patients presenting with the first episode of syncope, up to 33% of those with unexplained syncope, and up to 44% of patients with high PTP and/or positive D-dimer. Accordingly, the occurrence of this life-threatening complication should be promptly investigated in all patients with syncope, especially in those with an apparently unexplained episode.

PA 4.16-6

Performance of five clinical decision scores to rule out pulmonary embolism in primary care: a validation study

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Background: Multiple clinical decision rules (CDRs) have been developed to help physicians to rule-out pulmonary embolism (PE) in a hospital based setting. These CDRs are used (combined with D-dimer testing) to identify low-risk patients where PE can be safely excluded without referral for spiral CT scanning. Such a rule-out approach seems ideal for primary care, where most suspected patients are first evaluated. Yet, validation studies of these CDRs are altogether lacking, for a primary care domain.
Aims: To assess the ability to safely rule-out PE using five available CDRs (i.e. original and simplified Wells, revised and simplified revised Geneva, and Charlotte Rule) in suspected patients in primary care.
Methods: We prospectively collected data in primary care, including all items needed to calculate the score of all five CDRs. Patients were eligible for inclusion if the participating general practitioners (GPs, $n = 300$) had a suspicion of acute PE. A qualitative D-dimer test (Clearview Simplify) was performed by the GPs. All patients were subsequently referred to secondary care. Confirmation of the final diagnosis was based on a composite reference standard, including spiral CT scanning and 3 months of follow-up in primary care. Sensitivity and specificity, efficiency (defined as proportion of patients at low risk) and safety (proportion of patients with PE in this low risk category) of all CDRs (combined with a negative D-dimer) were calculated. A clinical calibration plot of each CDR (i.e. a graph in which the score of the CDR is plotted against the observed outcome of PE) was created.
Results: We included 598 patients with suspected PE in primary care (mean age 48 years, 71% females). PE prevalence was 12%. Sensitivity of the CDRs without D-dimer testing was low (range 52–88%), except for the Charlotte Rule (93%). In combination with a D-dimer test, sensitivity increased: range 88–96%. The efficiency of all rules combined with negative D-dimer testing was good and comparable (range 44–48%), except for the Charlotte Rule (low efficiency; 17%). Both the Wells and the Charlotte rule had an acceptable low failure rate (range 1.2–1.5%) whereas the Geneva rules had higher failure rates (range 2.7–3.1%). Visual inspection of the calibration plots showed good calibration (i.e. the higher the score, the higher the probability of indeed having PE) for the two Wells scores, whereas this was not the case for the Geneva rules.
Summary/Conclusions: PE can be safely excluded in primary care, notably using the original and simplified Wells CDR combined with qualitative D-dimer testing. Using these rules leads to an efficiency of around 40% and an acceptable low failure rate of below 2% in primary care. Hence, using such a rule-out approach, PE can be excluded in primary care in about four in every 10 suspected patients.

PA4.17 – DIC

PA 4.17-1

Increased levels of nucleosome in human plasma in septic patients with DIC

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Sepsis-related DIC is a life-threatening condition that is characterized by a whole-body inflammatory state (called a systemic inflammatory response syndrome, SIRS). Histones are essential for packing DNA into the cell nucleus and also play role in regulating gene expression but our study (last ISTH) have shown histones released into blood stream at the onset of sepsis-related DIC. However, western blot analysis is necessary for the detection of blood histone, and a result is not easily given. On the other hand, the nucleosome including histone is easily measurable by ELISA. Therefore, we measured the nucleosome in the sepsis-related DIC patient ($n = 15$) by ELISA (Cell Death Detection ELISA). Also, we investigated the nucleosome in the plasma from septic patients without DIC ($n = 5$). At the same time, extracellular histones in the plasma were examined by western blot analysis for histone H3 (H3).
The thrombin antithrombin complexes (TAT) levels were higher in septic patients with DIC as reported by others. We detected high levels

of nucleosome in the plasma of all septic patients with DIC by EISA. Also we recognized high levels of extracellular hisotones (H3) in the plasma of all septic patients with DIC by western blotting. However, in five septic patients without DIC, blood nucleosome became positive in two cases weakly, but was negative in three cases. Furthermore, in a sepsis-related DIC patient, blood nucleosome became negative when DIC was improved. Moreover, high-mobility group box 1 (HMGB1) concentrations in the human plasma were determined using ELISA kit (Shino-Test, Japan). Plasma levels of HMBG1 were negative for two cases in 10 septic patients with DIC. Also, Plasma levels of HMBG1 were negative in septic patients without DIC.
It was suggested that nucleosome in the plasma increased in the septic patients with DIC. Also these results suggest that assess of nucleosome in the plasma may be helpful in the making the diagnosis in septic patients with DIC.

PA 4.17-2

Dysregulation of inflammatory and hemostatic markers in sepsis associated disseminated intravascular coagulation

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Background: Disseminated intravascular coagulation (DIC) represents a complex pathophysiologic syndrome where marked alterations in the hemostatic system are manifested. As a result several inflammatory mediators are up regulated through multiple mechanisms.
Aims: The up regulation of inflammatory mediators such as anaphylatoxin C5a (C5a), procalcitonin (PCT), interleukin 6 (IL-6), interleukin 10 (IL-10), myeloperoxidase (MPO), C reactive protein (CRP), and circulating levels of hemostatic markers including protein C inhibitor (PCI), plasminogen activator inhibitor 1 (PAI-1), and protein C (Pr C) were evaluated in 741 subjects enrolled in a randomized, double-blind, placebo-controlled, Phase-2B study evaluating the safety and efficacy of recombinant thrombomodulin (ART-123) in subjects with sepsis and suspected DIC.
Methods: Thirty healthy male and female volunteers served as the control group. Commercially available ELISA methods PAI-1, AT and PrC (Diagnostica Stago, Parsippany, NJ), MPO (Assay Design, Ann Arbor, MI), IL-6 and IL-10 (R&D Systems (Minneapolis, MN), C5A (BD Biosceince, Franklin Lakes, NJ), CRP (Hyphen-Biomed, France), PCT (Brahms, Germany) and PCI (Technoclone, Austria) were used to measure the various mediators. Marked deviations in the circulating levels of these markers, as compared to controls, were observed.
Results: Subjects with sepsis associated DIC showed an increase in the circulating levels of most inflammatory markers. The percentage change from the normal controls of PCT (↑14.515%), IL-6 (↑6584%), CRP (↑1737%), IL-10 (↑836%), MPO (↑575%) and C5a (↑85%) where considerably higher in the DIC subjects whereas PCI (↓39%), Pr C (↓45%) and AT (↓42%) exhibited slight decreases. Wide individual variations were present. The PAI-1 (↑ 297%) levels were also increased in the DIC subjects.
Conclusion: These results clearly indicate that inflammation and impairment of fibrinolysis play a key role in the pathogenesis of sepsis associated DIC.

PA 4.17-3

Thrombin generation mediators and markers in sepsis associated coagulopathy and their modulation by recombinant thrombomodulin

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Background: Severe sepsis remains the most common cause of death in critically ill patients and thrombin plays a crucial role in the pathogenesis of sepsis associated disseminated intravascular coagulation (DIC).
Aims: The purpose of this study was to profile prothrombin fragment (F1.2), thrombin antithrombin complex (TAT) and D-dimer (DD) throughout the course of hospital stay in subjects identified with sepsis.
Methods: Patients were randomized to receive either thrombomodulin (ART-123) 0.06 mg/kg/day intravenously ($n = 371$) or placebo ($n = 370$), in addition to standard of care for 6 days. Blood samples were drawn for the biomarkers at baseline (day 0), 24 h after the first dosage (day 1), day 3 immediately before the next dosage (day 3 pre) and 24 h after the second dosage (day 3 post) and on days 7 and 14. Plasma samples were analyzed using the commercially available ELISA kits for prothrombin F1.2 complex and thrombin antithrombin complex (Dade Behring Siemens Miami, FL) and Asserachrome D-dimer (Stago, Parsippany, NJ).
Results: Administration of ART-123 resulted in a decrease in F1.2, DD and TAT levels. This decrease in F1.2 and DD was significantly different ($P < 0.001$) between treatment groups for days 1, 3 and 7. The TAT levels were significantly different from the placebo group on days 1 and 3 ($P < 0.05$) and on day 7 ($P < 0.001$)
Conclusion: While the data was widely scattered, these results show that DIC represents a hypercoagulable state along with other hemostatic abnormalities and the activation of the inflammatory process. Modulation of these activation processes through such targets as DD, F1.2 and TAT may play an important regulatory role in the pathogenesis of sepsis associated coagulopathy. Moreover, this study validates the hypothesis that thrombomodulin down regulates the thrombin generation mediators/markers in sepsis associated DIC.

PA 4.17-4

DIC and DIC in septic shock: myth or reality? (Why clinical trials failed to improve survival.)

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Background: For many years, in critically ill patients, DIC acronym has both significations: disseminated intravascular coagulopathy and death is coming. Many experimental studies point out the pivotal role of deregulated thrombin generation in the development of multiple organ failure and death.
Aims: To evaluate DIC scoring systems and clinician ability to diagnose DIC.
Methods: Two hundred and sixty-five patients with septic shock admitted in three French intensive care units were prospectively included in this observational study. DIC was diagnosed using ISTH 2001 'overt' and 'non overt' scores and JAAM 2006 score (reference score as specifically developed in critically ill patients). 'Non overt' score included antithrombin. Twelve clinicians unrelated to this study were asked for DIC diagnosis in 45 patients. After a 3-mounths period they were informed about DIC scores and asked a second time for the same patients. Statistical analysis uses k score to evaluate coherence between DIC scores and between clinicians and JAAM.

Results: DIC was present in 102 (38.5%), 92 (34.7%) and 91 (34.3%) patients according to JAAM, ISTH 'overt' and 'non overt' scores respectively. Nevertheless, k were 0.78 (JAAM/'overt'), 0.70 (JAAM/'non overt') and 0.69 ('overt'/'non overt') and only 50 patients were classified in DIC group by the three scores.
Clinicians poorly diagnosed DIC (k = 0.504 ± 0.115 [range: 0.305–0.728]) and only one used ISTH 'overt' score. In the second evaluation test, *k* was not significantly different despite scores supply. Clinicians did not calculate scores, or calculate 2 or 3 scores and were not able to make a choice. JAAM and 'non overt' were more frequently used by clinicians.
Mortality was not different regarding DIC diagnosis ($P = 0.053$, hazard ratio for no DIC 0.65 [0.43–1.01]). Then, we separate the 265 patients in three groups according to JAAM score and related haemostasis activation/deregulation: 1–2 (minimal, $n = 128$), 3–4 (intermediate, $n = 54$) and 5–8 (patent, $n = 83$). Patients in the third groups were more severely ill (SAPS2 and SOFA), had higher incidence of acute renal failure and renal replacement therapy requirement ($P < 0.05$) and 28-days mortality ($P = 0.006$, hazard ratio for no DIC 0.53 [0.30–0.75]). Prothrombin fragments 1 + 2 were increased regardless group confirming high thrombin generation in all septic patients, but fibrin monomers were higher and protein C lower in patients with higher JAAM scores (5–8). In multiple logistic regression models, only platelets count and D-dimers were associated with DIC diagnosis.
Summary/Conclusions: DIC remains difficult to diagnose without a rigorous scoring system and clinicians failed to recognise these patients. Moreover, mortality was not correlated with DIC but with JAAM score ≥ 5. A lower value was not associated with organ failure or mortality and targeted antithrombotic treatment would probably be inefficient if not deleterious as reported in large-scale randomised clinical trials with anticoagulants (TFPI, antithrombin and activated protein C) and one could suggest that only patients above this cut-off would be improved by specific therapies.

PA 4.17-5
New diagnostic strategy of sepsis induced disseminated intravascular coagulation (DIC)

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Background: Inflammation and coagulation are interrelated pathophysiologic processes that considerably affect each other. And various biomarkers such as proinflammatory cytokines, chemokines, adhesion molecules, tissue factor expression, platelet and endothelial activation are closely concerned with the complex interactions between inflammatory response and coagulopathy. However, there are different diagnostic criteria in sepsis and disseminated intravascular coagulation (DIC).
Aims: This study were to establish the diagnostic criteria of sepsis induced DIC.
Methods: A single center, prospective, observational study was carried out. Patients who had one or more systemic inflammatory response syndrome (SIRS) criteria were included in this study. The blood samples for measuring the markers were collected at the time of admission. Eighty two patients were enrolled for this prospective study from June 2010 to June 2011.
Results: Forty two patients (51.2%;42/82) were sepsis, severe sepsis or septic shock at the time of registration. In the receiver operating characteristics (ROC) analysis, the area under the curve (AUC) to distinguish sepsis was the highest for Procalcitonin (PCT) (0.91) followed by Presepsin (0.89), IL-6 (0.89), and CRP (0.84) as inflammatory biomarkers ($P < 0.0001$). Additionally, the AUC to distinguish sepsis was the highest for Protein C (PC) (0.83) followed by Thrombomodulin (TM) (0.81), Antithrombin (AT) (0.81) as coagulation biomarkers ($P < 0.0001$).
Logistic regression analysis that included PCT, Presepsin, IL-6, and CRP as inflammatory biomarkers identified only Presepsinlevel as independent predictor of the Japanese Association for Acute Medicine (JAAM) DIC and the optimal cut-off value was 899 pg/mL, and that included PC, TM, and AT as coagulation biomarkers that PC was only a predictor of JAAM DIC and the optimal cut-off value was 55%. On the other hand, the optimal cut-off value of sepsis in Presepsin and PC were 647 pg/mL and 47% respectively.
In the ROC analysis using both of Presepsin and PC, the AUC to distinguish sepsis and JAAM DIC were 0.911 and 0.913 respectively.
Summary/Conclusion: From these results, we defined the new diagnostic criteria of septic DIC which named sepsis induced DIC (SEDIC) as follow; Presepsin level > 900 pg/mL and PC < 45%. We strongly believe that this diagnostic criteria is very simply and useful.

PA 4.17-6
Heparin inhibits extracellular histones-induced HUVEC apoptosis

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Background: Sepsis is a potentially deadly medical condition characterized by a whole-body inflammatory state caused by severe infection, and its progression to disseminated intravascular coagulation (DIC) or multiple organ dysfunction syndrome (MODS) is a major cause of death in PICU. It has recently been shown that extracellular histones are major mediators of death in sepsis.
Aims: We aim in this study to explore whether extracellular histone level could serve as diagnosis indicator for sepsis severity and what can be used as potential treatment is not known.
Methods: We first determined the histone concentration in patients' plasma, and then investigated *in vitro* the impact of extracellular histones on HUVEC, the underlying mechanism and potential treatment.
Results: In the present study, we have found that plasma histone level in sepsis patients ($n = 33$) is much higher than control samples ($n = 30$), and the concentration is correlated with sepsis severity (i.e., patients with MODS have much higher plasma histone level than those without MODS), suggesting that extracellular histone level may be an indicator of sepsis progression. Consistently, treatment of HUVEC with histones in PBS or serum-free media dose-dependently induces HUVEC apoptosis, which starts as early as 15 min after treatment. Further studies have demonstrated that histone-induced HUVEC apoptosis depends on Toll-like receptors 2 and 4. Addition of fetal bovine serum (FBS) or human plasma to HUVEC significantly alleviated HUVEC apoptosis, suggesting that certain component(s) in serum could protect HUVEC from apoptosis against extracellular histones, consistent with the phenomenon that normal people could tolerate low dose extracellular histones in plasma without developing any syndrome like sepsis. More importantly, treatment of HUVEC with heparin, which is allowed to be used as clinical treatment in China, completely inhibits histones-induced apoptosis, strongly suggesting that heparin could be used as potential treatment in sepsis patients.
Summary/Conclusion: In summary, our studies clearly suggest that extracellular histone level could serve as an indicator of sepsis severity, and heparin may be routinely used in sepsis treatment.

PA4.18 – Inherited Risk Factors VT

PA 4.18-1

Venous thromboembolism risk assessment with a multilocus genetic risk score

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Background: In the development of venous thromboembolism (VTE) genetics contribute in a relevant manner. In clinical routine the presence of two mutations Factor V Leiden (FVL) and G20210A Prothrombin (PT) are analysed to evaluate this genetic contribution. However, new and relevant genetic variants have been associated with VTE. Though these genetic variants have not been properly validated neither translated to clinical practise.
Aim: To evaluate whether the use of Genetic Risk Scores (GRS) with these new variants provides a better assessment of the VTE risk than a model based only on FVL-PT.
Methods: Three panels of genetic variants were compared: FVL + PT; TIC panel (FVL, PT, ABO group A1 carriers, FV Cambridge, FV Hong Kong, rs2232698 SerpinA10 gene, rs121909548 SerpinC1 gene, 46C>T F12, and rs5985 F13 gene); and TIC panel plus F11 (TIC panel, rs2289252 and rs2036914 in F11 gene). All these variants influence the coagulation pathway.
For each panel a multi-locus GRS was computed for each individual as the sum of the number of risk alleles, after weighting them by its effect size published in the literature. The GRS was validated in two case-control studies. MARTHA: 1150 cases (347 males, 803 females; 38.0 ± 13.9 years old), 801 controls (383 males, 418 females; 47.4 ± 14.0 years old) designated to assess the association of FVL and PT with other risk factors; and Sant Pau (SP): a study with Spanish population with 249 cases (111 males, 138 females; 47.1 ± 14.0 years old), 248 controls (109 males, 139 females; 49.0 ± 14.9 years old).
All models were adjusted by age and sex. The predictive capacity was assessed by the discrimination of the different GRS calculating the c-statistic (AUC-ROC); and by the reclassification when using the new GRSs compared with FVL + PT calculating the NRI (net reclassification improvement) and IDI (integrated discrimination improvement). Informed consent was obtained and the studies were approved by recognised ethics committees.
Results: When compared to FVL + PT, the use of TIC or TIC + F11 panels significantly improved the capacity to discriminate VTE in both populations (AUC-ROC: 0.57 vs. 0.68 and 0.67 respectively, in SP and 0.56 vs. 0.57 and 0.58 respectively, in MARTHA population,). When compared to the risk stratification by FVL+PT, only the use of TIC panel improved the capacity to reclassify both cases and controls in both populations (NRI, 19.2, $P < 0.005$ and 4.9, P-value > 0.05, IDI 5.5, $P < 0.001$ and 3,2, P-value < 0.01 for TIC panel vs. FVL+PT in SP and MARTHA populations, respectively). Moreover, clinical sensitivity (number of cases where a genetic thrombophilia was demonstrated) increases very significantly in relation to FVL + PT (19.7% and 50.4% of the cases for FVL + PT, 87.3% and 95.1% for TIC in SP and MARTHA populations, respectively). TIC + F11 did not further improve the reclassification.
Conclusions: TIC panel significantly improves the predictive capacity of VTE risk, by improving discrimination and reclassification when compared to FVL + PT. Thus, our study suggests that the use of algorithms with a set of confirmed susceptibility loci (TIC) improves disease risk assessment and could be also an aid in the prevention, diagnosis and treatment of VTE disease.

PA 4.18-2

Incidence and risk factors for venous thromboembolism (VTE) amongst adults with sickle cell disease

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Background: Sickle cell disease (SCD) is caused by mutations in the ß-globin gene. Morbidity is due to vascular occlusion and hemolytic anemia. Recent studies suggest increased risk of VTE in SCD patients.
Aim: To examine the association between SCD and VTE.
Methods: Using the California Patient Discharge Dataset and Emergency Department Utilization database, we found adult (18–65 years) cases with SCD in the years 1990–2010. Using unique record linkage numbers, these cases could be followed longitudinally over serial admissions. We then determined incident VTE. To determine the odds ratio (OR) for VTE associated with SCD, each SCD case was matched with five non-SCD controls for race/ethnicity, sex, age (± 2), and year of index admission (± 2). Conditional logistic regression was then performed. To determine risk factors predictive for VTE within the SCD cohort, OR were determined for sex, disease severity, age (5 year increments), race, and co-morbidities. The effect of VTE on death in SCD patients was determined using matched (1:4) Kaplan-Meier (K-M) survival curves, stratified by disease severity (≥ 3 admissions or ED visits/yr or < 3 visits/year), and analyzed by log-rank.
Results: There were 4280 unique adult cases with SCD; mean age 28 years (± 10.5), 91% were African American, and 56% female. Forty-two percent had severe disease. Overall there were 361 incident VTE (185 PE; 172 DVT; four both) for a cumulative incidence of 8.4%. Forty-six percent of these events occurred within 30 days of an antecedent hospitalization or ED visit. Compared to non-SCD controls, SCD patients had an overall OR of 5.9 (5.1–7.0) for VTE; the OR was 3.8 (3.0–4.8) for less severe and 9.5 (7.5–12.1) for severe disease patients. Co-morbidity also increased the odds of VTE: OR 1.8 (1.5–2.5) for 1–2 co-morbidities vs. none; 3.0 (2.2–4.1) for ≥ 3 conditions. Amongst SCD patients, female sex (OR 1.4; 1.1–1.8) and severe disease (OR 1.9; 1.5–2.5) were associated with increased risk of VTE, adjusted for age, race, and co-morbidities. The median K-M survival for SCD patients that developed VTE was 149 months and had not been reached for SCD without VTE ($P < 0.0001$, log-rank). For severe SCD patients with VTE the median survival was 105 months and had not been reached for non-VTE matched patients ($P < 0.0001$). Less severe patients had not reached median survival, but the mean survivals (± SE) were 118 months (± 5.9) and 195 months (± 2.7) for VTE and non-VTE, respectively ($P < 0.0001$)
Conclusions: SCD is associated with an increased risk of VTE compared to a matched population that also required hospitalization. Nearly half the incident VTE occurred within 30 days of a previous hospitalization or ED visit. This suggests painful episodes, which are inflammatory events and the most common reason SCD patients seek medical attention, as a provocation for VTE. Relative immobilization may also play a role, and argues for robust thromboprophylaxis in hospitalized SCD patients. In contrast to other populations, females had increased OR for VTE. As shown for other populations with chronic diseases, incident VTE in SCD patients was associated with decreased survival.

PA 4.18-3

Replication and meta-analysis of the association between F11 genetic variants and the risk of incident venous thrombosis by statin use

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Background: Two *F11* single nucleotide polymorphisms (SNPs), rs2289252 and rs2036914, are known to be associated with the risk of incident venous thrombosis (VT). Statin use may be associated with a decreased risk of VT and may modify the association of the *F11* SNPs with VT risk.

Aims: To better inform the possibility of a drug-gene interaction associated with the risk of VT by presenting new findings from the Seattle HVH data and meta-analyzing with MEGA findings. We hypothesized that statin use would diminish the VT risk associated with the SNP risk alleles.

Methods: In the HVH population-based study, we identified cases of incident VT occurring from 1995 to 2009 and their matched controls. Participants with cancer were excluded. Genotypes for rs2036914 were available for 747 cases and 1721 controls; genotypes for rs2289252 were available for 693 cases and 3112 controls. Current statin use was determined using prescription fills. Separate logistic regression models for the two SNPs estimated the risk of VT associated with carrying the risk allele, stratified by statin use, adjusted for matching variables: index year, age, hypertension, sex, and race. Results were meta-analyzed with MEGA study findings.

Results: HVH cases and controls had an average age of 63 and 66 years, respectively; 74% of cases and 95% of controls were women. Statins were used by 16% of cases and 20% of controls. For rs2036914, in HVH, statin users who carried 1 or 2 C alleles had an increased risk of VT compared with carriers of TT alleles (OR = 1.7, 95%CI: 1.0–2.8, and, OR = 2.2, 95%CI: 1.1–4.6, respectively). Among non-users of statins, ORs for carriers of 1 or 2 C alleles were 1.3 (95%CI: 1.0–1.7) and 1.3 (95%CI: 0.9–1.8), respectively. There was no evidence of interaction between the SNP and statin use. For rs2889252, in HVH, statin users who carried 1 or 2 T alleles had no evidence of an associated VT risk compared with carriers of CC alleles (OR = 1.1, 95%CI: 0.6–1.9, and, OR = 1.4, 95%CI: 0.6–2.9, respectively). Among non-users of statins, ORs for carriers of 1 or 2 T alleles were 1.2 (95%CI: 1.0–1.5) and 1.5 (95%CI: 1.1–1.9), respectively. There was no evidence of interaction.

Meta-analyzed results for rs2036914 suggested no difference in risk between statin users (OR = 1.6, 95%CI: 1.1–2.3 for 1 C allele, and, OR = 1.5; 95%CI: 0.9–2.4 for 2 C alleles) and non-users (OR = 1.3, 95%CI: 1.2–1.5 for 1 C allele, and OR = 1.7, 95%CI: 1.5–1.9 for 2 C alleles). For rs2889252, meta-analytic results suggested modest differences in risk between statin users (OR = 1.1, 95%CI: 0.8–1.5 for 1 T allele, and, OR = 1.2, 95%CI: 0.7–2.0 for 2 T alleles) and non-users (OR = 1.3, 95%CI: 1.2–1.5 for 1 T allele, and, OR = 1.8, 95%CI: 1.6–2.0 for 2 T alleles).

Summary/Conclusion: Replication results were less pronounced than discovery results. In meta-analyzed results, risk of VT by rs2036914 genotype did not appear to differ by statin use. Risk of VT associated with rs2889252 appeared to be modestly blunted among statin users, compared to among non-users, for those carrying the risk allele.

PA 4.18-4

Height and risk of venous thromboembolism: a mendelian randomization study

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Background: Height has been identified as a risk factor for venous thromboembolism (VTE), but it is unclear whether the relationship is causal or explained by confounding factors. Mendelian randomization studies can be used to investigate causal relationships underlying observed associations between exposure (height) and outcome (VTE) in epidemiological studies. Because genetic risk influences height, but is unaffected by environmental confounders, a significant association would provide evidence that height is causally related to VTE, whereas a lack of association indicates that the relationship is due to unrecognized confounding factors.

Aims: We wanted to investigate the association between a genetic score for height and risk of VTE.

Methods: We utilized genetic sequence data generated through exome sequencing. Cases who had a first VTE (n = 407) and age- and sex-matched controls (n = 406) were identified from a population-based, nested, case-cohort study (the Tromsø study) comprising 78% (n = 27,158) of the adult residents of Tromsø in Norway. Exome sequencing was performed to an average depth of approximately 100× in exome regions and approximately 0.5× in non-targeted regions. Utilizing on and off-target reads, genome-wide SNP genotypes were inferred by imputation using Beagle. We calculated a weighted height genetic score using independent markers previously identified in a large GWAS study, taking into account the original effect estimate and the imputation quality. The genetic score was tested for association with VTE case status. In addition, a previously published case-control study of VTE (N = 2596) was used to replicate these findings. The study was approved by the regional committee for research ethics, and all participants gave their informed written consent to participate.

Results: Imputation of the exome data using the 1000 Genome Project European haplotypes resulted in effective estimation of genotypes for 120 of the 180 SNPs recently identified as associated with height. We recapitulated the association between height and VTE (OR = 1.31 for a 10 cm difference, P = 0.008) after adjustment for age and sex. We also showed that the established height genetic score was associated with height (R^2 = 0.04, 2.6 cm difference between upper and lower halves, $P = 4 \times 10^{-13}$). However, we did not observe a significant association between the height genetic score and risk of VTE. Thus, the height genetic score did not explain the relationship between height and VTE. Similar analyses using the GENEVA VTE study showed consistent results.

Conclusions: These results do not provide support that the association between height and risk of VTE is causal, but suggest that the effect could be mediated by other confounding factors. Due to the small amount of variation that the genetic score explains, our finding may be limited by power and would benefit from confirmation in a larger study.

PA 4.18-5

Not all factor V Leiden or prothrombin G20210A mutations are equal: results from a retrospective cohort family study

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Background: Factor V Leiden (FVL) and G20210A prothrombin gene mutation (PT20210) are risk factors for venous thromboembolism (VTE). In family studies the risk of VTE in relatives heterozygous or homozygous for FVL or PT20210 might be influenced by either the genotype or the clinical presentation of the proband. This risk might be higher in relatives selected from kindreds with heterozygous symptomatic probands than in those belonging to kindreds with homozygous asymptomatic probands, because in the former case the proband is symptomatic despite a lower risk of thrombosis is expected with heterozygous than homozygous mutation.

Aims: To assess whether or not the selection of kindreds according to genotype and clinical presentation of the proband influences the risk of VTE in relatives.

Methods: A retrospective cohort family study of 192 kindreds with at least one member with homozygous FVL or PT20210 were collected in five Italian centers, for a total of 886 relatives [380 males, 506 females; median age 39 years (IQR: 25–54 years); 129 heterozygous PT20210, 378 heterozygous FVL, 36 homozygous PT20210, 101 homozygous FVL, 38 combined heterozygous, 204 wild-type]. The proband of the family was heterozygous for FVL or PT20210 in 69 kindreds and homozygous or combined heterozygous in 123. Twenty-three probands were asymptomatic, 11 had had arterial thrombosis, seven obstetrical complications, and 151 venous thrombosis (29 superficial and 122 VTE). According to the genotype and clinical presentation of the proband, the study population was divided into four groups: relatives belonging to kindreds with (i) homozygous proband without VTE (reference), (ii) homozygous proband with VTE, (iii) heterozygous proband without VTE, and (iv) heterozygous proband with VTE. For each group, the annual incidence of VTE (and its 95% CI) was calculated. The follow-up started at the date of birth and ended at the date of either the first VTE or the first visit to the centers. The hazard ratio (and 95% CI) for VTE in each group compared to the reference was estimated by using a multivariable Cox's proportional hazard model, with adjustment for a possible confounding effect of age, sex, type of genetic defect and genotype of the relatives.

Results: A total of 27 objectively-documented episodes of VTE during 34,966 patient-years of follow-up were recorded, for an overall incidence rate of 0.77 × 1000 patient-years (95%CI:0.48–1.06). Twelve episodes of VTE were unprovoked (44%) and 15 (56%) secondary to transient risk factors. Excluding probands, the incidence of VTE (×1000 patient-years) in relatives was higher when the proband had heterozygous than homozygous mutation [1.22 (95%CI:0.71–1.86) vs. 0.45 (0.20–0.79)], and when the proband had had VTE instead of other clinical presentations [0.95 (0.57–1.42) vs. 0.50 (0.19–0.96)]. Compared to the reference group, the adjusted hazard ratio of VTE for relatives from kindreds with heterozygous proband with VTE was 3.97 (95% CI:1.12–14.09).

Conclusion: Both the genotype and the clinical presentation of probands influence the VTE risk in relatives with FVL or PT20210. This index event bias can in part explain the different risk of VTE in family members with the same genetic defect.

PA 4.18-6

Association of ABO blood type with cognitive decline: the REasons for Geographic and Racial Differences in Stroke Study

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Background: ABO blood group is associated with many forms of cardiovascular disease, including stroke, coronary heart disease and venous thromboembolism. This is likely due, in part, to the link between non-O blood groups and higher levels of the procoagulant proteins von Willebrand factor and factor VIII (FVIII). Many cardiovascular disease risk factors have been identified as also contributing to cognitive decline, but there are currently no published data on the relationship between ABO group and cognitive decline.

Aims: To study the association of blood types A, B, AB, and O with cognitive decline in the REasons for Geographic and Racial Differences in Stroke (REGARDS) study.

Methods: The REGARDS cohort consists of 30,239 black and white US individuals ≥ 45 years old, enrolled between 2003 and 2007, with 4.5 years follow up for cognitive function. Incident cases of cognitive decline were defined based on longitudinal scoring using three cognitive domain tests: list learning, list recall, and verbal fluency. ABO blood group was measured by SNP genotyping in a case-control sample of 495 cognitive decline cases and 587 controls. The odds ratios for cognitive decline by ABO status were calculated using logistic regression models, with blood group O as the reference group. All models were adjusted for age, sex, and race. FVIII was then added to this model as a continuous variable to examine its contribution to any observed associations. Because blacks have a higher stroke risk and differing blood group frequencies than whites, an interaction term between race and blood group was tested, with an interaction $P < 0.10$ considered statistically significant.

Results: All non-O blood groups showed significantly higher levels of FVIII compared with the O group (mean 104; 95% CI 100–107), with the highest level seen in type AB (mean 142; 95% CI 126–202). Blood group AB was associated with incident cognitive decline in a model that included age, race, and sex (OR 1.78; 95% CI 1.13–2.81), while blood groups A and B showed no association. When FVIII was added to the model, every 40 IU/dL higher FVIII was associated with cognitive decline (OR 1.22; 95% CI 1.08–1.37) and the odds ratio for group AB was reduced to 1.67 (95% CI 0.99–2.83). There was no significant difference in AB blood group association by race (P interaction = 0.95).

Summary: Blood group AB was associated with incidence of cognitive decline in this prospective study. This relationship was only partially mediated by FVIII. These data support the hypothesis that while blood group may impact cognitive function through its effects on FVIII levels, differences in the ABO glycosyltransferase that determines ABO blood group are likely to affect the glycosylation of other proteins, which may alter cognitive decline risk.

PA4.19 – TTP/Thrombotic Microangiopathies – II

PA 4.19-1

International registry for patients with hereditary thrombotic thrombocytopenic purpura (TTP) – upshaw-Schulman syndrome

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Background: Hereditary TTP, or Upshaw-Schulman syndrome (USS), is a rare, and recessively inherited disorder, due to homozygous or compound heterozygous mutations in the ADAMTS13 gene located on chromosome 9q34. TTP has a wide variation in clinical presentation from only mild thrombocytopenia to severe recurrent TTP episodes leading to end organ damage or even death. The first occurrence of USS is variable with onset in the neonatal period up to older age. Due to the rareness of hereditary TTP evidence based guidelines on prophylaxis with different ADAMTS13 sources such as fresh frozen plasma and knowledge of long-term outcome are lacking, which emphasizes the need of a multicenter cooperation.

Aims and Method: An electronic database system for USS patients and their interested family members (www.ttpregistry.net, ClinicalTrials.gov NCT01257269) has been established to gather baseline information and in patients long-term follow-up data. They contain the clinical courses, therapeutic as well as prophylactic treatment regimens and performed laboratory investigations in order to identify yet unknown triggers of TTP episodes and factors influencing the clinical course. Eventually this leads to improved treatment and better understanding of this potentially fatal disease.

Eligibility criteria are:

1 ADAMTS13 activity $\leq$ 10% on two separate occasions at least 1 month apart and
2 Absence of a functional ADAMTS13 inhibitor and
3 $\geq$ 2 ADAMTS13 gene mutations and/or a positive infusion trial (full recovery and plasma half life of 2–3 days of infused plasma) or
4 Being a family members of a confirmed patient.

Information is collected retrospectively up to enrollment as well as prospectively every 12 months and when acute TTP episodes occur. Analysis of ADAMTS13 related parameters including molecular analysis of the ADAMTS13 gene are free of charge to patients and their family members.

Results: Today, 30 participants from eight different countries (CH, CRO, CZ, NOR, PL, ESP, TU, USA) have been entered into the database and another eight countries (AUT, CAN, D, DAN, FIN, IND, IL, JP) have confirmed their participation with 1 to over 40 patients per study site. The data acquisition is in progress. A first point of interest is the investigation of episodes of thrombocytopenia during infections or pregnancies in a number of obligatory or confirmed heterozygotes, so far generally reported as being asymptomatic.

Conclusion: The international network and knowledge platform established to exchange information and experience on USS will help to improve diagnosis, treatment and prevention of acute episodes in affected patients. All Physicians are invited to contact us for diagnostics and enrollment of their patients with USS.

PA 4.19-2

Measurement and prevalence of circulating ADAMTS13-specific immune complexes in autoimmune thrombotic thrombocytopenic purpura

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Background: Autoimmune thrombotic thrombocytopenic purpura (TTP) is a thrombotic microangiopathy characterized by the presence of autoantibodies against the von Willebrand factor cleaving-protease, ADAMTS13. The formation of non-covalent antigen-autoantibody associations in the bloodstream (i.e. circulating immune complexes, CIC) occurs and is a pathophysiologic mechanism in several autoimmune disorders. CIC have a wide range of properties, including complement-system and leukocyte activation, which may promote inflammation and cellular damage in autoimmune conditions. Although autoimmune TTP is an autoantibody-mediated disease, the role of ADAMTS13-specific CIC in TTP has not been studied.

Aims: The goals of our study were (i) to develop a method to assess the presence of and quantify ADAMTS13-specific CIC in patients with autoimmune TTP and (ii) to evaluate the prevalence and the association of CIC with ADAMTS13- and anti-ADAMTS13-related measurements in patients with autoimmune TTP.

Methods: We developed an ELISA method for the detection of ADAMTS13-specific CIC. The linearity and specificity of the assay were validated by experiments including immunoprecipitation of CIC from normal and TTP plasma. Intra- and inter-assay coefficients of variation were calculated to assess the precision of the method. We measured ADAMTS13-specific CIC by ELISA in 36 patients with autoimmune TTP from the Milan TTP Registry (URL:http://www.ttpdatabase.org/) who had anti-ADAMTS13 autoantibodies at the time of measurement (acute disease for 15 and remission for 21 patients). Measurements were carried out in duplicate and results were expressed in optical density units (OD) standardized for a positive control. The cutoff for the presence of anti-ADAMTS13 CIC was defined as the 99th percentile of the distribution of normalized OD in 22 control subjects. The associations between ADAMTS13-specific CIC levels and ADAMTS13 activity, ADAMTS13 antigen and anti-ADAMTS13 IgG levels were assessed by linear regression.

Results: The anti-ADAMTS13 CIC ELISA test had intra- and inter-assay coefficients of variation of 5.3 and 9.6. The 36 patients with TTP included in the study had a median age at study measurement of 41 years (interquartile range [IQR]: 34–52 years) and were predominantly of female sex (n = 30, 83%). All 36 patients had severely deficient ADAMTS13 activity (< 10% of normal), a median value of ADAMTS13 antigen of 24% (IQR: 13–45%) and a median level of anti-ADAMTS13 IgG of 20% (IQR: 6–48%). The median normalized OD of ADAMTS13-specific CIC measurement was 0.37 (IQR: 0.28–0.45). The prevalence of CIC in TTP patients was 47% (n = 17; 95% confidence intervals [CI]: 32–63%). The normalized OD value was not associated with ADAMTS13 activity, ADAMTS13 antigen or with anti-ADAMTS13 IgG levels. In patients with acute TTP at measurement, increasing levels of ADAMTS13-specific CIC were associated with higher number of plasma exchange procedures required to attain remission (per 0.1 increase in normalized OD values, beta: 2.9; 95% CI: −0.7 to 6.5).

Summary/Conclusions: Approximately one half of patients with autoimmune TTP display ADAMTS13-specific CIC. Levels of ADAMTS13-specific CIC measured by this ELISA method are not associated with ADAMTS13 or anti-ADAMTS13 IgG levels. A thorough investigation of the prognostic relevance of ADAMTS13-specific CIC levels in patients with autoimmune TTP is warranted.

PA 4.19-3

Sequelae of chronic relapsing idiopathic thrombotic thrombocytopenic purpura

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Background: ADAMTS13 (a disintegrin and metalloproteinase with TSP-1-like domains) cleaves high molecular and prothrombotic von Willebrand factor (VWF) multimers into smaller molecules. Severely decreased levels of ADAMTS13 are in most cases caused by autoantibodies but also in rare cases by mutations leading to thrombotic thrombocytopenic purpura (TTP). Different trigger factors contribute to the clinical manifestation of TTP. These life-threatening acute episodes alternate with relatively symptom-free phases of remission. Acute episodes are characterized by consumptive thrombocytopenia, hemolytic anemia and spontaneous VWF-platelet aggregation leading to microvascular thrombus formation in arterioles and capillaries of different organs. The brain is frequently affected inducing different neurological abnormalities of varying severity. Further typical symptoms are bleedings, gastro-intestinal complaints, fever and hematuria, chest pain and weakness. Without treatment, TTP may result in many cases in deterioration and death. Presently, plasma exchange is still the mainstay therapy, often combined with immunosuppressive drugs. Aim of therapy is to achieve a persistent remission with complete recovery characterized by normal physical condition and regular laboratory data for platelets, hemoglobin and lactate dehydrogenase.
Aims: Aim of the study was to investigate risks of persisting neurocognitive impairments as well as anxiety and depression in consequence of relapsing idiopathic TTP.
Methods: Sequelae of 20 idiopathic TTP patients were analyzed by means of different questionnaires, medical records and reports.
The medical records/reports come either from a psychiatrist or from the TTP-treating hematologists and have been collected from first manifestation until now.
Three different standardized questionnaires were used. The questionnaire IRES (developed by Gerdes and Jäckel) was used as an indicator for rehabilitation; HADS/D evaluates anxiety and depression; mental performance was examined by the questionnaire for complaints of cognitive disturbances (FLei).
Results: Ten of 20 patients have been treated due to depression, and many of them need psychological help. One patient has been under inpatient psychiatric treatment. Furthermore, many patients complain of anxiety disorders. As a result of depression and anxiety disorders, these patients suffer from general fatigue, exhaustion, lag of drive, adjustment disorders, social anxiety disorder and chronic insomnia.
Neurocognitive impairments including brain-fag syndrome, lack of concentration, amnesic aphasia and dysphasia, disturbance of long-term and short-term memory occur frequently, as well.
Two patients are afflicted with epilepsy, a sequelae of stroke. Four patients developed renal insufficiency. Many of the patients suffer from side effects of cortisone and further immunosuppressive drugs, especially from Cushing's syndrome, cortisone acne, overweight, osteopenia and alopecia.
Most of the patients stated to be living seclusively and to possess only a reduced quality of life. They emphasized problems in their daily life, social communication and relationships. Their professional life is impaired to the point of a temporary or permanent disability.
Summary: Altogether TTP-patients recover well from their acute phase with regard to physical examination results and laboratory data, despite low ADAMTS13 activity and high inhibitor titer.
However, many of them suffer from neurocognitive impairments, anxiety disorders and depressions. As a result, they have to cope with a permanent restriction of their daily routine in private as well as in professional life.

PA 4.19-4

Characterization of hereditary thrombotic thrombocytopenic purpura (TTP) from one of blood centers in China

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Background: Hereditary thrombotic thrombocytopenic purpura (TTP) is characterized by abnormally disseminated thrombus due to the mutations of ADAMTS13, which can cleave its substrate von Willebrand factor (VWF) in normal conditions.
Aims: To characterize the clinical and genetic molecular features of ten patients with congenital TTP.
Methods: ADAMTS13 activities were analyzed by residual collagen binding assay (R-CBA) plus FRET-VWF substrate. And the inhibitors of ADAMTS13 were analyzed by 9:1 mixture of patient and pooled normal plasma followed by R-CBA. The intracellular location and secretion of recombinant ADAMTS13 mutants were studied.
Results: Ten patiens, among whom, eight patients with pregnancy aged between 25 and 31 years old and one girl aged 16 years old plus one male aged 39, were diagnosed with congenital TTP because their ADAMTS13 activities were < 5% (both R-CBA and FRET-VWF substrate) and absence of ADAMTS13 inhibitors. The following mutations were found in these patients, which included: R193W, R1095W, R349C, R498C, R692C, Y177C, E1005V, C265Y, M1260-S1264, Nt:1335G(Del), Nt:3677C(Del), Nt: 1435 + 2insG. In addition to the previously reported mutation of R498C, all the novel missense mutations (including C265Y, E1005V, Y177C) had the impaired secretion and retained in the HEK293 cells.
Conclusion: Among ten patients registered in our institution, eight patients were pregnant women, which implies that it may be necessary to screen ADAMTS13 activity in pre-pregnancy checkup for TTP prophylaxis.

PA 4.19-5

Safety and efficacy of cryosupernatant as a replacement fluid for plasmapheresis in thrombotic thrombocytopenic purpura: a single center restrospective evaluation

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Background: Thrombotic thrombocytopenic purpura (TTP) is a severe systemic disease caused by decreased activity of ADAMTS13, resulting in reduced clearance of ultra-large VWF multimers and thrombotic microangiopathy. Treatment of TTP is based on daily plasmapheresis (Px) with replacement with fresh frozen plasma (FFP), aiming to reestablish normal ADAMTS13 activity and VWF multimer distribution. Cryosupernatant (CSP), the remaining of a FFP unit after the removal of cryoprecipitate, is a plasma product with lower concentrations of large VWF multimers and similar amounts of ADAMTS13. It has been hypothesized that CSP might be preferable replacement fluid for Px in TTP patients. CSP is at least as efficacious as FFP in the treatment of TTP, but evidence of additional benefits has not been demonstrated, as showed in a meta-analysis of three randomized clinical trials comparing FFP with CSP. Besides, none of these studies reported on the frequency of adverse events of using CSP.
Aims: To evaluate the efficacy of Px in patients with TTP treated with CSP or FFP and the safety of either option.
Methods: In our center, both CSP and FFP are available as replacement fluid for Px, being the choice between these two options

performed before the 1st session at the assistant physicians'discretion. Once initiated, only one type of replacement fluid was used until the 1st remission. We retrospectively evaluate the efficacy of Px with either product based on the number of sessions, volume of plasma product exposure and frequency of acute exacerbations and relapses. In addition, we evaluated the safety of either option by reporting the frequency and nature of adverse events.

Results: From June 2007 to October 2012, 14 patients with newly diagnosed TTP were treated at our center. The proportion of CSP:FFP use was 5:9. There were no significant differences in age, ethnic background, initial platelet count, hemoglobin levels, LDH, or etiology of TTP (idiopathic × secondary) between groups. Regarding efficacy, we observed a trend towards a higher median number of Px sessions (15 vs. 7) and higher plasma exposure in CSP, compared to FFP-treated patients. Although the number of relapses was not significantly different between groups, acute exacerbations were more frequent among patients treated with CSP than FFP. Regarding safety, the frequency of adverse reactions was not different between CSP or FFP-treated patients. Mild allergic reactions were the most common treatment-related adverse event in both groups.

Summary: Our results confirm the lack of benefit of using CSP as a replacement fluid in TTP compared to FFP. In addition, our data raises the possibility that in newly diagnosed TTP, CSP use might be associated with worse outcomes, as evidence by a higher frequency of acute exacerbations during the 1st 30 days, and a trend towards a higher number of Px sessions and higher exposure to plasma products. Considering that obtaining large volumes of CSP to treat TTP requires special logistic actions and increases treatment costs, our data corroborate the concept that CSP should not be used as 1st line treatment of TTP.

PA 4.19-6

In vitro characterisation of two ADAMTS13 mutants (I143T, Y570C) identified in two patients with congenital thrombotic thrombocytopenic purpura (TTP)

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Background: Congenital TTP is a heterogeneous condition with respect to age of disease onset and need for plasma exchange/infusion. ADAMTS13 genetic defects have been identified in congenital TTP patients. Although over 100 mutations have been identified, only about 30% have been expressed *in vitro* and of these, the majority (84%), lead to reduced (< 50%) secretion of mutant recombinant ADAMTS13.

Aims: Two mutations present in different congenital TTP patients were studied *in vitro* for their effects on the secretion and sub-cellular location of ADAMTS13, in order to investigate the contribution of ADAMTS13 genotype to disease phenotype.

Methods: Two ADAMTS13 missense mutations (I143T, Y570C) were introduced into wild type (WT) ADAMTS13-cDNA separately by site directed mutagenesis and used for subsequent transient transfection of HEK 293T cells. The presence of WT or mutant ADAMTS13 within cell supernatant and lysates was investigated using western blotting. The quantity of protein in cell supernatant was measured using an antigen ELISA assay and the activity measured by FRET assay (detection limit 2.5%). Expression of the mutant proteins was compared to that of WT (assigned a value of 100%). Localisation of the WT and mutant proteins was investigated in the endoplasmic reticulum (ER) and Golgi by confocal microscopy.

Results: Both patients presented with acute TTP during adolescence having undetectable ADAMTS13 activity and antigen in plasma and receive regular prophylactic plasma exchange. Patient 1 was homozygous for the mutation I143T (metalloprotease domain) and patient 2 for the mutation Y570C (spacer domain). *In vitro* both mutants were detected within cell lysates, at a slightly greater quantity than WT. However in the supernatant the quantity of each mutant secreted was either undetectable ($n = 3$) or very low (2% of WT, $n = 1$). In both cases no ADAMTS13 activity could be detected. Confocal microscopy showed that both mutants localised within the ER ($n = 3$) and the Golgi ($n = 6$).

Conclusion: In conclusion *in vitro* expression of the I143T and Y570C ADAMTS13 mutants demonstrated a severe secretion defect. This correlates with the undetectable ADAMTS13 activity and antigen present in the patient's plasma. Within the cell both mutants localised within the ER and Golgi suggesting a potential defect in the transport from the Golgi to the extracellular environment. Further investigation is underway in order to determine if these mutants are degraded within the cell by the cell proteasome or lysosomes before they can be secreted.

A4.20 – Vessel Wall

PA 4.20-1

Platelet adhesion to collagen under flow conditions is increased in essential thrombocythemia patients

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Background: Essential thrombocythemia (ET) is a myeloproliferative neoplasm characterized by high incidence of both arterial and venous thromboembolic events. The acquired activation of vascular cells, particularly leukocytes and platelets, has been previously demonstrated in ET patients and suggested to promote the development of thrombosis.

Aim: This proof-of-principle study aimed to assess whether the increased activation of platelets from ET patients may lead to an increase in their thrombus formation potential *in vitro* under flow conditions.

Methods: Nine ET patients (mean platelet count 755×10^9/L, range 240–1409) and nine healthy control subjects (mean platelet count 244×10^9/L, range 216–323) were enrolled into the study after informed consent. Peripheral venous whole blood samples, withdrawn in sodium citrate, were recalcified and anticoagulated with heparin and perfused over a collagen coated surface at a shear rate of 1000/s. Platelet adhesion and thrombus formation was evaluated after 4 min with the EVOS fluorescence microscope system. In particular, platelets were stained with an anti-CD2P (P-selectin)-FITC antibody as an index of platelet activation, and annexinA5-Alexa Fluor 647 that provides a measure of platelets exposing a procoagulant surface (namely phosphatidylserine). After staining, images of adherent platelets in random fields were taken using phase contrast and fluorescence imaging. Results are expressed as mean ± SD of the percentage of area covered by all or fluorescently-labeled platelets.

Results: After 4 min of blood perfusion, the area covered by adherent platelets was found significantly greater in ET patients compared to healthy controls (38.7 ± 5.1 vs. 20.4 ± 3.1% coverage, $P < 0.05$). No statistically significant correlation between platelet count and percentage of coverage was found. However, in the four ET patients with a platelet count > 700×10^9platelet/L, the% coverage was significantly higher compared to the five ET patients with a platelet count < 700×10^9platelet/L (i.e. 54.9 ± 17.5 vs. 25.8 ± 11.3% coverage, respectively, $P < 0.05$). Similarly to what observed with the overall adherent platelets, the area covered by P-selectin positive platelets was significantly ($P < 0.05$) higher in ET patients vs. controls. On the

contrary, the coverage by annexinA5-positive adherent platelets was not statistically significantly different between ET patients and controls.

Conclusions: These preliminary results show that, compared to healthy subjects, blood from ET patients has an increased tendency of shear-dependent platelet adhesion at a collagen surface, indicative of a greater thrombus formation capacity, without increase in platelet-dependent coagulation. These results support an active role of platelet adhesiveness in the prothrombotic state of patients with ET. A possible influence of the patients' characteristics and treatment type is also under evaluation.

PA 4.20-2

The shear rate dependence of nitric oxide inhibition of platelet aggregation

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Background: The concentration of nitric oxide (NO) at an injury site and how it affects platelet function under flow is poorly defined. For example, reports of the NO concentrations necessary to inhibit platelet aggregation vary widely in the literature (5 nM–4 μM) (Marcondes et al., *PNAS*, 103, 2006). The signaling pathway of NO inhibition is also a source of debate. It is generally accepted that NO inhibition occurs primarily through soluble guanylyl cyclase (sGC)/cyclic guanosine monophosphate (cGMP)-dependent pathways (Dangel et al., *JTH*, 8, 2010). But, recent studies suggest that cGMP-independent mechanisms may also mediate inhibition at millimolar NO concentrations (Zhang et al., *Blood*, 118, 2011).

Aims: The first aim was to identify the shear rate dependent regulation of platelet aggregation by NO at a controlled NO wall flux. The second aim is to determine the relative role of sGC dependent and independent pathways.

Methods: A NO-releasing polymer containing an NO donor, dialkyldiamine-based diazeniumdiolated, was used to control the wall flux of NO into a whole blood flow assay. The NO flux from the polymer was determined by chemiluminescence. Whole blood collected by venipuncture into FPR-chloromethylketone (PPACK), was treated or not treated with the sGC inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) and perfused at 200, 500, and 1000/s over type I fibrillar collagen adsorbed to the NO-releasing polymer. The accumulation of fluorescently labeled platelets and volume of the resultant platelet aggregates was recorded by confocal microscopy. A computational model was developed to estimate the near wall NO concentration as a function of NO flux and shear rate.

Results: The NO-releasing polymer films produced fluxes ranging from 0.07×10^{-10} to 12×10^{-10} mol/cm^2/min, above and below reported fluxes from cultured endothelial cells (Vaughn & Kuo, *AJP*, 274,1998). A NO flux of 2.5×10^{-10} mol/cm^2/min was found to abrogate platelet aggregation, but not initial adhesion, on collagen at 200 and 500/s as effectively as the $\alpha_{2b}\beta_3$ antagonist abciximab. The dynamic range of NO fluxes found to induce measurable inhibition of platelet aggregation spanned from 0.33×10^{-10} to 2.5×10^{-10} mol/cm^2/min at 200 and 500/s. These fluxes correspond to near-wall NO concentrations of 230–2200 nM based on a computational model of NO transport. Preliminary studies show that a sGC independent mechanism may be shear rate dependent.

Conclusions: A NO-emitting material was built into a flow assay for quantitatively measuring platelet function as a function of NO flux and shear rate. NO begins to attenuate the height of the thrombus at approximately 230 nM, which interestingly corresponds to the equilibrium dissociation constant of sGC (Stone et al., *Biochemistry*, 35, 1996). At 1.5 μM NO, platelet aggregation was completely inhibited. Even at the highest NO concentration, platelet were able to adhere to collagen, suggesting that NO does not affect GP1b-VWF and GPVI-collagen interactions. We are in the midst of measuring the relative importance of sGC dependent and independent pathways under flow.

PA 4.20-3

Angiogenesis and permissiveness to invasion in the human endometrium: role of anticoagulant heparan sulfate distribution during the cycle and in tumorigenesis

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Background: Anticoagulant heparan sulfate (aHS) is present in blood vessel walls where its anti-protease activity is thought to limit tissue remodeling and confer stability to the vessels. Preliminary evidence in rodent ovary suggests that aHS are downregulated during angiogenesis. In human uterus, the endometrium undergoes constant remodeling under hormonal control during the estrous cycle, with elongation of blood vessels. Angiogenic factors, like VEGF induce vascular permeability and fibrin deposition in vessel walls serving as provisional pro-angiogenic matrix. Downregulation of endothelial aHS could facilitate fibrin formation. Endometrial carcinoma is a frequent cancer with variable clinical outcome for which currently available markers have limited predictive value of tumor aggressivity. aHS could serve as marker of tissue stability and the abundant aHS present in follicular fluid could modulate tissue invasion in the endometrium.

Aim: Study aHS distribution in normal endometrium and in endometrioïd cancer to reveal if modulations of aHS expression occur during the cycle and in cancer.

Methods: We study normal human endometrium during the estrous cycle and endometrioïd carcinoma grade G1, with approval of the local ethics committee.

Endometrium sections are stained with Alexa^{488}-coupled probes, Anti-thrombin for detection of aHS and endothelial markers CD-31 and D2-40 for blood and lymphatic vessels.

Results: During the menstrual cycle, aHS is present in blood and lymphatic vessels and in basement membranes of surface and glandular epithelium. Vascular aHS is present and does not vary during the cycle and the vessel numbers remain stable in the endometrium. In contrast, epithelial aHS is downregulated in superficial layers of the endometrium during the secretory phase.

In endometrioïd carcinoma, aHS is suppressed to undetectable levels, in the endometrial epithelium but also in the vasculature, as well as in tumoral tissue.

Conclusion: During endometrial regeneration in the proliferative phase, angiogenesis occurs by elongation of existing vessels involving minimal changes in the vessel walls and no alteration of aHS. In the secretory phase, aHS disappearance from the endometrial epithelium corresponds to the implantation window, during which the embryo invades the maternal endometrium. In endometrioïd cancer, the complete absence of aHS indicates active tissue plasticity. These data support our hypothesis that aHS is expressed in stable vascular and epithelial walls and is downregulated during tissue remodelling, angiogenesis and tumor invasion. Further experiments are underways to correlate the mechanisms of implantation and cancer invasion with the disappearance of aHS.

PA 4.20-4

Deep vein thrombus formation induced by flow reduction in mice is determined by venous side branches

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Background: Interaction between vascular wall abnormalities, inflammatory leukocytes, platelets, coagulation factors and hemorheology in the pathogenesis of deep vein thrombosis (DVT) is incompletely understood, pointing to the need to develop well defined animal models of human disease.

Methods and Results: We subjected male C57BL/6 mice to ligation of the inferior vena cava (IVC) as a flow reduction model to induce DVT. Thrombus size and weight were analyzed macroscopically and sonographically by B-mode, pulse wave (pw) Doppler and power Doppler imaging (PDI) using high frequency ultrasound (HFUS). Thrombus size varied substantially between individual procedures and mice, irrespective of the flow reduction achieved by the ligature. Interestingly, PDI accurately predicted thrombus size in a very robust fashion ($r^2 = 0.9734$, $P < 0.0001$). Distance of the insertion of side branches from the ligation suture significantly determines thrombus weight ($r^2 = 0.5597$, $P < 0.0001$) and length ($r^2 = 0.5441$, $P < 0.0001$) in the IVC with distances < 1.5 mm drastically impairing thrombus formation, regardless of the flow measured by pw-Doppler. Occlusion of side branches prior to ligation of IVC did not increase thrombus size, probably due to patent side branches inaccessible to surgery.

Conclusion: Venous side branches influence thrombus size in experimental DVT and might therefore prevent thrombus formation. This renders vessel anatomy and hemorheology important determinants in mouse models of DVT, which should be controlled for.

PA 4.20-5

Human platelet activation/secretion and a membrane-associated urokinase-like activity induce the activation of the latent form of platelet transforming growth factor-β1 (TGF-β1)

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Background: TGF-β1, a multifunctional cytokine, plays key roles in many physiological and pathological processes, i.e, cell proliferation and differentiation, immune response and tissue matrix deposition. Platelets store and transport most of the TGF-β1 in the circulation, released it when activated by various agonists. Nearly all platelet TGF-β1 is in its latent, inactive form and requires to be activated for biologic effect. Shear force in the circulation activates platelet TGF-β1; in tissues, proteolytic cleavage appears as a prominent activation mechanism. Plasmin, derived from urokinase activation of plasminogen, is the best known protease with TGF-β1-activating capacity.

Aims: To test if shear-independent platelet activation results in expression of active TGF-β1 and to explore possible mechanisms involved in this process.

Methods and Results: Human, leukocyte-free platelets express TGF-β1 mRNA (RT-PCR) and synthesize the protein (metabolic radiolabeling). A 12 kDa protein, presumably active form, was revealed in platelet membrane fractions of non-stimulated, TRAP and VWF/Ristocetin-stimulated platelets, but not in platelet releasates (Western blotting). Barely visible bands of the 12 kDA protein were observed in cytosolic fractions obtained after centrifuging the membranes. Activated platelets co-incubated (30 min) with human or mice fibroblast cell lines revealed rapid translocations of Smad-2 into fibroblast nuclei (IF of pSmad-2). After 2 h incubation fibroblast CTGF mRNA expression, a pro-fibrotic molecule downstream of TGF-β1, was observed in TRAP > VWF-Ristocetin > Non-activated platelets. Relative CTGF-mRNA expression in VWF-Ristocetin stimulated platelets was equivalent to that of fibroblasts stimulated with 5 ng/mL TGF-β1 and 3-fold higher in TRAP stimulated platelets. This TGF-β1 mediated effect required fibroblast-platelet contact, and was not observed when fibroblasts were cultured only with platelet releasates or in the presence of SB 525324 (specific inhibitor of TGF-β1 receptor kinase activity). Aspirin abolished the effect of TRAP, but not that of VWF-Ristocetin. Induction of CTGF-mRNA expression, independent of the agonist used, was abolished by pre-incubation of platelets with amiloride (urokinase inhibitor), though not by aprotinin (plasmin inhibitor). Synthesis of type I collagen and fibronectin observed in co-cultures (48 h) of human platelets with human or mice fibroblast cell lines was inhibited by SB 525324.

Conclusions: (i) Human platelets activate fibroblast signaling mediated by TGF-β1 present in platelet membrane requiring platelet-fibroblast contact. This activity increases substantially after PAR-1 (TRAP) or GPIbα (VWF-Ristocetin) platelet activation. (ii) Inhibition of platelet thromboxane A_2 synthesis abolishes the potentiating effect of TRAP. (iii) Fibroblast activation can be triggered by a short time (30 min or less) contact with platelets. (iv) An inhibitor of urokinase abolishes the TGF-β1 activity of platelets, suggesting a role for urokinase or plasmin on TGF-β1 activation. However, plasmin inhibition by aprotinin has no effect on TGF-β1 activity. This contrasts with the known activating effect of plasmin on TGF-β1 and needs further elucidation. (v) Our observations support the notion that platelet adhesion and activation by subendothelium components rapidly trigger the cascade of events ending in wound healing. So, platelets would fastly deposit their cargo at the correct site and at the right time.

Fondecyt No. 11100013, 1110404, 1130853.

PA 4.20-6

Plasma levels of intercellular cell adhesion molecule-1 (sicam-1) in elderly with and without Alzheimer disease

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Introduction: Alzheimer's disease (AD) is a multifactorial disease and the diagnosis is purely clinical. Discovery of a specific, sensitive and reliable biomarker may be crucial for early diagnosis. Microvascular alterations in the brain have been associated with AD and may precede neurodegeneration. In particular, plasma levels of biomarkers of microvascular lesions such as vascular cell adhesion molecule-1 (sVCAM-1) and intercellular adhesion molecule-1 (sICAM-1) are reported to be increased in AD (Breteler, 2000; Ewers et al. 2009).

Purpose: To evaluate plasma levels of intercellular adhesion molecule-1 (ICAM-1) in elderly patients with Mild Cognitive Impairment (MCI) or with AD, and in elderly individuals without cognitive impairment (controls).

Methods: Participants were selected from University Hospital at the Federal University of Minas Gerais, Brazil, after informed consent by themselves or relatives. Blood samples from a total of 159 individuals, including those with AD ($n = 48$), MCI ($n = 53$) and aged individuals without cognitive impairment ($n = 58$) and then plasma levels of sICAM-1 were measured using an enzyme-linked immunosorbent assay (ELISA – R & D Systems, Minneapolis, MN, USA). The results are shown as medians and interquartile intervals (II = Q3–Q1) and groups were compared using ANOVA and Kruskal Wallis test.

Results: The median value of sICAM-1 found in the control group was 272.95 (II = 102.69) ng/mL; for patients with MCI was 303.24

(II = 146.49) ng/mL and for patients with AD was 356.8 (II = 174.23 ng/mL. Patients in the AD group had values above those ones considered normal for healthy adult individuals (115–306 ng/mL). In the current study, plasma levels of sICAM-1 was shown to gradually increase in the three groups as follows: control, MCI and DA groups ($P < 0.0001$; DA × Control).

Conclusion: The comparison among the three groups showed that the elevated plasma levels of sICAM-1 in DA patients, a biomarker of microvascular injury, seem to be associated with the development of AD, which reinforces the idea that microvascular injury may precede neurodegeneration.

Supported by CNPq, CAPES, FAPEMIG – Brazil.

PB1.21 – Antiplatelet Agents: ADP Receptors – II

PB 1.21-1

Evolving pattern of platelet P2Y12 inhibition during maintenance therapy in acute coronary syndrome patients

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Background: Dual antiplatelet therapy with aspirin and clopidogrel has previously been the standard of care for patients with acute coronary syndromes (ACS) but international guidelines have been evolving over the last 5 years with the introduction of prasugrel and ticagrelor. Prasugrel was approved in October 2009 by NICE in the UK for use in patients with ST-elevation myocardial infarction (STEMI) undergoing primary PCI, diabetics with non-ST-elevation (NSTE) ACS undergoing PCI and patients with stent thrombosis and other ACS patients were to continue receiving clopidogrel. Ticagrelor was approved in October 2011 by NICE for use in patients with moderate-to-high risk NSTE ACS or STEMI undergoing primary PCI and was recommended in preference to clopidogrel in ESC guidelines. These recommendations were adopted in our region, constituting a population of 1.8 million, following each NICE approval.

Aims: To study the effect of changing patterns of $P2Y_{12}$ inhibitor usage on levels of platelet inhibition during maintenance therapy.

Methods: Patients admitted to Northern General Hospital, Sheffield, with NSTE ACS or STEMI managed with primary PCI were enrolled in this study over two periods of time: May 2010–November 2011 (T1); and October 2012–January 2013 (T2). Venous blood samples were obtained at 1 month after the onset of ACS when patients were established on maintenance dual-antiplatelet therapy. Platelet-rich plasma derived from citrate-anticoagulated blood was used for light transmittance aggregometry (LTA) with ADP 20 mM as the agonist and final aggregation response was determined. VASP phosphorylation assay, Multiplate ADP test and VerifyNow P2Y12 assay were also performed. Results were determined as mean ± SD and compared by unpaired t test.

Results: One hundred and sixteen patients were enrolled in T1 of whom 82 were receiving clopidogrel and 34 were receiving prasugrel. Twenty patients were enrolled in T2, all of whom were receiving ticagrelor. All except one patient in the clopidogrel group was receiving low-dose aspirin. Demographic characteristics of the ticagrelor and clopidogrel patients were similar whereas the prasugrel-treated patients had a lower mean age, more often were smokers at ACS presentation and had a greater proportion of STEMI reflecting the recommendations for prasugrel. Mean LTA results according to treatment with clopidogrel, prasugrel and ticagrelor were 50 ± 24%, 37 ± 23%, and 10 ± 8%, respectively. Prasugrel was associated with significantly lower platelet aggregation responses than clopidogrel ($P = 0.006$) and ticagrelor was associated with significantly lower platelet aggregation responses than both prasugrel and clopidogrel (both $P < 0.001$). The results of the other assays showed consistent patterns of $P2Y_{12}$ inhibition.

Conclusions: International guidelines and NICE approval have led to increasing levels of $P2Y_{12}$ inhibition in ACS patients in this UK centre between May 2010 and January 2013. Ticagrelor was associated with significantly greater $P2Y_{12}$ inhibition than both clopidogrel and prasugrel during maintenance therapy.

PB 1.21-2

Comparison of two platelet function analysers in patients having taken clopidogrel and awaiting cardiac surgery: single centre experience in 50 unselected patients

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Background: There is increased bleeding risk associated with continued clopidgrel treatment up to the point of cardiac surgery. Guidelines suggest patients who have been taking clopidogrel should stop for 5–7 days before cardiac surgery to limit the risk of bleeding complications. Approximately 30% of patients do not respond to clopidogrel treatment it is therefore reasonable to assume that these patients would benefit from earlier surgical intervention. There has been a rising demand for platelet function testing of patients that have taken clopidogrel to identify those patients at risk of perioperative bleeding and subsequent reoperation. There continues to be much debate regarding the use of platelet function analysis in patients on antiplatelet therapy and which assays are most appropriate.

Aims: We compared two platelet analysers Multiplate and VerifyNow that have been used in this field to determine results and respective cut-offs for patients going for cardiac surgery with an attempt to identify those with a minimal risk of bleeding due to their antiplatelet therapy and if the results between the two analysers were interchangeable.

Methods: The VerifyNow P2Y12 cartridge (ADP 20 μM) was used for clopidogrel analysis using a single Greiner citrate sample (0.109 M). A single Hirudin sample was taken for Mulitplate analysis using ADP reagent (6.4 μM). Cut-off values for surgery were defined as < 20% inhibition with the P2Y12 VerifyNow assay (therapeutic range > 40%) and values > 45AUC (therapeutic range 0–45AUC) for the Multiplate. Fifty patients awaiting surgery were tested. Our current practice is to use the VerifyNow in this setting. As no extra blood samples were required than would be normally taken this was seen as a service evaluation of best clinical practice requiring no ethical approval. We used Spearman rank correlation to compare the two tests.

Results: The 50 patient samples tested only 47 were suitable for comparative analysis due to errors in three samples on the VerifyNow analyser resulting in no results. Of the 47 patients tested Multiplate results were median 35.5AUC range 2–90AUC, VerifyNow median 23.5% range 0–93%. Of those patients tested with the Multiplate analyser 12 patients would be classed as fit for surgery and using the VerifyNow device 23 patients would be accepted for surgery. There was agreement between the analysers in 22 patients and discordant results in 25 patients. Using the VerifyNow device we would have classified 25.5% of the patients as clopidogrel non-responders and 19% with the Multiplate. Spearman correlation −0.4714 ($P = 0.001$).

Conclusions: There was some disparity of results between the two analysers. Some of this discrepancy is likely due to differences in ADP concentrations used in the tests with 3× greater ADP concentration in the VerifyNow. This may explain why the VerifyNow device appeared to classify more quickly those patients stopping clopidogrel at 3–4 days as having platelet function acceptable for surgery than the Multiplate. This raises the question of which test is suitable in this scenario. This small patient sample highlights the need for further work to be conducted on the appropriate assays used when measuring the effect of antiplatelet drugs.

PB 1.21-3

Clopidogrel therapy has additional inhibitory actions on cyclo-oxygenase and thrombin receptor-mediated pathways in platelets

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Background: Clopidogrel in combination with low-dose aspirin is an effective therapy after acute coronary syndromes. The principal mode of action is by irreversible inhibition of the platelet P2Y12 receptor, thereby preventing platelet activation and aggregation by adenosine diphosphate (ADP). Previously published work has suggested that thienopyridines also affect other platelet pathways when tested *in vitro* or at higher than recommended doses.
Aims: To investigate the effects of clopidogrel on other platelet pathways including cyclo-oxygenase and thrombin receptor-mediated pathways during therapy with the recommended therapeutic dose.
Methods: We studied 159 patients with stable coronary artery or peripheral artery disease who were taking part in a randomised controlled trial to investigate clopidogrel rebound. Patients taking 75 mg daily aspirin and statin, were randomised to 75 mg clopidogrel or placebo for 28 days. After informed consent, blood samples were taken for aggregometry with arachidonic acid (AA) and ADP; Fibrinogen binding with ADP or Thrombin receptor activating peptide (TRAP); P-selectin expression with ADP; Verify Now Aspirin; Verify Now P2Y12; and VASP-P, at baseline (aspirin alone), after 28 days on treatment, and at 7, 14 and 28 days after stopping the study medication.
Results: In patients taking clopidogrel plus aspirin there were, predictably, statistically significant decreases on-treatment compared with baseline (aspirin alone), in ADP-induced aggregation, ADP-induced P-selectin, ADP-induced fibrinogen binding, Verify Now P2Y12, and VASP-P (all $P < 0.01$, paired t-test). There were no statistically significant changes in unstimulated platelet P-selectin, fibrinogen binding, or AA-induced aggregation. However, there were statistically significant decreases in Verify Now Aspirin [baseline 436 (407–477) ARU; on-treatment 396 (385–430); median(iqr)] and TRAP-induced fibrinogen binding [baseline 41.8 (38.8–62.1)%; on-treatment 10.6 (4.64–23.4)%]. No significant changes were found in the placebo plus aspirin group. At 7 days after stopping clopidogrel, all parameters had returned to baseline levels [Verify Now Aspirin 7d post 422 (408–467) ARU; TRAP-fibrinogen 7d post 37.4 (25.2–54.8)%]. Values for Verify Now Aspirin and AA-aggregation were consistent with adequate aspirin inhibition in all but two subjects, both of whom were in the placebo plus aspirin group.
Summary/Conclusions: This study shows additional inhibitory effects of clopidogrel therapy on the cyclo-oxygenase and thrombin receptor pathways which could contribute to the drug's antithrombotic actions or towards increased bleeding. Further investigation is required to determine if there is direct antagonism, or whether the observed effects are due to prevention of amplification of aggregation by endogenous ADP.
The study was funded by Heart Research UK, and Ethics approval obtained (NOSRES 08/S0801/087).

PB 1.21-4

The effect of CYP4F2 G1347A polymorphism and of clinical factors on platelet reactivity in patients, treated with dual antiplatelet therapy

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Background: Dual antiplatelet therapy by aspirin and clopidogrel is used to prevent recurrent ischaemic heart events after stent implantation, myocardial infarction or in patients with unstable heart disease. Indeed, some patients are 'resistant' to antiplatelet therapy. It has been proved that platelet hyperreactivity is associated with stimulation of platelet surface membrane receptors by epinephrine. Through activation of these (α2 adreno-) receptors, the dense granule secretion is higher and the aggregatory effect of other agonists (such as ADP, collagen, thrombin) can be amplified. There is also a synergistic effect between epinephrine and thromboxane A2, as with the downregulation of autocrine agents (thromboxane A2) the potentiating effect of catecholamines is inhibited. The binding of thromboxane A2 to its target-thromboxane/prostaglandin H2 (PGH2) receptor leads to platelet activation, otherwise, this receptor is inhibited by binding of 20-hydroxyeicosatetraenoic acid, which is produced from arachidonic acid by a member of hepatic P450 enzymes – CYP4F2 monooxygenase.
Aims: The aim of the study was to reveal whether clinical factors and *CYP4F2* G1347A (*rs2108622*) polymorphism had a significant effect on platelet reactivity in patients, treated with aspirin and clopidogrel due to acute coronary syndromes.
Methods: Totally 89 patients, who had been hospitalized due to acute coronary syndromes at the Department of Cardiology, of 2nd Hospital in Kaunas, Lithuania, from September of 2009 to January of 2010 and who continued clopidogrel and aspirin therapy for at least of 14 days, were included into the further study. A standard 75 mg/day clopidogrel and 100 mg/day aspirin doses were prescribed to all of the represented patients. Blood was taken for genetic test and for platelet aggregation. Tests for platelet aggregation and for genotyping were performed in the laboratory of Molecular Cardiology of the Institute of Cardiology of Lithuanian University of Health Sciences. The polymorphism *CYP4F2* G1347A (*rs2108622*) was assessed by using commercial Taqman probes (Applied Biosystems, UK) on ABI 7900HT Fast Real-Time PCR Thermocycler (USA).
This study was done according to the Declaration of Helsinki. Written informed consent was obtained from all patients included in the study. Permission for this study was obtained from Regional bioethics committee of Kaunas (Lithuania) in 2008.11.05. The permission number is BE-2-51.
Results: Platelet aggregation with epinephrine was higher in CYP4F2 GA genotype carriers (56.65 ± 24.46) as compared to GG (46.26 ± 21.53) ($P = 0.04$) or AA (34.42 ± 18.15) ($P = 0.01$) carriers. The frequency of GA, GG, and AA genotype carriers in studied population was 45.5%, 46.6% and 7.9%, respectively. The patients, who used angiotensin-converting enzyme inhibitors ($n = 5$), had lower platelet aggregation induced by epinephrine vs. non-users (28.80 ± 13.25 and 51.15 ± 23.50, $P < 0.03$, respectively).
Summary/Conclusions: CYP4F2 G1347A genotype has an impact on the hyperreactivity and 'resistance' to antiplatelet treatment. The antiplatelet effect of both clopidogrel and aspirin is augmented by concomitant use of angiotensin-converting enzyme inhibitors.

PB 1.21-6

Antiplatelet activity, P2Y1 and P2Y12 inhibition, and metabolism in plasma of diastereomers of the Ap4A analog diadenosine-5′,5‴-P1,P4-dithio-P2,P3-chloromethylenetetraphosphate

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Background: Diadenosine 5′,5‴-P^1,P^4-dithio-P^2,P^3- chloromethylenetetraphosphate (Ap(S)pCHClpp(S)A), compound 5, an analog of the naturally occurring platelet dense granule constituent diadenosine

5′,5‴-P^1,P^4- tetraphosphate (Ap_4A), inhibits adenosine diphosphate (ADP)-induced platelet aggregation by inhibiting both platelet ADP receptors, $P2Y_1$ and $P2Y_{12}$. The modifications to Ap_4A in compound five result in chiral centers, giving rise to multiple diastereomers.

Aim: To determine whether the diastereomers of compound five differ in their inhibition of platelet aggregation, $P2Y_1$ and $P2Y_{12}$ function, and stability in plasma.

Methods: Four diastereomers of compound 5 (5.1, S_pS_p; 5.2, $S_pSR_p \equiv R_pSS_p$ or $S_pRR_p \equiv R_pRS_p$; 5.3, $S_pRR_p \equiv R_pRS_p$ or $S_pSR_p \equiv R_pSS_p$; 5.4, R_pR_p.) were separated by preparative RP-HPLC in a pH 7, 20 mM potassium phosphate/methanol buffer system. ADP 3 μM-induced platelet aggregation was evaluated by light transmission aggregation (LTA), $P2Y_{12}$ inhibition by the vasodilator-stimulated phosphoprotein (VASP) phosphorylation assay, and $P2Y_1$ inhibition by changes in cytosolic Ca^{2+} in FLUO-4 loaded platelets. Recovery of the diastereoisomers following 37 °C incubation in plasma was used to determine stability.

Results: The ability of the diastereomers to inhibit LTA and the platelet $P2Y_{12}$ receptor, and their stability in plasma strongly depended on the stereo configuration of the chiral P^1- and P^4-phosphorothioates, the S_pS_p diastereomer (5.1) being the most potent inhibitor and completely resistant to degradation in plasma, and the R_pR_p diastereomer (5.4) being least potent inhibitor and with lowest plasma stability. The inhibitory activity of S_PR_P diastereomers depended on the configuration of the chiral P^2,P^3- chloromethylene group, one of them being significantly more active than the other. Their plasma stability did not differ significantly, being intermediate to that of the 5.1 and the 5.4 diastereomers. The differences between the diastereomers in their inhibitory effect on platelet $P2Y_1$ receptors were less remarkable.

Conclusion: The S_pS_p diastereomer of diadenosine 5′,5‴-P^1,P^4-dithio-P^2,P^3- chloromethylenetetraphosphate (Ap(S)pCHClpp(S)A), compound 5.1, showed greater stability in plasma and greater inhibition of LTA and the platelet $P2Y_{12}$ receptor than other diastereomers of compound 5. These results will be useful for structural and mechanistic studies, development of novel antiplatelet agents that inhibit both $P2Y_1$ and $P2Y_{12}$ ADP receptors, and drugs for the therapeutically important fields of purinergic receptors and ecto-nucleotide pyrophosphatase/phosphodiesterase enzymes.

PB1.22 – Standardizing Platelet Function Tests

PB 1.22-1

A simplified approach to monitoring changes in VASP phosphorylation in platelets and other blood cells

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Background: Measurement of the degree of phosphorylation of vasodilator-stimulated phosphoprotein (VASP) is used as a surrogate measure for platelet cAMP. Recently we developed a cytometric bead assay for the sensitive and reproducible measurement of VASP-phosphorylation in platelets. This procedure has now been simplified by the development of a one-step solution which lyses, captures and fluorescently labels phosphorylated VASP (VASP-P) in platelets and other blood cells.

Aims: To validate the assay and demonstrate its use as a simple means of detecting the effects of various G_s-coupled agonists on platelets in platelet-rich plasma (PRP) or blood, and of assessing inhibition of the platelet $P2Y_{12}$ receptor.

Methods: Anticoagulated blood or PRP from healthy volunteers was incubated with the appropriate agents (e.g. PGE1, iloprost, ADP) for 5 min at room temperature (final volume 125 μL). An aliquot (5 μL) was removed and mixed with the one-step solution (25 μL). The sample was then incubated for 2 h in the dark at room temperature or frozen and stored at −20 °C for subsequent analysis. Samples were then analysed using a Becton Dickenson LSRII flow cytometer using FACSDiva acquisition software. The results are expressed as median fluorescence (mf).

Results: Values of VASP-P following platelet stimulation appeared to be lower in blood than PRP possibly because of dilution with VASP from other cells. Values were identical when expressed as percentage of maximal VASP-P. A strong linear relationship was obtained between the amount of VASP-P in the sample and the median fluorescence obtained ($R^2 = 0.99$). Intra-assay variation ranged from 1% to 6%, with a mean of 3%. Inter-assay variation ranged from 2% to 9%, with a mean of 6%. The flow cytometric analysis can be performed shortly after venepuncture or the samples stored for analysis at a later date. Once the one-step solution has been added to blood, samples may be stored until it is convenient to analyse them on the flow cytometer. Frozen samples remain stable for at least 2 months. Our approach to determining the effectiveness of P2Y12 antagonists as inhibitors of platelet function involves determining the increase in the level of VASP-P in platelets in blood by adding an agent such as iloprost and comparing this with the level attained with simultaneous addition of ADP. Effective inhibition by a P2Y12 antagonist results in persistence of VASP-P induced by iloprost even in the presence of ADP. Results demonstrate clear effects of the potent P2Y12 antagonist prasugrel or cangrelor with varying effects with clopidogrel depending on the donor used. The assay is not affected by aspirin.

Conclusions: A one-step method of measuring VASP-P in platelets has been identified and validated as described above. This novel assay provides a simple, sensitive, reproducible and flexible method for determination of VASP phosphorylation in platelets.

PB 1.22-2

Simultaneously measuring adenosine triphosphate (ATP) release and light transmission aggregation does not potentiate platelet aggregation in participants with clinically diagnosed bleeding disorders

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Background: Current guidelines recommend the use of lumi-aggregometry to simultaneously assess dense granule secretion with platelet aggregation when investigating platelet disorders, as there is evidence that dense granule secretion defects can be misdiagnosed if relying solely on platelet aggregometry. However, a recent publication has suggested that the addition of the commercial reagent Chronolume® to measure ATP release potentiates platelet aggregation responses and may mask a platelet defect in some patients, especially in response to epinephrine (adrenaline) (Thromb Haemost 2012;107:726–34).

Aims: We sought to investigate whether potentiation of platelet responses to epinephrine occurred in a cohort of patients with clinically diagnosed bleeding disorders.

Methods: Participants with clinically diagnosed excessive bleeding and healthy volunteers were recruited to the Genotyping and Phenotyping of Platelets study (GAPP, ISRCTN 77951167) from April 2012 to January 2013 from UK Comprehensive Care Haemophilia Centres. The study aimed to determine the prevalence of platelet function defects in this patient group. Platelet function testing was carried out by light transmission aggregometry alongside measurement of ATP secretion using a dual-channel Chronolog lumi-aggregometer (Model 460 VS). Platelet aggregation measurement with and without Chronolume® was carried out simultaneously. This study was approved by the National Research Ethics Service Committee and all participants gave written informed consent.

Results: One hundred participants were included ($n = 73$ with suspected platelet function defects and $n = 27$ healthy volunteers). A platelet defect was found on platelet function testing in 40% of participants with bleeding symptoms (60% had no demonstrable platelet defect, 18% had a Gi-type defect, 11% had a dense granule secretion defect, 7% had a defect in the thromboxane pathway and 4% had a

complex phenotype). Addition of Chronolume® did not result in a significant increase in maximal platelet aggregation in response to epinephrine 10 μM in healthy volunteers (Δ = 0.7, 95%CI −7.5 to 9.0, $P = 0.74$), or in participants with bleeding symptoms ($n = 57$, 78%) who had a sustained and biphasic response to epinephrine 10 μM (Δ = −1.0, 95% CI −4.0 to 1.9, $P = 0.48$). In 16 (22%) participants with bleeding symptoms, there was no secondary wave in response to epinephrine 10 μM. Addition of Chronolume® in this group resulted in a non-statistically significant increase in maximal platelet aggregation in response to epinephrine 10 μM (Δ = 10.6, 95%CI −1.8 to 23.1, $P = 0.09$). In four out of 16 participants who had no secondary wave in response to epinephrine, addition of Chronolume® induced a secondary wave of aggregation. However, out of 57 participants with biphasic aggregation, addition of Chronolume® resulted in abrogation of a secondary wave in three participants. Overall, the McNemar test showed no impact of adding Chronolume® on induction of a secondary wave in response to epinephrine 10 μM ($P = 1.0$).

Summary/Conclusions: Our data support the use of Chronolume® to simultaneously assess dense granule secretion with platelet aggregation when investigating patients with excessive bleeding, and suggest that this practice does not mask platelet defects including those that reduce platelet aggregation response to epinephrine.

PB 1.22-3

Label-free detection of platelet adhesion to collagenous substrates

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Background: Platelets use vessel wall collagens as both an anchorage and a stimulus during thrombus deposition on the damaged vessel wall. Discriminating the adhesive and activatory roles of collagens is not simple, since the native collagens combine both functions. Both collagens I and III are present in the vessel wall.

Aims: To develop a rapid, real-time means of identifying adhesive and activatory collagen motifs using the collagen Toolkits in conjunction with the Acea xCELLigence® impedance-based adhesion platform. Toolkits are libraries of triple-helical peptides used to map the sites where interacting proteins bind collagen, ideal for the homotrimeric collagen III, but less than optimal for heterotrimeric collagen I.

Methods: Washed platelets were dispensed into ePlates, 96-well plates containing micro-electrode arrays, onto which collagenous peptides were coated, providing real-time readout (called Cell Index) of thrombus accretion on the base of the well. Cell adhesion occurs over 5–60 min as platelets contact the surface, attach and spread. We optimised platelet numbers for this study. Sites were mapped onto Toolkit III, and compared with known sites for platelet receptor binding. We used the homotrimeric Toolkit II as a surrogate for collagen I, since the alpha1 chains are most highly conserved amongst the collagens. Wells were coated using 10 μg/mL of each peptide.

Results: We identified peptides specific for GpVI and for integrin α2β1, and could discriminate between activatory and adhesive peptides by including antagonists of αIIbβ3 in the suspending medium, which abolished fibrinogen-mediated accumulation of platelets on the growing platelet mass. Key α2β1-specific peptides could be identified by integrin blockade, and since they contained GxOGER or related recognition motifs. Sites for GpVI tended to be GPO-rich, as peptide III-30, and were blocked using anti-GpVI antibodies.

Summary/Conclusion: New platelet-reactive collagen peptides were identified rapidly and in real time. The method is suitable for multiwell aggregometry, provided that adhesive substrates (relevant proteins such as collagen, fibronectin or fibrinogen) are used. Data indicate a much closer apposition (faster kinetics and higher Cell Index) mediated by α2β1 than by GpVI.

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PB 1.22-4

Effect of platelet count on platelet aggregation measured by impedance aggregometry (Multiplate – analyzer) and by light transmission aggregometry

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Background: The *in vitro* evaluation of platelet aggregation is useful to characterize patients with defects of primary hemostasis. In some laboratories, it is also used to monitor antiplatelet treatment. Traditionally, platelet aggregation is measured by light transmission aggregometry (LTA), which measures the transmission of light through a sample of platelet-rich plasma (PRP) or platelet suspensions in buffer, which increases when platelets aggregate. Impedance aggregometry (IA), which measures the increase in electrical resistance between two electrodes brought about by platelet adhesion and aggregation, allows to measure platelet aggregation also in whole blood. We previously demonstrated that a platelet count within the range of 150–600 × 10^9/L does not affect the results of platelet aggregation measured by LTA. However, considering that the extent of platelet aggregation measured by IA is a function of the volume of platelet mass accumulating on the electrodes, it is likely that platelet count affects its results. As a matter of fact, a positive correlation between platelet count and the extent of platelet aggregation measured by IA (Multiplate™ analyzer) has been found in a study of patients on treatment with aspirin. No studies have so far measured the changes in the extent of platelet aggregation measured by IA under conditions in which platelet count is the only variable.

Aim: This study was to evaluate the effects of varying the platelet count in platelet suspensions on the extent of platelet aggregation, measured by IA (both in the presence and absence of red blood cells [RBC]).

Methods: Platelets from blood anticoagulated with acid-citrate-dextrose of 15 healthy subjects were washed and resuspended in Tyrode buffer containing apyrase. The platelet count was adjusted to 75, 150, 250, 500 and 750 × 10^9/L with Tyrode buffer. Platelet aggregation induced by collagen (3.2 μg/mL), thrombin receptor activating peptide-6 (TRAP, 32 μM) or ADP (20 μM) was measured by Multiplate™ (in absence and presence of 40% washed autologous RBC) and, for comparison, also by LTA.

Results: Platelet aggregation increased linearly as a function of the platelet count, when measured by Multiplate™ both in presence and absence of RBC. In the presence of RBC, the extent of platelet aggregation ranged between 576 (75 × 10^9/L) and 1737 (750 × 10^9/L) AU*min (TRAP), 359–1391 (collagen) and 119–434 (ADP). For every 50 × 10^9/L increase in platelet count, we observed an increase of 111.8 AU*min (TRAP), 80.1 (collagen), 27.3 (ADP). In contrast, collagen- and TRAP-induced platelet aggregation measured by LTA was similar at platelet counts ranging between 150 and 750 × 10^9/L, but was significantly reduced at a platelet count of 75 × 10^9/L. ADP-induced platelet aggregation measured by LTA was similar at platelet counts ranging between 150 and 500 × 10^9/L, confirming previous results, but was reduced at platelet counts of 75 and 750 × 10^9/L.

Conclusions: Slight variations in the platelet count within the normal range cause remarkable variations in the results of platelet aggregation, as measured by IA (Multiplate™). This effect should be taken in due consideration when platelet function of patients with bleeding disorders or on treatment with antiplatelet agents is measured by this technique.

PB 1.22-5

Thrombin generation and microparticalassociated procoagulant activity as new assays to characterize the hemostatic profile of platelet concentrates

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Introduction: The hemostatic quality of platelet concentrates (PC) is affected by preparation methods and storage conditions. Different *in vitro* techniques can be used to analyze PC quality, including platelet adhesion and aggregation assays. However, these tests are not able to characterize the procoagulant capacity of platelets. Thrombin generation (TG) and microparticle-associated procoagulant activity (MP-PCA) are two emerging assays to characterize the hemostatic profile of plasma samples.

Aims: In this study we evaluated the suitability of MP-PCA and TG assays to assess the procoagulant properties of PC prepared for transfusion use and the sensitivity of these assays to the different PC preparation methods.

Methods: Eighteen PC were obtained from pooled buffy coats of overnight stored whole blood. Of them, 10 were prepared by separation with the Fenwal System (FS) and eight by separation with the Terumo TACSI System (TTS). PC were sampled the day of preparation (D0) and after 3 days of storage (D3) at 22 °C. Supernatants of PC (S-PC), obtained by centrifugation, were tested for TG potential (CAT assay; Stago) and MP-PCA (PPPL kit, Stago).

Results: At D0, both TG and MP-PCA of S-PC displayed values similar to that measured in normal pool plasma (NPP). The analysis according to the method of PC preparation, showed TG values significantly ($P < 0.05$) higher in S-PC prepared by TTS compared to FS (ETP: 1400 ± 95 vs. 1134 ± 65 nM*min), while no differences were found in the MP-PCA levels. At D3, in S-PC prepared by TTS a statistically significant reduction in TG (ETP: 900 ± 180 vs. 1304 ± 191 nM*min; $P < 0.05$) occurred in parallel to a significant ($P < 0.05$) increase in MP-PCA. Differently, no significant modifications were detected in S-PC prepared by FS. Of interest, the lowest TG and the highest MP-PCA values were found in S-PC from B blood phenotype.

Summary/Conclusions: Our data support the validity of these assays in the assessment of procoagulant activity of PC. The increased MP-PCA observed in samples collected after 3 days of storage reveals the occurrence of platelet activation/apoptosis, particularly in S-PC prepared by TTS. These results provide background for the utility of these new tests to assess the quality of PC, in terms of capacity to contribute to restore a normal hemostasis in patients with severe bleeding complications.

PB 1.22-6

Subdivision according to size is necessary for correct interpretation of data regarding platelet expression of active GPIIb/IIIa and phosphatidylserine

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Background: Platelets analyzed by flow cytometry are often identified based on size and gated as a single population to investigate expression of activation markers. Platelets may also be divided into platelets and microparticles. In addition we have observed that activated platelets may form a third population of intermediate size.

Aims: To investigate how platelet size is affected by activation and how the expression patterns of active GPIIb/IIIa (α2bß3) and phosphatidylserine (PS) differ between different platelet subpopulations.

Methods: Platelets in heparinized whole blood were activated with PAR1-activating peptide (AP), PAR4-AP or collagen-related peptide (CRP) as single or combined agonists at high (30, 150 μM and 0.93 μg/mL) or low concentrations (2.5, 5 μM and 0.01 μg/mL). Flow cytometry was performed to detect platelets (GPIIb-RPE positive events) and their expression of the active conformation of GPIIb/IIIa (binding PAC-1-FITC) and PS (binding annexin V-APC).

GPIIb-RPE positive events were divided into different populations/gates based on size (forward scatter properties): 'normal-sized platelets' (approximately > 0.7 μm, lower limit excluding 1–2% of resting platelets), 'smaller platelets' (0.5 < ~0.7 μm), and 'platelet fragments' (< 0.5 μm) and was compared to a gate including all platelets. Expression of active GPIIb/IIIa and PS was then compared between these populations.

Results: Changes in platelet size depend both on the agonist used and the level of activation. With low as well as high concentrations of PAR1-AP together with PAR4-AP, approximately 90% of the platelets were located in the 'normal-sized platelets' gate. In contrast, when high concentrations of CRP in combination with PAR1-AP and/or PAR4-AP were present, the fraction of platelets located in the 'normal-sized platelets' gate decreased to 40–60%.

As an example, upon activation with high concentrations of PAR1-AP, PAR4-AP and CRP, 41.3 ± 13.8% of the platelets were located in the 'normal-sized platelets' gate, 37.3 ± 10.4% in the 'smaller platelets' gate and 13.4 ± 4.8% in the 'platelet fragments' gate ($n = 4$). With this stimulation the expression of active GPIIb/IIIa and PS in the 'all platelets' gate (36.9 ± 15.3% and 46.6 ± 16.8%, respectively) was distinctly different from that seen when the population was divided into 'normal-sized platelets' (77.0 ± 9.8% and 7.2 ± 6.3%), 'smaller platelets' (2.9 ± 1.7% and 77.1 ± 10.8%) and 'platelet fragments' (0.3 ± 0.1% and 86.3 ± 5.9%).

Summary/Conclusions: Upon activation with low concentrations of agonists, the use of a single platelet gate including normal-sized platelets or all platelets will give quite an accurate estimation of the expression pattern of the entire population. However, upon activation with high concentrations of CRP together with PAR1-AP and/or PAR4-AP a large fraction of platelets will turn into smaller platelets and platelet fragments. The different sized platelet populations show distinctly different expression patterns regarding active GPIIb/IIIa and PS, where normal-sized platelets primarily express an active GPIIb/IIIa and smaller platelets and platelet fragments primarily express PS.

Thus, the use of a single gate for all platelets will give an overall estimation of the platelet population but will not detect the characteristic expression patterns related to platelet size, which may make a correct interpretation of data more difficult. We therefore recommend that platelets are subdivided according to size before interpreting results investigating down-regulation of active GPIIb/IIIa and expression of PS.

PB1.23 – Platelet Integrins – I

PB 1.23-1

The role of the fibrinogen NGR motif in mediating platelet adhesion and activation

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Introduction: The integrin $\alpha_{IIb}\beta_3$ on resting platelets can bind to immobilized fibrinogen resulting in platelet spreading and activation but requires activation to bind to soluble fibrinogen. $\alpha_{IIb}\beta_3$ is known to interact with the general integrin-recognition motif RGD (arginine-glycine-aspartate) as well as the fibrinogen-specific γ-chain dodecapeptide; however it is not known how fibrinogen binding triggers platelet activation. The amino acid sequence NGR (asparagine-glycine-arginine) has been identified as an integrin-recognition motif and is present on both the β and γ chains of fibrinogen.

Aims: To determine if the NGR-sequence in fibrinogen can mediate an interaction with $\alpha_{IIb}\beta_3$.
Methods: Recombinant fibrinogens lacking either the β-chain (Fgβ$^-$) or γ-chain (Fgγ$^-$) NGR sequences were prepared by substitution with AAA. Platelet adhesion to recombinant fibrinogens was quantified by measuring acid phosphatase activity. Platelet spreading was characterized by confocal microscopy. Platelet adhesion was also quantified after blood was sheared over immobilized recombinant fibrinogen in a parallel plate flow chamber.
Results: Static, resting platelet adhesion to the mutant fibrinogens was reduced compared to plasma fibrinogen (pFg) (Fgβ$^-$: 38.7 ± 11.9% $P < 0.01$, $n = 14$ and Fgγ$^-$: 44.3 ± 16.4% $P < 0.05$, $n = 14$ reduction compared to pFg) and the percentage of spread platelets was also reduced (Fgβ$^-$: 16.1 ± 7.4%, Fgγ$^-$: 27.6 ± 11.4%,pFg: 55.1 ± 9.5% of total platelets spread, One way ANOVA, $P = 0.005$, $n = 8$) while the time to spreading was increased for Fgβ$^-$ (pFg 15.7 ± 1.3 min, Fgβ$^-$: 20 ± 2.5 min, $n = 3$, $P = 0.013$). When platelets were pre-activated with TRAP there were no longer any differences in the adhesion to recombinant fibrinogens and plasma fibrinogen. Under shear conditions (200/s) platelet adhesion was abolished ($P < 0.0001$, $n = 3$).
Conclusions: The NGR motif in fibrinogen specifically binds to resting $\alpha_{IIb}\beta_3$ and is responsible for triggering platelet spreading. It also plays a significant role in the adhesion of resting platelets to immobilized fibrinogen under shear conditions.

PB 1.23-2
Regulation of platelets collagen receptor a2β1 integrin and the possibility of intermediate affinity

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Background: Collagen-binding integrins (a1b1, a2b1, a10b1 and a11b1) form a class of I domain-containing integrin. They differ structurally from leucocyte integrins ($\alpha_M\beta_2$, $\alpha_L\beta_2$, $\alpha_X\beta_2$, and $\alpha_D\beta_2$) by the presence of a small helix of about five amino acids named the c-helix. Integrin a2b1 is a surface-expressed heterodimer, important in adhesion of platelets to damaged blood vessel wall, in the normal function of endothelium, and in the migration and development of vascular smooth muscle cells. Much progress has been made in unraveling the complexity of conformational states in integrins. At least three different activation states, represented by different quaternary conformations have been described: (i) a closed conformation (resting state), unable to bind its ligand; (ii) an extended conformation with closed headpiece (low affinity state); and (iii) an extended conformation with open head-piece (high affinity state). The two extended, active conformations are favored by outside-in and inside-out signaling and can bind their natural ligand. Molecular dynamic simulations showed that both aL and aM I domains can adopt three conformations where the b6-a7 loop moves successively between three ratchet positions, from closed to intermediate, and then to open. The same study suggested that collagen-binding integrins cannot adopt the intermediate conformation due to F-to-E substitution of the second ratchet residue at the top of helix 7. In a different study, two functional active conformations of a2b1 were identified on the platelet surface using a conformation-sensitive antibody.
Aim: To explore the collagen binding activity of the human and rat collagen-binding integrins, to investigate possible intermediate affinity states.
Methods: We designed a series of single and double amino acid substitutions in helix 7 and the c-helix. Site-directed mutagenesis was carried out in the I domain expressing vectors. Collagen II and III triple-helical Toolkit peptides of differing affinity for the integrins were used as adhesive substrates in solid-phase binding assays. Conventional ELISA-like methodology was used.
Results: The I domains display characteristic binding distribution across the Toolkit peptides, patterns of binding that reflect the affinity state of the I-domain
The data showed that a2 I domain is the only collagen binding I domain that can adopt an intermediate affinity.
Conclusions: The structure of a mutant that adopts intermediate affinity is equivalent to a closed conformation, as found in the crystal structure of the wild-type I domain, but with some differences around the MIDAS that must be responsible for its enhanced binding activity.
References:
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PB 1.23-3
Mass spectrometry of platelet lipid raft fractions reveals a substantial pool of active integrin aIIbβ3

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Background: Platelet membranes are divided into micro-domains in part by the formation of 'rafts' enriched with cholesterol and glycolipid, which can be isolated as detergent resistant fractions (DRFs). Most activatory signaling occurs in DRFs, yet integrin function is largely assumed to be outside the DRF, despite numerous reports that a small proportion of integrin αIIbβ3 can be detected in lipid rafts by western blotting. This led us to explore the possibility that this assumption is incorrect.
Aim: We hypothesised that integrin αIIbβ3 could be an important but overlooked component of DRFs.
Method: Resting or collagen receptor (GPVI) stimulated platelets from three healthy subjects were lysed with equivalent volume of ice cold 0.05% Triton X-100 buffer containing protease inhibitors. DRFs were isolated by mixing lysed samples with Optiprep (Sigma, UK) to a final concentration of 25% w/v iodixanol on top of which was layered a density gradient of 20%, 15% 10%, 5% w/v iodixanol, containing 0.025% Triton X-100, and centrifuged at 52,000 ***g*** for 90 min at 4 °C. Sequential 1 mL samples were collected from the bottom of each tube and DRFs were identified by the distribution of cholesterol, LAT and PAG-1. DRFs were analysed by MS/MS performed using HCT ion-trap (ESI) and peptides were analyzed by SwissProt_57.15.fasta.
Results: Using mass spectrometry to assess the relative abundance of proteins within DRFs we identified 149 proteins, dominated by GTPases, cytoskeletal and secretory proteins. In line with previous reports using western blotting, only 10% of total platelet integrin β3 was found within the DRF, yet because of the abundance of integrin αIIbβ3 on the platelet surface this represents a large number of molecules (approximately 8000 per cell). Using mass spectrometry to simultaneously assess the relative abundance of proteins within DRFs, we determined that contrary to the current dogma integrin αIIbβ3 was one of the major components in DRFs. Integrin αIIbβ3 and associated proteins accounted for 21%, by exponentially modified protein abundance index (emPAI), of the proteins identified in DRFs. Furthermore, β3 integrin within DRFs bound filamin in resting and stimulated platelets, and in resting platelets was bound to talin, an association that was reduced upon stimulation. These results suggest that integrin αIIbβ3 is functionally active within the DRF.
Summary/Conclusions: Knowledge of cellular location helps place proteins in their functional context, allowing more appropriate interpretation of functional, biochemical and physiological studies. In light of the data presented here the role of DRF resident integrin αIIbβ3 in controlling platelet response during haemostasis and its regulation should be re-evaluated.

PB 1.23-4

The fibronectin binding partner Msb2 of *Candida albicans* impairs the platelet-mediated host attack

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Background: Platelets can bridge innate and adaptive immunity. To attack microorganisms, platelets store microbicidal and immune stimulatory proteins in their α-granules. *C. albicans* can bind fibronectin which has specific binding sites for platelet integrins (α5β1, αvβ3, and αIIbβ3). The ability of *C. albicans* to grow in hyphae is a relevant factor of virulence *in vivo*. The *C. albicans* surface integrity during filamentation is sensed by Msb2, a transmembrane protein. Recently, it has been shown that Msb2 can be shedded.

Aims: We examined whether or not shedded Msb2 binds to fibronectin, providing a putative mechanism by which *C. albicans* can escape from opsonisation by fibronectin and subsequent platelet attack.

Material and Methods: To induce germination, *C. albicans* exponentially growing in complete medium at 30 °C and starved for 1 h, was diluted in PBS containing 10% FCS and incubated at 37 °C. Msb2 heme agglutinin-tagged purified by affinity chromatography was kindly provided by J. Ernst (PLoS Patho. 2012;8:e1002501). Plates (24 wells) coated either with purified Msb2 (10 μg/mL) or unlabeled fibronectin (10 μg/mL) were incubated with various concentrations fibronectin-alexa fluor 488 for 60 or 120 min. This fluorescent fibronectin was also used to study binding to *C. albicans* or platelets. The readout was performed by flow cytometry, confocal laser scanning microscopy (LSM, Zeiss) and fluorometry (Thermo Scientific) to quantify fluorescent fibronectin.

Results: Binding of 5, 10, 50, and 100 μg/mL fibronectin to 10 μg/mL immobilized Msb2 revealed saturation at 10 μg/mL fibronectin. Immobilized Msb2 bound 2.6-fold more fluorescent fibronectin than BSA ($P = 0.016$). Immobilized fibronectin bound 30% more fluorescent fibronectin than Msb2 and significantly more than BSA ($P < 0.01$). *C. albicans* germination over time at 30, 60, and 120 min generated 5–10%, 40%, and 90% hyphae, as determined by microscopy and confirmed by flow cytometry. Induction of *C. albicans* hyphae enhanced binding of fibronectin (40 μg/mL) from 1.7% positive yeast cells (baseline) to 6% at 30 min, 11.7% at 60 min, and 12.6% at 120 min ($P < 0.05$). As shown by LSM, *C. albicans* germ tubes were a target for fluorescent fibronectin. Germination of *C. albicans* yeast increased binding of platelets up to 5-fold after 30 min and 20-fold after 60 min ($P < 0.05$). Moreover, pretreatment of *C. albicans* hyphae with fibronectin (40 μg/mL) enhanced binding of platelets mediated by interaction of αIIbβ3 with fibronectin. Abciximab (2 μg/mL), a specific αIIbβ3 antagonist, blocked binding of platelets to *C. albicans* opsonized by fibronectin. Binding of fluorescent fibronectin was not homogeneously distributed on the cell surface of *C. albicans*. Upon addition of soluble Msb2, binding of fluorescent fibronectin decreased.

Conclusion: *C. albicans* hyphae, a relevant virulence factor, are attacked by platelets. Fibronectin, a ligand of the platelet integrins, opsonizes the hyphae of *C. albicans* and enhances platelet binding to *C. albicans*. Hence, platelets and fibronectin, with its intrinsic binding sites to integrins (α5β1, αIIbβ3, αvβ3) can support the platelet-mediated host defense against *C. albicans*. Msb2 is a novel fibronectin binding protein. Binding of fibronectin to *C. albicans* is impaired in the presence of soluble Msb2. Hence, shedding of Msb2 *in vivo* can protect *C. albicans* against the platelet-mediated host attack.

PB 1.23-5

Involvement of protein disulfide isomerase (PDI) in beta3 integrin-mediated adhesion of nucleated cells to immobilized fibrinogen

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Background: Extracellular protein disulfide isomerase (PDI) was previously shown to play a role in integrin-dependent platelet adhesion, aggregation and thrombus formation. PDI is secreted from platelets and endothelial cells, and its accumulation at the vascular injury site depends on the presence of β3 integrins. In addition to thiol isomerase and reductase activity, PDI has chaperone-like effect on tissue factor-dependent coagulation.

Aims: In this study we investigated the role of disulfide exchange-activity of PDI in β3 integrins-mediated adhesion of nucleated cells to immobilized fibrinogen.

Methods: We co-expressed in baby hamster kidney (BHK) cells αIIbβ3 and/or αvβ3 integrins together with WT-PDI or the inactive mutant in which the active-sites cysteines were replaced by alanine and serine respectively (ASAS-PDI) and tested the adhesion of these cells to immobilized fibrinogen. In addition, we examined the effect of exogenous PDI and of bacitracin, a membrane impermeable PDI inhibitor, on the adhesion.

Results: The presence of β3 integrins was necessary for BHK cell adhesion to fibrinogen, threefold increase was found in cells expressing integrin αIIbβ3 compare to αvβ3 alone. Bacitracin decreased the adhesion to 33% and to 54%, respectively. Over expression of WT-PDI enhanced the adhesion of BHK cells expressing αvβ3 to 322% while mutant ASAS-PDI enhanced the adhesion to 233%. In contrast, over expression of mutant ASAS-PDI had no effect on adhesion to fibrinogen of BHK cells expressing αIIbβ3and over expression of WT-PDI increased the adhesion only to 120% which was not statistically significant. Interestingly, simultaneously expressing of endogenous PDI and integrin αIIbβ3 resulted in 30%-50% decrease in integrin surface expression, suggesting that cell adhesion dependent on amount of both, PDI and integrin surface expressed. Bacitracin inhibited adhesion to fibrinogen of BHK cells expressing either WT-PDI or mutant ASAS-PDI together with both integrins αvβ3 and αIIbβ3. In the presence of exogenously added PDI, both cell types showed enhanced adhesion that was totally inhibited by bacitracin.

Conclusions: Adhesion of cells expressing integrin αIIbβ3 and/or αvβ3 to fibrinogen depends on surface-expressed cellular PDI. Over-expression of WT-PDI or increased concentration of exogenous WT-PDI increased adhesion, suggesting that surface-expressed PDI is a limiting factor in integrin-mediated adhesion. ASAS-PDI mutant (lacking oxidoreductase activity but retaining its activity as a chaperone), also increased adhesion mediated by αvβ3, suggesting that chaperone activity contributes to cell adhesion.

PB 1.23-6

Surface expressions of platelet glycoprotein Iba, GP?b/?a, and P-selectin are elevated in lung cancer patients

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Background: Thromboembolic events are common symptoms in patients with cancer, and the mechanisms are not totally understood. The platelet adhesion moleculars play important roles in the process of thrombus formation.

Aims: The aim of the current study is to investigate the surface expression of platelet glycoprotein (GP) Ibα, GPIIb/IIIa, and P-selectin in patients with lung cancer.

Method: Platelets were isolated from patients with lung cancer and normal volunteers, and then the surface expressions of platelet GP

Ibα, GPIIb/IIIa, and P-selectin were measured by Flow cytometry. The glycocalicin (GC) fragments in the peripheral blood of patients with lung cancer were tested by Western blot. The surface expressionof GP Ibα on normal volunteers' platelets incubated with the platelet-poor plasma (PPP) of patients with lung cancer was measured by Flow cytometry and Western blot.

Results: The surface expressions of platelet GP Ibα, GPIIb/IIIa, and P-selectin were obviously increased in patients with lung cancer compared with that of normal volunteers, and the GC fragments in PPP were also increased in patients with lung cancer. Incubation of control platelets with the PPP from cancer patients resulted in the elevationof the surface expression of GP Ibα in control platelets. Furthermore, the expressions of GP Ibα, GPIIb/IIIa, and P-selectin on platelets from patients with advanced stage of lung cancer (III–IV stage) were significantly higher than those with early stage (I-II stage) ($P < 0.05$). However, there were not significantly difference in the surface expressions of GP Ibα, GPIIb/IIIa, and P-selectin among the pathological types of lung cancer (squamous cellcarcinoma, adenocarcinoma, small cell lung carcinoma, large cell lung carcinoma) ($P > 0.05$).

Conclusion: The surface expressions of platelet GP Ibα, GPIIb/IIIa, and P-selectin were obviously elevated in patients with lung cancer, and the plasma from cancer patients is responsible for the elevations. These findings provide a new view to understand the pathogenic mechanisms of thromboembolic events in the patients with lungcancer.

PB1.24 – Platelet Apoptosis

PB 1.24-1

Arsenic trioxide induces platelet apoptosis

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Background: Arsenic trioxide, a component of Traditional Chinese Medicine, is known as an effective anticancer druge specially in the treatment of acute promyelocytic leukaemia (APL). APL has emerged as the most curable subtype of acute myeloid leukaemia since the widely use of arsenic trioxide-based chemotherapy. However, recent researches show that thrombocytopenia occurred in 79% of the relapsed or refractory APL patients treated with arsenic trioxide, and part of the APL patients had to be stopped treatment because of catastrophic bleeding, such asintracranial and pulmonary haemorrhage. Thrombocytopenia also occurred in 43% of the myelodysplastic syndromepatients treated with arsenic trioxide. Recently, arsenic trioxide has been proved to have a pro-apoptotic effect on various kinds of nucleated tumour cells or non-tumour cells. The effect of arsenic trioxide on enucleated platelet, however, still remains unclear.

Aims: The aim of current study is to investigate whether arsenic trioxide induces platelet apoptosis.

Methods: Washed platelets (3×10^8/mL) were incubated with different concentrations of arsenic trioxide or vehicleat 37 °C for 4 h. Then, mitochondrial inner transmembrane potential ($\Delta\Psi m$) and phosphatidylserine (PS) exposure were tested by flow cytometry. In the mean time, the treated platelets were analyzed by western blot for the expression levels of pro-apoptotic protein (Bax), and anti-apoptotic proteins (Bcl-2 and Bcl-XL). Activation of caspase-3 was also examined by western blot using an anti-caspase-3 antibody.

Results: $\Delta\Psi$ depolarization and PS exposure were dose-dependently induced in platelets incubated with different concentrations (2, 4, 8, 16 μM) of arsenic trioxide as detected by flow cytometry, and the lowest concentration of arsenic trioxide incurring $\Delta\Psi m$ depolarization and PS exposure was 4 μM. Simultaneously, arsenic trioxide induced up-regulation of Bax and down-regulation of Bcl-2 and Bcl-XL in a dose-dependent manner. Furthermore, 17 kD cleaved caspase-3 fragments were dose-dependently induced in platelets treated with different concentrations of arsenic trioxide indicating that caspase-3 was activated by arsenic trioxide.

Conclusions: Taken together, the data indicate that arsenic trioxide induces platelet apoptosis *in vitro*, which might suggest a novel pathogenic mechanism of thrombocytopenia in the patients who treated with arsenic trioxide.

PB 1.24-2

Caspase-3 activation regulates platelet lifespan but it is not involved in microparticle (MP) formation and phosphatidylserine (PS) exposure

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Background: Previous reports have indicated the possibility that activation of a key effector molecule of cellular apoptosis, caspase-3 (Casp-3), is involved in MP formation and PS exposure of platelets (Plts). Although this hypothesis is based on the morphological similarity observed during cellular apoptosis and Plt activation, much is not known whether Casp-3 activation is involved in MP formation and PS exposure. 2.

Aims: In the present study, by using *vav-bcl-2* transgenic (Tg) mice, whose Casp-3 is hardly activated by apoptotic stimulation, we investigated whether Casp-3 activation regulates Plt lifespan and is involved in MP formation and PS exposure. 3.

Methods: After stimulation of washed Plts prepared from WT, *vav-bcl-2* Tg mice, and healthy volunteers, MP formation and PS exposure were assayed by flow cytometry using annexin-conjugated FITC. Casp-3 activation in Plts was analyzed by western blotting using an anti-Casp-3 polyclonal antibody. Plt half-life was measured by tracing biotin-labeled Plts using flow cytometry after intravenous injection of sulfo-NHS-LC-Biotin into mice. 4.

Results: Pretreatment of healthy human Plts with a broad Casp inhibitor, z-VAD-fmk, affected neither MP formation nor PS exposure induced by A23187. Furthermore, there was no difference in MP formation and PS exposure between WT and Tg Plts stimulated with various concentrations of A23187. Combined with the finding that activation of Casp-3 was not detected in A23187-stimulated healthy Plts by western blotting, we concluded that Casp-3 activation is not involved in MP formation and PS exposure. In contrast, Plt half-life in vav-bcl-2 Tg mice was significantly longer than that in WT mice (68 vs. 56 h). In accordance with that, Casp-3 activation was detected by western blotting in healthy human platelets incubated at 37 °C for 1–3 days, and this activation was inhibited by adding z-VAD-fmk. 5.

Summary/Conclusion: These data collectively suggest that Casp-3 activation regulates Plt lifespan but it is not involved in MP formation and PS exposure.

PB 1.24-3

Inner mitochondrial membrane disruption is closely associated with both Bax/Bak and cyclophilin D-mediated platelet phosphatidylserine exposure

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Background: Two primary intracellular pathways regulate platelet phosphatidylserine (PS) exposure. In response to strong agonists, a sustained increase of cytoplasmic calcium levels initiates cyclophilin D (CypD)-regulated mitochondrial permeability transition pore formation and subsequent PS exposure. Treatment of platelets with Bcl-xL inhibitors initiates Bax/Bak dependent caspase activation and PS exposure. The role of mitochondrial events in regulating these platelet

pathways has primarily been investigated through evaluation of mitochondrial transmembrane potential (deltapsi(m)) using fluorescent lipophilic cationic dyes, such as TMRM or JC-1. A limitation of these studies is their inability to distinguish whether deltapsi(m) loss occurred due to disruption of the electrical gradient, such as occurs in response to protonophore treatment or respiratory chain inhibition, or through inner mitochondrial membrane (IMM) disruption, or pore formation.

Aims: Here we utilize novel assays to closely examine and compare the sequence of mitochondrial events regulating platelet PS exposure in response to physiologic agonists and the Bcl-inhibitor ABT-737.

Methods: To evaluate mitochondrial outer membrane permeabilization (MOMP), platelets were incubated with 0.01% digitonin for one minute for plasma membrane permeabilization. Cytochrome C retention was evaluated by cytochrome c immunofluorescence.

To directly assess IMM disruption, a calcein-cobalt fluorescence assay was utilized. Platelets were preincubatedwith calcein-AM and $CoCl_2$, and calcein fluorescence was assessed following agonist(s) stimulation. IMM disruption allows mitochondrial entry of cobalt, which quenches calcein fluorescence.

To evaluate mitochondrial calcium influx, or ROS generation, washed platelets were preincubatedwith 5 mM Rhod-2 or 5 mM H_2-DCFDA, respectively, and stimulated with 1 mM ABT-737.

Results: In both ABT-737 treated and physiologic agonist (thrombin and convulxin (T/C) stimulated platelets IMM disruption, not MOMP, was temporally associated with PS exposure. The sequence of events responsible for PS exposure was more closely examined in ABT-737-treated platelets. ABT-737 treatment caused rapid MOMP, occurring within 5 min. IMM disruption and PS exposure were closely associated and occurred approximately 30 min after ABT-737 treatment. In Bax/Bak double deficient platelets, mitochondrial inner and outer membrane disruption and high-level PS exposure were all markedly blunted. Caspase inhibition had no effect on MOMP, but both IMM disruption and PS exposure were markedly blunted. CypD's absence had minimal effects on ABT-737 initiated events. As in ABT-737-treated platelets, in T/C stimulated platelets IMM disruption, not MOMP, was closely correlated with PS exposure. Influx of mitochondrial calcium ($Ca^{2+}{}_{mit}$) and ROS, key regulators of mPTP formation, were examined in ABT-737-stimulated platelets. As in T/C-stimulated platelets $Ca^{2+}{}_{mit}$ increased significantly in ABT-737-stimulated platelets prior ro PS exposure. However, in contrast to T/C stimulated platelets, abrogation of $Ca^{2+}{}_{mit}$ entry neither decreased IMM disruption nor PS exposure.

Conclusions: Platelet PS exposure in response to both Bax/Bak and CypD-mediated PS exposure is temporally associated with IMM disruption. CypD and caspase-regulated events mediate IMM and PS exposure in response to T/C and ABT-737, respectively. Increased $Ca^{2+}{}_{mit}$ occurs in ABT-737 stimulated platelets, but is not required for PS exposure.

PB 1.24-4

Platelet apoptosis in uremic patients

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Background: Apoptosis, which serves for the regulation of cell life span and is long attributed exclusively to nucleated cells, has been well-documented in anucleate platelets. Diverse cell-external chemical and physical stimuli have been reported to induce transformation of resting platelets to apoptotic state. Uremia, a clinical syndromeassociated with retention of various solutes that would normally be excreted by the kidneys, is frequentlyaccompanied by bleeding tendency, and the mechanism remains unclear.

Aims: The aim of the present study is to investigate whether platelet apoptosis occurs in uremia patients.

Methods: Venous blood was drawed from 13 patients with end stage renal disease (ESRD) who exhibited uremia syndrome and 13 health controls. Platelet-rich plasma (PRP) was prepared and then was detected for apoptotic events including depolarization of mitochondrial inner membrane potential ($\Delta\Psi$m), phosphatidylserine (PS) exposure, variations of apoptotic Bcl-2 family proteins, activation of caspases-3 by Flow cytometry or Western-blot. Furthermore, normal washed platelets were incubated with the poor- platelet plasma (PPP) from uremic patients, and then were detected for apoptotic events. The comparisons of the data were made using paired Student's t-test.

Results: Compared with controls, $\Delta\Psi$m significantly depolarizated in platelets from uremic patients ($P < 0.05$). Furthermore, Bax was up-regulated, Bcl-2 and Bcl-X_L were down-regulated, and caspase-3 was activated inplatelets from uremic patients. However, there was not significant difference in PS exposure and P-selectin expression between the platelets from uremic patients and health contols ($P > 0.05$). Next, PPP from uremic patients was incubated with normal platelets, and the platelets presented $\Delta\Psi$m depolarization, up-regulation of Bax, down-regulation of Bcl-2 and Bcl-X_L, and caspase-3 activation.

Conclusion: The data demonstrate that platelets are incurred apoptosis in uremia patients. The finding might suggest a novel pathogenic mechanism for bleeding tendency in uremic patients.

PB 1.24-5

The effects and mechanisms of cyanidin-3-glucoside on platelet apoptosis

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Background: The platelet activation, aggregation and apoptosis are involved in the atherosclerotic development. It has been evidenced that the process of the platelet apoptosis is different from that of the activation and aggregation. Our previous studies have found that anthocyanins significantly inhibited platelet activation, adhesion and aggregation and induced platelet apoptosis both *in vitro* and *in vivo*. However, the effects and mechanisms of anthocyanins on platelet apoptosis were not fully understood.

Aims: In this study we aimed to explore the effects of anthocyanin cyanindin-3-glucoside (Cy-3-g) on platelet apoptosis and BCL-2/BCL-X_L mediated intrinsic mitochondrial apoptosis signaling pathway.

Methods: Cyanidin-3-glucoside (Cy-3-g), the predominantly bioactive compound of anthocyanin, was bought from Polyphenol AS Company inNorway. Purified gel-filtered platelets from healthy volunteers were incubated at 37 °C for 40 min with different concentrations (0.5, 5 and 50 mM) of Cy-3-g or PBS buffer as a control. The mitochondria membrane potential ($\Delta\psi$m) was detected with the dye JC-1, and the membrane phospholipid phosphatidylserine (PS) exposure was assessed with Annexin V-FITC by flow cytometry. The expression of anti-apoptotic protein BCL-2 and BCL-XL and pro-apoptotic proteins such as BAK, BID, BAX as well as gelsolin, caspase-3,8,9 were determined by Western Blotting.

Results: In purified gel-filtered platelets, Cy-3-g concentration-dependently stimulated dissipation of the mitochondrial membrane potential ($\Delta\psi$m), 50 μM Cy-3-g obviously stimulated dissipation of the mitochondrial membrane potential ($P < 0.005$). While 0.5 μM Cy-3-g and 5 μM Cy-3-g showed no significant differences. Five micromolar Cy-3-g significantly induced PS exposure ($P < 0.01$), 5 and 50 μM Cy-3-g significantly inhibited the expression of BCL-2 and BCL-X_L respectively ($P < 0.05$), while 5 μM Cy-3-g increased the expression of pro-apoptotic proteins such as BAX, BAK and BID ($P < 0.05$). The level of gelsolin was reduced by Cy-3-g in a dose dependent manner (0.5 μM vs. control, $P < 0.05$, 5, 50 μM vs. control, $P < 0.01$). The results showed that Cy-3-g markedly induced the caspase-3, 8, 9 activation in a dose dependent manner but this tendency need to be further confirmed.

Conclusions: Our primary data demonstrated for the first time that purified anthocyanin (Cy-3-g) induced platelet apoptosis through

BCL-2/BCL-XL mediated endogenous mitochondrial apoptosis signaling pathway.

PB1.25 – Platelet Disorders: Gain-of-Function

PB 1.25-1

Platelet apoptosis and agonist-mediated activation in myelodysplastic syndromes

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Background: Myelodysplastic syndromes (MDS) patients have a defect in the differentiation of bone marrow multipotent progenitor cells. Even when thrombocytopaenia is frequently observed in MDS patients, some of them may present normal or high platelet count. Thrombocytopaenia in MDS patients may be due to premature megakaryocyte death, but platelet apoptotic mechanisms may also occur.
Aims: This study aimed to study function and apoptotic state of platelets from MDS patients with different platelet count in order to elucidate whether they reflect anomalies corresponding to a stem cell disorder.
Methods: We recruited 75 patients with MDS, divided into three groups according to IPSS score: 39 with low, 27 with intermediate-1 and 9 with high risk, composed of intermediate-2 plus high risk patients. Sixty-eight healthy controls were included in the study. Reticulated platelets, platelet activation (measured through PAC1 binding and P-selectin surface expression after PAR-1 receptor stimulation with thrombin receptor-activating peptide 6, TRAP), activated caspases and annexin-V binding were evaluated by flow cytometry. Pro-apoptotic Bax and Bak proteins were determined by western blots, and plasma thrombopoietin by ELISA.
Results: High plasma thrombopoietin levels and low immature circulating platelet count showed a pattern of hypoplastic thrombocytopaenia. Regardless of platelet count, platelets from MDS patients bound less PAC1 and expressed reduced levels of P-selectin in comparison to controls after stimulation with TRAP. A significant correlation was observed between activation markers expressed on the surface of circulating platelets after stimulation with TRAP and platelet count (Spearman test $r = 0.354$, $P < 0.05$ for PAC1 binding and $r = 0.446$, $P < 0.0001$ for P-selectin exposure).
Platelets from all MDS patients, even those with a normal platelet count, exposed more phosphatidylserine (PS) than controls. To determine if PS exposure was related to apoptosis, levels of activated caspases-3, 7, -8 and -9 were evaluated in a group of MDS patients with normal platelet count. A significantly higher proportion of platelets from MDS patients contained activated caspases -3, 7, -8 and -9. Moreover, platelets from MDS patients showed increased Bax and Bak levels; Bax with a strong positive correlation with the AnnexinV binding values (Spearman test $r = 0.740$, $P < 0.0005$). On the other hand, AnnexinV binding and Bax expression showed an inverse correlation with platelet count (Pearson test $r = -0.255$ $P < 0.05$ and Spearman test $r = -0.712$, $P < 0.005$, respectively). Impairment in platelet function significantly correlated with platelet surface exposure of PS (Spearman test $r = -0.558$. $P < 0.001$).
Conclusion: Our results showed that platelet function was undermined in patients with MDS and that this impairment seemed to be related to increased apoptosis. Moreover, platelet dysfunction and apoptosis is more pronounced in patients with thrombocytopaenia, appearing to reflect that MDS is a stem cell disorder, and so both, number and function of progeny cells are affected.

PB 1.25-2

H2 haplotype of ADP-P2Y12 receptor gene is associated with increased platelet response to ADP in Taiwanese population

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Background: In Caucasians, carriers of the P2Y12 receptor gene H2 haplotype exhibited increased platelet reactivity, higher risk of arteriothrombosis, and lesser clinical response to antithrombotic agents, suggesting that patients with the H2 haplotype need intensive antithrombotic remedies. This useful information remains lacking in Taiwan.
Aims: To establish genetic markers that are associated with increased platelet reactivity in Taiwanese population, we examined the variability of ADP-induced platelet aggregation among healthy subjects and its relation to the P2Y12 receptor gene polymorphism.
Methods: Citrated platelet-rich plasma (2×10^8/mL) was prepared and subjected to light transmittance aggregometry with various doses of ADP (2.5–30 μM). The H1 and H2 gene haplotypes of P2Y12 receptor were determined by DNA sequencing of the corresponding PCR products. Statistic analysis was performed with Two-tailed Mann–Whitney U test. This study was approved by the Ethical Committee of the Tzu Chi Buddhist General Hospital and all the healthy blood donors, including 47 females and 38 males, aged between 20 and 50 years old, gave their written informed consent.
Results: The response of platelets to ADP (5 μM) varied markedly among individuals ($n = 85$) and the variation was shown associated with the gene polymorphism of P2Y12 receptor, carriers with the P2Y12 gene H2/H2 haplotypes ($n = 19$) exhibited higher platelet aggregation response to ADP as compared with the H1/H1 haplotypes. The individual variability of ADP-induced platelet aggregation become insignificant as the platelets had been pretreated with aspirin, an irreversible cyclooxygenase inhibitor.
Conclusion: There is a marked difference in ADP-induced platelet aggregation among Taiwanese healthy subjects and this variation might be attributed to the distinct level of thromboxane A2 formation in platelets following ADP activation. The H2 haplotype of P2Y12 receptor gene associated with enhanced platelet reactivity might be a useful genetic marker in distinguishing between patients who need more intensive antithrombotic treatment in Taiwanese population.

PB 1.25-3

Purified dietary anthocyanin inhibited platelet secretion in hypercholesterolemia

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Background: Platelet secretion has been established to be a safe and effective target to prevent atherosclerosis and thrombosis formation. Hypercholesterolemia is one of the major risk factors for CVDs, in which platelet secretion is increased and enhance the formation of thrombosis and atherosclerotic lesions. Anthocyanins are major phytochemicals abundant in plant food and have been shown to play a protective role against CVDs. Our previous studies showed that anthocyanin could inhibit platelet activation and attenuate thrombosis formation, however, the effects of anthocyanin on platelet secretion in hypercholesterolemia are still unclear.
Aims: To explore the effects and mechanisms of anthocyanin on platelet secretion in hypercholesterolemia patients *in vivo* and *in vitro* respectively.
Methods: *In vivo* study, 150 subjects with hypercholesterolemia consumed purified anthocyanin mixture (320 mg/day) or a placebo twice a day for 24 weeks in a randomized, double-blind trial. To estimate the platelet secretion levels, plasma levels of platelet factor-4(PF4), soluble

platelet selectin (sP-selectin), soluble CD40 ligand (sCD40L), regulated upon activation normal T cell expressed and secreted (RANTEs), transforming growth factorβ1 (TGF-β1), β-thromboglobulin (β-TG) were detected by ELISA, all of which are mainly derived from platelet granules. To confirm the effects, platelets from both healthy volunteers and hypercholesterolemia patients were then separated and purified and then incubated with purified anthocyanin mixture as well as the main two compounds of the mixture cyaniding-3-glucosides (Cy-3g) and delphinidin-3-glucoside (Dp-3-g) respectively *in vitro*. Platelet P-selectin and CD63 secretion induced by thrombin were detected by flow cytomety and the ATP release were measured in platelet Lumi-aggregometer. RANTES and serotonin contents were evaluated by ELISA.

Results: After intervention with purified anthocyanin mixture for 24 weeks in hypercholesterolemia patients, we observed significant reduction in the plasma levels of sP-selectin, sCD40L, RANTEs and β-TG as compared with the control group. PF4 and TGF-β1 levels were also reduced obviously though they didn't reach statistical significance. *In vitro* study in healthy volunteers, we found that purified anthocyanin mixture, Dp-3-g and Cy-3-g significantly inhibited platelet P-selectin and RANTES secretion, both of which stands for the α-granule secretion. The dense granule secretion levels evaluated by CD63, serotonin and ATP were also significantly reduced by purified anthocyanin mixture, Dp-3-g and Cy-3-g. Study in platelets separated from hypercholesterolemia patients showed similar inhibitory effects of these three compunds on platelet granule secretion though the effects were slightly weaker than that in healthy volunteers' platelets. We further found that Cy-3-g attenuated thrombin-induced P38 MAPK and ERK1/2 phosphorylation in platelet in a dose dependent manner.

Conclusions: Our data clearly demonstrated for the first time that purified anthocyanin markedly inhibited platelet secretion in both hypercholesterolemia patients and healthy volunteers, the P38 MAPK and ERK1/2 signaling pathway were possibly one of the mechanisms of anthocyanin.

PB 1.25-4

Polymorphism of human platelets antigens in Tunisian patients with acute ischemic stroke

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Background: The role of genetic risk factors in ischemic stroke is unclear. The polymorphism of human platelets antigens (HPA) has been implicated in the pathogenesis of arterial thrombotic diseases mainly the myocardial infarction.

In the context of the Identification of stroke susceptibility genes, we investigated the gene frequencies of five major human platelets antigens (HPA 1–5) in Tunisian population and to assess if polymorphism of HPA is associated with acute ischemic stroke (AIS) in Tunisian.

Patients and Methods: DNA was isolated from peripheral blood collected from 71 consecutive stroke patients and 100 healthy blood donors.

HPA 1–5 genotyping was performed by the polymerase chain reaction method using sequence-specific primers (PCR-SSP).

Gene frequency distribution was tested by Hardy-Weinberg equilibrium. Comparisons of HPA gene frequencies between the patient and control groups were made by chi-square test. Haplotype associations were tested by SNPStat software.

Results: The allelic frequencies of HPA-1, 2, 3, and 4 among patients and controls did not reveal significant differences. However, the HPA-5a allele was significantly more frequent in patients than in controls ($P < 0.002$).

The genotype distribution of HPA-4 polymorphism reveals that the HPA-4a4b genotype was more frequent in patients (67/71: 94.36%) than in controls ($P < 0.001$). The frequency of HPA-1b1b and HPA-3a3a were higher in control subjects than patients ($P = 0.009$ and $P = 0.01$ respectively).

The haplotype distribution of HPA-1, HPA-3 and HPA-4 (located on the same glycoprotein complex GPIIb/IIIa) showed that aab and abb haplotypes were statistically more frequent in patients than in controls ($P < 0.001$ and $P < 0.001$ respectively).

Conclusions: In the literature, the involvement of human platelets antigens in the occurrence of an ischemic stroke is conflicting.

In our population, a positive association between HPA-4a4b and stroke is found. The aab and abb haplotypes of HPA-1, HPA-3 and HPA-4 seem to be also implicated. HPA-1b1b and HPA-3a3a were identified as a protective genotype.

The value of HPA polymorphisms as useful markers for potential risk of ischemic stroke deserves to be confirmed on larger studies.

PB 1.25-5

Modeling and molecular dynamics simulations: structural comparison of the V33 variant of the integrin subunit β3 with its L33 (HPA-1a) and P33 (HPA-1b) forms

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The human platelet antigen (HPA)-1 system carried by the αIIbβ3 integrin is characterized by a leucine (allele 1a)-to-proline (allele 1b) substitution in position 33 of the mature β3 subunit. This alloantigenic system is the first cause of alloimmune thrombocytopenia in Caucasian populations and the allele HPA-1b may be a risk factor for thrombosis. The HPA-1 system presents a third variant defined by a valine in position 33, a variant not described as immunogenic (Santoso et al, Transfusion 46:790–799, 2006). Sera containing alloantibodies to the HPA-1a antigen react variably with the V33 form of β3. This suggests structural alterations of the V33 β3 although leucine and valine have similar physical and chemical properties; they differ only by the size of their side chain as valine lacks a -CH2- radical. In this work, we studied the structural modifications related to the L33V substitution, and compared the structures of the three β3 variants.

A 3D structural model of the V33 β3 extracellular domain was obtained by *in silico* mutagenesis of a L33 β3 model (Jallu, Poulain et al, PLoS One. 2012;7(11):e47304,). Molecular dynamics simulations showed that the high flexibility of the PSI, I-EGF-1, and I-EGF-2 domains is conserved. L33 and V33 β3 structural models share many characteristics although V33 β3 adopt specific features. We use a series of 16 small protein prototypes named Protein Blocks (PBs) to analyze precisely the local protein conformation (de Brevern et al, Proteins; 41:271–287, 2000). PBs analysis revealed in particular that the L33V substitution strongly displaces structural equilibriums toward a loop tightly maintained by hydrogen bonds for residues 27–31 of the PSI domain, and toward a β-turn with a start of a helical motif for residues 435–338 of the I-EGF-1 domain (more than 90% of occurrences in both cases). In L33 and P33 β3, these structures are in dynamical equilibriums with extended conformations (more than 40% of occurrences).

Despite common features between leucine and valine amino acids, our study showed that the L33V substitution effectively affects the dynamic equilibrium of the structural model of β3. Nonetheless, structural similarities between L33 and V33 β3 models underlie the variable reactivity of anti-HPA-1a alloantibodies with V33 β3. Unlike the L33P substitution, the L33V change is not expected to significantly affect the flexibility, if at all, of the PSI, I-EGF-1, and I-EGF-2 domains composing the β3 knee, suggesting that the functions of αIIbβ3 are not altered.

Characterization of HPA-1a epitopes is challenging, yet important regarding the high clinical impact of this platelet alloimmune system. Studies on HPA-1a epitopes are still needed to develop new techniques or applications and to understand underlying mechanisms.

PB 1.25-6

Diagnostic laboratory validation of platelet transmission electron microscopy

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Background: Among various hereditary qualitative platelet disorders, storage-pool deficiencies (SPD), such as dense granule (DG) deficiency (including Hermansky-Pudlak syndrome), are relatively common. Platelet whole mount (WM) and thin section (TS) transmission electron microscopic studies (TEM) are the gold standard Methods for diagnosing DG deficiency as well as other ultra-structural abnormalities. Nevertheless, platelet TEM tests are still largely research tools and have not been thoroughly validated and standardized as clinical tests according to current diagnostic laboratory quality standards and requirements. Furthermore, normal reference ranges of mean platelet DG counts/platelet (DGC) have not been adequately established.

Aim: Our goals were to validate platelet WM and TS TEM tests, and establish the normal reference ranges of platelet DGC.

Methods: Based on previously established methods (Blood, 33:598–606), whole blood samples collected in ACD-(A or B) tubes from healthy donors were employed for this study. We first optimized and standardized various pre-analytical, analytical and post-analytical procedures including sample matrix, sample stability, platelet-rich plasma (PRP) preparation, coated-grid options, platelet WM protocol, automated sample processing for TS, and lastly establishment of DG counting criteria. We started a large normal range (confident intervals, CI) study of the DGC, and have recruited 32 healthy donors to date. Statistical analyses by JMP 10 program were employed in this study.

Results: Of the two ACD tubes, ACD-B anticoagulated whole blood samples gave a more stable DGC and better TS ultra-structure quality than those of ACD-A samples. Both mean DGC and platelet ultra-structure were stable when ACD-B whole blood samples were stored at room temperature (RT) for up to 4 days. The optimal centrifugation setting for preparing PRP is 200 g for 20 min at RT. Interestingly, after centrifugation, platelets that were devoid of any DG were enriched in the upper 1/3 proportion of the PRP. The lower 2/3 proportion of PRP thus gave a more consistent DGC. Among various commercial grids, formvar-carbon coated 200 mesh grids (Electron Microscopy Science) gave the most reliable platelet adhesion and DGC. Platelet TS sample processing was automated by using an agar embedding procedure on a Leica processor. The DG counting criteria were developed based on previous publications and guidance from Dr. James G. White. Using the same WM TEM images and DG counting criteria, agreements among different technologists ($n = 5$) improved from 60% to 95%. Finally the on-going DGC normal range study (nine male and 23 female; age range 22–72 years; scoring 100–200 platelets from each donor) showed that the DGC distribution of each sample was positively skewed (skewness = 0.7–1.5) with on average 17% of platelets (95% CIs: 5–32) being empty. The mean DGC was 2.6 with 95% CIs at 1.6 and 4.0. Neither gender nor age was associated with the mean DGCs.

Conclusion: We investigated and validated platelet WM and TS TEM procedures. Our on-going normal range study of the DGC demonstrated a distinct distribution pattern. We also noticed that the DGC was procedure-dependent, further underscoring the importance of platelet TEM procedure standardization.

PB1.26 – Platelet Function in Disease

PB 1.26-1

Enhanced lipid peroxidation and platelet activation as potential contributors to increased cardiovascular risk in the low-HDL phenotype

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Background: Low HDL levels are major predictors of CV events, even in patients on statin treatment with LDL at target. In animal models HDLs protect LDL from oxidation and blunt platelet activation. Our study is aimed at examining whether HDL levels are related to *in vivo* oxidative stress and platelet activation, as determinants of atherothrombosis.

Methods and Results: Urinary 8-iso-$PGF_{2\alpha}$ and 11-dehydro-TXB_2, *in vivo* markers of oxidative stress and platelet activation, respectively, were measured in 65 CHD normocholesterolemic patients with HDL $\leq$ 35 mg/dL, and in 47 CHD patients with HDL > 35 mg/dL. The two eicosanoids were also measured before and after fenofibrate treatment in otherwise healthy subjects with low HDL ($n = 10$).

Patients with HDL $\leq$ 35 mg/dL showed significantly higher urinary 8-iso-$PGF_{2\alpha}$ [median (25th-75th percentile): 289 (189–380) vs. 216 (171–321) pg/mg creatinine, $P = 0.019$] and 11-dehydro-TXB_2 [563 (421–767) vs. 372 (249–465) pg/mg creatinine, $P = 0.0001$] than patients with higher HDL. A direct correlation was found between urinary 8-iso-$PGF_{2\alpha}$ and 11-dehydro-TXB_2 in the entire group of patients (Rho = 0.77, $P < 0.0001$). HDL levels were inversely related to both 8-iso-$PGF_{2\alpha}$ ($\rho = -0.32$, $P = 0.001$) and 11-dehydro-TXB_2 ($\rho = -0.52$, $P < 0.0001$). On multiple regression, only 8-iso-$PGF_{2\alpha}$ ($\beta = 0.68$, $P < 0.0001$) and HDL levels ($\beta = -0.29$, $P < 0.0001$) were associated to urinary 11-dehydro-TXB_2 excretion, independently of gender, age, smoking, hypertension, diabetes, previous myocardial infarction, total cholesterol, LDL, and triglycerides. Fenofibrate treatment significantly reduced the two eicosanoids in healthy subjects, in parallel with an HDL increase.

Conclusions: A low HDL phenotype, both in CHD patients and in healthy subjects, is associated with increased lipid peroxidation and platelet activation. These data provide novel insight into the mechanisms linking low HDL to increased CV risk.

PB 1.26-2

Platelet activation and thrombin-generation in paediatric patients with acute ITP, chronic ITP and chemotherapy-induced thrombocytopenia

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Background: Immune thrombocytopenic purpura (ITP) is a common hematologic disorder in children that can lead to severe bleeding symptoms in rare occasions. Yet no biological markers were identified to predict bleeding severity or the risk for life threatening bleeds. Platelet count helps to identify patients at risk, but does not help to predict severe bleeding events. Therefore we investigated platelet activation and thrombin generation in children with ITP.

Aims: First we aimed to identify differences in markers of platelet activation and thrombin generation (TG) among patients with ITP, thrombocytopenia induced by chemotherapy (cTP) and healthy

children and second, to correlate this to clinical symptoms and bleeding score results.

Methods: In a prospective study we investigated venous blood samples of 20 patients (median age 6.5 years, range) with newly diagnosed acute ITP, before and after treatment with IVIg. In addition we studied 20 chronic ITP patients. Severity of bleeding symptoms was assessed according to a paediatric bleeding score for ITP (Buchanan et al., 2002, *J Pediatr*; 141:683–8). Eleven children with oncologic disease and cTP and 18 healthy children served as controls. Platelets were identified as CD42-positive events by flow cytometry and analysed for exposure of CD62 (P-selectin), CD63 and PAC1 using fluorochrome labelled monoclonal antibodies, to identify activation of platelets before and after thrombin-stimulation. TG was measured by a chromogenic assay.

Results: Median bleeding score at presentation was 2–3 in acute ITP and decreased to 1–2.5 in patients with chronic ITP. All acute ITP patients were treated with max. Three doses of IVIg (0.4–0.8 g/kg/dose) and increased the platelet count above 30,000/mL. Platelets of ITP patients showed a higher expression of CD63 than platelets of other thrombocytopenic patients, even before thrombin stimulation (27.21 ± 5.35%; thrombopenic controls 9.29 ± 1.7%). After thrombin stimulation, expression of all activation markers CD63, CD62 and PAC1 rose significantly. Both, patients with ITP and cTP, showed reduced thrombin-stimulated expression of CD63, CD62 and PAC1 compared to healthy controls. IVIg improved platelet activation after thrombin-stimulation to values seen in healthy controls. TG was reduced in acute-, chronic ITP and in cTP patients. In children with acute ITP, TG normalized after IVIg, even without normalization of platelet counts. The lowest TG was seen in cTP patients, that was even lower than in acute ITP ($P < 0.05$). A higher bleeding-score did not correlate with lower TGA or lower platelet activation. A weak correlation ($r = 0.38$, $P < 0.01$) was found between platelet counts and TG.

Summary: Platelets of ITP patients are partly activated, more than platelets of patients with chemotherapy-induced thrombocytopenia. Hence this activation is not correlated to increased TG. While the lowest TG is seen in cTP, in children with acute ITP, platelet activation and low TG can be increased by IVIg to values similar to healthy children.

PB 1.26-3

A clinical pilot study to compare T2MR and VerifyNow P2Y12 platelet activity measurements in cardiovascular patients

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Background: Platelets play a critical role in well-regulated hemostasis, and the definition of adequate regulation can change with the clinical state of the patient. The challenges of developing clinically relevant and timely surrogates to monitor platelet function are well established. Light transmission aggregometry (LTA) is the only established method that correlates to clinical outcomes, but LTA requires expert technical skills to run, may require a variety of agonist conditions to get a true measure of the state of the patient, and the data can be difficult to interpret. More recent measures of platelet function that rely on highly-specific single activator tests, such as ADP, can monitor the pharmacodynamics of specific platelet receptor inhibitors, but such tests do not predict adverse clinical outcomes related to platelet dysfunction. We will present preliminary clinical results to demonstrate that a new T2MR detection method may correlate with ADP-induced platelet activity and clotting in interventional cardiovascular patients 90% of the time.

Aims: T2 Biosystems has developed a simple, novel method to monitor platelet function using a portable T2 magnetic resonance (T2MR) device and small volume samples. The methodology enables use of all standard platelet activators and inhibitors. Here we used an ADP activator cocktail to make a global T2MR hemostasis function test sensitive to P2Y12 inhibition by measuring platelet activity in the biological context of fibrin clot formation and platelet mediated clot retraction.

Method: In our prospective, non-blinded, single centered pilot study, 30 patients who presented with chest pain and underwent a diagnostic or interventional cardiac catheterization were enrolled over a 6 month period. The VerifyNow P2Y12 test was run at the discretion of the ordering cardiologist, with the T2MR ADP assay run in parallel. Test results were reported as either active or inactive platelet function. The VerifyNow P2Y12 test was interpreted as platelet-inhibited (VN−) for PRU < 213, and platelet-active (VN+) for PRU > 213. The T2MR platelet function test was interpreted as platelet-inhibited (T2MR−) if the second hemostasis peak in the T2 signal profile fell below 5% of the total T2 signal, and platelet-active (T2MR+) if this signal was > 5%.

Results: In a retrospective analysis of T2MR and VerifyNow test data and associated patient clinical records, we found that 75% of cases ($N = 17$ T2MR+/VN+ and $N = 6$ T2MR−/VN−) were in agreement, with no significant adverse hemostatic events noted. Of the $N = 7$ cases in which the test results did not agree, examination of patient clinical records revealed that all ($N = 3$) of the T2MR+/VN− patients showed normal clotting or had no adverse events, although the incidence of rethrombosis is expected to be low. On the other hand, all ($N = 4$) of the T2MR−/VN+ patients were either on prasugrel ($N = 2$) or showed evidence of post-operative bleeding ($N = 2$), consistent with the T2MR signature indicating low clotting potential.

Summary: These preliminary clinical results suggest that the T2MR ADP test has the potential to predict the hemostatic state of ADP-induced platelet activity and clotting in interventional cardiovascular patients.

PB 1.26-4

Markers of activation haemostasis in patients with psychosis influence of an antipsychotic treatment: findings from the ANTRE study

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Background: Patients with schizophrenia have an increased risk of venous thromboembolism (VTE), and this might be associated with the use of antipsychotics.

Aims: The primary goal of the present study was to replicate our previous finding of increased coagulation and thrombocytes activity in drug-naïve psychotic patients in comparison with healthy controls and ascertain whether the blood levels of thrombogenesis markers further increase over the course of a consecutive two-years antipsychotic treatment.

Methods: We investigated the plasma levels of markers indicating activation of coagulation (D-dimers and factor VIII) and platelets (soluble P-selectin, sP-selectin) in an antipsychotic-naive group of 27 men and 18 women with acute psychosis (27.7 ± 8.0 years, range 18–52), and 45 healthy volunteers matched for age and gender. In the patient group, we repeated these assessments after 3 and 12 months and again after 2 years of antipsychotic treatment.

Results: D-dimers (median 0.37 vs. 0.20 mg/L; $P < 0.0001$), factor VIII (median 142% vs. 116%; $P = 0.016$) and sP-selectin (median 155.4 vs. 112.4 ng/mL; $P < 0.0001$) plasma levels were significantly increased in the group of patients with acute psychosis prior to treatment compared with healthy volunteers. The plasma levels of sP-selectin varied significantly ($P = 0.016$) in the course of the 1-year antipsychotic treatment. The plasma levels of D-dimers and factor VIII significantly decreased during 1 year ($P = 0.016$ resp. $P = 0.046$.

Conclusions: We found increased markers of activation of haemostasis in patients with acute psychosis as well as in chronic stage. Our results

could explain possible pathological mechanisms of VTE in schizophrenia patients independently to antipsychotic treatment.

PB 1.26-5

Evaluation of aspirin use on platelet function in essential thrombocythemia and polycythemia vera patients

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Background: Essential Thrombocythemia (ET) and Polycythemia Vera (PV) are two myeloproliferative neoplasms (MPN) characterized by a hypercoagulable state and an increased rate of thrombotic complications. The treatment choice of these diseases is highly dependent on the patient thrombotic risk. Antiplatelet prophylaxis with low dose aspirin is effective in reducing the thrombosis rate in PV patients, while alleviates vasomotor (microvascular) disturbances, and prevents pregnancy-associated complications in patients with ET, particularly those carrying the JAK2V617F mutation. Although low dose aspirin is now largely utilized for thromboprophylaxis in both low and high risk ET and PV patients, however there are subjects on aspirin who show platelet activation and persistent thromboxane biosynthesis.
Aim: To characterize the effect of aspirin on platelet adhesive and procoagulant function in ET and PV patients.
Methods: In this study the PFA-100 assay in whole blood and the thrombin generation (TG) assay in platelet rich plasma (PRP) were performed in a group of 46 ET and 38 PV patients to evaluate the effect of aspirin on platelet adhesive and procoagulant properties.
Results: PFA-100 collagen-epinephrine closure time was significantly ($P < 0.01$) prolonged in patients compared to healthy controls. However, the PFA-100 showed flow obstruction in 20% of patients after collagen-adenosine diphosphate and in 15% of patients after collagen-epinephrine trigger, the majority of them while on aspirin. Higher platelet count and immature platelet fraction were associated with shorter collagen-adenosine diphosphate closure time ($R = -0.5$, $P < 0.05$ for both). The results from the TG assay showed significantly increased TG in PRP from patients compared to controls. Among patients on aspirin, JAK2V617F positive patients had higher TG compared to JAK2V617F negative patients. Within JAK2V617F positive patients, significant correlations between platelet and/or immature platelet count and TG ($R = 0.3$, $P < 0.05$ for both) were found.
Summary/Conclusions: This study demonstrates that PFA-100 and TG assays are potentially relevant methods for monitoring aspirin effectiveness in MPN patients. Elevated immature platelet parameters are suggested important factors influencing increased platelet adhesive and procoagulant properties. New prospective studies are warranted to evaluate the usefulness of PFA-100 and TG assay in identifying MPN patients at high risk for thrombosis.

PB 1.26-6

Inhibition of MRP4 down regulates platelet activation and prevents pre-clinical arterial thrombosis

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Background: Platelet activation needs to be tightly regulated to prevent excessive platelet activity. In physiological conditions, major cytosolic inhibitors of platelet activation are cyclic nucleotides, cGMP and cAMP. Cytosolic rates of these second messengers result from an equilibrium between their synthesis, their degradation by phosphodiesterases and their relocation. Consequently, molecules that reduce the rate of cytosolic nucleotides, such as ADP stored in dense granules, act as platelet agonists. Indeed, following granule secretion, ADP binds to P2Y12 receptor, resulting in an inhibition of adenylate cyclase and of cAMP generation. Any mechanism involved either directly on cytosolic cyclic nucleotide level or on granular ADP may interfere with platelet activity. Recent studies pointed to the role of MRP4, a transporter of cyclic nucleotides and of ADP, specifically located on dense granule membrane in platelets, as a good candidate to control these mechanisms.
Aim: We evaluated *in vivo* and *in vitro* the role of MRP4 in the modulation of platelet functions using a MPR4−/− mouse model.
Methods: The total knock-out mice MRP4 (MRP4−/−), originally derived in the laboratory of J. Schuetz (St. Jude Hosp, TN, USA) on a FVB background, were compared to FVB wild-type (WT) mice. *In vitro* tests included platelet adhesion in flow conditions and washed platelet aggregation. *In vivo* tests performed were bleeding time (tail cut) and carotid arterial thrombosis (15% FeCl3 patch).
Results: Whole blood platelets from the two animal groups showed a similar initial adhesion on collagen in flow conditions, but aggregates were reduced in number and size after 3 min for MPR4−/− compared to WT. MRP4−/− washed platelet aggregation was impaired in the presence of low PAR4ap concentration (50 μM), with mean maximal aggregation at 16% compared to 53% in WT ($P < 0.01$). No differences were denoted at high PAR4ap concentration (100 μM) that induces activation poorly dependent of secreted ADP. Similar results were obtained in WT platelets in the presence of the MRP4 inhibitor MK571. A defect in platelet function related to an impaired ADP storage in MRP4−/− was further confirmed by secretion-independent aggregations performed in response to ADP, which were similar in the two animal groups.
The defect in platelet function was confirmed *in vivo* by a prolonged bleeding time in MRP4−/− (330 s) compared to WT mice (152 s; $P < 0.01$), in line with a greater volume of elapsed blood, estimated by hemoglobin assay (3253 vs. 327 mg for MRP4−/− and WT, respectively; $P < 0.001$). Arterial thrombosis experiments showed a significant longer occlusion time in MRP4−/− vs. WT mice (926 vs. 663 s; $P < 0.001$), suggesting that MRP4 inhibition protects against thrombosis.
Conclusion: These results show an impaired platelet function in MRP4−/− mice, part of which is likely to be related to an impaired ADP storage in dense granules. Our findings support previous studies, suggesting MRP4 as a new target for an antiplatelet agent. Ongoing work is conducted to evaluate the role of MRP4 in cAMP compartmentation in platelets.

PB1.27 – Platelets and Cancer

PB 1.27-1

Resistance to aspirin in myeloproliferative neoplasms: is it a reality? Evaluation of low dose aspirin resistance in a pilot study including 54 patients

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Background: myeloproproliferative neoplasms (MPN) are associated with an increased risk of arterial and venous thrombosis varying from 2.6% per year to 4.9% according to the studies. Low dose of aspirin is recommended in primary and secondary prophylaxis of thrombosis in polycythemia vera. In the prospective ECLAP cohort (Marchioloi R et al, J Clin Oncol 2005), low dose aspirin is associated to a reduced incidence of cardiovascular events by 28%. However evaluation of biological efficacy of low dose aspirin is still questionable as incidence of biological resistance ranges from 1% to 65% depending on the method used and the delay after the ingestion.

Aim of the Study: To determine aspirin resistance by light transmission aggregometry in well-characterized MPN patients under low dose aspirin (75 mg/day), 24 h after the last ingestion of aspirin
Methods: we prospectively enrolled 54 patients with MPN identified in Saint Louis Hospital, Paris (Dr C Dosquet and Pr C Chomienne). All of them were under aspirin 75 mg/day. Resistance to aspirin was performed on platelet rich plasma by platelet aggregation induced by arachidonic acid 1.33 mM and tested 24 h after the last dose. Resistance to aspirin was defined by a maximal platelet aggregation over 20%.
Results: Only seven out of 54 patients were resistant to aspirin (AsaR). An increased daily dose of 100 mg during 7 days at least overcame this biological resistance in six out of seven patients. The other 47 patients in whom aspirin was efficient were noted as the AsaS group. Clinical characteristics could be summarized as follows: Polycythemia Vera was diagnosed in 29 and in 15 patients of the Asa S and of the Asa R group, respectively and Essential Thrombocythemia in five patients of the AsaS group and in two patients of the Asa R group. JAK2V617F mutation was present in 64% of the AsaS group and in 86% of the AsaR group. Platelet count was 331 ± 172G/L in the AsaS group and 378 ± 201G/L in the AsaR group
Interestingly, 71% of patients resistant to aspirin had a thrombotic event in the arterial circulation or microcirculation but not in venous area compared to only 34% of patients responder to aspirin (Asa S). Moreover, even though platelet count was slightly increased in the Asa R group compared to Asa S patients, it did not reach significance. The only patient resistant to 100 mg aspirin had no history of thrombosis and a normal platelet count at 316 G/L. Bleeding tendency consisted in ecchymosis, epistaxis and gingivorragia and was more frequent in Asa S patients (12/47) than in Asa R patient (1/7).
Conclusion: Increasing the dose of aspirin from 75 to 100 mg once daily seems enough to overcome aspirin resistance when tested in standardized conditions. Only one patient over 54 (1.8%) was still resistant to aspirin with 100 mg once a day. Moreover biological resistance to aspirin was associated to past history of arterial thrombosis in most of the patients. These results need to be confirmed in a larger study.

PB 1.27-2

Intraplatelet angiogenesis regulators more relevant than serum carcino embryonic antigen (CEA) to colorectal cancer (CRC)?

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Background: Serum CEA is the most widely used marker for CRC recurrence following curative resection, although the accuracy of CEA monitoring during the follow-up period of surgical resection is not always consistent. Furthermore normal serum CEA levels may be associated with early stages of tumor progression or with non-CEA producing tumor. The post-operative monitoring of CEA is useless for patients with normal pre-operative serum CEA. There is no biological marker to detect recurrence following curative surgery for these patients. Very early in the course of cancer development, variable concentrations of angiogenesis regulators can be taken up, internalized and concentrated in platelet alpha granules for a timely delivery to tumors through specific activation. This suggests an avenue for developping biomarkers to diagnose and monitor CRC. Prior to validate intraplatelet angiogenesis regulators (IPAR) as markers for CRC recurrence in patients with normal CEA, we must confirm the clinical significance of pre-operative IPAR and check platelet activation in these patients before surgery.
Aims: We compared concentration of two major angiogenesis regulatory proteins (VEGF, PDGF) in platelets of CRC patients with normal serum CEA to those of normal controls. We assessed platelet activation through plasmatic p-selectin concentration.
Methods: Blood sample from 43 patients with newly diagnosed CRC were analyzed for CEA before surgery, 31 patients (72%) presented with norman serum CEA (< 10 μ/L). Platelet contents of VEGF and PDGF, and plasma p-selectin were measured for these 31 patients and 31 controls free of CRC. Analyte concentrations were measured with quantitative sandwich enzym immunoassays (R&D system). VEGF and PDGF levels were adjusted to the number of platelets in the pellets by dividing the amount of analyte by the actin quantity. Variables with skewed distribution were log-transformed before being used as continuous variables in statistical analysis. Normal controls were younger than patients (mean age = 47.3 + 1.9 vs. 63.7 + 1.9 respectively), age was entered in a multivariate model.
The study was approved by the medical ethics committee, and informed consent was obtained from patients and controls.
Results: Thirty-one CRC patients stage I (n = 4), II (n = 7), III (n = 4), IV (n = 16) and normal serum CEA were compared to 31 controls. Before surgery CRC patients presented with significantly higher platelet VEGF and PDGF concentrations compared to controls (1.05 + 0.10 vs. 0.43 + 0.06; P = 0.022 and 2.5 + 0.09 vs. 2.07 + 0.04; P = 0.019 respectively). Platelet activation was higher for patients compared to controls (77.34 + 5.03 vs. 58.67 + 2.66; P = 0.036). Platelet counts were not different between patients and controls. We did not observe any difference in analyte concentrations according to the CRC stage.
Conclusion: We confirmed platelet activation in CRC. Pre-operative intraplatelet VEGF and PDGF seem to be more sensitive than CEA to CRC. these results provide further insights into CRC monitoring especially for patients with normal serum CEA prior to surgical resection. Next step will be to confirm these findings on a larger study and to mesure the effect of surgical resection on these parameters.

PB 1.27-4

Immune thrombocytopenia in patients with chronic lymphocytic leukemia treated with 2 – CdA-based regiments or chlorambucil

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Background: The relationship between treatment of Chronic lymphocytic leukemia (CLL) with cladribine (2 – CdA) or chlorambucil and immune thrombocytopenia (IT) have not been yet determined.
Methods: We retrospectively analyzed records of 777 patients treated in two randomized PALG (Polish Acute Leukemia Group) CLL programs with these agents.
Results: Immune thrombocytopenia occurred in 55 of 777 (7.1%) patients. No significant differences in IT prevalence was seen between patients on chlorambucil or 2-CdA – based regiments (P = 0.33). IT developed at a median time of 0.499 years (0.06–4.8) from the start of CLL therapy. This time was significantly longer in patients treated with chlorambucil (2.03 years, 95% CI: 0.06–4.22) in relation to patients treated with 2-CdA- based regiments (0.52 years, 95% CI: 0.34–0.69, P = 0.049). Overall survival (OS) of patients with IT and those without IT were not statistically different (2.65 vs. 3.2 years, P = 0.23) but the severity of bleeding was more pronounced in 2- CdA group. Response to IT therapy was 35%, 54% and 75% for steroids, chemotherapy and splenectomy respectively.
Conclusions: In this study we demonstrated unexpectedly high percentage of IT prevalence in patients with CLL requiring chemotherapy. Although no marked differences were seen in IT frequency in patients treated with 2 – CdA-based regiments compared to chlorambucil regimen the clinical course of haemorrhagic diathesis was more severe in 2 – CdA group. Also time that elapsed from study screening to IT diagnosis was significantly shorter in 2-CdA group than in chlorambucil group suggesting causative relation. The appearance of IT did not influence the median time of OS.

PB 1.27-5

Crosstalk between platelets and inflammation in nasopharyngeal carcinoma

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Background: Nasopharyngeal carcinoma (NPC) is an unique malignancy because of its geographical and ethnic patterns. South China and South East Asia have the highest incidence, while in Indonesia its incidence is about 5.66 cases per 100,000 populations per year. Complex interactions between tumor cells and circulating platelets play an important role in cancer growth and dissemination. Soluble Platelet-Selectin (sP-selectin) is a marker of *in vivo* platelet activation. Inflammation, marked by elevated Interleukin-6 (IL-6) concentrations, can have 'cross-talk' with platelets resulting in thrombosis. Thus we investigated the level of sP-selectin and platelet count in nasopharnyngeal carcinoma and whether they had correlation with IL-6.

Aims: Studying the 'cross-talk' between platelets and inflammation in nasophyarngeal carcinoma.

Methods: This was a cross sectional study including patients with newly diagnosed nasopharyngeal carcinoma at Cipto Mangunkusumo Hospital, Jakarta, Indonesia in period of May to November 2012. Soluble P-selectin levels in various stages of NPC were measured using a human sP-selectin Immunoassay (R&D Systems) following the manufacturer's instructions. Interleukin-6 concentrations were measured using a human IL-6 ELISA (Bender MedSystems). Platelets of every patients were counted by ABX Micros 60. SPSS version 13 software was used in this study.

Results: From 53 patients of NPC, 17 patients had distant metastatic sites. The mean age was 44.2 years with ratio of men to women was 3.4:1. The most prevalence histopathology was undifferentiated carcinoma (84.9%). The mean platelets count was 340,924/μL. Eleven percent of the patients had elevated platelet count (> 450,000/μL). The median level of sP-selectin was 46.6 ng/mL (inter quartile range: 42.07–58.67) while the median level of IL-6 was 17.02 pg/mL (inter quartile range: 10.45–25.31). The median level of sP-selectin was statistically significantly higher among patients with metastatic NPC than non-metastatic NPC (59.5 vs. 44.7; $P < 0.001$). Statistically, the platelet count in metastatic NPC was not different with non-metastatic NPC ($P = 0.717$). In metastatic NPC, IL-6 had correlation with platelet count ($r = 0.514$; $P = 0.035$) and sP-selectin ($r = 0.562$; $P = 0.019$). Platelet count also had correlation with sP-selectin ($r = 0.556$; $P = 0.02$). In non-metastatic NPC, IL-6 didn't correlate with platelet count ($P = 0.637$) or sP-selectin ($P = 0.751$) and there was no correlation between platelet count and sP-selectin ($P = 0.724$).

Conclusion: In metastatic nasopharyngeal carcinoma, there was an increased level of platelet activation than in non-metastatic NPC. Inflammation had significant contribution to platelet activation and platelet count in metastatic nasopharyngeal carcinoma.

PB 1.27-6

Role of thrombopoietin signalling in murine B-cell lymphoma

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Platelets have been linked to promotion of metastasis, and in some cases to supporting the tumor vasculature in solid tumors. However, the role of platelets in liquid tumors, such as lymphoma and leukemia, is largely unknown. Thrombopoietin mimetic drugs were recently approved and are currently in clinical trials for use in chemotherapy-induced thrombocytopenia. It is therefore important to better understand the roles of thrombopoietin and platelets in cancer, including hematopoietic malignancies.

We set out to investigate if changes in platelet number and signaling via thrombopoietin, the primary regulator of platelet production, would influence myc driven B-cell lymphoma progression. To test this, *Eμ-Myc* transgenic mice, a well-established model of human Burkitt's lymphoma, were mated with *Mpl−/−* mice that have severe reductions in platelets, megakaryocytes and megakaryocyte progenitor cells due to lack of thrombopoietin signaling through Mpl. In addition, we mated *Eμ-Myc* mice with *TpoTg*transgenic mice which over-express thrombopoietin leading to increased megakaryopoiesis and high platelet numbers. As previously described, *Mpl−/−* mice exhibit platelet counts approximately 10% of wild type levels, whereas *TpoTg* mice have 3.5 times higher platelet counts compared to wild type mice.

In transgenic mice Myc over-expression is under the control of the immunoglobulin heavy chain gene enhancer (*Eμ*). The central mechanism of *Eμ-Myc* induced tumorigenesis is early expansion of immature pro-B/pre-B cells, which further progress to a malignant state. We monitored differences in survival and the rate of tumor initiation by measuring pre-B cells in 4–5 week-old pre-malignant mice.

Remarkably, *Mpl−/− Eμ-Myc* mice had reduced survival as well as earlier onset of lymphomagenesis, based on increased splenic pre-B cell numbers and blood lymphocyte counts in 4–5 week-old mice compared to *Eμ-Myc* control mice. Conversely, our data indicate that elevated thrombopoietin signaling is protective.

Results of tumor transplant experiments of established *Eμ-Myc* tumors into *Mpl−/−* or wild-type mice, point to similar survival rates. This suggests that changes in platelet number do not influence the rate of lymphomagenesis after it has been initiated. Rather, thrombopoietin signaling through Mpl could be of relevance for the rate of tumor onset. In addition to regulating platelet counts, thrombopoietin is known to affect hematopoietic stem cell (HSC) number and quiescence. Mpl receptor deficient mice have increased cycling of HSCs. We propose that changes in HSC cycling or an altered environment arising from abnormal platelet number could be the mechanism(s) behind differences in tumor initiation and survival. Such studies currently are underway.

Our study shows that changes in platelet number and/or signaling via thrombopoietin influence tumor onset and survival in murine B-cell lymphoma.

PB1.28 – Platelets and Cancer

PB 1.28

Procoagulant microparticles in cancer patients: prognostic value

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Background: Microparticles (MPs) are small vesicles (100 nm–1 μm) which directly bud from the plasma membrane of different cells, including blood, endothelial and tumor cells. The procoagulant MP levels increase in cancer patients. Nevertheless, the potential prognostic value of MPs in cancer remains unclear.

The aim of this study was to evaluate the potential prognostic value of MPs and MP-mediated procoagulant activity in cancer patients.

Material and Methods: We studied: (i) 60 patients with non-small cell lung cancer (NSCLC) stages IIIB and IV before first line, platinum-based chemotherapy +/2 bevacizumab, and after the third cycle of treatment; (ii) 22 patients, newly diagnosed, with histologically proven glioblastoma (GB) who received radiotherapy plus concomitant metronomic temozolomide treatment at a daily dose of 75 mg/m^2 before the start of radiochemotherapy and during the last week of this treatment. Sixty healthy subjects were evaluated as controls. Total MPs were quantified by flow cytometry by labeling with FITC-AnnexinV. Samples were acquired for 120 s at high flow rate. Plasma procoagu-

lant activity was assayed by the automated calibrated thrombogram as the endogenous thrombin generation measurements (TG, nM thrombin) and by the clotting time due to procoagulant phospholipids (PPLCT).
Results: The results of the current study show that the MP count (36,210 ± 34,494 vs. 11,088 ± 5814 MPs/120 s; $P < 0.0001$) and ETG levels (209 ± 86 vs. 158 ± 59 nMol thrombin, $P < 0.01$) are elevated in patients with NSCLC in comparison with healthy controls. Chemotherapy did not change the levels of these markers. MP levels positively correlated with TG levels, both before (Spearman $r = 0.60$, $P < 0.001$) and after treatment (Spearman $r = 0.68$, $P < 0.001$). We have also found an association between high MP levels, both before and after treatment, and survival. In GB patients the mean pretreatment levels of MPs were significantly higher than healthy controls (48,210 ± 47,700 vs. 11,088 ± 5814 MPs/120 s; $P < 0.001$). MPs and TG decreased, and PPLCT increased significantly after treatment. MP levels positively correlated with both pre- and post treatment levels of TG (Spearman $r = 0.74$, $P < 0.01$), and inversely correlated with pretreatment levels of PPLCT (Spearman $r = -0.86$, $P < 0.01$). It is also shown that a TG level greater than the 25th percentile in patients, after treatment with radiotherapy and concomitant temozolomide, is associated with a shorter survival.
Conclusion: Our findings provide new data on the prognostic value of MPs in patients with NSCLC and GB. The role of MPs as potential biomarkers in patients with cancer, treated with conventional chemotherapy or targeted therapies, warrant further study, since a better understanding of their role can open new perspectives in the fields of diagnosis and treatment.

PB 1.28-1
Effects of ultraviolet radiation on platelet shape

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Background: The adoption of pathogen reduction technology (PRT) has been considered for the implementation of safer platelet transfusion. PRT treatment involves ultraviolet (UV) radiation in a wide wavelength range; however, the effects of UV radiation at different wavelengths on platelets have not been fully clarified.
Aim: In this study, we evaluated the effects of UV radiation with UV of different wavelengths on the platelet shape.
Methods: An apheresis platelet concentrate was diluted with SSP+ additive solution (Macopharma) and used as a measurement sample (residual plasma rate, 30%). Changes in the platelet shape were monitored in real-time during the radiation of the sample with UV of 300–370 nm wavelengths (0–0.15 J/cm^2) using a 90° scattered light. The change in the amplitude of the signal (*amp*) obtained by frequency analysis of the change in the intensity of scattered light was used as an index of platelet shape change. The level of generation of reactive oxygen species (ROS) in platelets upon UV radiation was measured using hydroxyphenyl fluorescein (HPF).
Results: For UV with wavelengths shorter than 330 nm, *amp*, which indicates the percentage of discoid platelets, decreased with the dose of UV radiation. The extent of the decrease in *amp* increased with decreasing wavelength at the same dose of UV radiation. For UV with wavelengths longer than 330 nm, *amp* remained unchanged with increasing UV radiation dose. For UV with a wavelength of 300 nm, generation of ROS in the platelets was observed depending on the dose of UV radiation. There was a good correlation between the platelet shape change and the level of ROS generation ($r = 0.90$, $P = 0.04$). The platelet shape change and the generation of ROS in the platelets as a result of UV radiation at a wavelength of 300 nm were significantly suppressed ($P < 0.05$) in the presence of ascorbic acid (2.5 mM), an antioxidant.
Conclusions: UV radiation at wavelengths shorter than 330 nm induced a platelet shape change depending on the dose of UV radiation. It was suggested that the generation of ROS in the platelets is related to the platelet shape change upon UV radiation.

PB 1.28-2
Reversible blockade of GPIIb-IIIa reduces platelet storage lesions

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Background: The storage-related decline in platelet function and viability is collectively referred to as platelet storage lesions or PSLs. It has been suggested that PSLs actually begin when the automated forces of product processing delivers the first assault on the platelets prior to storage. During storage platelets begin to lose the ability to aggregate, as well as matrix adhesion capabilities. Platelet activation markers, soluble CD40 ligand (sCD40L) and CD62 accumulate on the platelet membrane surface or in the plasma environment over time. Finally, modifications in the expression of platelet-specific membrane proteins GPIb and GPIIb-IIIa and structural changes to the platelet cytoskeleton have been associated with platelet storage.
Aims: This study focused on a novel mechanistic approach that tested the hypothesis that GPIIb-IIIa blockade by a reversible agent, eptifibatide (EPT), may improve platelet functionality over storage time.
Methods: Platelet aggregation in response to 5 μg/mL collagen and to 20 μM ADP was measured as well as EPT occupancy and CD62 expression during blood bank storage of platelet products either treated with EPT or saline (SAL) control. pH and glucose was also monitored over storage time.
Results: Day 1 testing demonstrated that the platelet products were functional and that the added EPT saturated the GPIIb-IIIa receptors. At Day 3 of storage and after removal of the inhibitory effect of EPT, platelet aggregation response to collagen and ADP of the EPT-treated product had a discernible shape change and typical aggregation profile. However, the SAL control product had no evidence of shape change and only a minimal aggregation response. On Day 1, CD62 binding to the platelet product in the absence of exogenous agonist demonstrated little activation. By Day 3, CD62 MFI in the SAL treated product was 9-fold higher whereas in the EPT treated product, the MFI was increased < 4-fold. Furthermore, with ADP or TRAP activation, the MFI in the SAL treated product was slightly increased by 1.0- to 1.2- fold, respectively, indicating 'exhausted platelets'. In contrast, the EPT product had a more robust reactivity and the MFI increased by 2.5- to 5-fold, respectively, upon activation by ADP or TRAP. Other readouts showed that pH decreased from 7.5 to 6.5 and glucose was POS at day 3 in the SAL group compared to a constant pH 7.0 and NEG glucose for the EPT group.
Summary/Conclusion: Our data suggest that this novel treatment mitigates key characteristics of PSL and may be a viable, new approach for improving platelet post-transfusion survival and function. Reversible blockade of GPIIb-IIIa has the potential to reduce platelet storage lesions and lessen adverse events associated with platelet transfusion in patients that require platelet products. These data also suggest that EPT treatment may inhibit transitions in pH and have some benefit in reducing effects of early bacterial contamination.

PB 1.28-3

Sonorheometry assessment of aspirin effects on platelet contributions to blood clot stiffness *ex vivo* in patients undergoing cardiopulmonary bypass procedures

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Background: Platelets contribute significantly to blood clot stiffness and their decreased function may underlie bleeding following cardiopulmonary bypass (CPB) supported cardiac surgery. Few quantitative measures exist to guide transfusion of platelets during and immediately following CPB, leading to a shotgun approach to the delivery of multiple hemostasis-promoting products to the patient. Sonorheometry (SR) is a novel ultrasound-based technology that rapidly quantifies hemostasis from a blood sample by measuring *ex vivo* clot stiffness (*S*) during surgery using acoustic radiation force.

Aims: To assess platelet contributions to *ex vivo* CPB patient clot stiffness and correlate clot stiffness with pre-surgical aspirin therapy and intraoperative transfusion risk.

Methods: Forty patients were enrolled following informed consent (University of Virginia IRB approved study no. 14050) in a prospective pilot study to assess the utility of SR testing during surgeries involving cardiopulmonary bypass (CPB). The subset of subjects on aspirin therapy (81–325 mg/day) stopped ingestion 1–3 days prior to surgery. During surgery, patient blood samples (2 mL each) taken from the A-line were divided with one sample treated with abciximab and the other serving as a control. Samples were activated by kaolin (5 mg/sample) and changes in blood clot stiffness (*S*) were measured by SR. Blood was sampled immediately before surgery and 10 min after protamine infusion after the end of CPB. The blood sample was held in a custom ultrasound transducer holder at 37 °C. The anesthesiologist and the surgical team were blinded to the results of SR testing.

Results: A differential measure named the Platelet Activity Index (*PAI*) was defined by measuring *S* with and without the platelet IIb/IIIa integrin inhibitor, abciximab. *PAI* measured prior to CPB (*PAIpreCPB*) was 34% lower in subjects who were on prior aspirin therapy than those not on aspirin therapy ($P < 0.028$). *PAI* determined immediately after CPB (*PAIpostCPB*) was 33% lower in subjects on prior aspirin therapy ($P < 0.010$). Patients not on aspirin therapy had a 27% chance of receiving a transfusion. Patients on aspirin therapy had a 77% chance of receiving a transfusion. ROC analysis of platelet-based transfusions showed a correlation of *PAIpreCPB* measures with the surgical team's decision (AUC = 0.73, $P < 0.01$). Similarly, *PAIpostCPB* measures also correlated with the surgical team's transfusion decision (AUC = 0.71, $P < 0.018$). Furthermore, *PAI* was significantly lower for transfused subjects than for non-transfused subjects ($P < 0.003$).

Conclusions: In a prospective, small-scale pilot study, CPB patients on aspirin therapy had lower platelet contributions to *ex vivo* blood clot stiffness than CPB patients not on aspirin therapy and a higher occurrence of intraoperative platelet transfusions, suggesting the existence of residual impairment of platelet function due to aspirin treatment. SR assessment of platelet activity may help predict platelet transfusion needs and lead to more selective usage of platelets during surgery. This study was conducted following the guidelines of the Declaration of Helsinki.

PB 1.28-4

Use of high-sensitivity flow cytometry for the characterization of a lyophilized platelet-derived hemostatic agent

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Background: Uncontrolled bleeding is the leading cause of potentially preventable death among trauma patients in the pre-hospital civilian and military setting. Lyophilized platelets have been proposed as a therapeutic agent for pre-hospital management of hemorrhages. Characterization of the particles present in lyophilized platelet-derived hemostatic agents is essential for understanding the mechanism(s) underlying clinical benefit, to standardize production of the agent, and thus, to achieve reproducible clinical outcomes.

Aims: To develop a method to reproducibly quantify particles in discreet ranges between 300 nm and 15 μm, and immunophenotypically characterize particles in lyophilized platelet-derived hemostatic agents within each range.

Methods: Unstained or CD41a−, CD42b− and annexin V-stained samples were analyzed using the high sensitivity Beckman Coulter Gallios flow cytometer. To detect the smallest particles, forward light scatter (FSC) was collected in wide-angle mode with a threshold of zero and the photomultiplier tube (PMT) amplification was set to a gain of 2.0 and 500 V. To detect larger particles, FSC was collected in narrow mode and the PMT amplification was set to a gain of 1.0 and 350 V. Results of the median FSC-Area of bead standards were used to establish limits of regions corresponding to the following calibrated plastic microsphere sizes: 0.294–0.482, 0.482–0.9, 0.9–2.53, 2.53–5.037, 5.037–7.83, 7.83–14.73, and > 14.73 μm.

Results: Intra- and inter-day variability was determined for regions 0.482–0.9 and 0.9–2.53 μm by repeated measurements of a single sample. Intraday coefficients of variation (CV)s for these regions were 0.80% and 1.08%, respectively, while the interday CVs were 1.06% and 3.78%, respectively. Particles in human lyophilized platelet preparations showed a bimodal distribution of sizes by ungated analysis of wide-angle light scatter, with peaks just below the 0.294 μm and the 0.9 μm marker. Percentages of events in specific regions were: < 0.294 μm: 20–27%, 0.294–0.482 μm: 20–27%, 0.482–0.9 μm: 22–30%, 0.9–2.53 μm: 12–15%, 2.53–5.037 μm: 9–11% and > 5.037 μm: 3%. Due to the high sensitivity of the Gallios, a large number of particles < 0.482 μm were detected in ungated samples. However, these particles were negative for CD41a and CD42b and thus were eliminated when samples were gated on the basis of positivity for these markers. Percentages in the specific regions for CD41a+/CD42b+ events were: < 0.294 μm: 0.1%, 0.294–0.482 μm: 0.1–0.2%, 0.482–0.9 μm: 30–34%, 0.9–2.53 μm: 32–35%, 2.53–5.037 μm: 26–27% and > 5.037 μm: 7–9%. Analysis by narrow-angle light scatter of particles of both ungated and CD41a+/CD42b+ gated samples showed 17–23% of particles in size ranges > 5.037 μm. Samples were > 73% positive for annexin V in regions > 0.482 μm, compared to an isotype.

Summary/Conclusions: Results demonstrate reproducible quantification and immunophenotypic characterization of platelet derived particles across a wide range of sizes (< 0.294–> 14.73 μm). Characterization and standardization of the size and procoagulant phenotype of lyophilized human platelets is important for achieving reproducible clinical efficacy.

PB 1.28-5

Refrigerated storage of platelet products for transfusion in dogs

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Background: Platelet products for transfusion are normally stored at room temperature, and the storage time is limited to only 5 days to prevent the growth of potentially contaminating bacteria. Although refrigerated storage would likely reduce the growth of most bacteria, thus prolonging the storage, the *in vivo* survival time of human platelets after transfusion is known to significantly decrease due to refrigeration.
Aims: Preventing the rapid clearance of refrigerated platelets is challenging, and it is difficult or unethical to examine the effects of modifications to the storage protocols in human volunteers. Therefore, we examined the effects of refrigeration of platelet products in dogs to determine whether dogs could be used as an alternative to humans.
Methods: Canine platelet rich plasma in polyolefin containers was stored for 7 days at room temperature (22 °C) or at 4 °C with continuous agitation on a flat bed shaker. Samples were taken aseptically 1 and 7 days after storage, and the *in vitro* characteristics were assessed. The *in vivo* recovery and survival of the autologous platelet products that had been stored for 7 days were also assessed using a biotinylation method.
Results: The aggregability induced by collagen and ADP were better maintained in the refrigerated platelet products than in the platelets stored at room temperature on day 7 (22 °C: collagen, 36.7 ± 20.8%; ADP, 14.8 ± 12.9%; 4 °C: collagen, 72.3 ± 21.2%; ADP, 55.9 ± 29.5%). Significant differences were also found in the platelet counts, pH, glucose concentration, lactate concentration and the extent of shape change. No significant differences were found in the mean platelet volume, hypotonic shock response or lactate dehydrogenase activity. On the other hand, although the *in vivo* recovery was not significantly different, the survival time after transfusion of refrigerated platelet products was obviously shorter than that of room temperature stored platelet products (22 °C: 5.2 ± 0.6 days, 4 °C: within 2 days).
Summary/Conclusions: These results indicate that the effects of refrigeration on canine platelet products, especially the rapid clearance after transfusion, were similar to those in humans. Therefore, dogs may be considered a valuable preclinical model for assessing the effects of different modifications on refrigerated platelets in order to extend their storage life.

PB 1.28-6

Pneumatic tube transport affects platelet function measured by multiplate electrode aggregometry

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Platelet activation and aggregation is an essential component of many thromboticevents. Multiple electrode aggregometry (MEA) is used to measure platelet function. Pneumatic tube transport systems (PTS) for delivery of patient samples to a centrallaboratory are often used to reduce turnaround time for vital analyses. We evaluated theeffects of PTS transport on platelet function as measured by MEA. Duplicate samples were collected from 32 healthy individuals, eight patients from anintensive care unit, four patients from a coagulation clinic and 14 patients from acardiovascular unit treated with aspirin, clopidogrel or both. One sample was sent usingthe PTS and the other was carried by personnel to the lab. Platelet function was measuredby means of a Multiplate® analyzer (Roche, Mannheim, Germany) using adenosinediphosphate (ADP test), arachidonic acid (ASPI test), collagen (COL test), ristocetin (RISTO test) and thrombin-receptor activating peptide-6 (TRAP test) as aggregationagonists. Samples transported using PTS showed a reduction of AUC-values of up to a 100% ofthe average as compared to samples carried by personnel and a majority showedreductions of AUC-values greater than 20% of the average. Bias ± 95% limits ofagreement for the ADP test were 26 ± 56% of the average. Bias ± 95% limits of agreementfor the ASPI test were 16 ± 58% of the average. Bias ± 95% limits of agreement for theCOL test were 20 ± 54% of the average. Bias ± 95% limits of agreement for the RISTOwere 14 ± 79% of the average. Bias ± 95% limits of agreement for the TRAP test were19 ± 45% of the average. We conclude that PTS transport affect platelet activity as measured by MEA. Weadvise against clinical decisions regarding platelet function on the basis of samples sentby PTS in our hospital settings.

PB1.29 – Megakaryocytes and Thrombopoiesis – I

PB 1.29-1

Purinergic signalling regulates human megakaryocytes function by inducing store-operated Ca^{2+} entry

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Background: During differentiation megakaryocytes migrate from the osteoblastic niche to the vascular niche of the bone marrow, where they convert the bulk of their cytoplasm into multiple long processes called proplatelets that assemble nascent platelets at their tips. Despite the well-defined mechanisms that drive proplatelet formation and architecture, less is known about the signals that trigger platelet production. Different findings highlighted the importance of autocrine signals, together with environmental factors, in the regulation of megakaryocyte behavior, however the exact mechanisms by which all these factors coordinate to promote platelet release are unknown. We previously showed that megakaryocytes, and not platelets, express the purinergic receptor $P2Y_{13}$ which interacts with constitutively released adenosine diphosphate (ADP) leading to proplatelet formation. ADP-dependent platelet activation relies on the increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$), accomplished by store-operated Ca^{2+} entry. Despite this knowledge whether or not this mechanism plays a role in platelet production is not known.
Aims: Store operated Ca^{2+} Entry (SOCE) has been described to be required in different human cell types to promote interaction with extracellular matrix components. The aim of this study is to demonstrate the role of ADP-induced SOCE activation in regulating Mk behavior within extracellular matrix environment.
Methods: In this project we took advantage of an *in vitro* human model to obtain megakaryocyte from human cord blood-derived progenitor cells. We employed Ca^{2+} imaging to investigate the expression and functionality of SOCE in fully differentiated megakaryocytes by either pharmacological (i.e. cyclopiazonic acid) or physiological (i.e. ADP) stimulation.
Results: We demonstrated that ADP binding to $P2Y_{13}$ elicits a rapid increase in $[Ca^{2+}]_i$, followed by a plateau, which is lowered in Ca^{2+}-free solution, thereby suggesting the involvement of store-operated Ca^{2+} entry. Therefore, we provided the first evidence that megakaryocytes express the major candidates to mediate store-operated Ca^{2+} entry, STIM1 and Orai1, which were functionally activated upon depletion of the intracellular Ca^{2+} pool. The mechanism was inhibited by a phospholipase C inhibitor (U-73122), an inositol-3-phosphate receptor inhibitor (2-APB), or a specific store-operated Ca^{2+} entry blocker (BTP-2). Finally, studies on the effects of these compounds on megakaryocytes behavior revealed that Ca^{2+} entry form extracellular media is primarily involved in the regulation of cytoskeleton rearrangement responsible for cell adhesion and motility on extracellular

matrix components. Conversely, only Ca^{2+} mobilization form intracellular stores is required to activate signaling cascades that trigger proplatelet formation.

Summary/Conclusions: These findings provide the first evidence that ADP utilizes store-operated Ca^{2+} entry to regulate human megakaryocyte functions and open new perspectives in the evaluation of the signals that control platelet release *in vivo*.

PB 1.29-2

Establishment of conditions for *in vitro* and *in vivo* production of genetically modified human megakaryocytes and platelets

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Background: In recent years, large-scale 'platelet-omics' studies and transcriptome screenings in healthy volunteers and patients suffering from thrombotic events have discovered a large number of differentially expressed platelet genes and proteins, whose function is largely unknown. Efficient methods for genetic manipulation and subsequent functional characterization of human megakaryocytes (MK) and platelets are however currently lacking due to a low number of human platelets produced *in vitro* and the need for a large number (35×10^6) of $CD34^+$ hematopoietic stem and progenitor cells (HSPC) for transplantation in immunodeficient mice and human platelet production *in vivo*.

Aims: We aimed to develop a method that allows efficient platelet-specific genetic modification of HSPC in combination with *in vitro* and *in vivo* production of human MK and platelets.

Methods: We initially tested three different cytokine cocktails (TSF16, TSF and TPO containing different concentrations of TPO, SCF, Flt-3-Ligand, IL-6 and IL-1β, for their ability to support *in vitro* differentiation. Differentiation of HSPC towards the megakaryocytic lineage was assessed by flow cytometry by determining expression of CD34, β_3 (CD61) and GPIbα (CD42b) and by light and transmission electron microscopy after 7 and 14 days. The best performing cytokine cocktail was then used for lentiviral transduction of HSPC with a vector containing the GFP gene under control of the α_{IIb} promoter (lenti-α_{IIb}-GFP-WPT), after which the percentage of GFP^+ MK was again assessed by flow cytometry. Finally, HSPC were prestimulated, transduced and transplanted in sublethally irradiated NOD/SCID/IL2R$\gamma^{-/-}$ (NSG) mice and the production of human GFP^+ platelets was assessed up to 6 weeks post-transplantation.

Results: In all three media tested, differentiation of HSPC was observed as an increase in the number of β_3^+ and GPIbα$^+$ cells concomitant with a decrease in the number of $CD34^+$ cells. Culturing of HSPC in TSF16 medium resulted in the highest number of β_3^+ ($69.7 \pm 5.5\%$; $n = 3$) and GPIbα$^+$ ($41.1 \pm 17.7\%$; $n = 3$) cells after 14 days. Successful differentiation of HSPC in this medium was furthermore confirmed by the detection of proplatelet-forming MK using light and electron microscopy. Following overnight prestimulation in TSF16 medium, lentiviral transduction of HSPC with lenti-α_{IIb}-GFP-WPT for 2×2 h at MOI 2.5 resulted in $29.7 \pm 5.0\%$ ($n = 3$) of α_{IIb}^+ cells successfully expressing GFP after 7 days of *in vitro* culture in TSF16 medium. Finally, we transplanted 5×10^6 lentivirally transduced HSPC into a sublethally irradiated NSG mouse. This resulted in the detection of GFP^+ human platelets in murine peripheral blood for up to 6 weeks post-transplantation.

Conclusion: We here report on the establishment of conditions that permit efficient lentiviral transduction of HSPC in combination with *in vitro* production of transgenic proplatelet-forming MK as well as *in vivo* production of transgenic human platelets in the circulation of NSG mice. Furthermore, our optimized protocol permits a sevenfold reduction in the number of cells needed for transplantation, compared to previously reported data. These results show that overexpression or knockdown of a gene of interest in human MK and platelets is feasible, thus paving the way to study genetically modified human platelets in mouse models.

PB 1.29-3

Protein synthesis in the late stages of megakaryocyte maturation triggers proplatelet formation

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Background: Platelets are essential for hemostasis, and thrombocytopenia (platelet counts $< 150 \times 10^9$/L) is a major clinical problem. Megakaryocytes (MKs) generate platelets by extending long, branching processes, designated proplatelets, into sinusoidal blood vessels.

Aims: While the mechanism of platelet production has been studied extensively, very little is known about what initiates and regulates proplatelet formation. We aim to identify and study proteins and corresponding signaling pathways that initiate proplatelet formation.

Methods: We used the IncuCyte high content live-cell kinetic imaging system to monitor proplatelet formation in cultured primary megakaryocytes differentiated from murine hematopoietic stem cells. Kinetic analysis of proplatelet formation over 24 h revealed maximum proplatelet formation after 8 h. Puromycin treatment was used to inhibit protein synthesis. Polysome profiling was used as a global measure of protein synthesis. To identify what proteins are dynamically changing during proplatelet formation, we compared the proteome of round vs. proplatelet-producing megakaryocytes by two-dimensional differential interference gel electrophoresis (2D DIGE).

Results: Inhibition of protein synthesis with puromycin significantly decreased proplatelet formation from $54 \pm 4.0\%$ to $17 \pm 3.8\%$, and significantly reduced the amount of proplatelets and platelets released. These data suggest proteins synthesized immediately preceding proplatelet formation are necessary for the transition from round to proplatelet-producing MKs. Polysome profiling of round MKs immediately preceding proplatelet formation revealed enrichment in the polysome fraction, showing protein synthesis is strikingly active during this time; the polysome fraction was significantly reduced following proplatelet formation. Interestingly, proteomic analysis between round and proplatelet-producing MKs revealed only a small number of differences, suggesting regulation of a subset of proteins is sufficient to drive proplatelet production.

We focused on one protein identified in the both 2D DIGE and polysome profiling analysis that was up-regulated in proplatelet-producing MKs, myristoylated, alanine-rich, C kinase substrate (MARCKS). Differential expression of MARCKS in MKs was confirmed by western blot and immunofluorescence. MARCKS is a protein kinase C (PKC) substrate that binds PIP_2 and crosslinks F-actin in its dephosphorylated form at the plasma membrane. To examine the role of the PKC pathway and MARCKS in proplatelet formation, we treated MKs with the PKC inhibitor and activator Ro 32-0432 and PMA, respectively. Ro 32-0432 treatment dose-dependently augmented proplatelet production up to 48%, while treatment of MKs with the PKC activator PMA dose-dependently inhibited proplatelet formation up to 84%, suggesting that MARCKS must be dephosphorylated to promote proplatelet formation. This is supported by western blots of MKs at different stages of development; MARCKS total protein increases over 3-fold in proplatelet producing MKs while there is a 1.8 fold decrease in phospho-MARCKS.

Conclusions: Our preliminary data suggest that the dynamic cytoskeletal process of proplatelet formation requires protein synthesis. We propose that MARCKS is enriched and dephosphorylated in round MKs, where it binds to and localizes PIP2 throughout the demarcation mem-

brane system, facilitating signaling necessary to initiate proplatelet formation. In the future, targeting MARCKS directly may be a viable therapeutic option to drive proplatelet production and initiate 'autotransfusion' of platelets from existing bone marrow megakaryocytes.

PB 1.29-4

Human pre-adipocytes differentiate into megakaryocytes and platelets using endogenous thrombopoietin

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The regulation of megakaryopoiesis and thrombopoiesis is poorly understood. Better understanding of the mechanism may help us establishing novel strategy for *in vitro* production of sufficient number of platelets. We previously reported that pre-adipocytes differentiate into megakaryocytes (MKs) leading to platelet production. Pre-adipocytes exhibited the gene expression relevant to megakaryopoiesis and thrombopoiesis, such as p45NF-E2, GATA-2, thrombopoietin (TPO), and its receptor c-mpl. Among them, there is little expression of TPO in hematopoietic stem cells. TPO is the primary cytokine for megakaryopoiesis and thrombopoiesis, and recombinant TPO has been used to generate enriched populations of MKs using *in vitro* differentiation systems. The present study was undertaken to examine whether pre-adipocytes express endogenous TPO and c-mpl of sufficient magnitude to drive megakaryopoiesis. We used primary human pre-adipocytes (Cell Applications, Inc.). Cells were cultured either in MK lineage induction (MKLI) medium composed of IMDM, supplemented with L-glutamine, BSA, low-density lipoprotein cholesterol, iron-saturated transferrin, insulin, 2-beta-mercaptoethanol, and nucleotides, or in the maintenance medium, as a control medium, composed of DMEM supplemented with FBS and non-essential amino acids. The gene expression of TPO during MK differentiation using MKLI medium was examined by quantitative real-time PCR with premade primers (Applied Biosystems), and the threshold value of TPO normalized with GAPDH was 16.5 ± 0.8 on Day 0, 15.9 ± 0.6 on Day 5, 16.0 ± 0.5 on Day 8, unmeasurable level on Day 12, and unmeasurable level on Day 14. The protein levels of TPO measured by ELISA assay (Human TPO Quantikine, R&D) in the supernatant from 10^6 pre-adipocytes cultured in 2 mL MKLI medium were negligible on Day 0 and 28 ± 14 pg/mL on Day 8. No measureable TPO was observed in supernatants from pre-adipocytes on Days 0 and 8 cultured in the maintenance medium. These observations indicated that TPO was released into the supernatants when the culture medium switched from maintenance medium to MKLI medium. We examined MK differentiation from pre-adipocytes in MKLI medium in the absence (TPO-) or presence of exogenously added recombinant TPO, 50 ng/mL (TPO+). The frequency of human CD41-postive/CD42b-positive pre-adipocyte-derived cells in culture after 14 days was approximately 15% in TPO (-). Similar result was observed in TPO (+). The DNA ploidy, as assessed by propidium iodide staining, was from 2 to 16 N in TPO (+/−). The effects of the anti-c-mpl blocking antibody AMM2 (10 μg/mL) on MK production were performed on mouse pre-adipocytes which were also shown to differentiate into platelets. We observed 10-fold more CD41-positive cells in the absence of AMM2 than in its presence. For a platelet function, binding of Alexa Fluor 488-labeled fibrinogen to human pre-adipocyte-derived platelet-sized CD42b-positive cells in the presence of stimulation (5 U/mL thrombin) was increased to be approximately 20% as compared with the binding in its absence in TPO (+/−). Taken together, these findings indicate that pre-adipocytes differentiate into MKs and platelets using endogenous TPO. Although the precise molecular mechanism of TPO production during differentiation of pre-adipocytes into MK remains to be elucidated, our findings suggest the pre-adipocytes are reasonable cell candidate in production of platelets for clinical use.

PB 1.29-5

Expression and functionality of toll-like receptor 3 in the megakaryocytic lineage

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Background: Beyond having a key role in hemostasis, platelets and megakaryocytes also regulate the immune and inflammatory response, partially due to the expression of Toll-like receptors (TLRs) a family of proteins, which recognize molecular components of pathogens. Among the TLRs, TLR3 recognizes double-stranded (ds) RNA associated with viral infection, and induces the activation of the transcription nuclear factor-κB (NF-κB) and the production of type I interferons (IFN-I). We have previously demonstrated that Junin virus (JV), an arenavirus causative of Argentine hemorrhagic fever, or Poly (I:C), a synthetic dsRNA, ligand of TLR3, impair thrombopoiesis by decreasing release of *in vitro* generated-platelets. Moreover, JV or Poly (I:C) increase the expression of functional IFN-I receptor and induce the synthesis/release of IFN-I in early progenitors and mature megakaryocytes. Although we have shown TLR3 expression in megakaryocytes by RT-PCR, its expression and functionality in the megakaryocytic linage has not yet been studied.

Aims: To study the expression and functionality of TLR3 in the megakaryocytic lineage.

Methods: TLR3 expression was determined in human hematopoietic progenitors cells ($CD34^+$), $CD34^+$-derived megakaryocytes and peripheral blood platelets by RT-PCR, immunofluorescence and flow cytometry (FC). The TLR3 signaling pathways were evaluated by western blot. Platelet functionality was assayed by lumiaggregometry and FC. IFN-β release was determined by ELISA.

Results: We found that TLR3 is expressed throughout the entire megakaryocytic lineage at both, mRNA and protein level ($CD34^+$cells: 95 ± 2, megakaryocytes: 76.5 ± 5.2, platelets: 21.5 ± 1.8% of positive cells FC, $n = 3$). Treatment of megakaryocytes with two synthetic dsRNA such as Poly(I:C) or Poly(A:U) (100 μg/mL), resulted in the activation of NF-kB pathway, evidenced by the degradation of its inhibitor I-κB and phosphorylation of the p65 subunit, as well as in phosphorylation of Akt, Erk1/2 and p38 MAPK, known downstream pathways of TLRs. Moreover, megakaryocyte activation by both dsRNA decreased platelet production *in-vitro* (Poly(I:C): 78.8 ± 2.9; Poly(A:U): 81.8 ± 3.5% of control $n = 4$, $P < 0.05$) and triggered IFN-β gene expression and release that were abolished by inhibition of PI3K/Akt pathway with Ly294002 (C:0; Poly(I:C): 770 ± 21; Poly(A:U): 650 ± 27; Ly+Poly(I:C): 0; Ly+Poly (A:U):0 pg/mL, $n = 2$).

Similar to megakaryocytes, TLR3 agonists induce the activation of NF-κB, Akt and Erk1/2 pathways in platelets. Although both TLR3 ligands did not induce platelet aggregation or degranulation, they potentiate the aggregation mediated by suboptimal concentrations of classical platelet agonists such as ADP (2.3 ± 0.8 fold of control), collagen (1.8 ± 0.8), arachidonic acid (1.3 ± 0.2) and thrombin (1.8 ± 0.5; $n = 4$, $P < 0.05$ for each agonist). This effect was correlated with an increased integrin-αIIbβ3 activation determined by fibrinogen binding (C: 2 ± 0.6; Thr: 50 ± 9.2*, Poly(I:C)+Thr: 87 ± 2.8*, Poly(A:U)+Thr: 84 ± 4.2*% of binding, $n = 4$, $^*P < 0.05$ vs. control), and inhibited by blockade of the Akt pathway (Poly(I:C)+Thr: 164.8 ± 14.2; Poly(A:U)+Thr: 148.3 ± 15.1; Ly+Poly(I:C)+Thr: 98.3 ± 4.9*; Ly+Poly(A:U)+Thr: 101.7 ± 8.2*% of Thrombin, $n = 4$, $^*P < 0.05$).

The potentiation effect was also observed in the ATP release ($n = 4$ $P < 0.05$), but not in P-selectin membrane exposure.

Summary/Conclusions: Our findings indicate that functional TLR3 is expressed through the megakaryocytic lineage and suggest a potential role of this receptor in the megakaryo/thrombopoiesis alterations observed in viral infections.

PB 1.29-6

Evidence for non-hematopoietic Cre activity in Pf4-Cre mice

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Background: Transgenic mice expressing Cre recombinase under the control of the Platelet factor 4 (Pf4) promoter are a powerful tool for genetic modifications in the platelet lineage. However, there has been recent indication of Pf4-Cre activity in hematopoietic progenitor and stem cells (Calaminus *et al*., *PloS one* 2012). In addition, our recent observation of tumors in descending colon in Pf4-Cre^{+} APC$^{flx/flx}$ (Adenomatous Polyposis Coli) mice suggests that the Pf4 promoter may even be functional in non-hematopoietic tissues.
Aims: The aim of our study was to evaluate expression of Pf4-Cre in several mouse organs and tissues.
Methods: PF4-Cre mice (C57BL/6-Tg(Cxcl4-cre)Q3Rsko/J) (Tiedt *et al*., *Blood* 2007) were crossed with a reporter mouse (B6.129(Cg)-*Gt(ROSA)26Sor^{tm4(ACTB-tdTomato,-EGFP)Luo}*/J) constitutively expressing the membrane-targeted tdTomato in all tissues. Following Cre-mediated recombination, the TdTomato gene is excised, allowing expression of the downstream eGFP cassette. Recombination was investigated in adult mouse organs or E14-15 whole embryos by confocal fluorescence microscopy and electron microscopy immunolabeling using anti-GFP antibody.
Results: As expected, all platelets exhibited recombination. Bone marrow and spleen also exhibited numerous recombined cells, some of which being identified by confocal microscopy as megakaryocytes. Electron microscopy immunolabeling of the bone marrow showed that these cells included stage I to stage III megakaryocytes, with a growing intensity according to cell maturity. The Pf4-Cre transgene thus appears to be expressed in the earliest detectable megakaryocytes. In addition, clusters of small recombined cells were also observed, which may be either megakaryocyte progenitors, or hematopoietic stem cells as previously suggested (Calaminus *et al*., *PloS one* 2012). A similar recombination of megakaryocytes and small cell clusters were present in fetal liver of E14-15 embryos. Recombined GFP-positive individual cells were also observed in whole embryo and adult tissues (heart, liver, lungs, pancreas, kidney, stomach, proximal intestine, caecum, colon, rectum), as infiltrating cells between tissue-specific cells. Some of these were macrophages as indicated by MOMA2- or F4/80-positive labeling. More surprisingly, we also observed eGFP expression in epithelial cells from both descending colon and rectum, but not in small intestine or ascending colon. This recombination displayed a mosaic pattern, and in most cases, only part of the gland was eGFP-positive, suggesting expression of Cre in some but not all stem cells. This expression of Cre in mouse colon and rectum may be at the origin of the tumor development in Pf4-Cre^{+} APC$^{flx/flx}$ mouse.
Conclusion: These data show that early recombination occurs in the megakaryocytic-lineage in PF4-Cre mice allowing to draw conclusion on genes important for megakaryocyte and platelet biology. In addition, we provide evidence that recombination may occur in other tissues that could result in unexpected phenotypes. Thus, care must be taken in interpretation of the data when performing integrated studies in whole animals.

PB1.30 – Microparticles and Disease – I

PB 1.30-1

Increased circulating microparticles and endothelial cells in patients with psoriasis

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Introduction: Psoriasis is a chronic pathology characterized by increased inflammation that can be associated with changes in the vascular endothelium.
Aim: We quantified the levels of circulating microparticles (MPs) and endothelial cells (CECs) in patients with psoriasis in order to analyze their relationship with endothelial and inflammation markers, subclinical atherosclerosis and microcirculation.
Patients and Methods: We studied 20 patients with psoriasis and 20 controls. Circulating markers of endothelial damage (MPs, CECs and von Willebrand factor, [vWF]) and inflammation (E-selectin, [E-sel]; Interleukin-6, [IL6] and C-reactive protein, [CRP]) were determined. Total MPs were quantified by flow cytometry by labeling with FITC-AnnexinV in an EPICS XL-cytometer. CECs were measured by an immunomagnetic technique and immunofluorescence microscopy. Subclinical atherosclerosis was assessed by carotid ultrasound to obtain intima-media thickness (IMT). Microcirculation was evaluated by nailfold capillaroscopy.
Results: CEC, MP, vWF, CRP and E-sel levels were significantly elevated in patients when compared with controls ($P < 0.05$). Ninety-four and 53% of patients had CEC and MP levels, respectively, higher than 99th percentile in controls. Forty-seven percent of patients simultaneously showed increased CEC and MP levels. MPs correlated with all inflammatory markers ($r = 0.68$–0.76, $P < 0.01$) and with IMT: mean IMT ($r = 0.76$, $P < 0.01$) and max IMT ($r = 0.60$, $P < 0.05$). CECs correlated with vWF ($r = 0.49$, $P < 0.05$) and with the number of capillaries per mm per mm ($r = 0.56$, $P < 0.05$).
Conclusion: Psoriasis patients show elevated CECs and MPs, as a sign of endothelial dysfunction which correlates with inflammation as well as some capillaroscopy findings and subclinical atherosclerosis.

PB 1.30-2

Chemotherapy and anti-angiogenic drugs affect composition and coagulant phenotype of cell-derived vesicles in cancer patients

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Background: The relationship between chemotherapy and circulating microparticles in patients with cancer is complex. First, release of cancer cell-derived microparticles may contribute to resistance of cancer cells to chemotherapy. Second, chemotherapy and angiogenesis inhibiting agents promote a prothrombotic state in cancer, and microparticles have been shown to exhibit both pro- and anticoagulant features.
Aim: The aim of the present exploratory study was to determine the effects of therapy on the composition and coagulant activity of circulating vesicles.
Methods: Blood was collected from 11 patients with glioma and 5 patients with lung cancer, at respectively 4 and 6 different time points before and after start of two different chemotherapy regimens. Age and sex matched healthy subjects ($n = 11$) were included for the glioma

patients. In glioma patients, temozolamide was combined with bevacizumab, an anti-angiogenic agent. Patients with stage IIIB or IV lung cancer were treated with either cisplatin and gemcitabine, or with daily radiotherapy and cetuximab. Procoagulant activity was studied in a fibrin generation test. For some experiments, the coagulant activity of exosomes and other small types of vesicles in plasma was determined by removing microparticles by centrifugation. Numbers of endothelial and tumour-derived microparticles were determined by flow cytometry.

Results: Treatment did not affect the overall procoagulant activity of vesicles in cancer patients ($P = 0.39$). Plasma of three patients had a detectable coagulant activity in the exosome fraction before therapy, compared to plasma from six patients after chemotherapy. Levels of endothelial microparticles ($CD62E^+$) tended to increase in the glioma ($P = 0.18$), but not in lung cancer patients ($P = 0.41$). Baseline levels of microparticles exposing vascular endothelial growth factor receptor-1 (VEGFR-1) were increased in cancer patients compared to healthy subjects ($P = 0.012$), and VEGFR-1-exposing microparticles decreased by 85% after anti-angiogenic therapy in glioma patients ($P = 0.021$). Finally, overall, no differences could be observed in the levels of mucine-exposing microparticles, except in two lung cancer patients which showed a clear increase two days after chemotherapy.

Summary/Conclusion: In this small explorative study, chemotherapy and anti-angiogenic therapy lead to specific changes in the composition of circulating vesicles, especially with regard to endothelial- and tumour-derived microparticles. These changes are markedly different between the two patients groups and even between subjects within one group, suggesting that such microparticles may be associated with prognosis or response to treatment. At baseline, the procoagulant activity was mainly associated with microparticles, whereas after chemotherapy also a part of the procoagulant activity is associated with smaller vesicles (exosomes) in some patients, suggesting a role for such vesicles in the prothrombotic state after chemotherapy.

PB 1.30-3

The role of breast cancer cell microparticles in thrombogenicity, angiogenesis and apoptosis following chemotherapy

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Background: Chemotherapy administered at the maximal tolerated dose (MTD) induces death of tumor cells and disruption of tumor blood vessels, as well as an increased incidence of thrombosis. MTD chemotherapy is followed by increased levels of microparticles (MPs) – vesicles of approximately 1 μm that are shed from cells membrane upon activation or apoptosis. Previous studies demonstrated that plasma of breast cancer patients with large tumor masses or distant metastases contains significantly more MPs than that of healthy subjects. MPs bearing tissue factor (TF) – the main activator of the coagulation cascade – play a role in pathogenesis of the pro-thrombotic state in cancer patients. In addition, MTD chemotherapy is followed by rebound angiogenesis facilitating tumor re-growth. We assume that post-chemotherapy tumor-MPs are involved in cancer thrombogenicity, angiogenesis and tumor invasion.

Study Aims: (i) Isolation and characterization of MPs obtained from breast cancer patients prior to and during chemotherapy administration and from human breast cancer cell lines in the presence or absence of chemotherapy drugs. (ii) Evaluation of the thrombogenic, angiogenic, invasive and apoptotic effects of these MPs on breast cancer cell lines and endothelial cells.

Methods: MPs were isolated from blood samples of the two groups during chemotherapy: (i) Patients after surgical removal of small tumors (chemotherapy is given as adjuvant treatment); (ii) Patients after biopsy only (chemotherapy is given as neo-adjuvant (pre-surgery) treatment). In addition, MPs were isolated from breast cancer cell lines: MCF-7 human breast adenocarcinoma and estrogen receptor (ER) positive and MDA231, ER negative, following exposure to starvation or chemotherapy. MPs of all types were characterized by cell origin, thrombogenic and angiogenic protein profile. MPs structure was evaluated by electron microscopy. In addition, MPs apoptotic effects on endothelial and breast cancer cell lines were assessed by the Tunnel assay and their angiogenic effects were evaluated by migration assay, and time laps microscopy.

Results: Breast cancer cell lines stimulation led to increased levels of cell apoptosis and release of MPs. Breast cancer MPs adhered to endothelial cells penetrated into the cells, affected cell hemostasis and distracted cells gap junctions. MDA231-MPs assessed after high-dose chemotherapy, showed increase in procoagulant activity and higher level of VEGF-receptor-1 compared to MPs obtained after low-dose chemotherapy. MDA231-MPs isolated following starvation adhered to endothelial cells, penetrated into the cells and affected cell angiogenesis by inducing tube formation, while MPs assessed after low-dose chemotherapy inhibited tube formation. Patients' MP TF/TFPI ratio, reflecting MPs thrombogenicity, increased during chemotherapy mainly as a result of decrease in the coagulation inhibitor TFPI on MPs surface.

Summary: We suggest that MPs of breast cancer patients may reflect the physiologic state and efficacy of the treatment and affect the hemostatic balance and angiogenesis. This research may clarify the role of tumor MPs in hypercoagulable states and angiogenic rebound associated with chemotherapy.

PB 1.30-4

Microparticle characterization in patients with acute leukemia at diagnosis and after induction therapy

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Background: Microparticles (MPs) are membrane vesicles shed from various cells that may express antigens reflecting their cellular origin. Tissue factor (TF)-bearing MPs play a major role in the pathogenesis of the prothrombotic state observed in patients with malignancies. Acute myeloid leukemia (AML) is characterized by rapid growth of abnormal blast cells that accumulate in the bone marrow and interfere with production of normal blood cells. AML patients can develop venous thromboembolism despite thrombocytopenia. We hypothesize that circulating MPs may serve as a biomarker, capable to reflect the change in blood cells population and predict thrombogenic states in AML patients at diagnosis and in remission.

Methods: Blood samples were collected from healthy controls and patients with newly diagnosed AML at three time points: diagnosis, 2 weeks after treatment initiation and at remission achievement.

Microparticle cell origins were characterized by specific fluorescent antibodies and analyzed by FACS. The fluorescent antibodies included: CD41 (platelet glycoprotein complex), CD 62P (P-selectin expressed on activated platelets); CD62E and CD144 (endothelial markers), CD11 (leukocyte marker), CD14 (monocyte marker) and Annexin V (for MPs expressing negative phospholipids on their surface). To distinguish MP from cancer cells, MPs were labeled by CD34, CD117, CD33, and HLA-DR (for leukemic blast cells).

In FACS analysis used to determine the pro- and anti-coagulant potential of microparticles in the study groups, each sample of MPs was labeled with fluorescent antibodies against TF, TF pathway inhibitor (TFPI) and markers of the protein C anticoagulant pathway – endothelial protein C receptor (EPCR). The TF/TFPI ratio, potentially contributing to detection of the hypercoagulable state was calculated.

Results: Twenty AML patients were enrolled in the study; 13 patients achieved remission following induction therapy. The average MP count in patients at diagnosis was higher than in controls and than

that observed at two other points of treatment (at nadir and remission), although the difference did not reach statistical significance. Conversely, the level of Annexin V expression was significantly higher in controls compared to patients at three time points of sampling.
The platelet marker CD41 was significantly higher in controls compared to the rates of patients at diagnosis (33.7% vs. 5.9%; $P < 0.05$), without changes in platelet activation markers. The endothelial marker CD144 appeared to be higher in patients' MP at diagnosis compared to controls (16.4% vs. 4.48%; $P < 0.05$). The blast cells marker CD34 was significantly elevated in patients at diagnosis compared to controls (3.15% vs. 0.0; $P < 0.001$) and to patients in remission (3.15% vs. 0.0; $P < 0.05$). TF expression was similar in controls and patients at three time points tested; however, the TFPI level was higher in controls' MPs compared to patients, without changes in MPs EPCR.
Conclusion: In AML patients, the MPs number was higher at diagnosis and reduced after treatment. MPs of AML patients at diagnosis express markers of blast cells and may serve as a biomarker for disease and remission. Increase in endothelial MP level at AML diagnosis may indicate vascular injury, which could result in TFPI reduction and lead to the hypercoagulable state.

PB 1.30-5
Anticoagulant activity of MP in patients with atherosclerosis of the vessels of the lower extremities

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Background: Activation of blood cells and endothelium at atherosclerotic diseases leads to microparticles (MP) release. MP contributes to the development of hypercoagulability due to expression tissue factor (TF) and procoagulant phospholipids (PPL) on their surface. It is known that endothelial MP are enriched with the molecules such as tissue factor pathway inhibitor (TFPI), thrombomodulin, endothelial protein C receptor and may have anticoagulant activity.
Aim: to determine if thrombin generation caused by PPL of MP is limited by anticoagulant activity of MP in patients with atherosclerosis of the vessels of the lower extremities.
Methods: This research included 29 patients (10 females and 19 males) at the age from 44 to 77 with atherosclerosis of the vessels of the lower extremities. Reference blood samples were obtained from 30 healthy controls. Thrombin generation was measured in platelet-free plasma with Calibrated Automated Thrombinogram Assay method (CAT). "PRP-reagent", containing rTF, (1 pM) and 'FluCa–kit' (Thrombinoscope BV, The Netherlands) were used as triggers. Following parameters were derived: endogenous thrombin potential (ETP, nM-min), peak thrombin activity (Peak, nM), rate of thrombin generation (R, nM/min) lag-time (LT, min) and time to peak (TTP, min). Data were described by non-parametric methods using the median (Me), 50% confidence interval (CI) and Mann-Whitney U test (Statistica 6.0). $P < 0.05$ was considered sighnificant.
Results: There were not any differences in ETP, Peak and R in patients as compared with controls (ETP: Me-508.00, CI: 372.50–623.50 vs. Me-471.84, CI: 384.25–564.00, $P > 0.05$; Peak: Me-16.89, CI: 11.66–21.68 vs. Me: 18.40, CI: 12.29–20.26, $P > 0.05$; R: Me-1.48, CI: 1.05–2.01 vs. Me-1.59, CI: 1.19–2.01, $P > 0.05$). LT and TTP were longer significantly in patients than in controls (LT: Me-11.33, CI: 10.50–13.83, vs. Me-8.93, CI: 8.28–9.53, $P < 0.0001$; TTP: Me-23.08, CI: 21.17–26.25 vs. Me-19.90, CI: 18.68–20.97, $P < 0.0001$). We suppose that TFPI expressed on the MP surfaces may be a reason for LT prolongation and limitation of thrombin generation in patients with atherosclerosis of the vessels of the lower extremities.
Conclusion: Anticoagulant activity of MP balances the procoagulant properties of PPL MP in patients with atherosclerosis of the vessels of the lower extremities. The nature of the anticoagulant activity is needed in further investigation.

PB 1.30-6
Granulysin and other inflammatory mediator induced procoagulant response in patients with Stevens Johnson syndrome/toxic epidermal necrolysis

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Background: Stevens Johnson syndrome and toxic epidermal necrolysis (SJS/TEN) are life-threatening adverse drug reactions characterized by blisters and skin necrosis often accompanied by ocular inflammatory manifestations. Currently, there is no effective treatment. Although granulysin and tumor necrosis factor-α have been reported to be highly expressed in the skin lesions, a systemic procoagulant response has not been reported. Because of the massive inflammatory state, it was projected that these patients may manifest a procoagulant response.
Materials and Methods: Following institutional review board approval of the clinical study, blood samples and swabs from ocular, oral mucosal and skin lesions were obtained from SJS/TEN confirmed patients ($n = 3$) and from patients ($n = 5$) with systemic dermatologic conditions and associated skin sloughing, in whom the diagnosis was biopsy confirmed negative, and were considered as abnormal controls. Samples from normal healthy volunteers ($n = 6$) served as normal controls. The citrated blood samples were centrifuged to obtain platelet poor plasma which was aliquoted and kept frozen at −70 °C. The plasma samples were thawed and analyzed to determine thrombin antithrombin complex (TAT., Dade-Behring®, Marburg, Germany), prothrombin fragment F 1.2 (F 1.2, Dade-Behring®, Marburg, Germany), plasminogen activator inhibitor-1 (PAI-1, Diagnostica Stago®, Parsippany, NJ), using ELISA kits and ZYMUPHEN platelet microparticle activity (MP) (Hyphen® BioMed (Neuville-Sur Oise, France), HEMOCLOT protein C and Stachrom antithrombin (Diagnostica Stago, Parsippany, NJ) using functional assays per manufacturer's instructions. The swabs collected were immediately frozen at −70 °C and thawed and the exudates were isolated following the addition of 0.25 mL of saline to each swab and double centrifugation. The exudates and plasma samples were analyzed using a SELDI-TOF, mass spectrometric technique for unique biomarkers.
Results: Analyses of the plasma samples revealed that, there was a marked increase in the TAT (6.3 ± 5.9 μg/mL), F1.2 (430.4 ± 202.4 pM), MP (13.1 ± 9.3 nM) and protein C levels ($90.5 \pm 63.4\%$) levels, a decrease in PAI-1 (53.3 ± 18.8 ng/mL) and antithrombin levels ($80.7 \pm 42.4\%$) compared to normal human plasma, confirming a procoagulant response. Mass spectrometric analysis of the swabs revealed the presence of unique biomarker peaks in some of the patients samples. The recombinant human granulysin exhibits a molecular weight of 15.4 kDa. In the protein chip array, it exhibited two major peaks at 14.2 and 15.6 kDa. In contrast to the controls, the skin and oral mucosal exudates showed distinct peaks in the same molecular weight range as that of granulysin. The ocular discharges did not exhibit any peaks in this range.
Conclusion: Since unique peaks were noted in the ocular, oral mucosal and skin discharges similar to granulysin, the observed peaks maybe related to this mediator. Analysis of plasma samples revealed a procoagulant state as demonstrated by the increase in the thrombin generation markers, protein C and MP, and decrease in the fibrinolytic inhibitor PAI-1. Additional clinical validation of these findings will provide guidance to risk stratification and development of novel therapeutic options including the specific antagonist to such mediators as granulysin and TNF-α.

PB1.31 – Endothelial Function

PB 1.31-1

Procoagulant activity at the margins of TNFa treated endothelial cells leads to pericellular fibrin deposition and anti-streptococcal function in flowing plasma

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Background: Inflammation causes endothelial cells to relax junctions with neighboring cells, to thicken the cell body, and to extend filopodia. We recently reported localized procoagulant activity on filopodia, related to focal phosphatidylserine exposure and high curvature. We asked whether this pattern would lead to pericellular fibrin deposition without clotting of bulk plasma.
Aims: Our aims were (i) to determine whether TNFa treated endothelial cells generate pericellular fibrin while maintaining anticoagulant function toward bulk plasma (ii) To determine which coagulant pathways regulate pericellular fibrin deposition and (iii) to determine the extent to which the pericellular procoagulant response contributes to innate immunity against group A streptococci.
Methods: Human umbilical vein endothelial cells were cultured to confluency in 30 mm microtiter dishes and in an 'endothelialized' microfluidic coated with fibronectin. Recalcified plasma or fresh plasma treated with corn trypsin inhibitor ± 0.1 μ/mL heparin was overlaid on cells and perfused through the microfluidic at shear rates of 10–100/s. Fibrin was detected with fluorescein-labeled mAb 59D8, specific for the beta chain of fibrin, or by adding fluorescent fibrinogen to the plasma. The quantity of fibrin was measured in the presence of specific inhibitors. *Streptococci pyogenes* strain 700294 from ATCC was grown according to standard techniques.
Results: Plasma did not clot over endothelial cells but did clot over control gelatin-coated coverslips. Confocal microscopy showed fibrin strands between TNFα-treated endothelial cells that were largely absent from untreated cells. Corn trypsin inhibitor-treated plasma infused at sheer rates of 10–100/s led to deposition of fibrin margins demarcating TNFα treated endothelial cells without deposition on or around untreated cells. Thus, the inflammatory response of TNFα-treated HUVEC's leads to pericellular fibrin deposition without clotting of bulk plasma. Fibrin was diminished by an anti-tissue factor mAb and by hirudin. Fibrin was increased by an anti-tissue factor pathway inhibitor mAb but not by corn trypsin inhibitor, anti-activated protein C mAb, anti-thrombomodulin mAb, or anti-factor VIII mAb. Thus, focal fibrin deposition is mediated by the extrinsic coagulation pathway.
Streptococcus pyogenes, was added to plasma at a concentration of 10^3 cfu/mL. The concentration of viable bacteria decreased > 90% in 1 h at 37 °C in the presence of TNFα treated endothelial cells. Addition of hirudin enabled Streptococci to grow at the same rate as in citrated plasma alone or re-calcified plasma incubated over quiescent endothelial cells. Confocal microscopy indicated that streptococci decorated the fibrin strands in the inter-endothelial space but not the intercellular matrix or the endothelial cell bodies.
Summary: TNFα treated HUVECs demonstrate pericellular procoagulant activity via the extrinsic coagulation pathway while maintaining cell-centered anticoagulant function. Pericellular fibrin contributes to innate immune response against streptococci pyogenes in the absence of phagocytes or platelets. This suggests a role of pericellular procoagulant activity in innate immunity. We are currently investigating the range of bacteria that may be influenced by this arm of innate immunity and the efficiency of this mechanism as a function of vessel dimensions and sheer rate.

PB 1.31-2

Effects of antiretroviral treatment on endothelial dysfunction and regeneration in HIV-positive patients: 1-year of follow-up

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Background: To evaluate the effect of antiretroviral therapy (ART) on the markers of endothelial dysfunction and regeneration, such as circulating endothelial cells (CECs) and endothelial progenitor cells (EPCs) in a population of HIV naïve positive patients.
Methods: Sixteen HIV+ patients naïve for antiretroviral drugs (M15, F 1) with a median age of 42 (22–65) years and 16 age- and sex-matched control subjects were enrolled in the study. Ten of 16 HIV positive patients were treated with ART therapy for 1 year. Circulating CECs were defined as CD146+/CD31+/CD45−/CD61−, while EPCswere defined as CD34+KDR+, CD133+KDR+ and CD34+CD133+KDR+.
Results: Before antiretroviral treatment HIV positive subjects showed a significant higher number of CECs and a significant lower number of EPCs with respect to the controls [CD146+/CD31+/CD45−/CD61− 8 (3–23) cells/10^6events vs. 3 (0–13) cells/10^6events $P = 0.021$; CD34+/KDR+ 7 (0–23) cells/10^6events vs. 16 (3–40)cells/10^6events, $P = 0.047$; CD133+/KDR+ 7 (0–23) cells/10^6events vs. 13 (7–43) cells/10^6events $P = 0.043$]. After 1 year, 8/10 (80%) patients in ART showed a marked decrease in CECs number and 6/10 (60%) in EPCs number, whereas in patients who did not receive antiretroviral therapy the numbers of CECs and EPCs decreased in 3/6 (50%) and 5/6 (83.3%) respectively. After 1-year of follow-up HIV+ patients who received antiretroviral therapy showed lower number of CECs with respect to HIV+ patients who did not receive the treatment [CD146+/CD31+/CD45−/CD61 2 (0–15) cells/10^6events vs. 10 (0–37) cells/10^6events $P < 0.05$], whereas EPCs number was similar in both groups.
Conclusions: Our data demonstrate the presence of an endothelial dysfunction, as documented by low EPCs and high CECs number, in naïve HIV positive patients with respect to a control population. Moreover present results suggest that ART is associated with an improvement of the markers of endothelial dysfunction.

PB 1.31-3

Regulation of endothelial cell proliferation and survival by collagen receptors

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Background: Collagen has been identified as a suitable biomaterial to be used in tissue engineering, a medical therapy that involves implanting a 3D scaffold seeded with cells *in vivo*. Vascularisation of an implant is a prerequisite for success. Various types of collagen promote or inhibit endothelial cell proliferation; however the distinct segments of collagen that supports endothelial cell proliferation and survival are yet to be identified.
Aims: To identify critical collagen segments that support and enhance endothelial cell proliferation and survival using the collagen Toolkits and other triple-helical peptides, to specifically target collagen receptors on HUVECs (human umbilical vein endothelial cells).
Methods: Twelve-well plates were coated with collagenous ligands and HUVECs were allowed to adhere and proliferate. Changes in the cell numbers were detected at various time points, evaluated by CellTracker, Hoechst 33342 and propidium iodide staining. At each end-point, simultaneous labeling with fluorescent Annexin V and propidium iodide were used to resolve cells undergoing necrosis and apoptosis.

Additionally, rapid real-time live cell monitoring was performed using Acea xCELLigence cell-index analyser.

Results: We identified a direct relationship between collagen-receptor affinity and endothelial cell proliferation and survival. Significant elevation of proliferation and prolonged survival was observed on endothelial cells cultured on high affinity collagenous ligands. Co-staining with Annexin V and propidium iodide revealed high affinity peptide such as GFOGER prevented necrosis and delayed apoptosis of HUVECs.

Conclusion: Neovascularisation is a critical step in wound healing and tissue repair. The response of endothelial cells to collagen is therefore of great importance in tissue engineering. We have identified collagen peptides that encourage endothelial cell proliferation and are central to delaying cell death using receptor specific ligands. This finding may offer vast improvements in tissue engineering where, as necessary, such ligands can be used as biomaterials to decorate 3D scaffolds.

Reference: 1. Farndale et al. (2008) Cell-collagen interactions: the use of peptide Toolkits to investigate collagen-receptor interactions. *Biochem. Soc. Trans.* **36**, 241–250

PB 1.31-4

A role of cilostazol in Alzheimer's disease treatment: induction of LRP1 expression in endothelial cells

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Background: Alzheimer's disease (AD) is a type of progressive neurodegenerative disease exhibiting the clinical cardinal symptoms of the impaired memory and cognitive function, observed with the extracellular deposition of beta-amyloid peptide (Aβ), neurofibrillary tangle due to the abnormal phosphosphorylation of tau protein, and neuronal cell death in the brain of AD patients. Vascular endothelial cells play a major role in controlling the Aβ level in the brain via low density lipoprotein receptor related protein 1 (LRP1) and receptor for advanced glycation end products (RAGE). In addition, the destruction of tight junction in the blood brain barrier is observed in AD. Hence, protecting function of vascular endothelial cells could exhibit preventive/improving effect in medical treatment for AD.

Cilostazol is an inhibitor of phosphodiesterase III (PDE3), which is a degrading enzyme of cAMP and the cGMP, and has a protective effect on endothelial functions.

Aim: This study clarified the therapeutic effect and mechanism of Cilostazol in AD.

Methods: Cilostazol was administered to amyloid precursor protein Swedish transgenic (APPswe) mice, and then learning and memory function was evaluated using a Morris Water Maze (MWM) test. The Aβ and LRP1 levels in the brain were detected by immunohistochemistry and Western blotting.

Using bEnd3 cells, vascular endothelium cell, the signaling pathway involved in the Cilostazol-induced LRP1 expression was analysed by western blotting.

Results: Memory and learning function was improved, and the intracerebral Aβ level was significantly declined due to the cilostazol administered. Cilostazol increased the expression of LRP1 in vascular endothelial cells, accelerated the clearance of intracerebral Aβ. The activation of JNK, ERK and Akt was inhibited by Aβ, but recovered by Cilostazol. Pharmacological analysis revealed Akt was involved in the Cilostazol-induced LRP1 expression. In addition, the cGMP pathway, but not cAMP pathway, mediated the induction of LRP1.

Conclusion: Cilostazol increases the expression of LRP1 in vascular endothelial cells through the cGMP and Akt pathway, thereby reducing the intracerebral Aβ level and improving learning and memory function in AD.

PB 1.31-5

Closely spaced thiols in integrin are involved in adhesion of endothelial cells

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Activation of integrins, transmembrane receptors that mediate cell adhesion and migration, is accompanied by a series of conformational rearrangements resulting in changes in affinity and avidity. Several observations indicate that conformational changes induced by ligand interaction with integrins lead to exchange of disulfide bonds within the integrin molecule, which stabilizes the altered conformation. Closely spaced thiols in proteins that interconvert between the dithiol form and disulfide bonds are called vicinal thiols. The purpose for this study was to examine whether vicinal thiols are involved in adhesion process of endothelial cell. The manganese ions are know to affect the thiol-disulfide balance and activate integrin to maximal affinity. In the present study, we attempt to explain whether activation of integrins in endothelial cells by Mn^{+2} might involve vicinal thiols. Human umbilical vein endothelial celle (HUVEC) were cultured in medium 200 supplemented with low-serum growth supplement. Labeling of sulfhydryl groups was performed using the poorly membrane-permeable maleimide reagent (MPB). Protein labeled with MPB were precipitated using avidin-Sepharose, electrophoresed and transferred on nitrocellulose. Biotinylated proteins were detected using streptavidin-horseradish peroxidase with chemiluminescent substrate. In some experiments, the MPB-labeled cells were used for immunoprecipitation performed with monoclonal or polyclonal antibody to αvβ3 integrin. For cell adhesion assay the plates were coated with fibrinogen, fibronectin or vitronectin. In some cases the cell were pretreated with phenylarsine oxide (PAO) and added to the plate. The binding of vitronectin or LM609 antibody to endothelial cells activated by manganese ion were evaluated by flow cytometry method. The studies ith membrane-impermeable reagent 3-N-maleimidylpropionyl biotin (MPB) demonstrate that exposure of endothelial cells to Mn^{+2} results in the appearance of surface protein thiol groups, which can found in αvβ3 integrin. Phenylarsine (PAO), a reagent that binds vicinal thiols inhibit adhesion of endothelial cells activated by $Mn^{+2.}$ Additionally, PAO inhibit sulfhydryl labeling of thiols in αvβ3 molecule. The αvβ3 contains vicinal thiols that provide sites for redox regulation of function of this integrin. Closely spaced thiols are involved in activation of αvβ3 integrin during adhesion of endothelial cells.

PB 1.31-6

Cocaine induces oxidative stress and decreased nitric oxide production in human endothelial cells: beneficial effect of atorvastatin

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Background: Cocaine abuse is associated with an increased risk of cardiac and cerebrovascular events. The underlying mechanisms leading to these complications are not fully understood although accelerated atherosclerosis is a prominent finding. We have recently demonstrated that chronic cocaine use is associated with endothelial dysfunction (Sáez et al. Thromb Res 2011; 128: 18) and RhoA/Rho kinase (ROCK) pathway activation (Blood 2012; 120:2177), a key event in the onset and progression of atherosclerosis. Several experimental studies have showed that activation of the Rho kinase pathway is related to an increase in oxidative stress. On the other hand, ROCK also mediates the down-regulation of endothelial nitric oxide (NO) synthase (eNOS) with decreased vascular NO bioavailability, the hall-

mark of endothelial dysfunction. Inhibition of Rho function by statins increases NO production.

Aim: The main aims of these studies were: (i) to test the hypothesis that cocaine-induced activation of ROCK in endothelial cells is associated with oxidative stress and decreased NO synthesis and (ii) to investigate the effect of atorvastatin on these effects *in vitro*.

Methods: Human umbilical vein endothelial cells (HUVECs) were cultured under standard conditions and supplemented for 5 h with plasma from chronic cocaine users, normal plasma, cocaine (10 μM) or vehicle. After media removal, HUVECs were lysed for determination of ROCK activity by western blot assessing the levels of phosphorylated to total myosin light chain phosphatase 1 (MYPT1-P/T). The production of NO and ROS generation were determined by fluorometric assay using DAF-2DA (5 μM) or DCF-DA (100 μM), respectively and the results expressed as arbitrary fluorescence units (AFU). Experiments were conducted in the presence or absence of atorvastatin (10 μM).

Results: HUVECs supplemented with plasma from chronic cocaine users showed: (i) an increase in ROCK activity by 25% (*P*: 0.039) and (ii) a significant decrease in NO production (*P*: 0.029). Exposure of cells to cocaine 10 μM resulted in: (i) a significant increase in ROCK activity by 95% (*P*: 0.04); (ii) an increment in ROS production as compared with vehicle treated cells (8.0 ± 0.3 vs. 2.72 ± 0.9 AFU, respectively; *P*: 0.0035) and (iii) a significant decrease in NO production with respect to cells exposed to vehicle (0.1 ± 0.04 vs. 0.3 ± 0.01 AFU, respectively; *P*: 0.017). Atorvastatin (10 μM) reduced significantly ROCK activity (*P*: < 0.01) and increased NO production (*P*: 0.048) in cells incubated with plasma from cocaine users. In cells exposed to cocaine, atorvastatin inhibited ROCK activity (*P*: 0.039); increased significantly NO production (*P*: 0.03) and showed no effect on ROS generation (8.0 ± 0.32 vs. 5.8 ± 1.8; *P*: 0.6).

Conclusions: Our results show that oxidative stress and impairment in NO production may play a key role in cocaine-associated endothelial dysfunction. The lack of effect of atorvastatin on the production of ROS by cocaine, suggest that their production probably involves mechanisms upstream of Rho/Rho kinase signaling pathway. However, the significant improvement in NO generation induced by atorvastatin may constitute a new therapeutic tool to prevent or ameliorate ischemic complications as part of the comprehensive management of cocaine addiction. (Fondecyt 110418).

PB1.32 – Atherosclerosis: Mouse Models

PB 1.32-1

Liver X receptor (LXR) agonist T0901317 induces regression of early and advanced atherosclerotic lesions under normolipidemic conditions in mice

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Background: Ligand-mediated activation of the liver X receptor (LXR) induces changes in gene expression, aimed at limiting build-up of pathogenic levels of cholesterol. LXR agonists have been shown to inhibit the initiation and progression of atherosclerosis. However, a key goal in the treatment of cardiovascular diseases is the regression of pre-existing atherosclerotic lesions.

Aim: In the current study, we evaluated the potential of LXR agonist T0901317 to regress both early fatty streak lesions and advanced collagen-rich atherosclerotic lesions.

Methods: Experiments were performed with C57BL/6 wild-type (WT) mice, LDL receptor (LDLr) knockout (KO) mice and apolipoprotein E (apoE) KO mice. C57BL/6 mice were fed with semi-synthetic cholate-containing cholesterol-enriched atherogenic diet containing 15% (w/w) cocoa butter, 1% (w/w) cholesterol, and 0.5% cholate for 16 weeks to induce atherosclerotic lesion development. LDLr KO mice were fed with semi-synthetic Western-type diet (WTD) containing 15% (w/w) fat and 0.25% (w/w) cholesterol for 6 weeks to induce atherosclerotic lesion development. ApoE KO mice were fed with WTD for 3 days or 3 weeks to induce the formation of initial or advanced atherosclerotic lesions respectively, and were subsequently transplanted with WT bone marrow (BM) to restore plasma apoE levels and normalize plasma lipid levels. After the formation of atherosclerotic lesions in all studies, diet was switched to regular cholesterol-free chow diet containing 4.3% (w/w) fat for 3 weeks (LDLr KO or WT mice) or 6 weeks (apoE KO mice), with or without supplementation of T0901317 (10 mg/kg/day).

Results: In LDL receptor knockout mice, T0901317 dramatically worsened plasma lipoprotein profiles and failed to induce lesion regression. In contrast, in wild-type C57BL/6 mice, chow diet in combination with T0901317 improved plasma lipoprotein levels and induced lesion regression (−43%, $P < 0.05$). BM apoE reconstitution in apoE KO mice dramatically improved plasma lipoprotein profiles and resulted in a marked regression of initial (−45%, $P < 0.001$) and advanced lesions (−23%, $P < 0.01$) Regression was associated with a dramatic decrease in the absolute macrophage content (−84%, $P < 0.001$) Treatment with T0901317 further decreased size of early (−71%, $P < 0.001$ vs. baseline; −48%, $P < 0.01$ vs. chow diet alone) and more advanced lesions (−36%, $P < 0.001$ and −17%, $P = 0.06$ respectively).

Conclusion: Our study shows that normal apoB-lipoprotein clearance is crucial to achieve lesion regression through LXR activation. Lesion regression induced by T0901317 is mainly the result of the disappearance of macrophages from atherosclerotic lesions ultimately leading to a highly significant reduction of the size of both early and advanced atherosclerotic lesions.

PB 1.32-2

Replacement of Apob peptide sequence with C5ar peptide sequence in a recombinant vaccine construct significantly increases the atheroprotective effect of immunization in Apobtm2SgyLdlrtm1Her/J mouse

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Background: Apolipoprotein B-100 (ApoB-100) is a major constituent of LDL and immune responses against ApoB-100 peptide sequences have been shown to reduce the development of atherosclerosis in $ApoE^{-/-}$ mice or $Apob^{tm2Sgy}Ldlr^{tm1Her}$/J mice (Schiopu A, et al Circulation. 2004; 110:2047–2052; Schiopu A, et al. J Am Coll Cardiol. 2007; 50:2313–2318; Lu X, et al. Atherosclerosis. 2010; 212:472–480) while immunization of mice with an N-terminal peptide has been shown to reduce atherosclerotic lesion formation through the induction of a specific Treg-cell response and blocking monocyte differentiation into macrophages (Lu X, et al. Arterioscler Thromb Vasc Biol. 2012;32:2358–2371).

Aim: To compare the effect of immunization with either C5aR peptide epitope or ApoB peptide epitope in a same recombinant construct on atherosclerotic lesion reduction in $Apob^{tm2Sgy}Ldlr^{tm1Her}$/J mice.

Methods: Six-week-old $Apob^{tm2Sgy}Ldlr^{tm1Her}$/J mice were immunized using a repetitive immunization multiple-sites strategy with GST-tagged recombinant constructs containing either C5aR epitope or ApoB epitope in a same protein scaffold. Mice were fed a high-fat diet for 10-week period. Lesions were evaluated histologically; local and systemic immune responses were analyzed by immunohistochemistry

of aorta samples and cytokine measurements in plasma samples and splenocyte supernatants.
Results: High levels of ApoB and C5aR antibodies were detectable 2 weeks after the first immunization with either ApoB epitope-containing or C5aR epitope-containing construct, respectively. Histological analyses demonstrated that mice immunized with C5aR epitope-containing construct showed significantly less lesion occupied area in the aorta sinus than that of mice immunized with ApoB epitope-containing construct (12% vs. 17% $P < 0.05$) when compared to the lesion occupied area in control group (38%). These results were further supported by significant differences in respect of the cellular and humoral immune responses between test animals.
Conclusions: Replacement of ApoB peptide sequence with C5aR peptide sequence in a recombinant construct may provide new antigenic and structural features which are favorable for its immunogenicity, thus leading to significantly reduced atherosclerotic lesion formation when used as an immunizing antigen. This approach offers a novel strategy for developing anti-atherosclerotic agents.

PB 1.32-3
Immune response and gene profiling at different stages of atherosclerotic plaque progression using mouse model

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Background: Atherosclerosis progression can be divided into several stages starting from early fatty streak formation to the matured plaque. The disease is complex in nature and involves expression of several genes during the development of the disease. We used ApoB/LDLr$^{-/-}$ to study the gene expression in different stages of lesion progression. The study was focused on MicroRNAs (small non-coding RNA's) that act as negative regulators of gene expression by inhibiting the translation or promoting the degradation of target mRNAs. Because individual microRNAs are responsible for regulation of the expression of multiple target genes with related functions, modulating the expression of a single microRNA instead of a single gene can actually influence an entire gene network.
Aim: To study the peripheral immune markers, progression of disease and gene profiling at different stages of plaque development in mice
Methods: Atherosclerosis was induced in groups of Apob^{tm2Sgy} Ldlr^{tm1Her}/J mice by feeding them with diet rich in cholesterol. Mice fed with normal chow diet were taken as control. Global gene expression was performed with RNA extracted from ascending aorta with atherosclerotic lesion at 4, 8, 14 and 20-week time points. Samples were hybridized in Agilent 8 × 60K mouse gene expression array slides and scanned. Genes that were twofold differentially regulated in high fat diet (HFD) samples in comparison to 'Chow' was obtained from Volcano plot. Significant Gene ontology categories and KEGG pathways were dysregulated using the software. Hierarchical clustering of differentially regulated genes was done for both entities and samples using Pearson uncentered algorithm with average linkage rule.
Results: The cDNA microarray analysis revealed a total of 62 miRNA genes which showed differential expression in high fat diet compared to the chow diet fed mice. Among these let-7/98, miR-1/206, miR-10, miR-125/351, miR-128, miR-129/129-5p, miR-137, miR-138, miR-141/200a, miR-149, miR-182, miR-185/882, miR-200bc/429, miR-203, miR-205, miR-208/208ab, miR-214/761, miR-218, miR-221/222, miR-23ab, miR-26ab/1297, miR-27ab, miR-28/28-5p/708, miR-33/33ab, miR-335/335-5p, miR-342/342-3p, miR-384/384-3p, miR-495/1192, miR-499/499-5p, miR-539 were significantly upregulated in mice fed with high fat diet compared to chow diet. It is interesting to note that most abundant miRNAs (with respect to the maximum number of targets) expressed in the murine aorta belong to the miR-340/340-5p and miR-218 (14%), the miR-19 (13%), the miR-196ab (11%) and let- 7/98 (8%) of all the total miRNAs. To validate the results, RT-PCR analysis of the samples was being done. We believe that further elucidation of the role of these miRNAs will help us to understand the disease progression leading to new therapeutic and preventive strategies.
Conclusions: This data opens up the possibility of identifying miRNA's as therapeutic target for prevention of atherosclerosis

PB 1.32-4
Oral administration of recombinant multi antigenic construct expressing three peptides induces tolerance to individual peptides and prevents development of atherosclerosis in mice

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Background: Atherosclerosis is a chronic inflammatory disease of the arterial wall characterized by accumulation of lipids and immune inflammatory cells. Inflammation mediated by a pathogenic T-cell response to self antigens like modified low density lipoproteins (LDL) and heat shock proteins (HSP) as well as exogenous antigens from pathogens, have been implicated in the initiation of an autoimmune response during atherogenesis. Induction of regulatory immune response to these antigens has shown protection against disease development. We believe that each of these self antigens has a distinct role to play in the pathogenesis of atherosclerosis and using multiple antigens would be more effective for a immune therapy against the disease than either of the individual antigens. We have earlier reported that a recombinant construct expressing multiple antigens can control atherosclerosis in mice.
Aim: The objective of the present study was to evaluate whether oral administration of a multi antigenic construct expressing epitopes from apolipoprotein B 100 (ApoB), heat shock protein (HSP60) and Chlamydia pneumonia outer membrane protein (Cpn), can induce immunological tolerance to individual peptides and prevent atherosclerosis development in mouse model.
Methods: ApoB, HSP60 and Cpn peptides were cloned and expressed as part of the dendroaspin protein scaffold. Groups of Apob^{tm2Sgy} Ldlr^{tm1Her}/J mice were given five oral doses of recombinant multi antigenic construct on alternate days. Mice were fed with high-fat diet for 10 weeks to induce development of atherosclerosis. Antigen specific tolerance was studied by Treg functional assay with individual peptides. Quantification of atherosclerotic lesions was carried out in the aortic sinus sections stained with Elastic van Geison. Immunohistochemical analyses were carried out by indirect immunofluorescence. Flow cytometry was used to study the specific immune cells in the lymphoid organs and aorta. ELISA was carried out to study antibody response to peptides
Results: oral administration of recombinant multi antigenic construct was found to induce antigen specific tolerance to ApoB, HSP60 and Cpn peptides as seen by reduction in effector cell proliferation in the presence of Treg cells isolated from splenocytes of tolerized mice. Regulatory T cells were found to increase in the lymphoid organs and the aorta of treated animals compared to control. Antibodies specific for the peptides were not detected in the serum of treated animals confirming immunological tolerance. Tolerance to the peptides resulted in 47.1% ($P = 0.002$) reduction in the development of atherosclerotic lesion in the aortic sinus compared to control. Protection against atherosclerosis was associated with decrease in macrophages infiltration (58.6%,$P = 0.03$) and increase in collagen content and IL10 in the developing lesion.
Conclusions: Our results suggest that oral administration of multi antigenic construct expressing ApoB, HSP60 and Cpn peptides induces tolerance to individual peptides and can prevent development atherosclerosis by reducing inflammatory mediators in the plaque.

PB 1.32-5

Immunization with a linear peptide derived from tissue factor and protease activated receptor-1 significantly reduces the development of atherosclerotic lesion in Apobtm2SgyLdlrtm1Her/J mice

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Background: Tissue factor (TF), an important protein in coagulation pathway, has been shown to be abundantly expressed in macrophages and the lipid rich core in atherosclerotic plaques and plays a pivotal role in thrombus formation associated with plaque rupture. The Thrombin signaling mediator protease activated receptor-1 (PAR-1) associated coagulation pathway which can be cleaved by thrombin, play critical roles in hemostasis and thrombosis, as well as in inflammatory, proliferative responses triggered by vascular injury and atherosclerosis. Stimulation of PAR1 in atherosclerotic plaques has been implicated in smooth muscle cell proliferation and exaggerated vasoconstrictory response.
Aim: To assess whether immunizing $Apob^{tm2Sgy}Ldlr^{tm1Her}$/J mice with the peptide epitopes derived from either TF or PAR-1 is effective in reducing atherosclerotic lesions.
Methods: Six-week-old $Apob^{tm2Sgy}Ldlr^{tm1Her}$/J (ApoB100 only, $LDLr^{-/-}$) mice were immunized using a repetitive immunization multiple-sites strategy with KLH-conjugated peptides derived from TF and PAR-1, respectively.
Results: Mice immunized with the peptide epitope derived from TF, PAR-1 and PAR-2 showed a greater reduction in lesion size compared to control mice immunized with KLH only, showing 26.5% ($P = 0.001$) and 27.4% ($P < 0.001$) for TF peptide- and PAR-1 peptide-immunized mice, respectively and these results were also in agreement with those in descending aortas compared with that in control showing significant decrease in lesion area in descending aortas (35.8% for TF and 42.2% for PAR-1). This effect on lesion reduction was associated with a shift in the cellular composition of plaques towards decreased inflammatory cell and increased regulatory T-cell content and increased production of anti-inflammatory cytokines and decreased secretion of proinflammatory cytokines demonstrated in plasma and in supernatant of stimulated spleen cells.
Conclusions: The immune response against epitopes derived from TF and PAR-1 has an effect in reducing atherosclerotic lesion formation.

PB 1.32-6

Aging- and activation-induced platelet microparticles suppress apoptosis in monocytic cells and differentially signal to proinflammatory mediator release

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Background and Aim: Platelet microparticles (PM) are the most abundant cell-derived microparticles in the blood, and accumulate in thrombo-inflammatory diseases. Platelets produce PM upon aging via an apoptosis-like process and by activation with strong agonists. We previously showed that long-term treatment of monocytic cells with apoptosis-induced PM (PM_{ap}) promotes differentiation towards resident macrophages. Here we investigated shorter term effects of various types of PM on monocyte signalling and function.
Methods and Results: Flow cytometry and scanning electron microscopy revealed that PM formed upon platelet aging (PM_{ap}) or ultrasonication (PM_{sonic}) expressed activated αIIbβ3 integrins and tended to assemble into aggregates. In contrast, PM formed upon platelet activation with thrombin (PM_{thr}) or Ca^{2+} ionophore (PM_{iono}) had mostly non-activated αIIbβ3 and little aggregate formation, but had increased CD63 expression. PM from activated and sonicated platelets expressed phosphatidylserine at their surface, while only the latter were enriched in the receptors CD40L and CX3CR1. All PM types expressed P-selectin, interacted with monocytic cells via this receptor, and were internalised into these cells. All PM types promoted actin cytoskeletal rearrangements and hydrogen peroxide production by monocytic cells. Markedly, both aging- and activation-induced PM types stimulated the phosphoinositide 3-kinase/Akt pathway, suppressing apoptosis induced by several agonists, in a P-selectin-dependent manner. On the other hand, the PM types differentially influenced monocyte signalling in eliciting Ca^{2+} fluxes (particularly PM_{ap}) and in releasing secondary mediators (complement factor C5a with PM_{ap}, and pro-inflammatory tumour necrosis factor-α with PM_{thr}).
Conclusions: In spite of their common anti-apoptotic potential via Akt activation, the aging- and activation-induced PM cause different Ca^{2+} signalling and mediator release events in monocytic cells. By implication, both aging and activated platelets may modulate monocyte function by forming different PM types.

PB1.33 – ADAMTS13: Clinical – I

PB 1.33-1

Quantitative PCR assay demonstrated exon deletions of ADAMTS13 in two unrelated patients with Upshaw-Schulman syndrome

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Background: Upshaw-Schulman syndrome (USS), also called hereditary thrombotic thrombocytopenic purpura, is associated with severe deficiency of plasma ADAMTS13 activity with an autosomal recessive trait of inheritance. In most of the patients with USS, homozygous or compound heterozygous mutations are identified in the ADAMTS13 genes. So far, more than 100 causative mutations have been identified worldwide by use of the direct sequencing method.
Aims: We previously analyzed the ADAMTS13 genes of 47 Japanese patients with USS in 41 unrelated families using direct sequencing. Of them, 44 patients in 38 families had homozygous or compound heterozygous mutations in ADAMTS13. As for the remaining patients, however, only one single missense mutation (two patients) or no mutation (one patient) was detected. Then, we intended to find more extensive defects of ADAMTS13 in these three patients.
Methods: The study protocol was approved by the ethical committee of the National Cerebral and Cardiovascular Center, and only subjects who provided written informed consent for genetic analyses were included. Genomic DNAs were prepared from blood cells and subjected to real-time PCR to quantitate the relative copy numbers of each exon in ADAMTS13. PCR primers were designed by the Primer-BLAST online tool (NCBI), and PCRs were performed using Quanti-Fast SYBR Green PCR Kit (QIAGEN). Fluorescent intensities were detected using Mx3000P QPCR System (Agilent Technologies), and each threshold cycle (Ct) was calculated by the MxPro software (Agilent Technologies).

Results: The Ct values implied that exon 8 of ADAMTS13 was heterozygously missing in one patient, who carries the c.1648G>A (p.Gly550Arg) mutation in exon 14. Similarly, exon 27 was heterozygously missing in another patient, who carries the c.841T>A (p.Cys281Ser) mutation in exon 8. Further analysis using PCR mapping and sequencing revealed that the loss of exon 27 was caused by 729-bp deletion ranging from the 36th nucleotide of exon 27 to the 587th nucleotide of intron 27 (c.3751_3892+587del). Assignment of the precise deleted region causing the loss of exon 8 is proceeding with difficulty, because of PCR amplification-resistant sequences around exons 7 and 8. The compound heterozygosity of the missense mutation and exon deletion was confirmed by PCR analysis of the parents' DNA samples. The other patient showed no abnormalities in the exon copy numbers of ADAMTS13.

Summary/Conclusion: The present study identified the patients with USS carrying the allele with exon deletion in ADAMTS13, which, as far as we are aware, has not been previously reported. One patient was compound heterozygous for c.1648G>A (p.Gly550Arg) and exon 8 deletion, and another was for c.841T>A (p.Cys281Ser) and c.3751_3892+587del (exon 27 deletion). Thus, the quantitative PCR assay provides an effective tool to identify the extensive deletion of ADAMTS13 in patients with USS. In our study, one patient did not show any abnormalities in either direct sequencing or quantitative PCR analyses, suggesting that the patient may carry uncommon types of defects in ADAMTS13 such as genetic translocation and inversion.

PB 1.33-2

Kinetics and half-life of plasma ADAMTS13 after plasma infusion in four patients with Upshaw-Schulman syndrome

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Introduction: Upshaw-Schulman syndrome (USS) is congenital deficiency of plasma ADAMTS13 activity caused by mutations in *ADAMTS13* gene, and characterized by repeated episodes of thrombocytopenia and hemolytic anemia that quickly respond to infusions of fresh froze plasma (FFP). Here, we investigate the kinetics and half-lives after single FFP infusion on routine infusions in order to estimate precious effect of preventive FFP infusion for USS patients.

Materials and Methods: We collected a series of plasma just before and 2 weeks after FFP infusion. We measured ADAMTS13 activity (:AC) by ADAMTS13-act ELISA, and ADAMTS13 antigen (:AG) by a sandwich ELISA and quantitative western blot analysis previously reported. Recovery values of ADAMTS13 level were calculated from maximum level of ADAMTS13:AC or AG after FFP infusion and volume of FFP infusion. The half-lives of ADAMTS13 levels were calculated from the time of half level decayed from maximum that predicted from the plots of ADAMTS13 level.

Patients: We analyzed four patients with USS (B3, W4, Q3, Q5) belonging to three families. USS-B3 was 20-year-old female with homozygous nonsense mutations of p.Q449*. She received FFP infusion every 2 weeks to maintain her platelet counts over 70×10^9/L. USS-W4 was 15-year-old female with compound heterozygotes for p.G550R and exon 8 deletion. She received FFP infusion upon episodes of thrombocytopenia and hemolytic anemia. USS-Q3 (24-year-old male) and Q5 (19-year-old male) were brothers and their ADAMTS13 gene mutations were compound heterozygotes for p.G227R and p.C908T. They received prophylactic FFP infusion every 2 weeks. The volumes of FFP infused in USS-B3, USS-W4, USS-Q3, and USS-Q5 were 240 mL (4.4 mL/kg), 320 mL (5.6 mL/kg), 160 mL (3.1 mL/kg), and 160 mL (3.3 mL/kg), respectively.

Results: The kinetics of ADAMTS13:AC and:AG showed similar pattern after FFP infusion. The maximum values of ADAMTS13:AC and AG were at 1 h after FFP infusion in USS-B3, W4 and Q3 and at 6 h in USS-Q5. After that, both levels of ADAMTS13:AC and AG gradually decreased. In USS B3 and Q5, both were no longer detected in plasma at 14 days after FFP infusion. The ADAMTS13:AC level at 14 days after FFP infusion of USS W4 was detectable, but severely decreased. In USS-Q3, both level were decreased into undetectable level at 7 days after FFP infusion. In USS-B3, W4, and Q5, platelet counts raised to maximum level at day 7 after FFP infusion. In USS-Q3, the maximum level of platelet was 77×10^9/L at 3 days after FFP infusion. The average of maximum value of ADAMTS13:AC and:AG after FFP infusion were 5.8 ± 1.8 and $5.8 \pm 2.6\%$ (mean $\pm$ 2 SD), respectively. The half-life of ADAMTS13:AC and:AG were 2.8 ± 0.6 and 2.7 ± 1.6 days, and the recovery of ADAMTS13:AC and ADAMTS13:AG after FFP infusion were $71 \pm 9.6\%$ and $70 \pm 15.2\%$, respectively.

Conclusion: We evaluated the efficacy of prophylactic FFP infusion in the patients with USS under remission status. Our results suggested that 5 mL/kg FFP infusion every 2 weeks was reasonable protocol for preventing relapses.

PB 1.33-3

The determination and characterisation of anti-ADAMTS13 autoantibodies

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Background: A deficiency of ADAMTS13, a multi-domain, anticoagulant protein, specifically responsible for the cleavage of von Willebrand Factor (VWF), can result in Thrombotic Thrombocytopenic Purpura (TTP). The acquired form results from inhibitory anti-ADAMTS13 autoantibodies directed against the protein's functional or catalytic domains. However, not all antibodies are inhibitory. Current methods designed to identify ADAMTS13 autoantibodies do not discriminate between the inhibitory and non-inhibitory forms.

Aims: This study aims to develop a method for screening patient plasma samples to determine the location of autoantibody binding sites, and their significance in TTP onset.

Methods: The individual domains of the ADAMTS13 protein were selected as antigens in an ELISA that was optimised for screening patient plasma samples. The individual peptide regions were selected from the corresponding sequence of a synthetic cDNA construct of the full ADAMTS13 coding sequence. A standard PCR was optimised using specifically designed primers. The peptides were then produced in a bacterial expression system. Three patient groups took part in the screening process; patients who contained high titres of anti-ADAMTS13 autoantibodies, yet did not develop TTP, stroke patients, and a group of TTP patients. All patient samples had aBethesdaassay performed to identify the presence of an inhibitor.

Results: OurBethesdaassay results demonstrated that all TTP patients contained an inhibitor, all of the healthy control patients who contained anti-ADAMTS13 autoantibodies did not contain an inhibitor. Interestingly the stroke patients exhibited a wide range ofBethesdaresults. From the peptides used in this study, the TTP patient autoantibodies were mainly directed at the catalytic spacer domain, where-as the non-TTP patient plasma samples were directed at the non-catalytic CUB 2 domain.

Summary/Conclusion: The TTP patients screened presented with autoantibodies directed at the spacer domain, further supporting the literature identifying this domain possessing the pathological binding sites. However, the CUB 2 domain may represent a target for the binding of non-inhibitory anti-ADAMTS13 autoantibodies and the pathogenesis, if any, of this subset of antibodies warrants further investigation.

PB 1.33-4

ADAMTS13 activity is a potential biomarker of thrombotic risk in systemic lupus erythematosus

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Background/Aim: A severe deficiency of ADAMTS13 activity results in the presence of VWF ultra large multimers with high affinity for platelets, causing thrombotic thrombocytopenic purpura. Other pathological conditions with mild to moderate ADAMTS13 activity levels exhibit a risk for thrombosis. We have explored the ADAMTS13/VWF axis in patients with Systemic Lupus Erythematosus (SLE) and its potential value as a thrombotic marker.

Methods: ADAMTS13 activity, VWF antigen and multimeric structure, and VCAM-1 were measured in plasma from 50 SLE patients. Anti-ADAMTS13 antibodies were evaluated in patients with low ADAMTS13. Parameters of disease activity (SLEDAI) and organ damage (SLICC), thrombotic events, and presence of antiphospholipid syndrome (APS-SLE) and antiphospholipid antibodies were registered.

Results: SLE patients showed reduced ADAMTS13 activity and high VWF levels (66 ± 27% vs. 101 ± 8%, $P < 0.01$, and 325 ± 151% vs. 81 ± 14%, $P < 0.001$, vs. control). ADAMTS-13 activity was below 60% and 40% in 40% and 22% of patients, respectively. VCAM-1 levels were superior in patients plasma than in control samples (range of 564–4991, vs. 596.25 ± 100 ng/mL, $P = 0.03$). ADAMTS13 activity was under 60% in APS-SLE patients and in all patients with thrombotic events. SLEDAI was > 6 in 65% of the cases.

Conclusion: Reduction of ADAMTS13 activity together with increased VWF levels could be ascribed to the chronic inflammatory state in SLE. These results were specially found in patients with higher disease activity index and with antiphospholipid antibodies. Therefore, ADAMTS13 activity, in combination with other laboratory parameters, may be used as prognostic biomarker of thrombotic risk in SLE.

PB 1.33-5

Decreased plasma ADAMTS13 activity during moderate to much consumption of ethanol in healthy volunteers: differences between normal and heterozygous mutant aldehyde dehydrogenase-2 alleles

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Background: Deficiency of ADAMTS13 activity (ADAMTS13:AC) results in the accumulation of unusually large von Willebrand factor multimers (UL-VWFM) and platelet thrombi formation. We demonstrated that enhanced production of UL-VWFM over deficient ADAMTS13:AC may contribute to the progression of liver injury and the development of multiorgan failure in patients with severe alcoholic hepatitis (Curr Drug Abuse Rev, 2008;1:188).

Aims: Little information is available on the effects of ethanol on plasma ADAMTS13:AC in healthy subjects. In this study, we determined changes in plasma ADAMTS13:AC following moderate to much consumption of ethanol in healthy volunteers who were either wild-type homozygous or mutant heterozygous for the aldehyde dehydrogenase (ALDH)-2 gene.

Methods: Eleven healthy volunteers homozygous for wild-type ALDH-2 genes (Group I) and nine heterozygous for wild-type and mutant alleles (Group II) were studied. All volunteers were co-workers who usually consumed moderate to much amounts of alcohol, and this study protocol was approved by the Ethics Committee of our university. Blood alcohol concentration (BAC), plasma levels of ADAMTS13:AC and VWF:Ag, and VWFM patterns were determined before and every hour for 5 h after ethanol consumption (60 g) with food (580 KCal). VWFM patterns were analyzed by SDS-0.9% agarose gel electrophoresis.

Results: BAC reached its highest levels 2 h after ethanol consumption in both groups (Group I 22 mM, Group II 24 mM), and thereafter tended to decrease more gradually in Group II than Group I. ADAMTS13:AC decreased by 20% from baseline between 2 and 5 h after ethanol consumption in Group II, whereas it decreased by 10% 4 h after ethanol intake in Group I. The incidence of subjects with > 20% decreases in ADAMTS13:AC was higher in Group II than in Group I (88.9% vs. 27.3%, $P < 0.05$). The largest decrease in ADAMTS13:AC after ethanol consumption was greater in Group II (−33.0 ± 9.0%, $P < 0.01$) than in Group I (−16.2 ± 10.8%, $P < 0.01$). Plasma inhibitor against ADAMTS13:AC was not detected throughout the study. VWF:Ag increased by 30% between 3 and 5 h after alcohol intake in Group II, but remained unchanged in Group I. As a result, the ratio of VWF:AG to ADAMTS13:AC increased by 55–79% between 2 and 5 h after ethanol consumption in Group II, whereas it was almost unchanged in Group I. VWFM patterns were normal in 9 (81.8%) and degraded (18.2%) in Group I, whereas they were normal in 2 (22.2%), degraded in 2 (22.2%), lacking in 2 (22.2%) and unusually-large in 3 (33.3%) in Group II.

Conclusions: Plasma ADAMTS13:AC significantly decreased in healthy volunteers with a mutant allele of the ALDH2 gene after moderate to much consumption of ethanol, resulting in increased levels of VWF:Ag. VWFMs showed lacking and unusually-large patterns in more than half of volunteers with mutant allele gene except normal and degraded patterns observed in those with wild-type genes. These subjects with mutant allele of the ALDH2 gene may be susceptible to platelet hyperaggregability after moderate to much consumption of ethanol.

PB 1.33-6

Levels of the ADAMTS13 metalloprotease and risk of myocardial infarction: review of the literature and meta-analysis

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Background: The circulating metalloprotease ADAMTS13 exerts an essential antithrombotic function by cleaving the ultralarge platelet-tethering multimers of von Willebrand factor. A severe deficiency in the plasmatic activity of ADAMTS13 causes thrombotic thrombocytopenic purpura, which is often associated with microthrombotic cardiac damage. Recent evidence from studies in humans and experiments in mice suggest that a reduced activity of ADAMTS13 might also be associated with the development of myocardial infarction (MI). A number of studies carried out in recent years investigated the association between plasmatic levels of ADAMTS13 and MI, yielding conflicting results.

Aims: To summarize and pool current evidence on the association between ADAMTS13 plasmatic levels and MI.
Methods: The study was conducted according to the principles of the PRISMA statement. We did not follow a pre-specified review protocol. In order to obtain material for the review, we conducted a Pubmed (URL: http://www.ncbi.nlm.nih.gov/pubmed/) search for articles published after 1980, using 'ADAMTS13' and 'heart' or 'myocardial' or 'coronary' as search terms (updated to September 12th, 2012). Articles were chosen for review based on the following criteria: (i) they concerned plasmatic ADAMTS13 levels in humans; (ii) they reported on controlled studies; (iii) the investigated disease was incident myocardial infarction. Studies were excluded from meta-analysis if they had too small a sample size to allow meaningful calculations (i.e. < 30 MI cases), chose controls from patients referred to the hospital for other diseases/conditions, had no frequency matching for age and sex, or did not provide any information on the distribution of patients and controls in tertiles/quartiles of ADAMTS13 activity. Data on the distribution of cases and controls in the tertiles or quartiles of ADAMTS13 levels were extracted from the reports. Studies suitable for meta-analysis were pooled by the Mantel-Haenzel method and by calculating unweighted mean to account for heterogeneity. The odds ratio for MI and its 95% confidence interval were the summary measures of choice.
Results: Seven case-control studies fulfilled the criteria for inclusion, after examination of 71 candidate manuscripts retrieved from the Pubmed repository. Selected studies most often concerned ADAMTS13 antigen levels rather than activity and measurements were carried out at different time periods from the acute episode. All studies had a case-control design, but yielded heterogeneous results regarding the association of ADAMTS13 with myocardial infarction. These encompassed protective effect, no effect and a risk-increasing effect of low ADAMTS13 levels. Of the seven studies, four could be used to obtain a pooled estimate. The unadjusted odds ratios for MI ranged from 0.62 to 1.27 in the four studies. The Mantel-Haenzel pooled odds ratio was 0.96 (95% confidence interval: 0.72–1.17) and the straight-averaged odds ratio was 1.01 (95% confidence interval: (0.72–1.30).
Summary/Conclusions: The results of this meta-analysis argue against a major influence of plasmatic ADAMTS13 levels on the risk of MI, although the large heterogeneity between studies suggests effects in specific groups.

PB1.34 – Fibrinolytic System: Clinical – I

PB 1.34-1
Increased N-terminal cleavage of alpha-2-antiplasmin in liver cirrhosis

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Background: Hepatic failure due to cirrhosis is caused by progressive fibrosis that ultimately results in nodular regeneration with loss of liver function. As the liver is the site of both synthesis and clearance of many proteins involved in hemostasis and fibrinolysis, including alpha-2-antiplasmin (a2AP), altered plasma levels of these factors are often found in chronic liver failure. The main function of a2AP, the inhibition of plasmin, is influenced by the proteolytic modifications the protein undergoes in the circulation. Approximately 35% of circulating a2AP is cleaved at its C-terminus, resulting in a form of a2AP that has lost most of its activity by losing its ability to bind to plasmin (ogen). Non-plasminogen-binding a2AP can still inhibit plasmin, but kinetically very slow. Additionally, a large part of circulating a2AP is N-terminally cleaved between residues Pro12 and Asn13 by the recently identified AntiPlasmin-Cleaving Enzyme (APCE), a soluble, circulating derivative of fibroblast activation protein (FAP). This cleavage turns the native Met-a2AP into Asn-a2AP. FAP is a type II integral membrane protease dominantly expressed in areas of tissue remodeling, e.g. by activated fibroblasts, myofibroblasts and activated hepatic stellate cells.
Aims: In this study we investigated whether liver cirrhosis, characterised by increased expression of FAP and therefore presumably also APCE, resulted in increased N-terminal cleavage of a2AP in the circulation.
Methods: We focused on the active plasminogen-binding form of a2AP, PB-a2AP, and on individuals homozygous for the common R-allele of polymorphism Arg6Trp, as this polymorphism strongly influences N-terminal cleavage of a2AP. New ELISA assays were set up to measure the antigen levels of both total PB-a2AP and N-terminally intact Met-PB-a2AP. With these data we calculated the percentage Met-PB-a2AP, reflecting N-terminal heterogeneity. a2AP antigen levels were measured in the plasma samples of 48 patients with liver cirrhosis with different types of etiology and 20 healthy control individuals. Informed consent was obtained and the study was approved by a recognised medical ethics committee. Patients were classified into three groups of increasing severity according to the Child-Pugh's score.
Results: As many other proteins synthesized in the liver, total PB-a2AP levels (mean ± SD) were reduced in the cirrhosis patients (30.0 ± 13.0 μg/mL) compared to the total PB-a2AP levels in the control individuals (55.1 ± 8.3 μg/mL; $P < 0.001$). The Met-PB-a2AP levels were also reduced in the cirrhosis patients (4.2 ± 4.0 μg/mL) compared to the Met-PB-a2AP levels in the control individuals (13.0 ± 3.3 μg/mL; $P < 0.001$). Interestingly, we found that, in addition to the reduction in a2AP levels, the percentage of Met-PB-a2AP was reduced in the cirrhosis patients (12.5 ± 6.4%) compared to the percentage of Met-PB-a2AP levels in the control individuals (23.6 ± 4.6%; $P < 0.001$). This indicates increased N-terminal cleavage of a2AP in the cirrhosis patients. All three variables significantly decreased with the severity of the disease.
Conclusions: We found an increase in N-terminal cleavage of a2AP in patients with liver cirrhosis which correlated with the severity of the disease. This may be of pathogenic importance in the frequently observed enhanced fibrinolytic potential of cirrhotic patients.

PB 1.34-2
Feasible mechanisms of fibrinolysis impairment in patients with antiphospholipid syndrome

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Background: The pathogenesis of thrombosis in patients with antiphospholipid syndrome (APS) is multifactorial. Numerous ways and mechanisms including an impairment of fibrinolysis by which antiphospholipid antibodies (aPL) may lead to the development of thrombosis in APS have been proposed. Possible mechanisms of aPL effects on activity of the fibrinolytic system are under study for the past years.
Aims: To study the relationship between thromboses, levels of aPL and fibrinolytic system components in APS patients.
Methods: The plasminogen (Pg) level was determined by the kinetic method in 78 patients with APS, 35 of them with systemic lupus erythematosus (SLE + APS) and 43 – with primary APS (PAPS). The PAI-1 antigen level measured by ELISA was assayed in 45 APS patients (21 with SLE + APS and 24 with PAPS). Positivity of aPL (LAC, aCL and $a\beta_2GP1$) was estimated. The thrombotic events were assessed clinically and confirmed by objective methods. A control group included 10 donors without autoimmune diseases and thromboses in anamnesis.

Results: Decrease in fibrinolysis may be caused by low level of Pg or high level of PAI-1 which neutralizes plasminogen activators. In the group of Pg study thromboses were registered in 67 APS patients (32 arterial and 53 venous thromboses, 14 patients had combined thrombi). Among 67 patients low Pg levels (0.37–1.49 μM) were found in 23 (34.3%) with nine arterial and 19 venous thromboses. The low Pg levels in patients with thromboses were associated with high-positive levels both of $a\beta_2$GPI (n = 16) and aCL (n = 9) and Pg levels were lower in patients with SLE+APS than in patients with PAPS.
In the group of PAI-1 study thromboses were registered in 43 (95.5%) APS patients (22 arterial and 33 venous thromboses, 12 patients had combined thrombi), 14 of them had increased (47.1–93.3 ng/mL) and 24 – high (102.0–463.3 ng/mL) levels of PAI-1 antigen which were associated with high-positive levels both of $a\beta_2$GPI (n = 20) and aCL (n = 11). One of possible mechanism of this interrelationship was considered. Patients with SLE + APS had higher PAI-1 levels than patients with PAPS. To test a possible contribution of genetic factors on the development of thrombosis, the genetic variants of PAI-1 in APS patients were determined by the method of polymerase chain reaction. It was found that arterial and, to a greater extent, venous thromboses are associated with the 4G/5G polymorphism of the PAI-1 gene and high plasma level of the inhibitor in 79% of APS patients.
Conclusion: The impairment of fibrinolysis are connected partially with decrease in Pg level and, to a greater extent, with increase in PAI-1 level both of which are associated with high-positive $a\beta_2$GPI and aCL levels at least in one third of APS patients with thromboses. The most of APS patients with thromboses and increased and high PAI-1 levels were carriers of 4G allele of PAI-1 gene. In APS patients venous thromboses occurred more frequently than arterial ones. The impairment of fibrinolysis due to aPL and PAI-1 gene mutation contributes to the development of thrombosis in APS patients.

PB 1.34-3

Topical and conjunctival use of fresh frozen plasma in patients with congenital plasminogen deficiency

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Background: Plasminogen plays an important role in fibrinolysis as well as wound healing, cell migration, and angiogenesis. Congenital plasminogen deficiency is rare autosomal recessive disorder that leads to development of pseudo-membranes on mucosal surfaces such as eyelids and mouth. Ligneous conjunctivitis is the most common presentation in which fibrin accumulation causes inflammation of conjunctiva and leads to thick, woody (ligneous) growths inside eyelids. The treatment options are few and the prognosis is poor especially in severely affected patients.
Aim: The aim of this report is to present our experience with systemic and conjunctival fresh frozen plasma treatment in our patients with congenital plasminogen deficiency.
Methods: Patients' files were evaluated retrospectively and 11 patients who were followed-up with a diagnosis of plasminogen deficiency were included.
Results: There were six girls and five boys. The most common clinical presentation was ligneous conjuctivitis (n:11) that was present in all patients, followed by ligneous gingivitis (n:5) and hydrocephalus (n:3). Further manifestations were hearing loss (n:2), genitourinary involvement (n:1), hepatomegaly (n: 1), epilepsy (n:1), recurrent respiratory tract infections (n:1) and Dandy-Walker malformation (n: 1). The median age of first clinical manifestation was 7 months (range: 0.5–24 months). All patients were Turkish descent. Most of the patients' parents were consanguineous (n:6). Two girls and two boys were sisters and brothers; in addition to that these four patients were related. Venous thrombosis did not occur in any of the patients. In nine patients eye lesions were surgically removed and in all of them there were relapses on follow-up after surgery. Eight patients were treated by intravenous and conjunctival fresh frozen plasma. One of them had an anaphylactic reaction during plasma infusion so it was discontinued. One girl passed away due to endophtalmitis shorly after presenting to our clinic. None of the patients treated by fresh frozen plasma had a recurrence on follow-up. Three patients have been followed-up for > 3 years now with a prophylactic use of fresh frozen plasma every 10–30 days.
Conclusion: Patients with congenital plasminogen deficiency are symptomatic early in life. Boys usually become symptom-free at 4–5 years of age, however girls suffer throughout adulthood. Our experience showed that the severity of symptoms are not parallel with plasminogen levels and the clinical progress is more severe in girls compared to boys. Hydrocephalus and hearing loss are important clinical manifestations and ophthalmologists treating ligneous conjuctivitis patients should be aware of these systemic complications. Treatment of ligneous conjunctivitis is challenging due to frequent recurrences. Surgical excision should not be performed unless there is proggressive, intractable fibrin deposition. Systemic and topical fresh frozen plasma can be used successfully for preventing recurrences and relieving symptoms. The long-term prophylactic application of fresh frozen plasma seems to prevent recurrences and has a protective effect on the vision. Local conjuctival use of fresh frozen plasma in combination with other eye medications such as cyclosporin and artificial tear drops may relieve the nuisance feeling of 'something in the eyes'.

PB 1.34-4

Effect of genetic PAI-1 polymorphisms 4G/5G, C428T and G429A on PAI-1 activity and clot lysis time

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Background: Plasminogen activator inhibitor type-1 (PAI-1) plays an important role in clot lysis and is influenced by both environmental and genetic factors. Data on cardiovascular disease (CVD) risk and genetic factors influencing PAI-1 levels in the black African population, in whom the prevalence of CVD is increasing rapidly is, however, scarce.
Aims: We determined the frequencies of three polymorphisms in the promoter area of the PAI-1 gene and their influence on PAI-1_{act} levels and clot lysis time in a black African population. We also investigated gene-environment interactions and their influence on PAI-1_{act} levels and clot lysis time (CLT) and whether urbanisation influenced these interactions.
Methods: Data from 2010 apparently healthy black men and women (aged 35–65 years) who participated in the South African PURE study were cross-sectionally analysed. Anthropometric and dietary intake data were collected, as well as blood samples for the determination of biochemical factors, including PAI-1_{act} and CLT determined using a turbidimetric assay, and DNA isolation and genotyping.
Results: The 5G homozygous genotype frequency of the 4G/5G polymorphism was 72.5% in this population. PAI-1_{act} was the highest in the 4G/4G group (6.82 U/mL) followed by the 4G/5G group (5.77 U/mL) and then the 5G/5G group (4.77 U/mL) in the urban subgroup, while PAI-1_{act} levels did not differ significantly across genotypes in the rural group. CLT did not differ across 4G/5G genotypes. PAI–1_{act} additionally increased across the 4G/5G genotypes in normal, overweight and obese participants as well as in centrally obese participants,

but not in those with normal waist circumference. CLT differed significantly across the 4G/5G genotypes in centrally obese participants only. The C428T and G429A polymorphisms did not influence PAI-1_{act} or CLT, except for higher PAI-1_{act} in the homozygous wild type group of the G429A SNP.
For the 4G/5G polymorphism we found significant gene-environment interactions for WC, BMI and triglycerides on PAI-1_{act}; and for fibrinogen and fibrinogen gamma prime on CLT. Significant interactions for the C428T polymorphism and PAI-1_{act} were found with triglycerides, total homocysteine, fibrinogen gamma prime and HDL-cholesterol levels, and for CLT with BMI. The G429A polymorphisms showed interactions with LDL-cholesterol, and fibrinogen on PAI-1_{act} and interactions on the determination of CLT with fibrinogen and blood pressure. Some of the gene-environment interactions also differed significantly between the rural and urban subgroups.
Summary/Conclusions: The high prevalence of the 5G allele may contribute to the low PAI-1_{act} levels seen in this population. The 4G/5G polymorphism significantly influenced PAI-1_{act} levels in urban but not rural participants. The influence of the 4G/5G polymorphism on PAI-1_{act} is apparent in normal, overweight and obese participants and those who are centrally obese. The C428T and G429A SNPs do not seem to play an important role in PAI-1 transcription, probably owing to these SNPs not being present within a known transcription site. The 4G/5G, C428T and G429A polymorphisms showed significant interactions with various CVD risk factors in determining PAI-1_{act} and CLT. Many of these interactions were, however, affected by urbanisation as differences were observed between rural and urban participants.

PB 1.34-5
The hyperfibrinolytic phenotype induced by short-term venous stasis is not triggered by activation of the clotting cascade or thrombin formation

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Background: Venous stasis is a major factor in the development of venous thrombosis. The venous occlusion (VO) test is used to simulate this pathomechanism by induction of short term venous stasis. Recently, we have shown that a procoagulant response is not induced by the VO test in healthy individuals.
Aims: To study if the VO test induces such a procoagulant shift in the presence of endothelial cell dysfunction or inherited thrombophilia we extended our study to patients with arteriosclerosis or hereditary thrombophilic risk factors.
Methods: The study population consisted of three groups: healthy individuals (n = 25, 14 females, mean age, range: 32, 20–58 years); patients with arteriosclerotic coronary artery disease confirmed by coronary angiography (n = 15, one female, 65, 48–77 years); and thrombophilic patients (n = 19, 13 females, 43, 18–64 years) with antithrombin deficiency (n = 2), protein C or S deficiency (n = 6), homozygous FV Leiden (n = 8) or FII G20210A mutation (n = 3). The study was approved by the local ethics committee and all patients gave written informed consent. Blood samples were taken before, during VO (12 min after start) at the stasis arm and the contralateral arm, and 15 min after VO. Blood samples were analyzed for plasma levels of free thrombin and activated protein C (APC), both measured using highly sensitive oligonucleotide-enzyme capture assays showing lower limits of quantification of thrombin and of APC of 0.039 and 0.116 ng/mL, respectively. In addition, tissue-type plasminogen activator (t-PA), plasmin-α2-antiplasmin-(PAP)-complexes, and thrombin-antithrombin-(TAT)-complexes were determined. All determinations were corrected for changes in hematocrit.
Results: In all three cohorts the majority of samples taken during VO showed thrombin and APC levels below the LLOQ. Thrombin levels above the LLOQ during VO were observed only in one patient in the arteriosclerosis group (7.468 ng/mL) and one patient with homozygous FV Leiden mutation (1.738 ng/mL). Consistent with previous reports the VO test induced a fibrinolytic response, indicated by a significant increase ($P < 0.05$) in plasma levels of t-PA from 1.5 ± 3.1 ng/mL (mean ± SD) to 8.2 ± 7.6 ng/mL in healthy probands, 2.0 ± 1.1–11.2 ± 4.7 ng/mL in arteriosclerotic patients, and 1.7 ± 0.9–11.4 ± 6.6 ng/mL in patients with thrombophilia. Plasma levels of PAP-complexes also increased significantly from 657 ± 476 to 1730 ± 1085 ng/mL in healthy probands, 609 ± 166–1562 ± 913 ng/mL in patients with arteriosclerosis; and 614 ± 371–1642 ± 975 ng/mL in thrombophilic patients. TAT-complexes did not change significantly in all three cohorts.
Summary/Conclusion: Activation of the clotting cascade is not triggered by short term venous stasis even in the presence of additional thrombotic risk factors such as endothelial dysfunction or inherited thrombophilia. Furthermore, our results indicate that the fibrinolytic response induced by the VO test is caused by mechanisms independent from coagulation activation and thrombin formation.

PB 1.34-6
Nicotinic acid/laropiprant modulates fibrinolytic system in patients with elevated levels of Lipoprotein(a)

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Background: Hyperlipidemia can be effectively treated either by niacin or HMG-CoA reductase inhibitor. In addition, elevated levels of Lipoprotein (a) may be, at least in part, reduced by nicotinic acid. Scarce data are available on the role of nicotinic acid/laropiprant on fibrinolysis.
Aims: To asses the behaviour of the fibrinolitic system in patients with elevated levels of Lipoprotein (a) treated with with nicotinic acid/laropiprant.
Patients and Methods: Fourteen consecutive unselected dyslipidemic patients, with previous adverse cardiovascular event (seven acute coronary syndrome, three stroke, two peripheral artery disease, one retinal occlusion, one sudden hypoacousia) and multiple comorbidities (six hypertensives, no diabetics), were treated daily with 2000 mg of nicotinic acid/40 mg Laropiprant and optimal statin therapy, in addition to cardiovascular medications according to current guidelines, for 6 months. The Clot Lysis Time (CLT) and the blood levels of triglycerides, HDL, LDL cholesterol and Lp(a), were determined before (T0) and after 6 months of treatment (T1). CLT was performed as lysis time of tissue factor-induced clots exposed to exogenous t-PA (Lisman et al., 2006); triglycerides, HDL, LDL levels were analyzed by chromogenic assays and Lp(a) by nephelometric measurement.
Results: The Clot lysis time (CLT) was significantly decreased after treatment (median (range) T0: 61 min (51–123.9) vs. T1: 56 min (44–76); $P < 0.05$). The average decrease of CLT was 11% (95%CI 4–18). The treatment reduced significantly the levels of triglycerides (median (range) T0: 98 mg/dL (65–201) vs. T1: 76 mg/dL [61–148]), LDL cholesterol (median (range) T0: 111 mg/dL (76–182) vs. T1: 90 mg/dL (54–118) $P < 0.05$) and Lp(a) (median (range) T0: 1155 mg/dL (560–1870) vs. T1: 677 mg/dL (202–1030) $P < 0.005$). As expected, HDL cholesterol levels significantly increased (median (range) T0: 53 mg/dL (33–96) vs. T1: 68 mg/dL (42–107);$P < 0.05$). The average decrease of concentration of triglycerides, LDL and Lp(a) was respectivly 27% (95% CI: 18–36%), 24% (95% CI: 16–32%), and 39% (95% CI: 26–51%) whereas the average increase of HDL cholesterol was 30% (95% CI: 11–50%).A significant correlation ($P < 0.05$) was found between CLT, triglyceride and LDL.
Conclusion: A treatment with nicotinic acid/laropiprant in patients with elevated levels of lipoprotein (a) may provide additional benefit on fibrinolysis.

PB1.35 – Haemophilia A: Clinical – I

PB 1.35-1

A fusion peptide binding to tissue factor pathway inhibitor (TFPI) inhibits both plasma- and platelet TFPI

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Background: TFPI is a Kunitz-type protease inhibitor that inhibits both FXa and TF-FVIIa and which is an important physiological inhibitor of the extrinsic coagulation pathway. Plasma contains various truncated forms of TFPI, which are poor inhibitors of FXa and FVIIa, and full length TFPI (0.25–0.5 nM) which is the physiologically important form of TFPI. Platelets also contain full length TFPI which is released to plasma when the platelets become activated thus increasing the full length TFPI concentration in plasma 50–75%. Direct inhibition of TFPI in hemophilia models with blocking antibodies, aptamers or peptide inhibitors mitigates bleeding complications and may become an important approach in hemophilia treatment
Aims: To compare the effectivity by which a TFPI binding fusion peptide with TFPI antagonist activity inhibits the anticoagulant activity of plasma- and platelet TFPI in model systems and in plasma.
Methods: Platelets isolated from blood samples of different donors were activated with convulxin and spun down by centrifugation. The TFPI present in the supernatant was quantified with an ELISA and was taken as source of platelet TFPI. Recombinant TFPI, either glycosylated or non-glycosylated, was taken as source of plasma TFPI. The functional activities of plasma- and platelet TFPI and the effect of fusion peptide as TFPI antagonist thereon were compared in model systems (FXa and TF-FVIIa-catalysed FX activation) and in plasma (TF-triggered thrombin generation).
Results: Detailed kinetic analysis showed that platelet-derived TFPI and recombinant TFPI were equally potent inhibitors of TF-FVIIa-catalysed FX activation in models systems. The TFPI antagonistic fusion peptide blocked the anticoagulant activity of plasma- and platelet TFPI in the model system with similar IC50 values that were in the low nM range. Recombinant TFPI and platelet-derived TFPI also inhibited TF-triggered thrombin generation in TFPI-depleted plasma with identical activities and the anticoagulant activities of both forms of TFPI were blocked by TFPI antibodies as well as the TFPI antagonistic fusion peptide. The fusion peptide also enhanced thrombin generation in TFPI-depleted plasma reconstituted with platelets which is indicative of specific inhibition of platelet TFPI. Fusion peptide titrations of thrombin generation in platelet-poor plasma (PPP) and platelet-rich plasma (PRP) showed that the peptide enhanced thrombin generation in PPP 3–4 fold with a half maximal effective concentration (EC50) of 2 nM and in PRP 2-fold with an EC50 of 8 nM. Thrombin generation in PRP to which an inhibitory FVIII antibody was added to simulate PRP of a haemophilia patient was enhanced threefold by both the fusion peptide and TFPI antibodies.
Summary and Conclusions: Plasma- and platelet TFPI exhibit similar anticoagulant activities in the inhibition of TF-FVIIa catalysed FX activation in a model system and in the down-regulation of TF-triggered thrombin generation in plasma. A TFPI antagonistic fusion peptide enhances thrombin generation in PPP and PRP both in the absence and presence of inhibitory FVIII antibodies by blocking the anticoagulant activities of platelet- as well as plasma TFPI. Our observation supports the notion that targeting TFPI with TFPI inhibitors is a promising novel strategy to mitigate the bleeding risk of haemophilia patients.

PB 1.35-2

The incidence and impact of intracranial hemorrhages within a hemophilia and non-hemophilia population

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Background: Intracranial hemorrhage (ICH), while rare, is a very serious complication of hemophilia. Understanding the incidence and outcomes of individuals with hemophilia who have had an ICH may help emphasize the importance of lowering ICH rates.
Aims: The objective of this analysis was to assess the incidence and outcomes of hemophilia patients who suffer an ICH compared to a non-hemophilia population to better understand the risk associated with ICH complications.
Methods: A retrospective, case-control analysis was undertaken using the *Truven Analytics Marketscan® Database*, which contains medical claims data from US commercial health plans representing approximately 130 million covered lives. Using data from years 2002–2011, male patients with ICD-9 diagnosis codes of 286.0 or 286.1 for hemophilia were selected and compared to a male only, random sample of non-hemophilia patients (excluded ICD-9 code 286.x). In addition to the incidence of ICH, inpatient visits, length of stay (LOS) and hospitalization costs were compared between the two groups. ICD-9 diagnosis codes 430, 431, and 432 were used to identify ICH.
Results: There were 11,267 persons with hemophilia in the database, of these 79% were Hemophilia A, 13% Hemophilia B, and 8% had codes for both hemophilia A and B. The non-hemophilia sample consisted of 103,620 individuals. Within the hemophilia sample, 217 (1.9%) had an ICH diagnosis, compared to 179 (0.2%) of the non-hemophilia sample ($P < 0.0001$). The mean age of patients with an ICH was 45.7 and 40.9 for the hemophilia and non-hemophilia population, respectively ($P < 0.05$). One hundred and forty (1.2%) patients in the hemophilia sample had an ICH inpatient visit with a median LOS of 7 days. In the non-hemophilia sample, 101 (0.1%) had an ICH inpatient visit with a median LOS of 6 days. The median (interquartile range) cost for an inpatient ICH visit among the hemophilia sample was $24,207 ($12,688–$68,728), while the median cost for the non-hemophilia sample was $19,255 ($11,804–$47,653).
Conclusions: These results indicate that individuals with hemophilia have approximately a 10-fold increased incidence of ICH compared to a non-hemophilia sample. This research also demonstrates the significant impact of ICH both in terms of hospital length of stay and cost. Given guidelines recommend ICH patients are treated with high-dose Factor concentrates for at least 10–14 days and are then placed on long-term prophylaxis, the hospitalization costs identified in this analysis do not reflect the full economic burden of ICH. Further research to better understand the long-term impact of ICH should be conducted to ensure that appropriate strategies are employed to lower the relative incidence of ICH in a hemophilia vs. non-hemophilia population.

PB 1.35-3

Variation in effect of DDAVP in mild haemophilia patients with an Asn637Ser mutation

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Background: In mild haemophilia desmopressin acetate (1-deamino[8-Sarginine]-vasopressin, DDAVP) can be used for the treatment of bleedings. DDAVP increases FVIII and von Willebrand factor (VWF) 2–6 fold through endogenous release. Effect of DDAVP varies between patients and but it is suggested that it is dependent on the mutation causing haemophilia.

In our haemophilia centre a large cohort of patients with Asn637Ser (1910A>G) mutation causing mild haemophilia A is known. In individual patients the effect of intravenous DDAVP is routinely tested

Aim: To retrospectively investigate the variability in FVIII raise after DDAVP infusion in this cohort of patients.

Material and Methods: All male patients known with mutation Asn637Ser, and their relatives from maternal side suffering from mild haemophilia treated at the Van Creveldkliniek were included. Baseline characteristics including residual FVIII level, VWF level and blood group, FVIII raise after 30–60 min of intravenous infusion of 0.3 μg/kg, DDAVP age at test, were collected. DDAVP was infused in 20–30 min. When FVIII levels were measured at two time points between 30 and 60 min after end of infusion, the highest levels was used for analysis.

Results: In 77 patients with mild haemophilia caused by mutation Asn637Ser, a DDAVP test has been done, of which 68 could be analysed. The lowest baseline FVIII level varied greatly between 0.06 and 0.36 IU/dL, mean VWF was 0.101 IU/dL (IQR, 0.76–112). Forty-nine percent had blood group O, 23% A, 7% AB and 5% blood group B. Low VWF levels were associated with lower FVIII baseline levels. No effect of blood group on FVIII levels was found. The mean age at DDAVP test was 21.7 years (IQR 7–34). After DDAVP the mean level was 101.7 IU/dL (IQR 80–124)

The mean raise in FVIII levels was 0.78 IU/dL (IQR 57–98). Effect of DDAVP varied greatly from 2 to 10 fold increase of FVIII levels 30–60 min after end of infusion. Higher baseline FVIII was associated with a higher absolute raise in FVIII after DDAVP. After correction higher age appeared to have a positive effect on outcome, no effect of baseline VWF and blood group on outcome was seen.

Summary/Conclusion: In this group of patients with mild haemophilia caused by mutation Asn637Ser, baseline FVIII levels varied greatly. The effect of intravenous DDAVP varied between patients and was associated with baseline FVIII and age. Patients with higher baseline FVIII and older patients showed a better response to DDAVP

PB 1.35-4

Major surgery in haemophiliacs: Istanbul experience

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Background: Surgery in patients with haemophilia can present a range of challenges to physicians.

Aim: To determine the perioperative management and outcome of heamophilia A and B patients during major surgical procedures.

Methods: Data pertaining to major surgeries from 1997 to 2012 at our center were retrospectively analysed. Patients with inhibitors were excluded from the study. All operations were elective and a plan for management of hemostasis was prepared for each patient.

Results: During this period 80 major surgeries were performed in 56 haemophiliacs (52 with haemophilia A and four with haemophilia B). Their median age was 29 years and range was 3 months–72 years. Most of the haemophiliacs (34/52 haemophilia A and 2/4 haemophilia B) had severe disease (Factor level < 1%). The majority of the surgeries were orthopedic procedures (n:64); there were eight urological and three neurosurgical procedures, three patients had adeno-tonsillectomy, one had abdominal abscess drainage and one had apendectomy. Eight orthopedic surgeries and one urologic surgery included two different operations at the same procudere. All patients were treated with intravenous bolus factor replacement therapy pre- and post-operatively. No severe life threating bleeding occured in any of the patients, 4% of the patients had mild bleeding after surgery. One severe haemophiliac developed post operative hematoma on related knee after total endoprosthesis of hip and left knee at post-operative 24th day while he was still on daily factor replacement treatment; the bleeding was controlled with extra factor infusion. Another haemophilia A patient (moderate) developed macroscopic haematuria after left kidney nephrolithiasis operation at 15th day of surgery but resolved in 2 days with an extra factor therapy. One severe haemophilia A patient who bled at the 7th day of left knee total endoprosthesis developed low responding inhibitor following operation. The bleeding was controlled with by-passing agents. One right knee prothesis insertion operation had a infection complication at the operation site which ended up with amputation of the affected leg. Another knee prothesis insertion operation was also infected and resulted with extraction of prothesis and arthrodesis of the affected ankle.

Conclusion: Our results showed good hemostasis plan with no major bleeding. Inhibitor development is currently the most severe complication in patients with haemophilia treated for surgical procedures. Three haemophiliacs developed inhibitors, two of them were low titer inhibitors, one haemophiliac needed long-term use of by-passing agents. The incidence of inhibitor development following intensive treatment for surgery was 5%. Wound infections are also an important problem after major surgeries, postoperative infection occured in two of our patients (2.5%) and resulted with amputation in one of them. Major surgical operations can be performed in haemophiliacs with a right hemostasis plan and the incidence of hemorrhage is low. Experienced physicians and centers have key role in operation of heamophiliacs and operations should be done without delay.

PB 1.35-5

What we can learn from real-life clinical experience data from a post authorization safety surveillance in PUPs treated with antihemophilic factor (recombinant), plasma/albumin free method in Japan

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Background: The treatment of young hemophilia A patients has been improved by the availability of safe FVIII products and the adoption of routine prophylaxis, but a major complication still remains the development of inhibitors to FVIII. Data on previously untreated patients (PUPs) is valuable in delineating the natural history of hemophilia treatment. The real-life clinical practice during the early treatment phase of hemophilia A in Japan has been documented by ADVATE (rAHF-PFM) PUPs study since 2007 under Japanese ordinance Good Post-Marketing Study Practice (GPSP).

Aims: To investigate safety ie, adverse events namely inhibitors, and efficacy in PUPs.

Methods: This prospective, multicenter, open-label, observational surveillance cohort study was initiated at 63 sites on February 2007 to investigate PUPs of any age and disease severity, with ≤ 3 exposure days (EDs) at study entry, who were prescribed rAHF-PFM. Data were collected every 6 months for over 2 years using the electronic data-capture system (EDC).

Results: As of 31 December 2012, data for 116 PUPs (0 ED:97, 1–3 EDs:19) out of 119 enrolled patients from 63 sites were available. Fifty eight (50%) of 116 patients had undergone a greater than two-year observation period. Eighteen patients dropped out within 2 years of observation (adverse events:7, lost to follow up:4, death:1, others:6). Seventy-four percent of patients had FVIII < 1%, 8% had 1 ≤ FVIII ≤ 2, 6% had FVIII > 2–5% and 12% had FVIII > 5%.

The median age at entry was 0.8 (range 0–81 years). All are Asian male. Forty-two percent of 116 patients had a family history of hemophilia and 8% had a family history of inhibitors. The median observation periods were 24.3 months. Fifty-eight percent had over 50 infusions (median: 80, 1–2092 infusions) on study.
The median age of diagnosis of hemophilia A was 0.33 in subjects who developed inhibitors (Inh) and 0.66 in subjects with no inhibitor (no Inh). The median age of first bleeds was 0.58 (Inh), 0.61 (no Inh) and the median age of severe bleeds was 0.37(Inh) and 1.13 (no Inh). The median age of first exposure to rAHF-PFM was 0.68 (Inh) and 0.88 (no Inh).
Ninety-four (81%) of 116 started on-demand and 57 (61%) of 94 moved to prophylaxis. At the worst efficacy evaluation during observation period, excellent/good rating was observed for 88.6% on prophylaxis and 90.7% on demand.
At the time of last report, 20 patients (17.24%, 95% CI: 10.86, 25.36) developed an inhibitor (seven high-, 13 low titer at inhibitor diagnosis). In nine of 20 inhibitors have disappeared. The adjusted odds ratio in risk of inhibitor development were family history of hemophilia: 0.91 (0.240–3.462), family history of inhibitors:12.518 (2.561–61.178), ICH:2.600 (0.684–9.887), severe bleeds: 2.040 (0.595–6.993), catheter insertion: < 0.001 (< 0.001–> 999.999), hemophilia severity:1.132 (0.194–6.589).
Conclusions: The safety profile of rAHF-PFM in Japanese PUPs appears consistent with previous reports with an inhibitor rate of 17.24% in this report. The adjusted odds ratio in inhibitor development was significantly higher in subjects with a family history of inhibitor.

PB 1.35-6
Costs and utilization of haemophilia A and B patients with and without inhibitors

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Background: Haemophilia is a costly disease resulting in bleeding complications, joint damage, and use of expensive factor therapies. Descriptive analysis of patient populations may provide valuable insight into the delivery of care and health resource utilization.
Aim: To evaluate the characteristics and health system costs among patients with haemophilia A and B with and without inhibitors over a 5 year period.
Methods: This was a retrospective, observational study utilizing medical and pharmacy electronic medical records and administrative encounters/claims data tracking US patients between 2006 through 2011 for up to 5 years. Patients with International Classification of Diseases, Ninth revision (ICD-9) diagnosis codes only for haemophilia (ICD-9 code 286.0 for haemophilia A (factor VIII deficiency) and 286.1 for haemophilia B [factor IX deficiency]) were identified. Eligible patients must have received clotting factors at least twice during the study period. Inhibitor patients were characterized by the utilization of one of two bypassing agents: activated prothrombin complex or factor VIIa on two or more distinct dates. Severity was classified as mild (5–40% normal factor activity), moderate (1–5% normal factor activity), or severe (< 1% normal factor activity). Research data was derived from an approved Naval Medical Center, Portsmouth, VA IRB NMCP.2012.0016 protocol.
Results: Overall, there were 160 haemophilia A patients and 54 haemophilia B patients identified. Of this group, seven were designated as inhibitor patients (five with haemophilia A and two with haemophilia B). The mean age (SD) in years was 2.6 (± 3.9), 8.6 (± 8.8), 11.2 (± 12.2) for patients with inhibitors, patients with haemophilia A without inhibitors, and patients with haemophilia B without inhibitors, respectively. Haemophilia A patients without inhibitors reported 65 (41.9%) as being severe, 19(12.3%) as moderate, and 71 (45.8%) as mild. Haemophilia B patients without inhibitors reported 9 (17.3%) as being severe, 1(14.3%) as moderate, and 2 (28.6%) as mild. All inhibitor patients had been hospitalized in the previous 5 years compared to 64 (41.3%) with haemophilia A without inhibitors and 22 (42.3%) with haemophilia B without inhibitors. The median aggregate cost per year (including factor and health resource use) was $325,780 for patients with inhibitors compared to $98,334 for haemophilia A patients without inhibitors and $23,265 for haemophilia B patients without inhibitors.
Summary/Conclusions: The results of this analysis suggest that while the frequency of inhibitors within the haemophilia cohort was low, there was a higher frequency of hospitalizations and the associated median aggregate costs per year are three-fold higher than those patients without inhibitors. In contrast, haemophilia B patients experience less severe disease and account for lower aggregate yearly costs compared to either patients with haemophilia A or patients with inhibitors.
The views expressed in this article are those of the authors and do not necessarily reflect the official policy or position of the Department of the Navy, Department of Defense, or the United States Government.

PB1.36 – Haemophilia A: Clinical – II

PB 1.36-1
Cognitive dysfunctions and cerebral microbleeds in adult patients with haemophilia A and B: the role of cardiovascular disease

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Background: In children with haemophilia a decrease in intellectual function and in their face-spatial and motor skills was described. Moreover, a lack of language and language-related activities was reported. All these cognitive dysfunctions seemed to be related to previous cerebral microbleeds (CM). Studies that provide information about the cognitive condition of adult patients with haemophilia are lacking.
Aims: To measure the neuropsychological profile in a group of adult patients with haemophilia A and B; to verify the presence of asymptomatic CM; to identify some correlation between CM and cognitive dysfunctions; to assess the possible role of several contributing factors (i.e. severity of haemophilia, type of therapy, comorbidity) in determining cognitive dysfunctions.
Methods: After informed consent, 49 adult (age, 42 ± 15 years) patients with haemophilia A and B (31 severe and 18 mild), without inhibitors and consecutively evaluated at the Haemophilia Centre of Padua, were enrolled. Each patient underwent: (i) a 'Short Neuropsychological Test' assessing the following cognitive functions: learning, episodic memory, working memory, psychomotor speed, selective and divided attention, concentration, reasoning, and executive functions; (ii) a Nuclear Magnetic Resonance (NMR) of the brain to evaluate changes in signal intensity within the white matter on T2-weighted images, areas of brain atrophy, or haemorrhagic lesions. Results were standardised on a control group matched for age, sex, and cultural profile.
Results: Patients suffering from hemophilia A and B presented with a reduction of the overall cognitive performance (OCP), expressed by the Z-PSI index (−1.18 CI 95%: −1.34 and −0.03). In particular, the most compromised aspects were lack of both memory and verbal fluency. According to the OCP, no significant difference between severe and mild haemophilia was observed. Nevertheless, the score of the memory tests tended to be worse in severe haemophilia patients than in patients with mild haemophilia. The OCP was similar between

patients undergoing 'on demand' treatment and patients undergoing 'prophylactic' treatment (Z-PSI index -0.32 ± 0.48 vs. -0.13 ± 0.63; $P = 0.37$). According to risk factors for cerebrovascular disease, the OCP was significantly correlated with the presence of coronary artery disease ($P = 0.02$). As for NMR findings, available in 40 patients because nine of them refused to give informed consent, the presence of CM was associated with a worse OCP. Specifically, the CM episodes were inversely related to the OCP ($R = -0.32$ $P < 0.05$). CM was significantly associated with the presence of cerebrovascular risk factors (Fischer Test $P = 0.018$). Finally, patients undergoing 'prophylactic' treatment showed a better OCP than patients undergoing 'on demand' treatment.

Conclusions: In adult patients with hemophilia A and B a high prevalence of cognitive dysfunctions was observed. Moreover, a correlation between OCP reduction and CM was found. Hence, identifying each individual cerebrovascular risk in order to tailor the most adequate treatment to the needs of each patient is of crucial importance.

PB 1.36-2

Decreased FVIIIa stability of mild Hemophilia A mutation R527W explains discrepancy between two chromogenic method applications using different FX activation times

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Several mutations for mild Hemophilia A are reported to cause discrepant results between one-stage clotting methods: vs. two-stage clotting and chromogenic methods.

In an oligocenter study on plasmas from patients with mild Hemophilia A, two sites used the chromogenic Coatest SP4 FVIII kit, utilizing either 5 or 10 min FX activation times. There was an overall high agreement in results but the site using a 5 min FX activation time obtained significantly higher FVIII activities for ten samples, representing the five different mutations R527W, R531H, R531C, G479R, V663A.

Six of the ten samples were sourced from individuals with the R527W mutation and mean (SD) FVIII activities were 0.25 ($\pm$ 0.03) and 0.12 ($\pm$ 0.01) IU/mL when using 5 and 10 min FX activation times, respectively. Hence, a 5 min activation time resulted in about two-fold higher FVIII activities.

A study on FXa generation vs. time demonstrated that the time to reach a plateau level of FXa, indicating completely inactivated FVIIIa, on analysis of two R527W plasma samples was about half as compared to a normal reference plasma and a T295A plasma, the latter showing similar FVIII activity assignment at the two sites.

Using a 10 min FX activation time will therefore result in relatively more FXa being generated for normal plasma as compared to R527W plasmas and accordingly to a lower FVIII activity assignment of R527W plasmas.

FVIII:Ag was 0.75 ($\pm$ 0.09) IU/mL for the six R527W samples and hence within the normal range, whereas FVIII:Ag was 0.10 and 0.12 IU/mL for two T295A samples.

The R527W mutation is located in the A2 domain and a plausible explanation for the more labile R527W-FVIIIa is a faster dissociation of the A2 domain from FVIIIa. This has been shown by Pipe et al (Blood, 1999) to be the case for the R531H mutation. One R531H sample was included in this study, with assigned FVIII activity of 0.15 and 0.07 IU/mL when using 5 and 10 min activation times, respectively, and hence a similar two-fold difference in FVIII activity as for R527W.

Our results demonstrate that in patients referred for FVIII activity analysis as a follow-up from global screening or on suspicion of mild Hemophilia A due to the clinical phenotype, caution has to be made in interpreting FVIII activity results. In this study, FVIII activities were reported for the R527W mutation with two chromogenic method applications using 5 or 10 min FX activation times and for both applications the results were concordant with mild Hemophilia A. This may, however, not necessarily be the case for alternative methods.

PB 1.36-3

Clinical outcome of hemophilia patients undergoing major orthopedic surgery without pharmacological thromboprophylaxis

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Background: Hemophilia patients are generally considered at low risk for venous thromboembolism. However, orthopedic surgery for hemophilic arthropathy exposes them to well established major risk factors for venous thrombosis due to the procedure itself, long immobilization, and factor replacement therapy. In contrast to non-hemophilia patients, pharmacological prevention of thromboembolism for hemophilia patients is not well studied nor standardized and largely depends on local practice. A multicenter European survey reported that 50% of centers are using anticoagulant prophylaxis. At our center, in agreement between orthopedic surgeon and hematologist, no pharmacological prevention of thromboembolism is given to patients undergoing orthopedic surgery.

Aims: The present study analyzes the safety and feasibility of this approach.

Methods: Retrospective analysis of all orthopedic operations performed in hemophilia patients of our center between 1978 and 2012. The study was approved by the local ethic committee, no patient informed consent was obtained as the retrospective study only involved anonymous patient record analysis.

Results: A total of 166 major orthopedic operations were performed in 47 patients. Forty-three (91.5%) had hemophilia A and 4 (8.5%) hemophilia B. Thirty-one (66%) patients suffered from severe, 14 (29.8%) from moderate and 2 (4.3%) from mild disease, respectively. One patient (2.1%) had a combined FVIII/Factor V deficiency. Median age was 44.9 years (IQR 34.9–55.0). Orthopedic interventions were divided into surgery of the lower extremity ($n = 138$, 83.1%%) and the upper extremity ($n = 21$, 12.7%%), for 7 (4.2%) data are missing. Follow-up information 9 weeks after surgery could be obtained for 124 operations (74.7%). No clinical signs of venous thromboembolism were observed in all cases. One (0.6%) intervention in a mild hemophiliac was complicated by a life-threatening perioperative myocardial infarction. Data on factor consumption was available for 101 interventions (60.3%) with a total dose per hospitalization of 45,000 IU (IQR 33,250–60,250) given to patients (mean body weight of 71.3 kg). Transfusions of red blood cells were needed in 12.3% of all interventions.

Conclusions: In this cohort of hemophilia patients undergoing orthopedic surgery without pharmacological prevention of thromboembolism followed over an overall period of 34 years, no symptomatic deep venous thrombosis or pulmonary embolism was observed. Prospective trials incorporating a systematic assessment for thrombosis are needed to confirm these encouraging data.

PB 1.36-4

Integrated analysis of safety data from 12 clinical interventional studies of a plasma- and albumin-free recombinant factor VIII (rAHF-PFM) in persons with hemophilia A (HemoA)

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Objectives: To update evaluation of the safety of rAHF-PFM by encompassing the results from 12 clinical interventional studies. rAHF-PFM is approved in the USA for perioperative management, control and prevention of bleeding episodes in both children and adults with HemoA. It is manufactured free of exogenous human or animal proteins using a non-hypersensitivity-inducing monoclonal antibody.

Methods: Four hundred and eighteen treated subjects (363 PTPs, 55 PUPs, all with baseline FVIII levels ≤ 2% of normal) from all Baxter Phase I–IV studies excluding PASS studies had a median age of 18.7 years (range: 0.07–72.3) who received at least one infusion of rAHF-PFM. Study analysis subsets comprised immune tolerance induction, PK assessments, prophylaxis, recovery, and surgical prophylaxis.

Results: The 418 subjects received a total of 122,281,528 IU of rAHF-PFM from 1276 lots in 63,188 infusions. 88.5% of subjects received their infusions as prophylaxis (82.8% of total infusions). Overall, subjects received a median of 97.0 exposure days (range: 1–709), with a median of 98 infusions per subject (range: 1–711) and a median of 118,778 IU (range: 467–1,572,194 IU) per subject. Three thousand three hundred and seventy-six adverse events (AEs) were reported in 314 of the 418 subjects (75.1%) with 106 events being serious. However, the majority of all AEs were non-serious (3270/3376) and judged by the investigator to be unrelated to IP (3283/3376). Only 93 AEs in 45 subjects (10.8%) were related to rAHF-PFM, with no predominant type or cases of recurrence in subsequent infusions. Of these, only Factor VIII inhibition, headache, and pyrexia were considered to be in the ADR Frequency Category of 'common'. Of 106 serious AEs (SAEs), that occurred in 69 subjects (16.5%): most commonly FVIII inhibition (4.1%), device-related infection (1.0%), or pyrexia (0.7%) were detected. Seventeen SAEs that were considered related to rAHF-PFM included the development of FVIII inhibitors in 16 PUPs (seven high-titer (> 5 BU), nine low-titer (≤ 5 BU), in 29% of PUPs) and 1 PTP (low titer). Eleven of the 16 PUPs with inhibitors underwent immune tolerance induction, with 72.7% success. Overall, the incidence of any FVIII inhibitor in PTPs was 0.4%, similar to reports of other FVIII products. No deaths and no cases of hypersensitivity related to rAHF-PFM occurred during the conduct of these studies.

Conclusions: The integrated safety analysis confirms previously demonstrated safety and tolerability in children and adults with moderately severe or severe HemoA in a wide variety of clinical settings, and reveals no new safety signals.

PB 1.36-5

Clinical study in children with severe haemophilia A investigating efficacy, immunogenicity, pharmacokinetics, and safety of human-cl rhFVIII

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Background: Human-cl rhFVIII is the first recombinant factor VIII concentrate expressed in a human cell line (Human Embryonic Kidney 293F cells). Studies in previously treated adult and adolescent patients with severe haemophilia A demonstrated that Human-cl rhFVIII is bio-equivalent to a full length rFVIII concentrate and safe and effective in preventing and treating bleeding episodes (BE).

Aims: The objectives of this GCP study were to evaluate the pharmacokinetics (PK), efficacy, safety, and immunogenicity of Human-cl rhFVIII in previously treated children between 2 and 12 years of age.

Methods: First, all patients were to undergo an *in-vivo* recovery (IVR) investigation with Human-cl rhFVIII. In a subset of patients, also the PK of Human-cl rhFVIII was assessed in comparison to the patient's previously used FVIII product. After an injection of a nominal dose of 50 IU/kg, blood samples were collected up to 48 h for PK analysis and up to 2 h for IVR. IVR was repeated in all patients after 3 and 6 months. FVIII coagulant activity (FVIII:C) was measured by chromogenic substrate and one-stage clotting assay in a central laboratory. All patients were to be treated prophylactically with Human-cl rhFVIII every other day or three times weekly with 30–40 IU Human-cl rhFVIII per kg for 6 months. Human-cl rhFVIII was also to be used in case of breakthrough bleeds. Inhibitors were measured before, at defined time points during and at the end of the study by modified Nijmegen Bethesda assay in a central laboratory. Adverse events were recorded throughout the study.

Results: The study was approved by the Ethics Committee of each participating institution and informed consent/assent was obtained from the parents/legal guardians or patients, if applicable, prior to any trial-related activity. Fifty-nine patients (29: 2–5 years; 30: 6–12 years) were enrolled from 15 sites in Europe. Thirteen children of each age group participated in the comparative PK investigation. Mean PK parameters of Human-cl rhFVIII were similar to those of the previous FVIII product (20 full-length rFVIII, six plasma-derived FVIII), both for the chromogenic and the one-stage assay: AUC_{norm} 0.234 vs. 0.25 h IU/mL/[IU/kg]); IVR 1.61 vs. 1.59%/IU/kg; $T_{1/2}$ 12.5 vs. 13.1 h (one-stage assay). IVR remained stable throughout the study. There were a total of 129 BEs in 39/59 patients during the study, 108 of which were treated with Human-cl rhFVIII. The majority of treated BE were traumatic (60.2%) and minor (56.5%). The mean ± SD monthly rate of all types of BEs/patient was 0.34 ± 0.43 (spontaneous BEs: 0.12 ± 0.27; traumatic BEs: 0.19 ± 0.29). No patient discontinued the study because of an AE. There were no related serious and two possibly related AEs (mild headache, mild back pain) in two patients. No child developed an inhibitor.

Conclusion: The data indicate that Human-cl rhFVIII is efficacious and safe in preventing and treating BEs in previously treated children. The PK of Human-cl rhFVIII and the previous product were very similar to each other. A study in previously untreated patients was initiated.

PB 1.36-6

Evaluation of the hemostatic potentials in a mild hemophilia A with a novel factor VIII mutation Thr677Ile

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Hemophilia A (HA) is classified by the factor (F)VIII activity (FVIII:C) as severe (FVIII:C < 1 IU/dL), moderate (1–5 IU/dL) and mild types (> 5 IU/dL). Some mild/moderate HA patients show the discrepancies in FVIII:C between a one-stage clotting assay and chromogenic assay. The patients with FVIII mutations such as Arg531Cys, Arg527Trp, Ser1791Pro and Leu1978Phe show the higher FVIII:C by one-stage assay than chromogenic assay. On the other hand, the mutations at Arg1639His and Arg1689His in the A3 domain of FVIII are known to show the higher FVIII:C by chromogenic assay than one-stage assay. We had a case of mild type HA associated with a Thr677Ile mutation in the A2 domain of FVIII, not enrolled in the HAMSTeRs database, hospitalized with iliopsoas muscular hemorrhage, exhibiting higher FVIII:C by one-stage assay (32 IU/dL) than chromogenic assay (18 IU/dL) and positive cross reacting materials (FVIII:Ag 92% in ELISA). To investigate the hemostatic potential in this case, the standard control plasmas constituted with FVIII-deficient plasma and serially diluted recombinant FVIII (final concentration 0.2–100 IU/dL) were prepared, and the thrombin generation test (TGT) and the clot waveform analyses (CWA) were performed. The parameters obtained (peak thrombin and time to peak thrombin) in the case by TGT were compared with those in standard plasmas. The respective parameter was 128 nM and 15 min, equivalent to FVIII:C 8–10 IU/dL in standard plasmas. Also, a parameter (min^2), acceleration rate of the clotting, in the case by CWA was equivalent to that of FVIII:C 5 IU/dL in standard samples. The haemostatic potential evaluated by TGT and CWA reflected the clinical phenotype, and was similar to FVIII:C measured by chromogenic assay rather than one-stage assay. To examine the mechanism(s) of the discrepancy of FVIII:C in this case between one-stage and chromogenic assays, the concentration of the aPTT reagent consisting of phospholipids (100 μg/mL) and ellagic acid (0.1 mM) used in one-stage clotting assay was varied. Dilution of aPTT reagent had little effect on FVIII:C in normal plasmas, whilst FVIII:C of the case was reduced by approximately 20% using the fourfold diluted aPTT reagent. The concentration of the phospholipids showed an inversed correlation with aPTT, but no significant difference was observed between in the normal and the case plasma. Surprisingly, the concentration of the ellagic acid also was inversely correlated with aPTT, but the rate of shortening of aPTT in the case plasma was approximately 1.3-fold greater than that in the normal plasma. Thus, the hemostatic potential in the case might be overestimated with the excess amounts of ellagic acids, and the combination of coagulation assays as TGT and CWA would be important to evaluate the clinical haemostatic potential of mild/moderate HA with discrepancy in FVIII:C.

PB1.37 – Haemophilia A: Clinical – III

PB 1.37-1

Molecular genetics of inherited bleeding disorders. External quality assessment identifies errors in genotyping and interpretation

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Background: In the field of molecular genetics, investigation of the causative mutation in individuals and their families with haemophilia and other inherited bleeding disorders is common practice. The results of genotypes are unequivocal with no borderline values but failure to correctly identify a mutation or candidate mutation, or to misinterpret its significance, can have major implications for an individual, their family and offspring. Participation in an external quality assurance programme is a fundamental part of laboratory practice and in theU-Kis a requirement for laboratory accreditation. Experience from other genetic external quality assessment schemes has highlighted that errors may occur, both in the correct identification of mutations and in their subsequent interpretation.

Methods: An external quality assurance programme for the Molecular Genetics of Haemophilia was established in 1998 by UK NEQAS for Blood Coagulation. Preliminary exercises explored the ability of laboratories to identify the presence or absence of the *F8* intron 22 inversion mutation; subsequent exercises have included samples with point mutations in the *F8*, *F9* and *VWF* genes. In each exercise, participants are provided with a clinical scenario and are asked to carry out genotyping and associated interpretation in order to answer a clinical question. Numerical scores are assigned on anonymised reports by a panel of expert scientists and clinicians. In this way, the clerical accuracy, diagnostic capability with respect to genotyping, and interpretative ability of the centre is assessed.

Results: During the course of the 20 exercises to date there has been marked improvement in the quality and consistency of laboratory reports, prompted by advice and comments from the expert panel. Enhancements to reporting have included use of standard gene names and promotion of mutation nomenclature recommended by the Human Genome Variation Society. In the majority of exercises, the genotype failure rate of laboratories is low (~1%), although failure due to errors in the interpretation of data are more frequent (7%). However, an exercise in November 2011 revealed a failure rate of 4/22 (18%) in the genotypic detection of a *F8* intron 22 inversion mutation in an affected female. Three of these four failures were associated with long rangePCR methods. The material employed in this exercise was immortalised cell line material from the National Institute of Biological Standards and Controls (NIBSC, Potters Bar, UK). Investigations are ongoing into the reason for the unusually high failure rate in this exercise.

Conclusion: The scheme now has 26 participants primarily in the UK/Europe but also including a small number of laboratories outside of this geographical area. Given the large number and international distribution of laboratories performing genetic screening for inherited bleeding disorders it is likely that many centres do not participate in EQA, and thus fail to benefit from the improvement in diagnostic accuracy and reporting that such participation can bring.

PB 1.37-2

Efficient typing of copy number variations in HA and HB families with large deletion/insertion mutations using multiplex competitive amplification

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Background: Large deletion/insertion mutations are found in approximately 5% of patients with severe haemophilia. Though the patients with these kinds of mutations can be confirmed, carrier identification in affected families is indefinable by direct sequencing.
Aims: The aim of this study was to do the carrier and prenatal diagnosis in haemophilia families with the large deletion/insertion mutations using multiplex competitive amplification.
Methods: The APPT, PT, TT, Fg, FVIII:C, FIX:C were detected to make phenotypic diagnosis. LD-PCR and PCR were adopted for the screening of the intron 22 and 1 inversion respectively. The F8/F9 gene coding and boundary sequences were analyzed by direct sequencing. Seven STR sites related to F8/F9 gene were combined together to do the linkage analysis. The new CNV genotyping method, AccuCopy, based on multiplex competitive amplification of F8/F9 gene respectively was used to investigate eight HA families and three HB families with large deletion/insertion mutations.
Results: Laboratory tests indicated that all of the HA and HB patients had severe deficiency of related factors. Large deletions of exon 4, exon14, exons 2–9, exons 7–9, exons 2–6, exons 5–7 were confirmed in seven HA patients whose relative segments' copy number variations were zero respectively. One insertion of exons 11–12 was detected in one patient whose CNV of these segments was 2 while the normal male control's was 1. The carriers and prenatal diagnosis could be confirmed by the CNV detection of these segments with AccuCopy method. For the carrier, the CNV of the relative exons was 1, while the normal control female's CNV was 2. In the three HB families, it was failed to amplify the exons 1–6, exons 2–8 and exons 1–8 segments of the index respectively. The results of AccuCopy method manifested that the copy number variations of the relative exons were zero in all of the patients and one copy in their mothers who were female haemophila carriers respectively. The females who requested for the carriers detection were detected to have two copy numbers of the segments respectively. Furthermore, the linkage analysis results were coincident with the genetic analysis in these HA and HB famillies.
Conclusions: This report illustrated that multiplex competitive amplification method represented an efficient method to determine the carrier status and do the prenatal diagnosis in haemophilia families with large deletion/insertion mutations.

PB 1.37-3

Ongoing prospective rAHF-PFM immune tolerance induction registry (PAIR): success rates continue to support published literature

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Background: Prospective recombinant anti-hemophilic factor plasma/albumin free method (rAHF-PFM) Immune Tolerance Induction (ITI) Registry (PAIR) is a global, ongoing, non-interventional, post-authorization safety surveillance registry designed to collect treatment information on rAHF-PFM safety/effectiveness in ITI therapy. Several registries have reported ITI success rates ranging from 55% to 78% for rAHF-PFM and other products (Mariani, 2003; Hay, 2012).
Aims: To present interim results for patients who completed ITI at the time of data transfer on September 6, 2012. Specifically, to assess incidence of adverse events (AEs) related to rAHF-PFM during ITI therapy. Secondary objectives were to collect information on the incidence of central venous access device-related complications, and to evaluate success rates of ITI therapy.
Methods: Patients with hemophilia A of any severity and an inhibitor were eligible for study. Investigators determined dosing regimens and monitoring schedules. Maximum observation period for ITI was 33 months with a 12 month follow-up period.
Results: Enrollment took place from July, 2007 to April, 2011. Forty-four patients were enrolled from 10 countries. At the time of data transfer, 32/44 patients (72.7%) completed ITI therapy, 25 of which completed the 12 month follow-up. The following rAHF-PFM dosing regimens were administered for the full analysis set ($n = 44$): 4 (9.1%) patients received ≥ 200.4 IU/kg/day; 3 (6.8%) patients received 131–199 IU/kg/day, 26 (59.1%) patients received 90–130 IU/kg/day, and 11 (25%) patients received < 90 IU/kg/day. During the observation period, 262 AEs, excluding bleed events, occurred for all enrolled patients ($n = 44$). Of these, 52 (18%) AEs were serious and unrelated while 15 (7%) were considered non-serious and at least possibly related. None of the SAEs were considered related to the treatment. In the full analysis set, 294 bleeding episodes occurred. Central venous device complications were common: one patient was hospitalized, 10 experienced line infections, 10 had line insertions, five reported line malfunctions, four experienced line removal, and one patient reported pain following portacath bleed. While six patients withdrew prior to completing ITI therapy, 32 'completers' were categorized as follows: 14 patients had successful disappearance of inhibitor, seven achieved partial success, four were deemed successful by investigator, six were not anticipated to be successful within 33 months, and one patient reached the end of the study period at 33 months. Inhibitor titer level results for 28/32 completers were the following: 16 patients achieved negative titer levels, two experienced a high to low titer conversion, five failed to achieve negative titer, and five were unassessable due to missing titer data or discontinuation. After 12 months therapy, Kaplan–Meier estimate of success for achievement of first negative titer was 74.9% (asymptotic 95% CI: 56.7–89.8, $n = 32$) for the completer group. Kaplan Meier success rates were similar for the per-protocol (79.3%, CI 61.8–92.4%, $n = 27$) and lower in the full analysis set (61.1%, CI 45.6–76.9%, $n = 44$).
Summary/Conclusions: These interim results are consistent with previously reported PAIR analyses and with published data on rAHF-PFM and other commercially available FVIII products used in ITI (Mariani, 2003; Hay, 2012). PAIR continues to prospectively document rAHF-PFM safety and effectiveness in ITI and will contribute to understanding prognisticators of ITI success.

PB 1.37-4

Adherence to clotting factor treatment among patients with haemophilia A or B

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Background: Two limitations of conducting retrospective analyses related to outcomes and adherence for haemophilia patients include the fragmentation of care between home and hospital, and the inability to track episodic vs. prophylaxis treatment. However, such data are important for healthcare providers.
Aim: To evaluate adherence to clotting factor treatment and associated outcomes for patients with factor VIII or factor IX deficiencies using an integrated delivery system database for haemophilia patients.
Methods: This was a retrospective, observational study utilizing medical and pharmacy electronic medical records and administrative encounters/claims data tracking patients between 2006 through 2011

for up to 5 years. Patients with diagnosis codes for haemophilia (ICD-9 code 286.0 for haemophilia A and 286.1 for haemophilia B) were identified. Eligible patients must have received clotting factors at least twice during the study period. Severity was classified into mild (5–40% normal factor activity), moderate (1–5% normal factor activity), or severe (< 1% normal factor activity). Bleeding and complication rates were annualized over the study period. Medication adherence was assessed using prescription claims for clotting factors by examining sequential time periods of 180 days for each patient's continuous enrollment. Adherence within the time period was calculated using days supply for all clAims divided by 180 days. For each patient up to 10 observational windows were evaluated. Under the assumption that severe haemophilia patients should be treated continuously, patients were considered adherent within the time period if the ratio of days supply to observed days was 60% or greater. The approach was validated using haemophilia severity as a proxy. Research data was derived from an approved Naval Medical Center, Portsmouth, VA IRB NMCP.2012.0016 protocol.

Results: A total of 207 patients (74.9% and 25.1% haemophilia A and B, respectively) met the inclusion/exclusion criteria. The mean age (± SD) was 9.2 years (± 10.0). There were 101 (48.8%) mild, 32 (15.5%) moderate, and 74 (35.7%) severe haemophilia patients. The percentage of time periods where adherence to clotting factors was 60% or greater was 14% (SD = 28%) for patients with mild disease, 21% (SD = 32%) for moderate disease, and 51% (SD = 36%) for severe disease. Among patients with severe disease, 27 (36.5%) were adherent 30% of time periods or less, 22 (29.7%) adherent 31–70% of the time periods, and 25 (33.8%) were adherent between 71% and 100% of the time. Any bleeding episodes, joint bleeding episodes, and hospitalizations were uncommon events.

Summary/Conclusions: This analysis provides a novel approach for measuring adherence to clotting factor treatment through a retrospective database analysis. Adherence varied by severity. Among patients with severe disease, the majority (66.2%) were adherent < 70% of the time. The infrequent bleeding events and sample size limit analysis by severity. Future studies should explore the reasons for non-adherence and understand the implications as it relates to patient-reported breakthrough bleeding.

The views expressed in this article are those of the authors and do not necessarily reflect the official policy or position of the Department of the Navy, Department of Defense, or the United States Government.

PB 1.37-5

P.I.S.A. – safety, immunogenicity and efficacy of a full length DNA rAHF-PMF in patients with hemophilia A in the Italian post-marketing surveillance study

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Background: Postmarketing surveillance studies of FVIII concentrates have been recommended [1], [2] to confirm the favorable efficacy and safety profiles of already approved medicines.

Aim of the Study: To assess safety, immunogenicity and efficacy of ADVATE (full length DNA rAHF-PMF) in Italian daily routine practice.

Patients and Methods: This was a retrospective-prospective, multicenter, 12-month observational study carried out in subjects with severe, moderately severe or moderate hemophilia A who gave informed consent. At enrollment, all subjects should have been treated with Advate for at least 6 months, according to any regimen determined by the physician and based on national SPC. Each subject has thus been retrospectively evaluated for this period of at least 6 months and prospectively observed for 12 months. The primary endpoints were incidence of non-serious and serious adverse events (AEs) at least possibly related to ADVATE, and incidence of high-titer, low-titer and transient inhibitor development during the course of ADVATE treatment. The main secondary endpoint was the overall assessment of the hemostatic efficacy for all bleeding episodes treated with ADVATE.

Results: During the period of surveillance, a total of 287 patients and 1452 bleeding episodes were treated with ADVATE. The median annualized bleeding rate (ABR) at baseline was 4 (IQ25–75 1–15). At the end of the period of surveillance the median ABR was 1.0 bleed year-1 (IQ25–75 0–5.2) for the entire cohort. The median ABR was the lowest for those continuously on prophylaxis (0 bleeds year-1, IQ25–75 0–2.7); higher median ABR was observed for subjects treated on-demand (5.4 bleeds year-1, IQ25–75 1–16.1)

At baseline nine subjects (3.2%) had FVIII inhibitors. The titer was < 1 BU in four subjects, between 1 and 5 in one subject, and > 5 in four subjects. Exclusion of subjects with a history of FVIII inhibitor (seven subjects) from this group yielded to an incidence of 2/276 (0.72%) patients at baseline who developed inhibitor during the previous 6 months retrospective phase. During the surveillance no new inhibitors developed in subjects without a history of inhibitors. A total of 40 AEs were observed in the 287 subjects treated with ADVATE during the period of surveillance. Three AEs were serious (retroperitoneal hematoma, cholecystitis and seizures). All AEs were deemed unrelated to the study drug by the investigators.

Conclusions: The results of this Italian surveillance confirm safety, efficacy and low immunogenicity of Advate in daily clinical practice.

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PB 1.37-6

The influence of co-morbidities on annualised bleeding rates in patients with severe haemophilia A: experiences from the pivotal turoctocog alfa prophylaxis trial (guardianTM1)

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Background: Individual bleeding rates in patients with severe haemophilia A often show a high variability. This may be due to differences related to biological and behavioural patterns as well as differences in co-morbidities and regional treatment practice. Novo Nordisk has developed turoctocog alfa, a new recombinant FVIII with a truncated B domain. In the guardian™1 trial, 150 (24 adolescents and 126 adults) previously treated patients with severe haemophilia A were given prophylaxis with turoctocog alfa. This trial included patients from 15 countries across the world. Interestingly, a large variation between countries in the annualised bleeding rates was observed in spite of following the prophylactic dosing regimen specified in the protocol.

Aim: To investigate the potential influence of frequent co-morbidities on the annualised bleeding rate as a possible explanation of the variation between countries and patients.

Methods: Patients with severe haemophilia A (≤ 1% FVIII activity), aged from 12 years and above, with no history of inhibitors and with at least 150 exposure days to other FVIII products were included. Informed consent was obtained from all patients before any trial related activity and the trial protocol was approved by appropriate ethics committees or institutional review boards. The trial was conducted in accordance with the declaration of Helsinki and the principles of good clinical practice. At baseline, the patients' concomitant illnesses were recorded and a physical examination was performed. For each patient, we will assess two frequent co-morbidities, i.e. joints with haemophilic arthropathy and hepatitis C status. The association between these co-morbidities and individual annualised

bleeding rates during prophylaxis with turoctocog alfa will be investigated.
Results: A total of 499 treatment-requiring bleeds were reported by 105 patients, of which 389 were joint bleeds reported by 94 patients. The remaining 110 bleeds were non-joint bleeds reported by 51 patients, while 45 patients did not experience any bleeds during the trial. There was a large variation in the country-specific bleeding rates, and for some countries the rates were significantly different. The country-specific annualised bleeding rates ranged from 1.3 bleeds/patient/year (95% CI: 0.5–3.3) based on six bleeds in nine patients to 23.2 bleeds/patient/year (95% CI: 14.6–37.0) based on 58 bleeds in five patients. The association between each of the two frequent co-morbidities and the individual annualised bleeding rates will be explored graphically and statistically.
Conclusions: Several potential factors may have influenced the variation between countries in annualised bleeding rates. Here we will analyse the impact of two frequent co-morbidities as potential explanations for the observed variation in the country-specific bleeding rates.

PB1.38 – Haemophilia A: Clinical – IV

PB 1.38-1

The mechanism of action of prophylactic administration of recombinant factor VIIa may be explained by the presence of hemostatically active FVIIa in plasma throughout the time frame of prophylaxis

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Background: Recombinant factor VIIa (rFVIIa) is registered for treatment of patients with inhibitor-complicated hemophilia A and B, in which it effectively treats bleeding episodes at a 90 μg/kg dose administration. Recently it has been shown that a prophylactic administration of 90 μg/kg rFVIIa once daily was successful in reducing the number of bleeding events. These results suggest that a single dose of rFVIIa has a pro-hemostatic effect of up to 24 h, which is difficult to explain given its half-life of ~2 h.
Aims: To assess potential mechanisms by which rFVIIa may exert a prolonged hemostatic effect, we analyzed FVIIa plasma levels and associated hemostatic activity of blood samples from non-bleeding pigs receiving a 90 μg/kg bolus administration of rFVIIa.
Material and Methods: Six adult pigs received a 90 μg/kg bolus administration of rFVIIa, and were followed up for 24 ($n = 3$) or 48 h ($n = 3$). Plasma was collected at baseline and at 5, 15, 30 min, 1, 2, 4, 6, 8, 12, 16, 24, and 48 h post-administration. Plasma FVIIa levels were measured using a commercially available FVIIa-specific clotting assay (STACLOT VIIa-rTF). Plasma haemostatic potential was estimated by a prothrombin time (PT) assay and thrombin generation assays (TGA) using calibrated automated thrombography.
Results: Plasma FVIIa levels reached their maximum at 5 min post-administration (30445.5 ± 1022.2 mU/mL [mean ± standard deviation]) and decreased to pre-administration levels over time with a half-life of 2 h. Elevated plasma FVIIa levels were detected up to 24 h post-administration (41.3 ± 14.1 mU/mL at 24 h vs. 7.5 ± 15.6 at baseline), although differences measured beyond 4 h did not reach statistical significance. Immediately after administration of rFVIIa, PT values decreased, and remained shortened compared to baseline until 48 h post-administration (10.0 ± 0.1 s at 48 h vs. 10.6 ± 0.5 at baseline, $P < 0.05$). In post-administration plasma samples, thrombin generation curves were altered compared to baseline, with the most profound effect in the lag time. These lag times were significantly shortened up to 12 h post-administration (1.9 ± 0.2 min vs. 2.4 ± 0.2 at baseline, $P < 0.05$), but only returned to pre-administration levels at 48 h.
Conclusions: Following administration of a 90 μg/kg bolus of rFVIIa to non-bleeding pigs, elevated plasma FVIIa levels remained detectable up to 24 h post-administration, despite a half-life of approximately 2 h. These low FVIIa levels are hemostatically active, as shown by shortened clotting times in PT and TGA assays. This, as yet unrecognized, prolonged pro-hemostatic effect of FVIIa may explain the prophylactic efficacy of a once daily rFVIIa treatment. We propose that only low plasma levels of FVIIa are required for prevention of bleeding, whereas much higher levels are needed for treatment of active bleeds.

PB 1.38-2

TF-initiated thrombin generation associates with bleeding phenotype in patients suffering from haemophilia A

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Background: Although there have been improvements in the management of haemophilia A, there is no validated laboratory test that predicts bleeding tendency in patients suffering from severe haemophilia A. It is known that patients suffering from severe haemophilia A have a variable bleeding risk. Thrombin generation is increasingly recognized as a sensitive and reliable way to get an indication of a patients bleeding risk.
Aim: The aim of our study is to investigate whether thrombin generation is a method to get an indication of the bleeding risk in patients suffering from haemophilia A.
Methods: To investigate this we included 30 adult haemophilia A patients (FVIII ≤ 5 IU/dL). Patients were not on primary prophylaxis thereby not influencing coagulation measurements. Patients were assigned as bleeder when they had a Pettersson radiologic score between 3 and 4 with frequent spontaneous bleeding episodes. Factor VIII levels were determined via a recently published assay based on activation with FIXa and used thrombin generation as read out. Plasma samples were tested applying the Calibrated Automated Thrombograph method. Coagulation was initiated using 1pM of tissue factor (TF).
Results: From the 30 haemophilia A patients 21 patients were assigned as having a bleeding phenotype based on the Pettersson radiologic score. Using FIXa as initiator no difference was observed between bleeders and mild-bleeders ($P > 0.05$). When thrombin generation was initiated via 1 pM of TF, we observed a significant difference ($P < 0.001$) in ETP between bleeders and mild-bleeders (429 ± 163 vs. 787 ± 192 nM*min).
Conclusion: The endogenous thrombin potential (ETP) measured in low TF driven thrombin generation is a strong predictive thrombogram parameter of bleeding phenotype among patients with < 5 IU/dL FVIII.

PB 1.38-3

Thrombin generation assay by calibrated automated thrombogram (CAT): application to monitoring severe hemophilia A treatment

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Background: Severe hemophilia A (HA) has been traditionally considered when dosage of factor VIII (FVIII) measured by the one-stage clotting test is < 1 IU/dL. However, patients with FVIII < 1 IU/dL show an evident clinical heterogeneity. About 10–15% of patients classified as severe do not show a significant haemorrhagic incidence. This can be due to other procoagulant factors that compensate the FVIII deficiency. International guidelines for prophylaxis in severe HA recommend maintaining FVIII trough levels slightly above 1 IU/dL. The thrombin generation assay (TGA) is a global haemostatic technique capable of detecting the influence of small levels of coagulation and anticoagulation factors.

Aim: The aim of this study was to determine whether TGA can offer valuable information for proper monitoring of the prophylactic treatment in severe HA patients.

Methods: We included 103 samples of 44 severe HA patients with bleeding phenotype (more than two spontaneous hemarthrosis in the same joint in < 6 months) that were in prophylactic treatment biweekly. Blood samples were obtained every 3 months on the trough levels, 72 and 96 h after the last FVIII infusion. In our protocol the goal was to maintain the patient between 1.5 and 2 IU/dL of FVIII:C. Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were prepared for testing TGA and FVIII measurement. FVIII was quantified by one-step coagulation test (FVIII:C, IU/dL). TGA was measured by the calibrated automated thrombogram (CAT, Thrombinoscope BV) and three parameters were assessed: latency time (min), endogenous thrombin potential (ETP nMxmin) and peak height (nM Thrombin). All determinations were performed on frozen samples, as we have previously shown that freezing PRP not significantly affect TGA (Haemophilia. 2012 Nov 23. doi: 10.1111/hae.12044). Statistical comparisons were performed using the paired t test, and the bivariate correlations were analyzed by Pearson's test.

Results: TGA in PRP and PPP correlated significantly (for both peak height and ETP $r > 0.7$; $P < 0.01$). As expected, PRP showed higher thrombin generation than PPP (peak 78%; ETP 25% higher in PRP). However, there was no correlation between the number of platelets and the TGA (peak $R = 0.024$, $P = 0.541$). Overall, FVIII:C correlated significantly with the PRP-TGA peak height ($R = 0.77$, $P < 0.001$) and ETP ($R = 0.50$, $P < 0.001$) but there was no correlation with the latency time. When samples with FVIII:C < 1.5 IU/dL were analysed (22% samples), these correlations disappeared (peak $R = -0.317$, $P = 0.162$; ETP $R = -0.294$, $P = 0.221$). Meanwhile, samples with FVIII:C > 1.5 IU/dL maintained a good correlation (peak $R = 0.73$, $P < 0.001$; ETP $R = 0.34$, $P = 0.007$).

Conclusions: FVIII levels higher than 1.5 IU/dL correlate well with the TGA in PRP, especially with peak height, but lower levels do not have any significant correlation. The TGA as overall hemostatic test is especially useful when FVIII levels are very low, because this assay may detect small traces of haemostatic factors undetectable by one-step coagulation test. As prophylactic FVIII through levels may be at these low ranges (< 1.5 IU/dL), it would be helpful to implement the TG test for monitoring doses and frequency of these treatments.

PB 1.38-4

Patient reported outcomes in clinical hemophilia practice

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Background: Children with congenital bleeding disorders may experience Health Related Quality of Life (HRQOL) problems. Two multicenter studies measuring the effect of electronic Patient Reported Outcomes (ePROs) in clinical pediatric practice pointed out that discussing HRQOL during consultation increases the psychosocial items discussed and the identification of emotional problems. It also increased the satisfaction of the pediatricians with their provided care.

Aims: The aim of this abstract is to describe the implementation of ePROs in pediatric hemophilia practice with the use of the KLIK website.

Methods: In September 2011, ePROs were introduced as part of the standard care for children at the Hemophilia Treatment Center AMC. Children (8–18 years) or their parents (if the child is < 7 years) with congenital bleeding disorders complete online HRQOL questionnaires (TNO-AZL Preschool children Quality of Life; TAPQoL or Pediatric Quality of Life Inventory; PedsQL) at the website www.hetklikt.nu before regular consultation. The answers are converted into a KLIK ePROfile, and discussed during consultation with the pediatric hematologist. Before multi-disciplinary consultations, patients also complete a behavioral problems questionnaire (Strengths and Difficulties Questionnaire; SDQ), which is discussed by a psychologist, and parents complete the distress thermometer (Distress Thermometer for Parents; DT-P), discussed by a social worker.

Results: So far, 106 children (out of 157 patients with congenital bleeding disorders; 70%) completed the online questionnaires and discussed the KLIK ePROfile during consultation. Children, parents and pediatric hematologists are positive about the use of the KLIK ePROfile. With the use of the internet, the KLIK ePROfile is easy to implement in clinical practice and helpful in facilitating communication about HRQOL. Moreover, KLIK helps to detect psychosocial problems at an early stage and to provide tailored interventions. Other Dutch Hemophilia Treatment Centers will start using the KLIK ePROfile in the near future.

Summary/Conclusions: The implementation of the KLIK ePROfile in daily clinical hemophilia practice appears to be feasible. To see an example of the KLIK ePROfile, log in as a doctor on www.hetklikt.nu with 'PRO' as username and 'KLIK' as password. The website is also available in English. On the website you can find an informative movie about KLIK.

PB 1.38-5

Adherence to prophylaxis in the Netherlands: a multicentre study

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Background: To prevent bleeding and joint damage in severe haemophilia and other congenital bleeding disorders, adherence to prophylactic treatment with clotting factor concentrates is of great importance. To be able to provide adequate support and to formulate recommendations for haemophilia care, studies with regard to adherence and the possible risk factors for non-adherence are of significance.

Aim: To assess adherence and to quantify deviations from the treatment schedule in patients with congenital bleeding disorders on prophylactic home treatment.

Methods: In three Dutch haemophilia treatment centres, semi-structured interviews were embedded in the routine consultation by

the haemophilia nurse. Interviews included questions on the timing of infusions and deviations from prescribed therapy over the last 2 weeks. High adherence was defined as a minimum of 90% adherence rate: no deviation from the prescribed schedule and skipping of prophylactic infusions no more than once per month. Parents who infuse their child and patients on self-infusion were analysed separately.

Results: In total, 243 patients were included (Utrecht $n = 186$, Amsterdam $n = 43$ and Rotterdam $n = 14$), comprising 71% of the total population on prophylaxis in these centres. Median age was 19.9 years (range 2.0–76.9), with 86% severe haemophilia A, 10% severe haemophilia B and 4% other bleeding disorders. Parents who infused their child ($n = 75$), deviated significantly less from prescribed treatment than the 168 patients who infused themselves (high adherence: 71% of the parents vs. 50% of the patients, $P < 0.01$). Skipping or forgetting an infusion was rare; parents skipped 1.2% infusions and patients 9.2% infusions. Both parents and patients were aware (93%) of the importance of infusing in the morning. However, 29% of the parents and 44% of the patients did not administer the clotting factor concentrate at this time point. Only 5.3% of patients changed the prophylactic dose on their own initiative.

Conclusion: In this study 50% of all patients on prophylactic home treatment demonstrated a high adherence, 71% of all parents. These results are slightly better than adherence rates reported in other chronic diseases. However, adherence rates decreased with increasing age. Both groups mainly deviated from prescribed therapy with regard to timing, instead of skipping infusions. Preferences and barriers for adherence to prophylactic therapy, as well as effects of non-adherence on bleeding and clotting factor consumption should be assessed to further improve haemophilia care.

Keywords: adherence, compliance, prophylaxis, haemophilia.

PB 1.38-6

Immuno-monitoring of patients with severe hemophilia A

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Background: Immune tolerance induction (ITI) is the only available treatment that allows efficient eradication of FVIII inhibitors in patients with hemophilia A. ITI consists in the repeated administration of therapeutic FVIII. It is successful in 60–80% of the patients. However, ITI is complicated by the facts that it requires an extreme compliance of the patients and that the cost of treatment may reach more than 0.2 million euros/patient/year. It is thus essential to identify clinical and biological parameters that are associated with ITI success or failure. We initiated an immuno-monitoring study as a satellite study of the Observational Immune tolerance Induction (ObsITI) research program to follow immunological parameters in patients undergoing ITI.

Aim: To follow the evolution of CD4+ T cells and B cells during ITI in patients with hemophilia A.

Methods: Blood was collected from nine patients with severe hemophilia A (< 1% FVIII activity) before initiation of ITI, and 2 weeks, 4, 9, 12 and 18 months later. Percentages of different cell populations were quantitated by flow cytometry: naive (CD45RA) and memory (CD45RO) CD4+ T cells, $CD25^{low}$ and $CD25^{high}$ CD4+ T cells, and naive (IgD+IgM+) and memory (IgD−) B cells. The evolution of inhibitory titers, FVIII recovery and FVIII half-life were also measured to assess success/partial success/failure of ITI.

Results: Median age of patients at start of ITI was 5 years (range: 2–18). All patients received Octanate (Octapharma) for ITI. Seven patients had first intention ITI and two patients had third intention. Inhibitor was eliminated (< 0.6 BU) in 6 (67%) patients. Five patients had complete ITI success (inhibitor titre < 0.6 BU, FVIII IVR ≥ 80%, FVIII $t_{½} \geq 7$ h) after a median time of 7 months (range: 5–12), one ongoing patient has partial success and three patients failed ITI (two of whom were in third ITI course). No clear trend in evolution of naive or memory T and B cells was associated with ITI outcome. In particular, levels of memory B cells did not differ between patients with ITI success (three patients tested out of 5: 16%) and the two patients with ITI failure (10% and 25%). Likewise, increases in CD4+CD25+ T cells, that encompasses regulatory T cells, did not occur in all patients, and were found in some failure as well as ITI success patients.

Summary/Conclusion: Several parameters have been associated with ITI outcome or with the duration of treatment until ITI success. These parameters include the age of the patients at start of ITI, the high or low dose FVIII treatment, the recombinant or plasmatic origin of the FVIII used for ITI, and the properties of the FVIII inhibitor. Our results on the immuno-monitoring of a small cohort of severe hemophilia A patients before and during ITI does not confirm that patients with ITI success have higher levels of memory B cells, as compared to patients with partial success or failure. Likewise, we do not find that tolerance induction is associated with a consistently increase of CD4+CD25+ regulatory T cells.

PB1.39 – Haemophilia B – I

PB 1.39-1

Treatment of haemophilia B- comparison study of the *in vitro* activities of plasma derived and recombinant factor IX

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Background: Haemophilia B results from a deficiency of coagulation factor IX (FIX) and is treated effectively by either recombinant (r-FIX) or plasma derived (pd-FIX) although these have been noted to display some differences in behaviour. FIX is activated *in vivo* by both FXIa and tissue factor (TF)-FVIIa, however conventional APTT-based assays assess only activation by FXIa.

Aims: To examine the differences between pd-FIX and r-FIX concentrates in terms of their activities in thrombin generation and activation by different proteases.

Methods and Results: For equivalent labelled potency, FIX ELISA showed the antigen content of pd-FIX concentrate was ~1.6 fold greater than rFIX. Using calibrated automated thrombography (CAT), triggered by TF (1.5–5 pM) and 90 nM FIX antigen, the peak thrombin produced by r-FIX was > 1.5 fold that by pd-FIX. A similar but less marked difference was seen after contact activation. We noted that r-FIX stimulated significant thrombin generation in the absence of TF or contact; whereas, pd-FIX did not. Chromogenic FIXa assay demonstrated the presence of FIXa in r-FIX was ~10 fold that of pd-FIX at equal antigen concentrations, which may explain the above findings. Because we expect FIXa to be cleared rapidly following infusion, this may also explain the lower *in vivo* recovery observed for r-FIX. Next, we analysed the activation of the two FIX concentrates in a purified system. Over the first 10 min of activation, the amount of r-FIXa generated by FXIa was 1.53x that of pd-FIXa, whereas no difference in activation was observed when triggered by TF-FVIIa. No accumulation of FIXα was observed, suggesting the initial interaction and cleavage is limiting. To investigate the reason for the different activation rates we studied the effect of posttranslational modifications of FIX in the two preparations. The Km for Ca^{2+} was similar for pd- and r-FIX which suggests a lack of effect of the varying profiles of GLA domain γ-carboxylation between pd- and r-FIX as a cause of their distinct activation rates. Removal of N-linked glycans and terminal sialic acids in O-linked glycans by treatment of FIX with PNGase F and Neuraminidase respectively, did not alter the activation rate of pd- or r-FIX regardless of activation by FXIa or TF-FVIIa. This indicates that differences in glycosylation profile between pd- and r-FIX do not influence FIX activation. Furthermore, dephosphorylation of pd-FIX did not reduce the difference in activation rates between the two FIX concentrates.

Conclusions: The only remaining posttranslation difference is sulfation. More than 90% of pd-FIX is sulphated at tyrosine155, compared to < 15% in rFIX but it is not possible to manipulate this *in vitro*. Protein sulfation is important for protein-protein interaction and remains a possible explanation for the difference in pd- and r-FIX activation by FXIa. In conclusion, clear differences are observed between pd-FIX and r-FIX concentrates including the proportion of FIXa and the activation by FXIa. These may explain some of the discrepancies observed clinically and suggest that the APTT may not reflect their resultant *in vivo* properties.

PB 1.39-2

Restoration of coagulation factor IX function impaired by different splicing mutations by a unique exon-specific U1 small nuclear RNA (snRNA)

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Background: Limitations of replacement therapy encourage research toward alternative strategies for coagulation deficiencies. The U1snRNA, the component of the spliceosomal U1snRNP with a key role in exon definition during the earliest pre-mRNA splicing step, represents an attractive molecule because of its ability to rescue splicing impaired by mutations, a frequent cause of coagulation factor disorders (~15%). Though an engineered U1snRNA, designed to restore complementarity to the mutated donor splice site, we have previously demonstrated in cellular models the rescue of coagulation factor VII mRNA splicing and protein function impaired by the IVS7+5G/A mutation.

Aims: To explore the ability of modified U1snRNAs to rescue different splicing mutations in coagulation factor IX (FIX) gene, and associated to moderate/severe Hemophilia B.

Methods: Creation of plasmids for the expression of splicing-competent FIX cDNA constructs and of modified U1snRNA in Human Embryonic Kidney (HEK293) cells. Evaluation of FIX mRNA (RT-PCR) and protein (ELISA. aPTT-based coagulation assays, Western Blotting) levels in conditioned medium.

Results: We investigated the aberrant splicing mechanisms caused by the IVS5-2A/T, IVS5-2A/C, IVS5-2A/G, IVS5-1G/T and IVS5+1G/A mutations at the donor splice site (5′ss) and by the IVS5-8T/G, IVS5-9T/G changes in the polypyrimidine tract of the acceptor splice site (3′ ss) of *F9* exon 5.

All mutations almost exclusively induced exon 5 skipping from the mature FIX mRNA, thus predicting the synthesis of a FIX variant lacking the EGF2. Studies at the protein level demonstrated that this deleted FIX variant was secreted with reduced efficiency as compared to FIXwt, and did not elicit any appreciable coagulant activity. Co-expression of these FIX minigenes with a U1snRNA designed to bind by complementarity to the normal FIX IVS5 5′ss (U1-FIXwt) was able to restore exon 5 inclusion in the presence of the 5′ss IVS5-2A/T, IVS5-2A/C and IVS5-2A/G mutations. Noticeably, the U1-FIXwt also corrected both the IVS5-8G and IVS5-9G at the 3′ss. Rescue of FIX mRNA splicing was paralleled by secretion of the full-length FIX with normal coagulant activity.

To improve specificity for *F9* gene we tested a panel of U1snRNAs, named Exon Specific U1snRNA (ExSpeU1), targeting non-conserved intronic sequences downstream of the 5′ss. Intriguingly, we found a gradient of rescue efficacy, which decreased with the distance from the 5′ss. The best ExSpeU1, targeting intronic positions +9 through +18, rescued exon 5 inclusion (from undetectable to 70–80%) in the presence of the mutations IVS5-2A/T, IVS5-2A/C and IVS5-2A/G at the 5′ss or of the IVS5-8G and IVS5-9G at the 3′ss, and resulted in remarkable increased secretion (~2–3 fold) of FIX molecules with procoagulant activity (from undetectable to ~100%).

Conclusions: These results demonstrate for the first time that a unique ExSpeU1 is able to restore gene expression impaired by different splicing mutants, and the extent of rescue of the procoagulant function, if achieved in patients, would be far beyond the therapeutic threshold. These data further highlight the therapeutic potential of the U1snRNA-mediated approach. Study to assess rescue of FIX expression in mouse models are underway.

PB 1.39-3

Improvement in health-related quality of life with recombinant factor IX prophylaxis in moderately-severe or severe hemophilia B patients: results from the BAX326 pivotal study

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Background: Health-related quality of life (HRQoL) benefits of prophylaxis have been largely drawn from the experiences of patients with hemophilia A. Clinical research has yet to establish the impact of prophylaxis on HRQoL in patients with hemophilia B.

Aim: To assess the impact of BAX326 (recombinant Factor IX) prophylaxis on HRQoL in adult patients with moderately-severe or severe hemophilia B who participated in the pivotal clinical trial.

Methods: A Phase I/III multicenter clinical trial prospectively evaluated the outcomes of patients with moderately severe ($1\% \leq$ FIX level $\leq 2\%$) or severe (FIX level < 1%) hemophilia B who were treated with BAX326 on-demand or prophylactically. Written informed consent was obtained from each participant and the study was approved by Independent Ethics Committee (IEC) of each participating institution. HRQoL data were collected from subjects aged ≥ 17 years using a generic HRQoL instrument, the Short Form-36v2 (SF-36). Baseline and 6 months follow-up HRQoL data in the prophylaxis arm were analyzed. Changes in HRQoL between baseline and follow-up in each of the SF-36 domains were tested for significance using paired t-tests. To further study the benefit of BAX326 prophylaxis, subgroup analysis was conducted to examine HRQoL changes among patients who had switched to BAX326 prophylaxis from either intermittent prophylaxis or on-demand treatment. Changes in SF-36 domain scores were compared to the previously established minimally important difference (MID) to determine clinically meaningful changes.

Results: Fifty-nine males participated in the prophylaxis arm of this study. Of these, 55 completed the SF-36. The median age for those who completed the SF-36 was 33.0 years. At Week 26, subjects reported statistically significant and clinically meaningful improvements in overall physical health-related quality of life, as measured by the Physical Component Score (PCS) (mean change = 2.6, $P = 0.018$), Bodily Pain (3.4, $P = 0.014$), and Role Physical domains (3.4, $P = 0.016$). Patients who switched to prophylaxis from intermittent prophylaxis or on-demand ($n = 42$) experienced more pronounced benefits, and reported statistically significant and clinical meaningful improvements not only in the PCS (3.21, $P = 0.014$), Bodily Pain (3.78, $P = 0.026$), Role Physical (4.43, $P = 0.008$), but also in the Vitality (3.72, $P = 0.04$), Social Functioning (5.06, $P = 0.002$), and General Health domains (3.4, $P = 0.009$).

Conclusions: Patients with hemophilia B experienced both statistically significant and clinically meaningful improvements in physical HRQoL after 6 months of BAX326 prophylaxis treatment. Furthermore, patients who switched to prophylaxis from intermittent prophylaxis or on-demand experienced not only larger improvements in physical HRQoL, but also statistically significant and clinical meaningful improvements in mental HRQoL.

PB 1.39-4

Complex gene rearrangement combining both large F9 gene duplication and exon 6 deletion in severe hemophilia B patients

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Background: Hemophilia B is an X-linked coagulation disorder caused by a wide range of mutations in the factor IX (*F9*) gene, mainly point mutations. Whole *F9* gene deletions and large *F9* gene deletions encompassing one or several exons are also reported and represent about 5% of the total defects. Identification of the causative mutation is not necessary for the diagnosis of the disease but is essential for genetic counselling.
Aim: Here, we report a complex rearrangement of *F9* gene associated with a same exon 6 deletion in severe non related hemophilia B families. Surprisingly, these families are originated from the same geographic region, Indian Ocean (La Reunion Island and Madagascar).
Methods: Exon 6 deletion was detected by PCR amplification in male patients and the breakpoint identified by Long Range-PCR and sequencing. Potential carriers in the families were further studied by either Multiplex Ligation Probe Assay (MLPA) and/or quantitative fluorescent multiplex-PCR (QFM-PCR) and array CGH for one family. These quantitative assays were also used to study the hemophilia patients.
Results: The breakpoints of the 6467 bp exon 6 deletion have been identified and are the same in all seven families c.[521–286_724-31497del]. The quantitative assays revealed a *F9* gene duplication encompassing all exons excepting exon 6, in three of seven of these families. Investigation by array CGH revealed that the duplication covers 514 kb and affects four other genes: *MCF2*, *ATP11C*, *MIR505* and *CXorf66*.
Conclusion: This is the first report of a complex *F9* gene rearrangement combining both gene duplication associated with deletion. The exon 6 deletion might be considered as causative event of hemophila B. Whether the duplication can be considered as deleterious or as a copy number polymorphim (CNP) is in discussion. Indeed, an Xq28 duplication has been recently reported in patients with intellectual disability without low factor VIII levels. A founder effect is suspected to have occurred in all these families bearing the same deletion. The genetic mechanism explaining this association is still unknown. This study suggests that a quantitative study should be realized in all patients with a deletion in *F9* gene to detect complex rearrangement, as shown in these families.
Acknowledgements: Thanks to the practitioners for addressing these families: P Lutz (Pédiatrie, Strasbourg), A Rafowciz (Centre de Traitement des Hémophiles Kremlin Bicêtre), F Cartault (Génétique Ile de La reunion).

PB 1.39-5

An amazing 10th century old mutation with a high prevalence in haemophilia B patients of the Rhone-Alpes region, France

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Background: The g.30150G>A substitution in the exon 6 of the *F9* gene (p.Ala279Thr) is responsible for mild haemophilia B, a X-linked recessive bleeding disorder caused by a deficiency or a functional loss of blood coagulation factor IX. Whereas this missense mutation is observed in different geographical areas with a low prevalence (37 times/2891 *F9* mutations, 1.3%) as estimated from the Haemophilia B database, its prevalence is much higher in Rhône-Alpes region, France (50 times/209 *F9* mutations, 23.9%, $P < 10^{-80}$).
Aim of this Study: to understand the high prevalence of this p.Ala269Thr missense mutation in Rhône-Alpes.
Methods: Thirty four unrelated patients carrying the g.30150G>A substitution and 50 control individuals were studied. Four Single Nucleotide Polymorphisms (SNP) -*Xmn*I (rs438601), *Taq*I (rs398101), *Mnl*I (rs6048) and *Hha*I- and one insertion/deletion – *Dde*I – within or in the near vicinity of the *F9* were tested by restriction fragment length polymorphisms along with 5 short tandem repeats (poly(AC)) – DXS1061, DXS1211, DXS1192, DXS1232 and DXS984 -flanking the *F9* gene on a 3 cM genetic interval. ESTIAGE program was used to estimate the age of the most recent common ancestor of mutation carriers.
Results: all males carrying the g.30150G>A mutation had the same haplotype at the closest polymorphic markers. Moreover, this haplotype was totally absent from the 50 control DNA demonstrating a founder effect. Concerning the combination of alleles, the probability of not being due to a founder effect displayed by the ESTIAGE software was very low (10^-59). ESTIAGE estimated that the most recent common ancestor lived about 56 generations ago (range from 42 and 77 generations). With an average generation every 20–25 years, the mutation presumably appeared at about the Xth century.
Summary and Conclusions: Seventy one percent of mild haemophilia patients in the Rhône-Alpes have the g.30150G>A mutation. Interestingly, all are descendant of a common ancestor who lived approximately at the Xth century. Founder effect has already been described in haemophilia B but in this study, we used extragenic flanking poly (AC) repeats to enable an estimation of dating the most recent common ancestor. The very ancient appearance of this mutation is an unexpected observation based on the estimated lifetime of a mutation in X-linked recessive disease, which is usually much shorter. This observation suggests that the Rhône-Alpes founder mutation in the *F9* gene could give a fitness benefit to hemizygous male patients or/and to heterozygous female carriers. It is also possible that a mild haemophilia B does not impair reproductive fitness of male carriers.

PB 1.39-6

Superiority of the chromogenic assay specific for activated factor IX over the non-activated partial thromboplastine time (NAPTT) clotting assay in detecting FIXa in recombinant FIX preparations

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Background: Due to the risk of thrombogenicity in hemophilia B patients, FIXa impurities in FIX concentrates should be minimal. Cur-

rently, two methods for detection of FIXa in rFIX preparations are available: a chromogenic assay specific for FIXa, and a one-stage clotting assay recommended by European Pharmacopeia (EP), which is sensitive to activated coagulation enzymes.
Aims: In this study we evaluated the suitability of a chromogenic FIXa and an NAPTT assay for measuring FIXa impurities in rFIX drug substance and drug product preparations.
Methods: We used a commercially available chromogenic FIXa assay in kit format (Rossix). FIXa activity was quantified specifically against an FIXa standard provided in the kit whose potency was assigned vs. a WHO International Standard for FIXa. FIXa was normalized for rFIX, which was tested by a standard clotting assay (APTT), and calculated as % FIXa (IU FIXa/IU FIX x 100). The NAPTT assay was performed as a one-stage clotting assay in human plasma by addition of phospholipids (tachostyptan, Baxter). Clotting was initiated with Ca^{2+} after addition of sample or buffer. The NAPTT assay readout was considered valid when meeting the criteria specified in EP (> 200 and < 350 s for the blank). Samples were measured undiluted and in serial dilutions ranging from 1:2 to 1:4096. The shortest NAPTT readout in seconds within this dilution series was related to the corresponding blank value for each sample by calculating a ratio.
Results: The FIXa content of 20 rFIX preparations with FIX-concentrations ranging from 50 to 3000 international units (IU) per mL (BAX326 drug substance and drug product, and licensed rFIX [Benefix, Pfizer]) was measured by chromogenic and NAPTT assay. NAPTT ratios ranged from 0.85 to 1.02, chromogenic results from 0.003% to 0.07% FIXa; values for each sample did not correlate. Preparations with the highest FIXa content did not have the lowest NAPTT ratios, as would have been expected, and vice versa.
Summary/Conclusions: A correlation between FIXa levels quantified by chromogenic assay and the ratios of sample to blank NAPTTs in rFIX drug substance and drug product preparations was not found over a broad range of FIX concentrations. The NAPTT assay was of limited use in determining the amount of FIXa in FIX products, therefore, the use of NAPTT only for product release would lead to possible release of batches with higher FIXa content.

PB1.40 – Heparin-Induced Thrombocytopenia: Clinical – I

PB 1.40-1

Evaluation of fondaparinux for the treatment of serotonin release assay positive heparin-induced thrombocytopenia

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Background: Diagnosis and management of heparin-induced thrombocytopenia (HIT) remains challenging. The current standard of therapy involves rapid initiation of intravenous direct thrombin inhibitors (DTI) with transition to oral anticoagulation. Due to the coagulation assay interference and high cost of DTI therapy, alternative cost effective and clinically appropriate therapies have been explored for many years. Fondparinux represents a potential treatment option because of a clinical lack of cross reactivity to heparin-PF4 antibody and lack of coagulation assay interference. However, clinical experiences with fondaparinux, especially in serotonin-release assay (SRA) positive patients is limited and requires further research.
Aims: To evaluate the clinical outcomes of fondaparinux therapy in patients with SRA positive HIT.
Methods: The Beth Israel Deaconess Medical Center Institutional Review Board (IRB) approved this evaluation prior to initiation. Pharmacy and hospital databases were used to identify patients who received fondaparinux for a minimum of 24 h between January 2005 and December 2012. Data collection included patient demographics, admitting diagnosis, admitting medical or surgical service, comorbidities associated with elevated intrinsic coagulant activity (malignancy, hepatic diseases), recent surgical procedures, previous anticoagulant treatment, baseline platelet count, INR, hematocrit, hemoglobin, serum creatinine and calculated creatinine clearance(CLcr), and results of platelet-factor 4 (PF4)-Heparin dependent antibody and SRA tests. Medical charts and medication administration records were reviewed for dose and duration of fondaparinux therapy, time to platelet count recovery (i.e. Platelet count > 100,000 mm^3 or return to patient baseline), day of warfarin initiation, warfarin doses administered, duration of dual fondaparinux and warfarin therapy, and incidence of recurrent venous thromboembolism (VTE) at 6 months, and major bleeding.
Results: Thirteen patients with SRA positive HIT, who were treated with fondaparinux rather than DTI, were identified and evaluated. No patients experienced active thrombosis as part of the diagnosis of HIT. Mean age was 63 years old and 57% were female, and the mean CLcr was 72 mL/min. The most common admitting services were surgical, followed by medical. PF4 Antibody and SRA testing were performed in all patients. Fondaparinux was consistently dosed at 7.5 mg once daily, and the mean duration of therapy was 6 days. Patients were transitioned to warfarin, usually beginning on day 2. During the follow-up period, there were no cases of thrombotic complications, recurrent VTE at 6 months, or major bleeding.
Conclusion: Fondaparinux was safe and effective, although infrequent, in the management of SRA positive HIT at our institution. Further studies with larger patient populations are needed to confirm these findings.

PB 1.40-2

Recombinant thrombomodulin as an alternate anticoagulant in the long-term management of patients with heparin-induced thrombocytopenia. Laboratory validation

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Introduction: Recomodulin® represents a recombinant form of human thrombomodulin, which has been used in Japan for the management of disseminated intravascular coagulation (Saito et al. Journal of Thrombosis and Haemostasis, 5: 31–41). This antithrombotic drug has also been tested in a clinical trial for the management of post-orthopedic surgical prophylaxis of DVT (Kearon, C et al. Journal of Thrombosis and Hemostasis, 3:962–968). Recomodulin produces its antithrombotic effects by multiple mechanisms, including the complexation with endogenous thrombin and mediating the conversion of protein C to activated protein C. It has been reported to have anti-inflammatory effects. At relatively low dosages, 0.3–0.45 mg/kg, this agent has been found to be effective in the management of post-surgical DVT. The structure and mechanism of action of this agent differed from heparins and can be used in the management of heparin-compromised patients. The purpose of this study was to determine if recomodulin cross-reacts with HIT antibodies in various settings.
Materials and Methods: Commercially available Recomodulin® was obtained in the lyophilized powder form from Asahi Kasei Pharma Corp. (Tokyo, Japan) and reconstituted in sterile saline to obtain 1 mg/mL solution. Working dilutions were prepared at 100, 10 and 1 μg/mL. The effect of recomodulin on HIT antibody mediated platelet aggregation was studied using platelet aggregometry. Platelet rich plasma preparations from normal donors (40) were supplemented at graded concentrations of 10, 1 and 0.1 μg/mL. The effect of HIT antibody was studied using individual and pooled HIT sera as a source of antibodies. Parallel positive controls were run using heparin at 10, 1 and 0.1 μg/mL. Saline served as a negative control. Recomodulin and heparin were incubated with whole blood and platelet factor 4 release was measured using an ELISA method.

Results: In a concentration range of 1–10 μg/mL, recomodulin did not facilitate HIT antibody mediated aggregation of platelets. In both the pooled and individual HIT sera studies, in contrast to recomodulin heparin mediated HIT antibody aggregation of platelets at 10 and 1 μg/mL in all PRP collected from various donors. At 0.1 μg/mL a slightly weaker aggregation response was observed in 30/40 donors' PRP. Incubation of heparin produced a measurable release of platelet factor 4 from platelets; whereas, recomodulin did not produce any release of PF4.
Discussion: These results suggest that recomodulin can be used as an alternate anticoagulant for the management of such heparin compromised patients as the heparin induced thrombocytopenia group. These studies also suggest that recomodulin does not interact with HIT antibodies and will not mediate platelet aggregation in HIT patients. Moreover, recomodulin does not release any platelet factor 4 and their subsequent complexation with heparin or heparin like substances. Since at relatively low dosages this agent has been found to be effective for extended prophylaxis of DVT, these studies indicate that recomodulin may be a useful option for the management of patients with heparin-induced thrombocytopenia.

PB 1.40-3

Variations in the prevalence of HIT antibodies during the period 2004–2012. Relevance to heparin contaminants

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Introduction: The generation of anti-heparin platelet Factor 4 antibodies depends on several factors of which the quality of heparin in terms of its composition plays a major role. Heparins contaminated with other glycosaminoglycans and varying sources produce variable immunogenic responses. During the period of November 2007–April 2008 several batches of unfractionated heparin were recalled due to unexpected adverse reactions. These heparins were characterized to contain such contaminants as oversulfated chondroitin sulfate and high molecular weight dermatan sulfate. Other heparin-like glyocaminoglycans were also reportedly present in these preparations. Several centers also reported a high prevalence of heparin induced thrombocytopenia (HIT) during this time. In order to compare the prevalence of HIT antibodies in patients undergoing maintenance hemodialysis, plasma samples from different time periods were analyzed during the period spanning 2004–2012.
Materials and Methods: In conjunction with an ongoing program to investigate the prevalence of HIT antibody (HIT Ab) in ESRD, blood samples were collected from patients (n = 53–139) on maintenance dialysis were retrospectively analyzed for the presence of these antibodies during the period 2004–2013. Specified cut off levels were used to determine the positive and negative results. An ELISA method (GTI.Brookfield, WI) was used to quantitate the HIT antibodies.
Results: The prevalence of the HIT Ab during the period 2004–2011 was < 15% with the exception of 2007 where it was 35% and in 2008 it was 30%. A decreased prevalence was noted during the period 2009–2011 (13–15%). The samples collected in November 2012 showed an even lower prevalence (7%) of these antibodies. The antibody titers as quantified by optical density measurement were relatively higher during the year 2007 and 2008. Moreover, the subtyping of these antibodies in 2007 and 2008 showed a higher proportion of IgG subtype. Interestingly the platelet counts did not differ in the blood samples collected in different years. Similar trends were noted in the prevalence of HIT antibodies when other methods: to detect HIT antibodies were used.
Conclusions: The oversulfated glycosaminoglycans such as oversulfated chondroitin sulfate and other non-heparin GAG derivatives are most likely to contribute to this increased prevalence and seroconversion of HIT antibodies in 2007–2008. These observations suggest that heparin contaminants have contributed to the higher prevalence of HIT antibodies during the period 2007–2008. The improved quality measures resulting in more purified heparins after this recall have resulted in a decreased prevalence of these antibodies.

PB 1.40-4

Improving specificity for diagnosing heparin-induced thrombocytopenia through repeated testing

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Background: Prompt recognition and appropriate treatment of heparin-induced thrombocytopenia (HIT) is required to reduce the risk of serious thrombotic events. Because of the challenges of clinical diagnosis, physicians rely heavily on laboratory testing. Investigation of HIT is common in patients undergoing cardio-pulmonary bypass surgery-due to frequent development of thrombocytopenia post-surgery on a Background: of heparin exposure. 2012 BCSH Guidelines suggest that a cut-off point for a positive test should be used when using an immunologic ELISA to look for HIT antibodies, rather than simply reporting a positive or negative.
Aim: This study looked at the pattern of HIT antibody requesting in a reference laboratory, and the trend of sequential quantitative results obtained using the ELISA immunoassay.
Method: This was a retrospective study of all HIT antibody requests received over the 12-month period December 2011–2012. Results were reported as a quantitative optical density (OD) value. An OD ≥ 1 was deemed a clear positive result, 0.7 to < 1 high negative and < 0.7 clear negative.
Results: Four hundred and fourteen requests were received over 12 months for total 238 patients. 255/414 (62%) requests came from the cardio-thoracic unit and cardiothoracic ITU. Repeat testing (next day) was noted in 86/238 (36%) patients, of which 80/86 (93%) were from cardiac patients. For this sub group, all 51/80 (64%) patients with an initial clear negative test result showed no increase in their OD > 1 with sequential testing. For patients with an initial high negative OD, 14/24 (58%) showed a progression in their OD to ≥ 1. This was statistically significant using Fishers Exact Probability test, $P < 0.001$.
Conclusion: Sequential testing for HIT is common in cardiac patients. Initial high negative OD values 0.7–1.0 have a significant chance of becoming clear positive with repeat testing. High negative ODs should be repeated and such a strategy incorporated into diagnostic algorithms to improve the specificity of the ELISA for the diagnosis of HIT.

PB 1.40-5

Further studies on the pro-inflammatory and thrombotic mediators in patients with suspected heparin-induced thrombocytopenia

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Background: Heparin-induced thrombocytopenia (HIT) represents a complex pathophysiologic syndrome in which inflammatory processes, thrombogenesis and vascular manifestations contribute to the morbidity and mortality outcomes. Inflammatory mediators including CD40L, adhesion molecules, tissue factor (TF), microparticles, and vonWillebrand factor (vWF) contribute to the pathogenesis. In addition, several other mediators of inflammation are generated which are not fully characterized at this time. This study was undertaken to

profile some of the mediators of inflammation and thrombogenesis along with the biomarker profiling using a mass spectrometric method.

Material and Methods: Forty (40) baseline samples from patients with suspected HIT were collected at theKarolinskaHospital, Stockholm, and frozen at −70 °C. The control represents plasma samples from 40 healthy normal males and females who have apparently never received heparin or related drugs. These samples were analyzed for HIT antibodies using two ELISA methods from GTI (Wisconsin, USA) and Hyphen (Paris, France). Serotonin release assay (SRA) was also carried out on all samples. vWF, TF antigen and functional microparticles quantitated by annexin trapping were measured by assays from Hyphen. CD40L, P-selectin and ICAM antigens were measured using assays from R&D Systems (Minnesota, USA). Protein chip array was carried out on a SELDI-TOF mass spectrometer (PCS 4000; BioRad, California, USA) employing a gold chip array in the molecular range of 3–150 kDa.

Results: Thirty-eight out of 40 patient samples were positive in the GTI and 37 out of 40 samples were positive in the Hyphen methods. Nineteen samples were positive in the SRA. None of the controls showed positive response in any of the HIT antigen assays or SRA. P-selectin levels were the same in patient and control groups. TF (16.8 ± 4.3 vs. 6.8 ± 3.2 pg/mL) and microparticles levels (25.2 ± 22.1 vs. 4.8 ± 3.8 nM) were higher in the HIT group. CD40L (135 ± 119 vs. 82 ± 48 ng/mL) and ICAM (416 ± 43 vs. 180 ± 20 ng/mL) levels were higher in the HIT group. vWF was increased in the HIT group (1.6 ± 1.0 vs. 0.7 ± 0.3 U/mL). In the SELDI-TOF analysis 32 out of 40 samples showed a unique peak at 11.6 kDa which was absent from the normal samples. Additional peaks at 15.1 and 15.8 kDa were also present in the HIT samples.

Conclusion: These studies further validate that both the inflammatory and hemostatic activation processes are involved in the mediation of the pathogenesis of HIT antibody associated complications. The increased levels of TF, microparticles, vWF, along with the up regulation of CD40L and ICAM may further augment the vascular manifestations. The presence of unique biomarkers also suggests protease dysregulation in this complicated syndrome and require additional characterization of these unique peaks. These studies also suggest that the heterogeneous group of HIT antibodies in this syndrome plays a central role in the regulation of inflammatory and hemostatic activation processes.

PB 1.40-6

Suspicion heparin induced thrombocytopenia in internal medicine: how appropriate are the prescriptions of anti-PF4 antibodies tests?

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Background: Predictive scores (4T's and HEP) to derive a probability for type II heparin induced thrombocytopenia (HIT) have been developed but their real impact in everyday practice is uncertain, particularly in internal medicine poly-morbid patients.

Aims: To describe the clinical management of patients suspected of HIT and evaluate to what extent the 4T's score and the HEP score might safely decrease anti-PF4 antibodies testing in internal medicine patients clinically suspected of type II heparin induced thrombocytopenia (HIT).

Methods: Internal medicine patients treated with unfractionated heparin (UFH), low molecular weight heparin (LMWH) or fondaparinux in whom anti-PF4 were assayed were included. Non inclusion criteria were absence of thrombocytopenia or fall of platelets count and transfer from another hospital.

Results: We included 74 patients over a 29-month period with a mean age of 68 ± 16 years. Twenty-five (34%) were transferred from the intensive care Unit. Forty-three (58%) were treated with UFH, 31 (42%) with LMWH and none with fondaparinux. Indication was thromboprophylaxis in 45 (61%) and therapeutic in 29 (39%). Eleven patients (15%) tested positive for antiPF4 and 4 (5.4%) had confirmed HIT. Clinicians documented the 4T's score in only 10 (14%) patients and the HEP score was never used. The retrospectively calculated 4T's score was < 4 in 54 patients (73%) who would not have needed antiPF4 testing. HEP score was < 2 also implying no antiPF4 testing was necessary in 34 patients (46%, $P < 0.0001$ compared to 4T's). Both scores had a sensitivity of 100% (no patient with a score below the cut-off had HIT). Specificity of 4Ts was 77% and that of HEP was 48%. Following anti-PF4 testing, clinicians stopped heparin in 48 patients (65%) among which 25 (34%) were switched to fondaparinux while the remaining had either a transient or definitive anticoagulant arrest. In three patients, a heparin-induced platelet aggregation (HIPA) test was performed (one despite a 4T's score < 4). At 3 months of follow-up none of the patients were readmitted for a HIT-related condition.

Conclusions: Validated and recommended scores for HIT were vastly underused in this internal medicine population while they could have avoided 73% of the requested antiPF4 tests (4T's) or 46% (HEP) and have a high sensitivity and safety using the appropriate cut-offs.

PB1.41 – Rare Bleeding Disorders – II

PB 1.41-1

Update on GGCX sequence variations causing combined deficiency of vitamin K-dependent coagulation factors (VKCFD) type 1 with a new case of compound heterozygosity

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Background: Hereditary combined deficiency of vitamin K-dependent (VKD) coagulation factors is a rare autosomal recessive bleeding disorder, usually characterized by an early age of onset. Clinical symptoms of VKCFD vary mainly in accordance with pro-coagulant factors levels. VKCFD is genetically heterogeneous since VKCFD is caused by mutations in two distinct genes: the gene encoding for gamma glutamyl carboxylase (*GGCX*) and the gene encoding for the subunit 1 of vitamin K epoxide reductase complex (*VKORC1*). Both enzymes are involved in the vitamin K cycle and lead to post-translational modification of VKD factors to yield their biologically active form, which is an essential prerequisite for blood coagulation.

Aims: We have studied the genetic background of a full-term French male infant presenting a typical phenotype of VKCFD.

Methods: The infant, born with a forceps assistance showed retro-auricular haematomas and a cephalhematoma. He was referred to the Regional centre of bleeding disorders for clinical and biological exploration. After exclusion of acquired forms of the disorder, the two VKCFD related genes were studied using direct sequencing. *In silico* programs were applied to predict the functional significance of the variants. We finally summarized and presented an updated list of mutations associated with the phenotypic VKCFD1 background.

Results: The proband displayed an icterus having required extracorporeal phototherapy but did not show facial dysmorphy, organomegaly, nor infectious syndrome. No drug intake during pregnancy was reported. He is the first offspring of healthy non-consanguineous parents. The family history was negative for bleeding disorder.

Blood coagulation tests revealed a moderate deficiency of both VKD procoagulant factors and inhibitors. FV, VWF and FVIII levels were strictly in the normal range. A relative resistance to parenteral vitamin-K therapy led to explore genetical analysis for VKCFD.
Sequence analyses revealed a *GGCX* compound heterozygosity for a missense mutation of paternal inheritance p.Pro61Leu which is located in the first amino acid of the first transmembrane domain of GGCX, and for a frameshift mutation of maternal inheritance p.Tyr690Cysfs*39 in exon 14 which may give rise to a truncated protein. This was the eleventh case of VKCFD type 1 reported without PXE-like disorder associated, and the sixth involving a new compound heterozygosity.
Searches for Panther families and trees showed that Pro61 and Tyr690 are located in a highly conserved region. Interestingly, as predicted by JPred algorithm, both variants, if ever secreted, may impact the VKD protein site of interaction region of GGCX designed by Wu *et al*. (Blood 1997;89: 4058).
The follow-up of the child at 23 months of age displayed that the level of VKD coagulation factors returned to near-normal following treatment with 2 mg doses of vitamin K, three times a week, and there were no sign of skeletal abnormalities.
Summary/Conclusions: We identified a new *GGCX* compound heterozygosity (NP_000812.2: p.[Pro61Leu];[Tyr690Cysfs*39] associated with VKCFD1 in a French male infant. Functional impairment of the two variants was deduced from *in silico* studies. The collection of all genetically characterized VKCFD1 cases is also presented and could be useful for further genetic studies.

PB 1.41-2

Arg69Pro is a novel mutation in factor VII with defective binding to rabbit thromboplastin

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Background and Aim: Heritable Factor VII (FVII) deficiency is a rare autosomal recessive disorder with highly variable phenotype. It is caused by mutations in the *F7* gene located at position 13q34. *F7* gene contains 9 exons (1a, 1b and 2–8) of 12.8 kbp that are translated into a 466 primary polypeptide. Many functional polymorphisms and mutations are described which can explain the variable phenotype. Therefore characterisation of the underlying molecular defect in these individuals facilitates diagnosis, informs clinical management and provides information about structure -function relationships in FVII.
Patients and Methods: Fifteen patients with reduced FVII levels were screened for mutations in the *F7* gene. Mutations were found in 13 patients. History were unknown for four cases. 6/9 patients had mild bleeding symptoms while 3/9 patients were asymptomatic and detected following incidental finding of a prolonged prothrombin time. Except for two siblings, cases were unrelated probands. The FVII:C assay was based on Quick's one stage clotting assay performed on the ACL 3000 coagulometer using Hemosil PT-Fibrinogen HS reagent (both from Instrumentation Laboratory, USA). Genomic DNA was amplified by PCR using M13 tailed primers (Eurofins MWG Synthesis, GmbH.) as previously described. The DNA was amplified in 35 cycles with denaturation at 96 °C (30 s), annealing at 67 °C (1 min) and extension at 72 °C (1 min). Amplicons were analysed by direct sequencing in AB3100 Avant Genetic analyser (Applied Biosystems, UK). Chromatograms were scanned for variations using the Staden package (http://staden.sourceforge.net/). Variants were modelled on the crystal structure of tissue factor (TF) complexed with FVIIa (PDB Id: 1DAN) using PyMOL (DeLano Scientific LLC).
Results: Mutations and polymorphisms were detected in 13 out of 15 patients all in the heterozygous state. One novel missense mutation p.Arg69Pro in exon 2 encoding the Gla domain of FVII molecular structure was found. This patient's FVII activity was discrepant with a markedly lower level of 7 IU/dL when assayed with rabbit thromboplastin compared with 44 IU/dL with human thromboplastin. The defective binding to rabbit thromboplastin has been described previously in patients with mutations in serine protease domain (p.Arg364Gln) and in EGF1 domain (p.Arg139Gln). Both the mutations, p.Arg139Gln and p.Arg364Gln are in the region that directly binds with TF and which explains the discrepancy. It is not clear how Arg69 might interact with TF as the resolved structure only contains the extracellular domain. It is likely that Arg69 disrupts interaction with the phospholipid component of the thromboplastin reagent which may then have a secondary effect on TF binding.
Other findings included four previously described missense mutations, two synonymous variations, two donor splice site and one nonsense mutation. Several polymorphisms were found with three being functional polymorphisms which is known to effect FVII levels namely g.73G>A, IVS7 and p.Arg413Gln.
Conclusion: A novel mutation identified and the effects on protein structure predicted. Previously described mutations found in these individuals may help in greater understanding of the clinical phenotype and for delivering the evidence based treatment and management choices.

PB 1.41-3

Influence of coagulation factor XIII on the severity of the skin affection and endothelial dysfunction in patients with progressive systemic sclerosis

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Background and Aim: The postulated ability of factor XIII (FXIII) to influence the synthesis and resolution of collagen led to its usage in the treatment of progressive systemic sclerosis (PSS). Though an alleviation of the skin affection was described in the nineties, a further substitution therapy was given up due to the HIV epidemic. The fact safe FXIII concentrates are available today, allows us to reconsider the above-mentioned thesis.
Methods: After verification by the local ethical committee and with informed written consent, since March 2010 data from 142 patients could be collected. Blood clotting status, blood levels of disease-specific, acute phase and inflammation parameters (like VEGF, DLCO), medical results like the Modified Rodnan Skin Score (MRSS) and a Quality-of-Life-Questionnaire (Scleroderma Health Assessment Questionnaire) have been documented.
Results: Current interim results are presented. Fifty-seven patients (50 f, seven male) with PSS reflecting the broad spectrum of disease severity were included as well as 85 patients (60 f, 25 m) with rheumatic diseases without vascular involvement (R). The median age in both groups was alike (PSS: 52.5 ys R: 53.5). No correlation between FXIII and most of all examined parameters could be detected. Only in the group of patients with elevated FXIII (78% quantiles, $n = 16$) a not significant correlation ($P = 0.156$) to pulmonary arterial hypertension (PAP) was evident. A notable correlation was found between VWF:Ag and MRSS ($r = 0.66$, P: 0.036) as well as vWF:Ag and VEGF ($r = 0.52$, $P = 0.038$). Further, VEGF correlates with DLCO ($r = -0.62$, $P = 0.0558$).
Conclusion: The results of this interim analysis could give pointer of an influence of FXIII on endothelial damage and medical results, but could not yet confirm a significant correlation. Interestingly enough there were found a number of patients with elevated levels of FXIII in both groups (PSS>R). Considering these patients only, we could find an inverse correlation between FXIII and PAP, a severe complication in PSS.

PB 1.41-4

Intracranial hemorrhage (ICH) in Egyptian children with rare coagulation disorders: a single center experience

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Background: Rare Bleeding Disorders (RBDs) (such as afibrinogenemia, factors II, V, V+VIII, VII, X, XI and XIII deficiencies) represent 3–5% of all the inherited deficiencies of coagulation factors with ICH being the most serious complication. It is reported with a higher frequency in FVII, FXIII and FX deficiencies and a lower frequency in afibrinogenemia, FII, FV with overall prevalence ranging from 0% in FXI deficiency to 25% in FXIII deficiency.
In view of the rarity of these disorders, many questions remain unanswered making treatment of these life threatening episodes and subsequent prophylaxis a challenge because of the lack of experience and paucity of data on these disorders especially in children aim to prospectively collect data on children affected with RBDs who had CNS bleeding over a 7-year period, to establish incidence of recurrence, death rate, neurological sequences, efficacy of treatments and prognosis.
Methods: All patients with RBD were given instructions that in case of any head trauma or abnormal symptoms to immediately come for haematological consultation even if the child is well. All were subjected to a questionnaire to include all details of episode including triggering factors and replacement was started immediately according to the UKHCDO guidelines in rare coagulation disorders but in view of severe resource constraint. ICH was identified by clinical features, neurological examination and confirmed by computerized tomography scan of the brain. Neurosurgical consultation was done to identify those who could benefit from surgery. In children presenting initially with unexplained ICH, a full coagulation screen was done and patient was managed by hematologist.
Results: ICH occurred in 16/67 children (23.9%) of rare inherited coagulation disorders; 4 (25%) afibrinoginemia, 2 (12.5%) severe FV deficiency, 5 (31.3%) FX, three mild deficiency and two severe, 4 (25%) severe FVII Deficiency and one severe FXIII (7.7%) deficiency, a total of 36 episodes spontaneous in 83.3% and eight children having recurrent episodes. Their age ranged from 7 months to 10 years and 62.5% males whilst their age on intial presentation with ICH ranged from 1 month to 5.5 years (median: 5 months). ICH was the initial presenting symptom in 62.5%. All patients received replacement therapy with only six episodes (16.7%) requiring surgical evacuation. All children recovered their episodes of ICH but six developed neurocognitive deficit, two required shunt where one died postoperatively. Seven patients are on prophylaxis with three developing subsequent episodes whilst on prophylaxis. Intially patients presented with pallor, vomiting, fits, coma, high pitched cry but then subsequent episodes were usually of milder nature and patients outgrew the episodes as they became older . On CT scan, all had intracranial hemorrhage which was mainly subdural conclusion FX, FVII and FI deficiencies were associated with the highest prevalence with higher tendency of recurrence in those with FVII and X deficiencies. Surgical evacuation was not required in most patients and prognosis was usually good if the attacks were recognized within few hours of manifesting and replacement started immediately.

PB 1.41-5

Clinical and laboratory manifestation of bleeding diathesis in Noonan syndrome

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Background: Noonan Syndrome (NS) is a relatively common genetic disorder with an estimated incidence of 1:1000 to 1:2500 live births. Several genetic mutations have been described in these patients leading to different phenotypical presentations. NS is associated with congenital heart defects, hearing loss, skin and facial abnormalities, and growth retardation. Bleeding symptoms and altered laboratory coagulation test results are described in 20–89% of affected patients. The most commonly cited coagulopathies are related to deficiencies of factors VIII, IX and XI, thrombocytopenia, von Willebrand factor deficiency and platelet function defects. The severity of clinical bleeding symptoms is variable.
Aim: To elucidate the incidence of clinical bleeding symptoms and its relation to normal and abnormal coagulation test results in patients with NS at our institution.
Method: Clinical features, bleeding history, blood smear and laboratory coagulation parameters were retrospectively reviewed in patients with NS at our institution. We evaluated the frequency of clinical bleeding symptoms with a questionnaire.
Result: Nineteen patients (seven female, 36.8%) with NS (genetically confirmed in 5, 26.3%) and a median age of 7.4 years (1.5–26.3 years) were included. In nine patients (47.4%) either a positive bleeding history (with a wide variety of bleeding symptoms) and/or abnormal laboratory test results (thrombocytopenia, von Willebrand disease, and abnormal platelet function test) were noted. Altered tests of haemostasis were present in most patients with bleeding symptoms.
Conclusion: The presence of various bleeding disorders within one syndrome is unusual but might be explained by the heterogeneity of several mutations on different genes described in NS. A standardized questionnaire, clinical assessment and laboratory evaluation of haemostasis can help characterize the pathophysiology of bleeding manifestations in NS. Due to the young age of patients at which invasive procedures are undertaken, the bleeding history can be unremarkable at initial presentation despite abnormal laboratory findings. As serious complications can arise and invasive procedures are frequent in these patients, a thorough evaluation of all patients with NS is highly recommended to ensure optimal management for surgery and other risk situations.

PB 1.41-6

Prospective data collection on patients with fibrinogen and factor XIII deficiencies: design of the PRO-RBDD project

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The author is submitting the following abstract on behalf of PRO-RBDD project group.
Background: The cross-sectional project entitled 'Establishment of an European Network of the Rare Bleeding Disorders (EN-RBD)' funded in 2007 by the European Community was established to answer unmet questions on rare bleeding disorders (RBDs). The results of this project increased knowledge on frequency and distribution of each coagulation defect. The EN-RBD database enabled to explore the relationship between the laboratory phenotype and clinical severity of each deficiency, showing that only observed in fibrinogen, FX and FXIII deficiencies there is strong association between plasmatic factor levels and bleeding severity. The main limitation of this retrospective study was the lack of detailed data on the diagnosis setting and the chronology between diagnosis and bleeding episodes. Therefore there is still a gap in knowledge of annual incidence of either disorders and the bleeding manifestations as well as on what is the minimum coagulant activity level able to prevent spontaneous and trauma/surgery/pregnancy related bleeding and provide an adequate hemostasis.
Aims: To design a project to capture prospective data on patients with the most severe RBDs, fibrinogen and FXIII deficiencies being both conditions suffering from under-reporting.

Methods: A dedicated group consisting of specialists in the field and clinical epidemiologists have worked with informational technology team to implement the existing RBDD interactive web-base to include new variables for reaching the current objectives and to allow for data collection at sequential checkpoints (prospective design, PRO-RBDD).
Results: The new database captures data at specific time points for variables including: demographics, racial origin and family studies, genetic, laboratory studies, prophylaxis, bleeding/thrombotic manifestations and their sequelae, obstetric data, surgical bleeding and perioperative data, treatment efficacy and safety. A new section has been added to the database in order to allow prospective data entry. Over 3 years, there will be six data entry time points per patients (i.e. biannual update), although data will be collected also in any other non-scheduled visits. Seventeen participating centers from 10 countries (Germany, Greece, India, Iran, Italy, Pakistan, Serbia, Switzerland, Turkey, UK) have started the baseline data collection in January, which is performed at hospital/clinic. Follow-up visits will consist of initial screening contact by telephone, performed by experienced clinical study physicians/nurses, and personal check-up in case of report of (suspected) bleeding episodes or other medical events.
Conclusions: The PRO-RBDD collect prospective data on patients with the two most severe RBDs, fibrinogen and FXIII deficiencies. Once data will be available for these two disorders, hopefully the same model will be extended to different disorders. This endeavor shall provide essential information on the course and optimal management of these orphan diseases. Moreover the prospective cohort design will allow to define appropriate surrogate end-points to adequately evaluate treatments and particularly the design of new clinical trials and the creation of guidelines for standardized therapeutic approaches.

PB1.42 – von Willebrand disease: Clinical – I

PB 1.42-1

Iatrogenic bleeding is the presenting symptom in children with moderate or severe von Willebrand disease – from the WiN study

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Background: von Willebrand Disease (VWD), the most common inherited bleeding disorder, is caused by reduced levels or activity of von Willebrand factor (VWF). Clinical expression of VWD in children may differ from adults. Currently limited information is available on the bleeding phenotype in children with VWD.
Aims: To evaluate the occurrence and severity of various bleeding symptoms in a large cohort of children with different types of VWD and to compare it with bleeding symptoms in adults with VWD.
Methods: We included 140 children with type 1 (n = 70), 2 (n = 51) and 3 (n = 19) VWD and 664 adults (≥ 16 years old) with type 1 (n = 389), 2 (n = 247) and 3 (n = 28) VWD in a nation-wide cross-sectional study among patients with moderate and severe VWD, defined as VWF levels ≤ 30 U/dL (Willebrand in the Netherlands – WiN study). Informed consent and ethical approval was obtained. Bleeding severity was determined using the validated adult Tosetto Bleeding Score (BS) with the extension of additional pediatric-specific bleeding symptoms (umbilical stump bleeding, cephalohematoma, post-circumcision bleedings, post-venipuncture bleeding and macroscopic hematuria) (*Bowman et al, JTH 2009*).
Results: Median age was 7 years in children (range 0–16 years) and 45 years in adults (range 16–85 years). In children median BS was 5.5 (IQR 2.0–9.0). When pediatric-specific bleeding symptoms were included it was 6.0 (IQR 2.0–10.0). In adults the BS was significantly higher with a median BS of 11.0 (IQR 6.3–17.0) ($P < 0.001$). The most frequent bleedings in children were: cutaneous, minor wounds, tooth extraction and menorrhagia. Compared with adults with VWD, children had significantly less bleeding from minor wounds, oral cavity and gastro-intestinal tract, bleeding after surgery and tooth extraction, muscle hematoma and hemarthrosis ($P < 0.05$). Pediatric-specific bleeding symptoms were reported in 41 children (14/140; 29%), occurring in 12 (12/19; 63%) type 3 VWD, 17 (17/51; 33%) type 2 VWD and 12 (12/70; 17%) type 1 VWD patients ($P < 0.001$). Thirteen patients reported more than one pediatric-specific bleeding symptom. The most observed pediatric-specific bleeding symptoms were: post-vaccination bleeding (24/140; 17%), post-venipuncture bleeding (17/140; 12%), post-circumcision bleeding (5/140; 4%) and cephalohematoma (5/140; 4%). Eight of 31 index cases (26%) had a pediatric-specific bleeding symptom as presenting symptom. A iatrogenic bleeding was the presenting symptom in almost half of the index cases (n = 13/31; 42%). These iatrogenic bleeding symptoms were cephalohematoma after vacuum extraction (1/31; 3%), umbilical stump bleeding (1/31; 3%), post-surgery (5/31; 14%), post-circumcision (1/31; 3%) and post-vaccination bleeding (5/31; 16%).
Summary/Conclusion: A large proportion of children with VWD reported pediatric-specific bleeding symptoms. Iatrogenic bleeding is the presenting symptom of VWD in almost half the index case patients.

PB 1.42-2

Prenatal diagnosis in severe von Willebrand disease using intron 40 markers of VWF gene and phenotypic assays

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Background: von Willebrand disease (VWD) is the common hereditary coagulation abnormality arising from deficiency of von Willebrand factor (VWF), a multifunctional, multimeric protein that is required for platelet adhesion and carrier of Factor VIII. Type 3 is the predominant subtype (60%) followed by (19%) type 2 and (18%) type 1 reported at our center. Diagnosis, genetic counseling, carrier and antenatal diagnosis play an important role in the comprehensive management of these cases. Genetic diagnosis in VWD by direct mutation detection is a complex and laborious procedure due to large gene size, high heterogeneity of mutations and 97% homology to a pseudogene in chromosome 22.
Aim: To offer genetic diagnosis to affected severe VWD families using intron 40 markers in VWF gene and by phenotypic assays.
Methods: Sixteen families with severe VWD were referred for antenatal diagnosis. Chorionic villus sampling was done in 10–12th week of gestation and cordocentesis was done between 18th and 19th week of gestation. PCR amplifications of VWF1, VWF2 and VWF3 markers (entire VNTR amplified and digested using AluI restriction enzyme) of intron 40 followed by polyacrylamide gel electrophoresis reveals several alleles. Phenotypic assays included FVIII:C, FIX:C and von Willebrand factor antigen (VWF:Ag) assays.
Results: Antenatal diagnosis was offered by intron 40 VNTR analysis in 12 VWD families by chorionic villus sampling. Four families were offered the diagnosis by cord blood sampling. In two of these families,

none of the VNTR markers were informative, while two were referred late during pregnancy; so cordocentesis was done by phenotypic analysis. Among the 16 fetuses three were found to be affected and 13 unaffected.

Conclusion: Antenatal diagnosis could successfully be offered in all the sixteen VWD families referred using both genotypic and phenotypic investigations. RFLP analysis with the aid of intragenic markers is a simple accurate method for genetic diagnosis of severe VWD families, when both the parents are informative. Phenotypic assays are still not obsolete techniques for prenatal diagnosis of VWD and other bleeding disorders, wherein the genes are large and mutations are highly heterogeneous.

PB 1.42-3

Vicenza bleeding score is correlated with postoperative bleeding and blood transfusion requirement in total knee replacement operation

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Background: The Vicenza bleeding score is a validated research tool to discriminate between healthy subjects and those with von Willebrand disease. Standard preoperative bleeding assessment comprises history taking and physical examination whereas blood test is usually performed in the operation with a high bleeding risk. Whether Vicenza bleeding score is predictive for postoperative bleeding and postoperative blood transfusion requirement is not known.

Aims: To study the correlation of bleeding score and postoperative bleeding and blood transfusion requirement in total knee replacement operation.

Methods: Prospective study has been performed from 1 March 2012 to 31 January 2013. A total of 270 patients admitted for total knee replacement operation due to osteoarthritis of knee had been interviewed with bleeding score questionnaire. The amount of postoperative bleeding and blood transfusion given in hospital were recorded. Other collected data included preoperative CBC, coagulogram, creatinine, medication and underlying disease.

Results: Of the 270 patients (230 females, 40 males), the median age is 70 years old (range 46–90). There is no perioperative thromboprophylaxis. All patients received perioperative tranexamic acid injection. The median (range) of bleeding score are −1 (−3 to 9) in women and −1 (−2 to 4) in men, respectively. The mean + SD amount of postoperative bleeding is 424.8 + 267.1 mL. High bleeding score more than zero (score > 1) is statistically significantly associated with higher amount of postoperative bleeding (median 375 vs. 485 mL, $P = 0.01$ (Mann–Whitney *U*-test)). Postoperative blood transfusion requirement is significantly associated with both bleeding score ($P = 0.004$), preoperative hematocrit level ($P < 0.001$, (Mann–Whitney *U* test)), and amount of postoperative bleeding ($P < 0.001$, (Mann–Whitney *U* test)). Bleeding score is not associated with preoperative hematocrit level ($P = 0.119$). The amount of postoperative bleeding is not associated with preoperative hemoglobin level, platelet level, creatinine clearance level, medication and any underlying disease. There is no symptomatic venous thromboembolism during admission.

Conclusion: High Vicenza Bleeding score more than zero (score > 1) is associated with postoperative bleeding and blood transfusion requirement in total knee replacement operation.

PB 1.42-4

Screening for type 2 von Willebrand disease in the pediatric population

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Background: Diagnosis of Type 2 VWD can often be challenging. von Willebrand Factor (VWF) panel, especially VWF activity to VWF antigen ratio (VWF: RCo/Ag) is used as an initial screening tool for Type 2 VWD often followed by a confirmatory multimer analysis. Commonly, VWF: RCo/Ag of < 0.5–0.7 cut-off is used by pediatricians to select patients for multimer analysis. However, such a cut-off is established by adult studies and no pediatric specific range has been suggested thus far.

Aim: Our aim was to determine a cut-off point for VWF: RCo/Ag that best discriminates pediatric patients as suspicious for Type 2 VWD prompting multimer analysis vs. having type 1 VWD.

Methods: We performed a retrospective chart review of 300 children with VWF panels and multimer analysis seen at our tertiary referral hospital from 2009 to 2012. Patients without multimer analysis were excluded from the study population. A receiver operating characteristic (ROC) analysis was done to determine the optimal VWF: RCo/Ag cut-off value that identifies patients with ratio below the cut-off value as suspicious for Type 2 VWD prompting multimer analysis.

Results: Out of 300 patients (median age 14 years; range 11 months–20 years), 230 (77%) were female, of which 150 (50%) had menorrhagia at presentation. Fifty eight patients (19%) experienced epistaxis, four presented with intracranial bleed, 12 showed easy bruisabilty, six each showed post-operative bleeding and other symptoms such as hematochezia and hemoptysis while four had bleeding after dental extraction. Thirty one (10%) patients did not report bleeding symptoms at presentation and in 22 patients (7%), bleeding symptoms were not documented.

Twenty one patients out of 300 had abnormal multimer analysis of which 18 results were suggestive of Type 2 or acquired VWD and the remaining three were suggestive of artifact and/or inconsistent with Type 2 VWD.

At a cut-off value of 0.70 for VWF:RCo/Ag ratio, the sensitivity to detect possible type 2 VWD was 83% with a positive predictive value of 90%, specificity of 45% and a negative predictive value (NPV) of 98%. When the cut-off value was increased to 0.80, the sensitivity increased to 100%, but the specificity decreased to 25%. These results were essentially similar in a subset of female patients with menorrhagia. At a cut-off value of 0.70, the chance of missing a true positive patient was 17%. This contrasts with a 39% (< 0.6 cut-off) and 72% (< 0.5 cut-off) chance of missing a true positive patient with the currently employed cut-off values used by clinicians in determining the need for multimer analysis.

Summary/Conclusion: The current recommended VWF: RCo/Ag ratio below which multimer analysis is performed can be as low as 0.5. However, our study showed that VWF: RCo/Ag ratio of 0.7 is the optimal cut-off for screening children for abnormal multimers to detect Type 2 VWD. This is the first study to validate the role of VWF: RCo/Ag ratio as a screening tool for Type 2 VWD in the pediatric setting.

PB 1.42-5

von Willebrand factor genotyping for validation of type 2N von Willebrand disease subtitle: the relation between genotype and phenotype in von Willebrand disease type 2N

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Introduction: von Willebrand disease (VWD) type 2N is characterized by a defective binding of Factor VIII to the von Willebrand factor (VWF) resulting in lowered plasma factor VIII level and a clinical phenotype mimicking mild hemophilia A. Several mutations in the factor VIII binding site on von Willebrand factor have been reported, which were confirmed by the VWF – factor VIII binding assay (BC). This study aims to examine the effect of genotype on clinical phenotype in a cohort of VWD-2N patients.

Methods: In a retrospective study, results of genetic screening in the VWF molecule from 173 patients were analyzed. Patients with at least one 2N mutation were included in this study. Clinical phenotype including bleeding scores (BS) were obtained and analyzed using independent T tests and Mann–Witney *U* tests.

Results: Forty-three VWD-2N patients were included. Median age was 40 years. Six patients were homozygous, 37 patients were heterozygous. Eighteen patients had a secondary mutation outside the FVIII binding domain. Four different 2N mutations were reported. A strong correlation was observed between FVIII and VWF-FVIII binding capacity (slope = 0.735, $P = 0.000$). Typically affected 2N patients, defined as having a VWF-FVIII BC < 20%, were compared with heterozygous carriers (with a VWF-FVIIIBC > 20%). Significant differences were found in FVIII level ($P < 0.001$), VWF-ristocetin activity ($P = 0.048$) and VWF-antigen levels ($P = 0.003$). Median BS was 7 in typical 2N patients compared to 1 in carriers. There was an inverse correlation between vWF-FVIII:BC and BS (slope = −0.450, $P = 0.016$). Hemarthrosis (31%), muscle hematoma's (22%), and postpartum hemorrhage (60%) were only reported in typical affected patients but not in the group of type 2N carriers.

Conclusions: VWD type 2N patients form a heterogeneous group. Carriers of VWD 2N have an insignificant BS unlike typically affected patients. Bleeding symptoms of affected patients are comparable to mild hemophilia A. Classical Hemophilia A symptoms are absent in heterozygous carriers. We suggest genotyping as a powerfully tool in the diagnostics and sub classification of type 2 VWD.

PB 1.42-6

Safety and efficacy of a von Willebrand factor/factor VIII concentrate (Wilate®): a single centre experience

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Background: von Willebrand disease (VWD) is a highly heterogeneous bleeding disorder characterised by a defect in primary haemostasis. The majority of patients with severe disease will require treatment with a VWF containing concentrate in the event of bleeding or surgery. A new VWF/fVIII concentrate (Wilate®) has been available for usage in the United Kingdom. This concentrate has dual viral inactivation (SD and Permaheat) and provides a physiological ratio of VWF:fVIII (1:1). We present a large cohort of usage of this concentrate in an adult population.

Aims: Retrospective review of indications for Wilate® over 5 years (2007–2012) at a large haemophilia treatment centre. The primary outcome measure was efficacy for the treatment of bleeding and usage in the peri-operative period.

Methods: Clinical and laboratory data of all patients issued with Wilate® during the study period were reviewed.

Results: One thousand two hundred and eighty-seven evaluable doses of Wilate® were issued to 46 patients (25 male/21 female). Median age was 42.5 years (18–83). Eleven patients had type 1 disease, 26 patients had type 2 disease, eight patients type 3 disease and one patient acquired vWS. One hundred and twelve infusions were issued for surgery over 63 treatment episodes. Median number of infusions used peri-operatively was 1 (1–13), with a median dose prior to surgery of 3600 Units (1800–7200), corresponding to 42.6 U/kg (11.8–117.5). In patients receiving more than one dose the median follow up dose was 2250 Units (1800–4500) equivalent to 26.5 U/kg (17.9–68.2). Efficacy was rated as being excellent or good in 94%, fair in 6% and poor in 0%. Two hundred and forty-six doses were issued for the treatment of 39 hospital treated bleeding events. The median number of infusions received per bleeding episode was 2 (1–92). The median dose used was 2700 Units (900–8100) corresponding to 38.5 U/kg (11.8–95.7). Efficacy was rated as being excellent or good in 97%, moderate in 3% and poor in 0%. One treatment episode (type 3 VWD) required 92 inpatient treatments for haematuria due to underlying bladder pathology and subsequent prophylaxis. Thirty-nine infusions were issued for other indications such as delivery and single dose prophylaxis. Six patients were on home therapy programmes using 890 infusions. Of these patients, two switched from an alternative VWF/fVIII containing product. Similar efficacy was seen for Wilate® in comparison to the 6 month period on prophylaxis prior to switching. Six adverse events (0.005% of all infusions) were documented. This included four hypersensitivity reactions requiring medical treatment and subsequent product switching. No thrombotic or infective complications were described in the study period. There were no treatment failures. No demonstrable accumulation of FVIII was seen in patients receiving treatment for > 3 days.

Conclusions: A retrospective review of 1287 doses of Wilate® shows safe and well tolerated usage covering a wide range of indications. Within this study period efficacy was excellent or good in 94% or greater of all treatment episodes. In patients treated with repeat doses of Wilate® no accumulation of FVIII occurred.

PB1.43 – Von Willebrand Disease: Clinical – II

PB 1.43-1

von Willebrand disease patients with associated risk factor(s) of venous thromboembolism: efficacy and safety of a von Willebrand factor product with a low factor VIII content

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Background: The main objective of von Willebrand Disease (VWD) treatment is the control of bleeds with normalization of von Willebrand factor (VWF). Because VWF is the carrier of factor VIII (FVIII), exogenous added to endogenous FVIII may cause very high levels of FVIII coagulant activity after repeated infusions (FVIII:C). Multiple clinical risk factors as well as biomarkers have been identified as predictive of venous thromboembolism. These risk factors include patient-related factors e.g. advanced age, obesity, inherited thrombophilia and medical condition-associated factors e.g. surgery, immobility, pregnancy/puerperum or cancer. High plasma levels of FVIII:C are confirmed as a risk factor for VTE.

Aims: To present the efficacy and safety of WILFACTIN (VWF with low FVIII content) in different clinical settings in a large cohort of VWD patients with risk factors of VTE.

Methods: Data from four clinical studies and one post-marketing survey, carried out between 1999 and 2009, were pooled.

Results: Across clinical studies, data displays mainly three sets of patients:

- Elderly patients ($n = 21$) between 65 and 85 years old (1 type 3, 16 type 2, 4 type 1 VWD) were treated with WILFACTIN for 42 bleeds, 31 surgeries including six major risk orthopaedic procedures. Four patients were on long-term prophylaxis initiated for recurrent gastrointestinal bleeds for more than 2 years. Treatment was judged by investigators as 'excellent/good' in 90.9% of the 11 bleeding episodes and in 100% of the 26 surgical procedures for which the efficacy information was available. A 'moderate' response was observed for one bleed. The prophylaxis regimen (mean dose: 37 IU/kg 2.2 times per week) was successful in all patients; the annual number of bleeds was 1.2 (range 0–2.8).
- Obese patients ($n = 14$), including one elderly, had BMI > 30 kg/m^2. The body weight ranged from 82 to 135 kg (mean 99) at time of therapy. There were three patients with type 3 VWD, 8 type 2, 3 type 1; 86% were female. Coexisting thrombotic risk factors included surgery (13) or caesarean section (5). Efficacy was rated as excellent/good in all procedures (11) and major bleeds (5) in 11 patients treated for more than 3 days. No bleeding occurred in the elderly patient on long-term prophylaxis.
- Pregnant women ($n = 22$), including four obese patients, were treated for vaginal (9), caesarean (15) deliveries, or therapeutic abortion by caesarean (1). A 28–103 IU/kg/day dosage was used with a median of 374 (range 103–1114) IU/kg per procedure over a 9.0 (range 1–18) days therapy. Results showed that 95% of episodes (22 with available data) were treated successfully. No relevant safety information was reported in either mother or newborn.

No thrombotic events occurred in all these patients with risk factors for thrombosis.

Conclusion: The results obtained from the analyses of several clinical studies clearly show that WILFACTIN, a VWF concentrate almost devoid of FVIII, can be used frequently and intensively also in VWD patients with older age and/or with relatively high risk of thrombosis. Therefore, WILFACTIN should be always considered by haematologists among the available VWF products useful for VWD with those phenotypes.

PB 1.43-2

Validation of a new panel of automated chemiluminescence assays for von Willebrand factor antigen and activity in the screening for von Willebrand disease

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Background: von Willebrand disease (VWD) is one of the most common inherited bleeding disorders and its diagnosis largely depends on the results of von Willebrand factor (VWF) antigen and activity. Although used as a surrogate for the classical ristocetin cofactor activity (VWF:RCo), current latex immunoassays measure indirectly the VWF activity and do not reflect binding of VWF to GPIba or collagen. Recently, a new automated VWF:RCo assay on Acustar was developed. The HemosIL AcuStar VWF:RCo assay is a ristocetin-dependent, platelet-free chemiluminescent immunoassay. Recombinant GPIbα (rGPIbα) is coated on magnetic particles through a highly specific monoclonal antibody. VWF present in the test sample binds to the rGPIbα in the presence of ristocetin.

Aims: The assay panel on AcuStar combines the analysis of VWF:Rco with a new antigen (VWF:Ag) test. In this study, both chemiluminescence tests (HemosIL VWF:Ag and VWF:RCo) were evaluated.

Methods: Imprecision, limit of detection and linearity were evaluated. Method comparison with the currently used VWF:Ag latex assay, VWF activity (VWF:Act) and VWF:RCo by aggregometry was performed and diagnostic performance of the new test panel was examined by analyzing 61 patient plasma samples. As the new assays are calibrated against the current WHO-standard, the international standard was analyzed as well.

Results: The imprecision was 7% and the LOD was 0.2% for both assays. Dilution series showed a large linearity for both HemosIL VWF:Ag (0–300%) and VWF:RCo (0–200%) and method comparison studies revealed good agreement with the currently used VWD panel. The results of the WHO-standard were in accordance with the expectations. The new panel showed adequate diagnostic performance: diagnostic sensitivity was 100% and diagnostic specificity 82% compared to the VWF:Ag latex assay and VWF:RCo by aggregometry. In addition, the new HemosIL Acustar VWF:Ag and HemosIL Acustar VWF:RCo are more sensitive for VWD than the currently used assays.

Conclusions: The HemoSIL Acustar VWF:RCo demonstrated adequate diagnostic performance, improved precision (also in the lower range), a good sensitivity to low VWF activity and a large linearity. Furthermore this test can be considered as a true VWF ristocetin cofactor activity assay independent of the variability present in human platelets. The VWF:Ag test showed equally good performance and a lower LOD compared to the VWF:Ag latex assay. This new VWD test panel has adequate laboratory characteristics and allows fully automated and simultaneous analysis of the VWF:Ag and VWF:RCo.

PB 1.43-3

Analysis of factor VIII:c/VWF:Ag ratio in the Brno-von Willebrand disease study

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von Willebrand Disease (VWD) is an autosomally inherited bleeding disorder caused by a quantitative or qualitative defect of von Willebrand factor (VWF). VWF is important for platelet adhesion to collagen, platelet aggregation, and for binding to Factor VIII through the D'/D3 domain. FVIII degrades rapidly when not bound to VWF. Generally, except for type 2N patients, FVIII:c values mirror those for VWF:Ag.

In the Brno-VWD study blood was collected from 205 patients representing 95 families with suspected VWD. FVIII:c, VWF:Ag, VWF:RCo, VWF:CB, VWFpp, VWF-FVIII binding (if indicated), VWF multimers and molecular analysis were performed in all patients.

Within the Brno-VWD study we performed a separate analysis of the results for FVIII:c in this group of VWD patients. There were no type 2N patients in the Brno-VWD cohort

Mean FVIII:c/VWF:Ag ratio in the whole cohort van 1.13 (CI_{95} 1.04–1.21) with a statistically significant difference between patients with type 1 VWD (mean 1.31, CI_{95} 1.18–1.45) and type 2 patients (mean 0.91, CI_{95} 0.82–1.02). Two thirds of the patients had a ratio between 0.5 and 1.5. High ratios > 1.5 were found in 40/205 patients (19.5%) and 27/95 families in which sofar 10 mutations have been found (molecular analysis ongoing), mainly type 1 and some type 2A/IIE. Low FVIII:c/VWF:Ag ratios < 0.5 were found in 19/205 patients (9.3%) and 13/95 families in which sofar 6 mutations have been found (molecular analysis ongoing), mainly type 2A, 2A/IIE and 2M, with only 3 type 1 patients which are all compound heterozygous for the type 1 mutation p.P812Rfs (X31) together with the type 2N mutation p.R854Q .

Although the majority of VWD patients have FVIII:c/VWF:Ag ratios between 0.5 and 1.5, around one third may present with either very low or very high ratios. A FVIII:c/VWF:Ag ratio < 0.5 does not always lead to the diagnosis of a type 2N VWD but may be present in other type 2 mutations. High ratios are mostly seen in type 1 VWD.

PB 1.43-4

Efficacy and safety of Wilate® following an en masse switch for patients with inherited von Willebrand disease

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Background: Wilate is an albumin-free, high purity, double virus inactivated concentrate of von Willebrand Factor (VWF) and factor VIII (FVIII). Previous clinical trials have reported high levels of Wilate efficacy in the treatment of acute bleeding episodes and surgical procedures. A National Tender process in Ireland in 2010 resulted in the introduction of Wilate as the treatment of choice for patients with von Willebrand disease (VWD) who fail to respond to DDAVP.

Aims: This study aimed to assess the efficacy and safety of Wilate for the treatment of unselected Irish patients with VWD following an en masse switch in treatment product.

Method: A retrospective review of the clinical records of all patients treated with Wilate between September 2011 and September 2012 was undertaken. The following datasets were reviewed: demographics, VWD diagnosis, treatment indication, haemostatic efficacy, adverse events and laboratory data including pre and post FVIII, VWF antigen (VWF:Ag) and Ristocetin co-factor activity (VWF:Ricof) levels.

Results: Wilate was used on 39 occasions in 28 patients with the following subtypes of VWD: Type 1 (n = 14), Type 2 (n = 11), Type 3 (n = 2) and acquired VWD (n = 1). Of 29 invasive procedures, six were major surgeries, eight were minor surgeries and 15 were invasive dental procedures. The median (range) of Wilate doses used were 49 IU/kg (40–53.1 IU/kg) for major surgery, 42.5 IU/kg (28–53.1 IU/kg) for minor surgery and 30 IU/kg (20–43 IU/kg) for dental procedures. Wilate was used to treat acute bleeding on five occasions and to prevent postpartum bleeding on two occasions. One patient with acquired VWD received Wilate on three occasions to treat acute bleeding. Wilate doses ranged from 24 to 50 IU/kg to treat acute bleeds with higher doses used to treat the patient with acquired VWD (48–75 IU/kg). Haemostatic efficacy was rated as excellent or good in 28/29 surgical procedures and in all episodes of acute bleeding. Three mild adverse events were noted including dry cough, an anxiety reaction and an urticarial reaction with hoarseness. Post infusion, laboratory analysis demonstrated that the FVIII and VWF:Ag levels were in the normal range for all patients. The VWF:Ricof was < 0.5 IU/mL in five patients who received doses of Wilate of < 30 IU/kg. It was notable that the VWF:Ricof levels in all patients were significantly less than the VWF:Ag levels. No accumulation of FVIII was noted in patients receiving repeated doses but two patients with type 2A VWD required increasing doses of Wilate post-op due to sub-therapeutic VWF:Ricof levels. Importantly, no new clinically significant VWF inhibitors were identified following this en masse switch in treatment product.

Conclusion: Wilate has been found to be clinically effective with a satisfactory safety profile, following an en masse switch in Ireland. Of note, a significant number of patients with types 1 and 2 VWD have been treated which adds to the limited clinical data previously published on the use of Wilate in these subtypes. Our findings suggest that optimal laboratory assays and target levels for monitoring patient response following Wilate administration requires further evaluation.

PB 1.43-5

Biologic response to desmopressin is there predictable in patients from the same family?

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The use of desmopressin (DDAVP) has been largely recommended in the treatment of the von Willebrand Disease (VWD). As the DDAVP biologic response is dependant of the type and the severity of the VWD, its assessment contribute to verify its efficacy before surgery and also contribute to classify the subtype of VWD. Previous studies have shown that in type 2A and 2N only, the genotype could be helpful to predict the individual response. And in patients with severe type 1, a variability of the response could be observed despite the same type of mutation.

The aim of the study was to evaluate if an identical response to DDAVP could systematically be observed in all members from the same family, in order to know if a test have to be always performed.

Methods: We have retrospectively analyzed the biologic response to DDAVP in 87 patients with VWD from 37 families, referred to our centre between January 2003 and December 2012. Intravenous infusions of 0.3 μg/kg body weight DDAVP was given in all patients. Venous blood was withdrawn at baseline and at 0.5 or 1, 2 and 4 h after the infusion ended.

Biologic response to DDAVP was analyzed at time 0.5 or T1 h (T1H) and at time 4 h (T4H). Patients with VWF: RCo and FVIII:C higher than 50 IU/dL was defined as complete responders. Patients with VWF: RCo and FVIII:C lower than 50 IU/dL but increased at least three-fold was defined as partial responders, and if neither criterion obtained as non responders.

Results: Biologic response was evaluated in 58 members of 25 families with Type 1 VWD and 29 patients (12 families) with Type 2 (16 with type 2A, 4 with type 2B (New York), 4 type 2M and 5 unclassified), including 50 children (aged 2–16 years). The mean age was 18 ± 15 (mean SD) years.

In Type 1, 98% of patients had a complete response at T1H and 93% at T4H. At T1H, one patient with severe type 1 (1.7%) had no response, in discrepancy with the complete response observed in the other member of family.

At T4H, an absence of response was observed in four patients (69%) from four families, in discrepancy with the results of the others members.

In Type 2, 65% of patients had a complete response at T1H and only 45% at T4H.

At T1H, among the 10 patients (who had partial or no response), two patients with type 2A had discrepant partial response with the complete responses observed in the other members of the family.

At T4H, a discrepant additional response was observed in a family with type 2M VWD.

Conclusion: Within a family who present a moderate type 1 VWD, the biologic response to DDAVP is identical between the members and thereby the test could probably be delayed or cancelled in other members especially in children. As expected the lower rate of response and its variability in type 2, must to lead us to systematically perform a test in each members.

PB 1.43-6

Low-dose ristocetin induced platelet aggregation. Which dose is low enough?

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Low-dose ristocetin induced platelet aggregation (RIPA) is used to characterize Type 2B (VWD2B) and platelet-type (PT-VWD) von Willebrand disease variants, evidenced by an enhanced aggregation. A 0.5 mg/mL dose is classically applied but recent studies indicate that some VWD2B patients may need up to 0.80 mg/mL of ristocetin to evidence this behavior.

The aim of this study is to evaluate the effect of different doses of ristocetin on platelet-rich-plasmas (PRP) of healthy individuals and to determine the dose to be used in the screening diagnosis of variants with enhanced response to low doses of this agonist.

One hundred and fifteen normal PRPs of healthy individuals (median age 33 year old; range: 19–65, 65 blood group O and 50 blood group non-O) were analyzed testing RIPA at different doses of ristocetin from 1.00 to 0.40 mg/mL. All assays were performed using the same batch of ristocetin in order to avoid batch-to-batch variations. Maximal percent aggregation was measured. All individuals had no history of bleeding symptoms or drug use or thrombocytopenia. Twenty out of 65 (30.8%) of the blood group O and 19/50 (38.0%) of the blood group non-O individuals presented > 30% maximal aggregation at 0.80 mg/mL. Four out of 65 (6.2%) of the group O and 10/50 (20.0%) of the non-O group individuals presented > 30% maximal aggregation at 0.70 mg/mL. No individual presented RIPA at doses of 0.60 to 0.40 mg/mL. We found that a percentage of normal individuals had aggregation at doses > 0.7 mg/mL of ristocetin.

Different responses to the ristocetin doses were observed in healthy individuals according to their blood group. It is necessary to establish a local normal reference range for RIPA and the lowest dose of ristocetin able to differentiate enhanced PRP aggregation, in order to avoid under or over-diagnosis of VWD2B and PT-VWD variants.

PB1.44 – Von Willebrand factor – I

PB 1.44-1

Alterations in aberrant and alternative endothelial splicing of von Willebrand factor under high laminar shear stress

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Background: von Willebrand factor (VWF) mutations have been identified which affect splicing leading to von Willebrand disease (VWD). Although alterations in splicing are not always pathogenic, no alternative splice variants have been described for VWF thus far. Shear stress is an important environmental factor contributing to endothelial cell phenotype, altering many cellular processes, including the expression of transcription factors.

Aim: To investigate the effect of shear stress on the production of aberrant and alternative VWF splice variants in blood outgrowth endothelial cells (BOEC) from type 1 VWD patients and normal controls using an *in vitro* flow system.

Methods: BOEC were derived from two type 1 VWD patients; one with the consensus splice site mutation, c.5842+1G>C, and one with the putative exonic splicing mutation, c.3538G>A, as well as three normal controls. Platelet and BOEC-derived mRNAs were reverse transcribed and sequenced to identify VWF transcripts which were quantified by qRT-PCR. Additionally, BOEC were subjected to high laminar shear stress (30–50 dynes/cm^2) for 48 h and the ratios of the transcripts were compared to those from BOEC grown under static conditions.

Results: Three VWF transcripts were identified from heterozygous c.5842+1G>C BOEC: wildtype, in-frame exons 33–34 skipped, and exon 33 skipped, comprising 50%, 28%, and 22% of the patient's VWF mRNA respectively. The exon 33 skipped transcript was transcribed from the patient's wildtype allele, and represented 13% of VWF mRNA in normal BOEC and platelets, suggesting it was produced by alternative splicing. Under high laminar shear stress patient and normal BOEC significantly up-regulated this alternative transcript 2.6 fold; exon 33 was skipped in 68% of the patient's ($P < 0.05$) and 34% of normals' ($P < 0.005$) VWF mRNA. The proportion of the double exon skipping transcript relative to wildtype remained comparable regardless of shear stress. Therefore, the up-regulation of the alternative transcript shifted the patient's total VWF transcript composition to 22% wildtype, 68% exon 33 skipping, and 10% exons 33–34 skipping.

Four in-frame VWF transcripts were identified in the heterozygous c.3538G>A BOEC grown under static conditions: wildtype (2%), exon 23 skipped (22%), exon 26 skipped (64%), and exons 23 and 26 skipped (11%). The patient's platelet VWF RNA exhibited these transcripts in the same ratios. RNA from statically cultured normal BOEC contained exon 23 skipped or exon 26 skipped VWF transcripts at levels between 15% and 25%. Exposure to high laminar shear stress increased wildtype expression making the exon 23 skipped transcript negligible and decreasing the exon 26 skipped transcript to 3% in normal ($P < 0.05$). This shift was also observed in the patient BOEC under high laminar flow where expression of the exons 23/26 skipped transcript was also abolished under high laminar shear, producing a modified patient VWF population of 93% wildtype and 7% exon 26 skipped ($P < 0.0001$).

Conclusions: This study identified three potential alternative VWF splicing variants and found VWF mRNA in platelets is similar to that from static BOEC. Additionally this study highlights the influence of laminar shear stress on the regulation of VWF alternative and aberrant splicing and expression.

PB 1.44-2

Potential thiol isomerase activity of conserved CXXC motifs in D3, D4 and C1 domains of von Willebrand factor

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Background: A CXXC motif constitutes the active site of thiol isomerases. VWF propeptide contains two CGLC motifs. Whilst the isomerase activity of the propeptide has not been demonstrated directly, there is indirect evidence that these motifs are active in the Golgi, promoting interchain disulphide bonds to form multimers. We noted that VWF contains 14 other CXXC sites, three of which are similar to these motifs in the propeptide (^{994}CGLC in D3, ^{2085}CGIC in D4 and ^{2304}CGLC in C1). Because the -XX- residues are important in determining thiol isomerase activity, we chose these three for further study. In silico analysis showed that they are conserved across domains and species. We postulated that these motifs might either be important for the intracellular synthesis of VWF or in the extracellular promotion of thiol-disulphide exchange suggested to meditate VWF lateral self-association.

Aims: To examine whether the conserved CG (L/I)C motifs in D3, D4 and C1 possess thiol isomerase activity by expressing recombinant mutants and studying their structure and function.

Methods: Two complementary types of full-length VWF mutants were made:

1. D2 replacement mutants. The D2 domain (thiol isomerase activity essential for mutlimerisation) was replaced by either the D3 or D4 domain.
2. Glycine insertion mutants. Each motif was modified by insertion of a glycine (to form CXGXC), which is known to disrupt thiol isomerase activity, creating the mutants GlyD2, GlyD3, GlyD4, GlyC1. Additionally GlyD3 was made in the D'D3 fragment of VWF. The constructs were expressed in HEK293T or HEK293 cells.

Results: Neither of the D2 domain replacement mutants secreted.

GlyD4 and GlyC1 secreted and showed similar expression levels, collagen binding activity, and multimer pattern to wild-type (wt) recombinant VWF. However they both showed a relative increase in free thiols compared to wt VWF. When perfused over collagen, GlyD4 and GlyC1 showed similar platelet capture to wt over a range of shear (800–3000/s).

GlyD2 and GlyD3 were retained intracellularly, although a small amount of GlyD2 was secreted which was largely dimeric.

Wt D'D3 expressed as a monomer, detected in both media and lysate. Interestingly whilst GlyD3 in D'D3 did not secrete, the lysate contained higher order oligomers which were reduced to monomers by β-mercaptoethanol. The multimers were more marked when the intracellular pH was elevated by monensin or ammonium chloride. Co-expression with wt D'D3 did not rescue the secretion of GlyD3 in D'D3.

Summary: These data demonstrate that neither D3 nor D4 can substitute for the D2 domain. Whilst disrupting CG (L/I)C motifs in D4 and C1 resulted in increased free thiols, the integrity of these motifs is not essential for secretion, normal multimer structure, static collagen binding activity or platelet binding. Conservation of D3-CGLC is essential for secretion, suggesting that it has a critical intracellular role. The higher order oligomers in the lysate of GlyD3 in D'D3 is compatible with D3-CGLC regulating aberrant disulphide bond formation in the endoplasmic reticulum. A similar role has previously been reported for the CGLC motif in the D3 domain of submaxillary porcine mucin.

PB 1.44-3

An evaluation of the age-related quantitative and qualitative pathophysiology of von Willebrand factor

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Background: Plasma levels of von Willebrand factor (VWF) are influenced by a variety of both genetic and environmental factors. Several studies have shown that VWF levels increase with age, but the mechanisms mediating these age-related changes are unknown.
Aim: To determine the changes in VWF during aging in healthy humans and mice, and to begin to characterize the underlying mechanisms.
Methods: VWF antigen (VWF:Ag), VWF propeptide (VWFpp), Factor VIII (FVIII:C), ADAMTS13 activity, platelet count, Ristocetin cofactor (VWF:RCo) and total carbonyl content were measured in three normal human populations comprised of 172 individuals; 52 young (7 ± 5 years), 42 middle-aged (41 ± 6 years) and 78 old individuals (70 ± 7 years), similar in gender and ABO blood type. Additionally, we measured murine VWF:Ag, VWFpp, FVIII:C and ADAMTS13 activity in a minimum of 10 normal C57BL/6 mice at 9-, 55- and 97-weeks of age, as well as in young and old ADAMTS13 KO and VWF KOs (9- and $\geq$ 55-weeks). Infusions with 200 U/kg of recombinant or plasma-derived murine VWF were performed in young and old VWF KO mice to evaluate VWF clearance.
Results: VWF levels increased progressively with age in humans and mice ($P < 0.0001$), and no difference was observed with gender ($P = 0.55$). VWF levels were positively correlated with increased FVIII:C and reduced ADAMTS13 activity in the human population ($P < 0.0001$). VWFpp levels were only increased in the old human population ($P < 0.0001$), while no difference was observed in mice throughout aging ($P = 0.83$). Therefore, the VWFpp/VWF:Ag ratio decreased progressively with age in both humans and mice ($P < 0.0001$), suggesting a reduction in VWF clearance. Although no difference was observed in the clearance of recombinant mouse VWF in young and old mice ($P = 0.95$), VWF glycan composition may be a key determinant in this process as initial studies following murine pd-VWF infusions have shown marked prolongation of the VWF half-life. In the human population, a significant difference was observed in the increase in VWF levels with age in individuals with blood types A and B, compared to type O subjects ($P < 0.005$). In type O subjects, VWF levels increased minimally with age. ADAMTS13 was not associated with the increase in VWF levels, as similar levels were observed in ADAMTS13 KO mice compared to age-matched normal mice ($P = 0.40$). However, a decrease in ADAMTS13 activity with age and the aforementioned increase in VWF levels were both positively correlated with a decrease in platelet count in humans ($P < 0.05$). VWF levels were also correlated with an increase in VWF:RCo ($P < 0.0001$), however, there was no difference in the VWF:RCo/VWF:Ag ratio between the three populations ($P = 0.49$). Although no correlation was observed between VWF and total protein carbonyl levels ($P = 0.17$), higher carbonyl levels were observed in older individuals ($P < 0.0005$), suggesting that oxidative stress could contribute to altered processing and function of VWF in later life.
Summary/Conclusions: Our findings suggest that the age-related increase in VWF levels occurs progressively due, at least in part, to reduced clearance from plasma, possibly mediated through VWF glycan content. The results also show that there is enhanced VWF activity and secretion in later life.

PB 1.44-4

High-resolution, functional mapping by phage display of VWF residues required for platelet binding

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Background: Binding of von Willbrand factor (VWF) via its A1 domain to the platelet receptor, GPIb, initiates platelet-VWF interaction. This interaction is influenced by the residues in and around the A1 domain, particularly the cysteines (C1272 and C1458) responsible for forming the intramolecular disulfide loop within the A1 domain.
Aims: To define a minimal VWF fragment required for optimal platelet binding and to test the effect of the C1272-C1458 disulfide bond on platelet binding.
Methods: M13 filamentous phage libraries constructed with random VWF cDNA fragments (400–100 bp or 300–1000 bp) were composed of 8.6×10^5–2.7×10^6 independent clones. Accounting for VWF content (3/4), frame (1/3 at the 5′ and 3′ ends), orientation (1/2), and the fraction of VWF fragments large enough to encompass C1272 and C1458, these libraries were expected to display 754–2073 independent peptide fragments of the A1 domain. Phage clones were also constructed to display either a wild type VWF A1 fragment, a mutant VWF A1 fragment in which the C1272-C1458 disulfide bond was disrupted by alanine substitutions (C1272/1458A), or a wild type VWF A3 fragment. Phage libraries or phage clones screened against formalin-fixed platelets were subsequently analyzed by Sanger or high-throughput DNA sequencing.
Results: Following a single-round screen in the presence of botrocetin, DNA sequencing of randomly selected phage clones revealed a significant enrichment of VWF fragments containing the A1 domain (7/24, $P < 10^{-16}$). Surveying larger sample sizes of phage clones (~1x10^5) by high-throughput DNA sequencing resulted in ~1.24×10^7–1.35×10^7 reads. Applying *in silico* filtration for VWF cDNA and for correct orientation and reading frame at the VWF-phage cDNA junctions identified a minimal, overlapping VWF segment spanning V1252-C1458 (> 9-fold enrichment over pre-screened phage) and found no evidence for VWF segments outside the A1 domain. No specific VWF fragments were enriched in the absence of botrocetin. All enriched VWF fragments encompassed C1272 and C1458, consistent with a requirement for formation of this intramolecular disulfide bond to confer an optimal A1 domain structure for platelet binding. Further analyses of a mutant VWF A1 fragment (C1272/1458A) vs. a wild type VWF A1 fragment by competitive screening against platelets in the presence of excess VWF A3 fragment confirmed this observation. Formation of the C1272-C1458 intramolecular disulfide bond in *E. coli* was verified by western blotting.
Summary: These results demonstrate the utility of phage display for functionally mapping VWF residues with high resolution and confirm the importance of the C1272-C1458 intramolecular disulfide bond in VWF-platelet interaction.

PB 1.44-5

Investigation of the effect of CLEC4M on plasma von Willebrand factor level in the general population

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Background: von Willebrand factor (VWF) is a large multimeric plasma glycoprotein with an important role in haemostasis. VWF levels vary considerably (between 50 and 200 IU/dL in 95% of the

general population) with low levels associated with von Willebrand disease (VWD) while high levels are associated with increased risk of thrombosis, stroke and myocardial infarction. Several factors have been associated with VWF plasma level including ABO blood group, age, and genetic variations in *VWF* and other novel genetic loci identified by the CHARGE genome wide association study. These include *CLEC4M* which is expressed in liver endothelial cells and lymph nodes and encodes L-SIGN, a transmembrane receptor involved in pathogen adhesion and recognition, which binds glycosylated products. The *CLEC4M* intronic single nucleotide variant (SNV; rs868875, c.631+73A>G) and a 23 amino acid variable number tandem repeat (VNTR; ranging from 4 to 9 repeats in size) have both been reported to be significantly associated with VWF plasma level in the general population and type 1 VWD patients respectively.

Aim: To further investigate the contribution of *CLEC4M* genetic variation on VWF plasma level in the general population.

Methods: *In silico* prediction tools were used to identify SNVs in strong linkage disequilibrium (LD) with c.631+73A>G (Haploview v4.2) and to assess the effect of *CLEC4M* SNV on splicing (NetGene2 and NNSPLICE v0.9). SNVs were genotyped in healthy controls (HC) from the MCMDM-1VWD study, for whom extensive phenotypic data (including VWF:Ag level and ABO blood group genotype) was available. The VNTR was genotyped on an ABI 3730 and the size of the amplified product containing the repeat determined using Peak Scanner v1.0. The association of the SNVs and the VNTR with VWF level was investigated using Mann–Whitney and Kruskal–Wallis tests.

Results: c.631+73A>G was not predicted to influence *CLEC4M* splicing or L-SIGN expression. However, SNV rs2277998 (c.718G>A) in exon 5, in perfect LD with c.631+73A>G, altered an amino acid (p.Asp240Asn) present on the outer surface of the L-SIGN receptor suggesting a possible influence on ligand binding sensitivity. c.718G>A accounted for ~5% variation in VWF level in 921 genotyped HC with the non-reference allele associated with higher plasma levels (GG: 93.0 IU/dL; GA: 96.0 IU/dL; AA: 98.0 IU/dL; $P > 0.05$). The VNTR also accounted for ~5% variation in plasma level in 928 genotyped HC when divided into small/small (4–6/4–6; 95.5 IU/dL), small/large (4–6/7–9; 97.0 IU/dL) and large/large (7–9/7–9; 101.5 IU/dL) repeats ($P > 0.05$). These observations between c.718G>A and the VNTR with VWF level while failing to reach significance remained after correcting for ABO blood group. c.718G>A and the VNTR demonstrate ~74% LD.

Conclusion: Both the VNTR and SNV in CLEC4M may account for ~5% variation in VWF plasma level within the general population. LD between both variants may indicate a combined influence.

PB 1.44-6

Comparative pharmacokinetic analysis of 1200/500 IU vWF/FVIII and 900/800 IU vWF/FVIII concentrate in patient with type 3 vWillebrand disease

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Background: von Willebrand disease (vWD) is the most frequently inherited bleeding disorder, its incidence is of 1–2%. Type 3 is the most serious form of vWD with an autosom recessive inheritance, having a FVIII activity of < 1–2%. These patients have a haemophilia type bleeding tendency with joint bleeds. Our type 3 vWD patient was a 46 year old caucasian male scheduled for an elective hip prosthesis surgery. The baseline level of his Factor VIII procoagulant activity (FVIII:C) and vWF Ristocetin cofactor activity (vWF:RCo) were 3% and < 1% respectively.

Aim: For the determination of a proper dose of perioperative bleeding prophylaxis dose, we compared the pharmakokinetics (PK) of two plasma-derived coagulation factor concentrates composed of coaglation factor VIII (FVIII) and von Willebrand factor (vWF). Concentrate A contains FVIII/vWF in 1: 2,4, whereas concentrate B in 1:1 ratio, similar to that of normal plasma.

Methods: Simplate II bleeding time, FVIII:C and vWF:RCo activities were followed for 72 h. Baseline, then post bolus determinations were done at 15, 30, 60 min, respectively thereafter each hour untill 12, then at 24, 48 and 72 h. The wash-out period was 15 days between the administration the two concentrates.

Results: The calculated vWF:RCo activity for achieving 100% was 97 U/kg, i.e. 3492 IU from concentrate A and B as well. The measured peak vWF:RCo activity at 30 min after bolus infusion was 103% (recovery: 103%) in the case of concetrate A and 117% (recovery:117%) in concentrate B. The calculated activity of FVIII in the case of concentrate A was 41.6%, in concenrate B 109%. The measured FVIII activity was 40% (recovery: 95.45%) in the case of concentrate A and 98% (recovery: 89%) in the concentrate B. The half-time life of concentrates was the following: with concentrate A of vWF:RCo was 13 h that of FVIII was 44 h. For concentrate B the half-life of vWF:RCo was 10.5 h and that of FVIII was 48 h.

Conclusion: At both concentrates the recovery and the half-life (vWF:RCo, FVIII) was sufficient, similar and long enough, respectively. At concentrate B with higher FVIII level and higher peak activity, the half-life was the same as with concentrate A, having lower FVIII level. Both concetrates can be administered safely in the treatment of type 3 vWD. The pharmacokinetic examination is useful and advised at the planning of a personalized treatment strategy.

PB1.45 – Anticoagulant Agents – II

PB 1.45-1

Does anticoagulant treatment duration vary by the risk of venous thromboembolism recurrence in clinical practice?

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Background: Although the American College of Chest Physicians (ACCP) guidelines on venous thromboembolism (VTE) treatment provide recommendations for anticoagulant treatment duration based on patient-specific risk factors, it is unclear how well these recommendations are adopted in clinical practice.

Aims: This retrospective observational study examined whether anticoagulant treatment duration varies by risk of VTE recurrence among patients with a first observed VTE.

Methods: Data from the HealthCore Integrated Research Database, a commercial health plan database representing 45 million US members, were used. Study patients were identified by a first observed VTE event between June 2007 and September 2011; all patients were naïve to anticoagulants (including warfarin, low molecular weight heparin, unfractionated heparin, and fondaparinux). The index date was the date of the first observed VTE event. Patients who filled ≥ 1 anticoagulant within 7 days of the index event, were ≥ 18 years old, and had ≥ 12 months pre- and post-index health plan eligibility were included. Patients with a prior VTE event, atrial fibrillation, mechanical valve replacements, or rivaroxaban or dabigatran use were excluded. Based on the 8th ACCP guidelines (current at the time of this study), VTE patients were categorized into three risk categories for anticoagulant treatment duration: provoked VTE (secondary to a transient risk factor); VTE associated with active cancer (cancer-related); and unprovoked VTE. Anticoagulant treatment duration was defined as the period from initiation to discontinuation of anticoagulant treatment, based on prescription fills with an allowable gap of 90 days or an INR test of every 42 days. Kaplan–Meier curves and Cox proportional hazards models were performed to evaluate how VTE recurrence risk categories were associated with anticoagulation duration, while

controlling for baseline characteristics and bleeding risks (recent major bleeding, creatinine levels > 1.2 mg/dL, anemia, cancer, clinically overt pulmonary embolism, and age > 75 years).

Results: A total of 2002 patients were identified (52.3% males, mean age 57 ± 15 years), including: 21.4% provoked, 16.4% cancer-related, and 62.1% unprovoked VTE. The average anticoagulant treatment duration was 306 ± 269 days (mean follow-up period: 734 ± 245 days). A total of 20.2%, 22.0%, and 24.9% of patients discontinued anticoagulant therapy within 90, 180, and 365 days, respectively. Patients with provoked, cancer-related, and unprovoked VTE had anticoagulant treatment durations of: 266 ± 243, 309 ± 277, and 319 ± 275 days, respectively. After adjusting for demographics and clinical characteristics, provoked and cancer-related VTE patients were 30% (95% CI: 12–52%, $P < 0.001$) and 35% (95% CI: 6–71%, $P = 0.037$) more likely to discontinue anticoagulants than were unprovoked VTE patients, respectively. No differences were observed between provoked and cancer-related VTE patients.

Summary/Conclusion: The observed anticoagulation duration for VTE in clinical practice may not be concordant with guidelines, which recommend a longer duration of anticoagulant treatment for cancer-related and unprovoked VTE and a shorter duration for provoked VTE. While patients with unprovoked VTE had the longest treatment duration, the average length of therapy was only about 2 months longer compared with provoked VTE. Moreover, cancer-related VTE did not have a discernibly longer treatment duration than provoked VTE. Further studies are needed to explore potential reasons.

PB 1.45-2

Differential stimulation of fibrinolysis by vitamin K-antagonists alone and associated with low-molecular-weight heparin

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Background and Aim: Treatment with Vitamin K antagonists (VKA) has been shown to reduce fibrinolytic resistance of blood and plasma clots through the inhibition of thrombin-mediated TAFI activation. Because low-molecular weight heparin (LMWH) is co-administered with VKA during initiation of anticoagulant treatment, we evaluated the impact of LMWH and VKA combination on fibrinolytic resistance.

Patients and Methods: Three different groups were studied: (i) patients on stable warfarin treatment for more than 1 month ($n = 35$);(ii) patients starting oral anticoagulant therapy, who were evaluated both during the co-administration of enoxaparin and warfarin and 3–10 days after the withdrawal of enoxaparin ($n = 30$); 3) healthy subjects ($n = 15$). In patient groups, only the samples with an INR > 2 were considered. The resistance of tissue factor-induced clots to t-PA-induced fibrinolysis was evaluated in blood and plasma by thromboelastography (TEG) and turbidimetry, respectively. Results were expressed as fibrinolysis time.

Results: In healthy subjects, the median fibrinolysis time of blood clots (TEG) was 42 min [IQR: 36–58]. Patients on VKA only displayed a significantly shorter fibrinolysis time ($P < 0.001$), regardless of whether they were in group 1 (32 min [27–37]) or group 2 (32 min [26–39]). In patients receiving VKA and enoxaparin, fibrinolysis time amounted to 24 min [14–29] and was significantly shorter than the values recorded in the same patients after enoxaparin withdrawal and in patients on stable warfarin ($P < 0.005$). INR values were similar in all patient groups (2.7 [2.4–3.1]). Moreover, there were no differences in red blood cells, hematocrit, leucocytes and platelets. Concerning other TEG parameters, the clotting time (R) was slightly longer under dual anticoagulant treatment as compared to VKA only (8.8 min [7.3–12.2] vs. 7.6 min [6.0–8.9], $P = 0.03$) whereas the maximal amplitude was visibly smaller (22.5 mm [8.3–34] vs. 34.7 mm [27–42], $P = 0.0003$), suggesting that co-administration of enoxaparin markedly reduced clot strength. Thrombin generation assay in platelet-poor plasma showed that dual anticoagulation had no effect on lag time as compared to VKA only (7.0 min [6.3–9.8] vs. 7.3 min [5.4–9.9]) but reduced both the thrombin peak (22.4 nM [5.7–42] vs. 69 nM [41–101], $P < 0.0001$) and ETP (442 nM*min [35–590] vs. 559 nM*min [439–727], $P = 0.01$). Rather surprisingly, fibrinolysis time of plasma clots, at variance with blood clots, was similar in patients receiving enoxaparin+VKA and VKA only (47 min [44–56] vs. 56 min [46–66], $P = 0.08$). Most likely, thrombin generation in these groups was below the threshold concentration required to activate anti-fibrinolytic amounts of TAFI, as suggested by the fact that PTCI, a specific inhibitor of TAFIa, did not shorten the fibrinolysis time in either group.

Conclusions: These data show that the co-administration of LMWH in patients under VKA treatment has a marked effect on the fibrinolytic resistance of blood but not plasma clots, suggesting a cell-mediated mechanism. Further studies are warranted to assess the clinical implications of these findings.

PB 1.45-3

Evaluation and introduction of direct thrombin inhibitor assay to assess dabigatran anticoagulant effect in patients undergoing direct current cardioversion

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Background: Oral direct thrombin inhibitors (DTI) are now increasingly being used in clinical practice as anticoagulant of choice in reducing thrombotic risk. Whilst outcome of clinical trials negates requirement for regular monitoring in there are clinical situations where assessment of DTI anticoagulant activity is required to ensure adequate anticoagulation and assessment in bleeding complications. Traditionally patients undergoing direct current cardiversion (DCCV) would require warfarin prior to procedure to reduce the risks of thrombo-embolism, however this is associated with need for monitoring and frequent cancellation of DCCV if not in range. The use of dabigatran following risk assessment offers advantages over warfarin both in procedure scheduling and monitoring removal. Patients were prescribed a standard dose of dabigatran150 mg bd unless contraindicated.

Aims: To evaluate and establish a quantitative assay for measuring the anti-IIa activity of the DTI dabigatran and to assess the presence of an anticoagulant effect in patients, prior to undergoing DCCV.

Methods: Biophen DTI chromogenic assay (DTIChr) and the Hemoclot Thrombin Inhibitors clotting assay (DTICt – Hyphen BioMed) for dabigatran were established and optimised on the Destiny Max coagulation analyser (Stago). Citrated samples ($n = 28$) were collected as part of routine pre-operative assessment from dabigatran patients prior to undergoing DCCV. Activated partial thromboplastin time (APTT) using TriniCLOT aPTT HS (Stago) and thrombin time (TT) using TriniCLOT thrombin time (Stago) were performed. Plasma was frozen and DTI assays were performed in batches. Samples were collected randomly prior to DCCV which commenced 4–6 h post last dose of dabigatran provided prolongation of TT and APTT.

Results: There was good correlation between the APTT and DTIChr $r = 0.92$ ($r^2 = 0.84$) and DTICt $r = 0.90$ ($r^2 = 0.80$) and between the DTIChr and DTICt $r = 0.97$ ($r^2 = 0.95$) although DTICt gave lower results (median = 0.19 and 0.09 respectively) there was no statically significant difference $P = 0.21$. The TT was over sensitive with 'no clot' detected above levels of 0.03 μg/mL. The DTIChr had greater sensitivity to both low and high levels of dabigatran compared to the DTICt. Within the patient group lowest level detectable was 0.03 μg/mL by DTIChr, APTT and TT, however TT at this level was > 180s. The APTT below 0.03 μg/mL gave a normal clotting time. Dabigatran concentration in patient group ranged from 0.0 to 0.52 μg/mL with

mean 0.16 μg/mL. At time of testing based on PETRO trial data for maximum and trough levels of dabigatran 90.5% by DTIChr and 81.0% by DTICt were in the range 0.03–0.44 μg/mL with corresponding APTT and TT times.

Summary/Conclusions: APTT prolongation demonstrated a dose response in patients that had detectable levels of dabigatran by the DTIChr and would provide a rapid test for anticoagulant effect. TT can also be used but is oversensitive. The DTIChr offers improved sensitivity for quantifying dabigatran effect over a wide dose range. To minimise cancelling DCCV, testing prior to day of procedure 4–6 h post dose would potentially identify patients with no anticoagulant effect and establish a dabigatran dose range for procedure.

PB 1.45-4

Outcome of patients treated with plasma or prothrombin complex concentrates for warfarin related intracerebral hemorrhage

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Background: Prothrombin complex concentrates (PCC) can rapidly normalize prolonged prothrombin time, induced by vitamin K antagonists (VKA). It is unclear whether this rapid reversal of VKA coagulopathy would lead to improved survival compared to transfusion of plasma in patients with warfarin-related intracerebral hemorrhage.

Aims: To investigate whether the use of PCC improves the outcome of patients with warfarin-related intracerebral hemorrhage as compared to plasma.

Methods: We conducted a retrospective study at three centers in as many countries (Canada, The Netherlands and Sweden). We included consecutive patients with warfarin-related intracerebral hemorrhage treated either with plasma (mainly in Canada) or PCC (The Netherlands and Sweden) for the reversal of the anticoagulant effect of warfarin between 2002 and 2010. Data on age, sex, indication for anticoagulation, concomitant antiplatelet therapy, diabetes, and international normalized ratio (INR) on admission were collected. The volume of intracerebral hematoma was calculated from the first computed tomography (CT) scan. Bleeding localization, intraventricular hematoma extension and surgical evacuation were also documented. The unadjusted and adjusted odds ratio for 30-day all cause mortality in both treatment groups was calculated using logistic regression.

Results: Patients who received plasma ($N = 35$) had a significantly higher prevalence of diabetes, more concomitant use of antiplatelet therapy, and more frequent intraventricular hemorrhage on the initial CT scans as compared to patients who received PCC ($N = 100$). The median INR on admission was 3.0 (interquartile range 2.4–3.9) The volume of intracerebral hematoma was larger in the group treated with a median of 4 units of plasma compared to the group treated with a median of 1750 units PCC (hematoma, median 37.1 vs. 22.9 cm^3; $P = 0.063$). The unadjusted odds ratio for all-cause 30-day mortality in the PCC group was 0.40 (95% confidence interval, 0.18–0.87; $P = 0.021$) compared to the plasma group. After adjusting for the hematoma volume, bleeding localization and age, the effect of PCC on mortality became non-significant.

Conclusion: Treatment with PCC for warfarin reversal in patients with intracerebral hemorrhage does not seem to reduce the 30-day all cause mortality compared to plasma. Further studies are needed to clarify whether certain subsets of patients might benefit from warfarin reversal with PCC.

Keywords: warfarin, intracerebral hemorrhage, prothrombin complex concentrates, plasma.

PB 1.45-5

Therapeutic concentrations of dabigatran inhibit factor XIa

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Background: As the thrombin inhibitor dabigatran inhibits APTT (two-fold increase at 250 ng/mL dabigatran) stronger than it inhibits PT (two-fold increase at 465 ng/mL dabigatran), we suspected additional effects to thrombin, particularly on enzymes of the intrinsic coagulation pathway.

Aims: Therefore we evaluated possible pleiotropic effects of dabigatran *in vitro*.

Methods: Purified enzymes were purchased and measured on their advised chromogenic substrate. A factor Xa generation assay using plasma was developed, excluding thrombin participation by adding excess hirudin. The test was started with micronized silica and calcium/lipids. Thrombin generation was done with the TGA-RCH® from Technoclone.

Results: Inhibition tests with purified enzymes of the intrinsic coagulation pathway using chromogenic substrates showed that dabigatran only significantly inhibited factor XIa and not factor XIIa, kallikrein or factor IXa. The inhibition of factor XIa (Ki = 1400 ng/mL) was well above the therapeutic range of dabigatran. We subsequently utilized the Xa-generation test using contact activation of whole plasma for factor XIa generation to investigate more of its physiological roles such as reciprocal activation of factor XII, while in complex with HMW-kininogen. The chromogenic test excluded thrombin involvement by excess hirudin, and this was confirmed by benchmarking the test with prothrombin deficient plasma. The Xa-generation test showed a Xa peak which was confirmed by using factor X- and factor IX-deficient plasma, showing inhibition by rivaroxaban, and the production of a similar Xa peak when purified factor IXa was used as starter.

Addition of dabigatran showed a dose dependent shift in time to peak and in peak level with an IC50 for peak height of 450 ng/mL. Reducing factor XI level by mixing depleted plasma with normal plasma showed a delayed and lower factor Xa peak with IC50 at 10% factor XI. When using the thrombin generation test, dabigatran (IC50: 420 ng/mL) caused significant peak reduction, while this could not be achieved with hirudin and bivalirudin at comparable concentrations.

Summary/Conclusion: Dabigatran in therapeutic concentrations showed *in vitro* significant inhibition of factor XIa in factor Xa generation with an IC50 for FXI inhibition equivalent to 10% residual factor XI function. This inhibition is similar to peak reduction in the TGT test confirming inhibition potency of around 400–450 ng/mL dabigatran. Although the inhibition of factor XIa *in vitro* may not represent the actual inhibition in the clinic, the additional effect of therapeutic concentrations of dabigatran on factor XIa may be physiologically relevant. This may regard efficacy and bleeding as observed in patients with factor XI deficiency and should be investigated further.

PB 1.45-6

Assessment of dabigatran activity using the activated clotting time assay with concomitant heparin *in vitro*: comparison of two different point of care tests

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Background: Intraprocedural anticoagulation (AC) in patients receiving dabigatran or warfarin is achieved using unfractionated heparin (UFH), with or without discontinuation of the oral anticoagulant. The level of AC during procedures such as catheter ablation or angioplasty is usually monitored using activated clotting time (ACT). These assays

have been validated for use with heparins, however, it is unknown if the combination of dabigatran and heparin has additive effects on clotting time as shown by these assays.
Aims: The purpose of this study was to determine whether there is an interaction between dabigatran and unfractionated heparin (UFH) in ACT assays as measured by commonly used PoC systems.
Methods: Human whole blood (no anticoagulant) from healthy volunteers ($n = 4$) was obtained and increasing concentrations of heparin (UFH, 0.5–2 U/mL, ratiopharm®), dabigatran (125–500 ng/mL) or a combination was added. Samples were immediately tested in Hemochron Signature Elite®, Hemochron Response® or i-Stat® Point of Care (PoC) systems, using both kaolin and celite ACT assays. Assay results were obtained in seconds, and additive effects of known amounts of heparin and dabigatran were compared to each alone.
Results: Baseline clotting ranged between 115 and 122 s for kaolin tests and 124–125 s for celite-based tests. There was a concentration-dependent increase in all PoC assays with increasing concentrations of dabigatran and UFH alone, with i-Stat kaolin assay showing the greatest sensitivity to dabigatran (ratio of 3.8 and 5.1 over baseline, respectively, for 500 ng/mL dabigatran and 2 U/mL UFH). When dabigatran and UFH were combined in whole blood, measured increases were similar to the calculated additive effect when each was measured alone. At the lower concentration of UFH (0.5 U/mL), dabigatran activity tended to be slightly overestimated. At 1 and 2 U/mL UFH, this effect was no longer seen.
Conclusions: This study shows there is minimal interaction between UFH and dabigatran *in vitro* in these PoC assays. These results suggest that additive AC activity can be measured via ACT in dabigatran-treated patients given UFH, i.e. during intraprocedural AC. However PoC test results with concomitant warfarin and UFH treatment may respond differently than dabigatran and UFH. Particularly if dabigatran is stopped before the intervention, more UFH may be required to achieve a similar level of AC than in warfarin-treated patients due to the shorter half life of dabigatran.

PB1.46 – Anticoagulant agents – III

PB 1.46-1

Oral and parenteral antithrombotic agents differentially inhibit tissue factor mediated generation of thrombin in prothrombin complex concentrates

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Introduction: Several parenteral (hirudin, angiomax, argatroban) and oral antithrombin agents (ximelagatran and dabigatran) have been developed for various clinical indications. While the parenteral agents do not require any endogenous activation, the oral agents do require activation. Both groups of these agents inhibit thrombin at varying intensities. However their effects on the generation of thrombin are not fully explored. All of these agents have been reported to be weaker inhibitors of thrombin generation. The purpose of this investigation was to differentiate between the parenteral and oral thrombin inhibitors in biochemically defined systems.
Materials: Hirudin, Angiomax and bivalirudin were obtained from commercial sources. The active form of dabigatran exetilate was obtained from Selleckchem (Houston, TX) whereas the melatagran was of synthetic origin. All agents were reconstituted at 100 μg/mL. Cofact brand of prothrombin complex concentrate (PCC) was purchased from Biotest (Drerich, Germany) and reconstituted to 10 U/mL. Recombiplastin was purchased from Instrumentation Laboratory. The PCC was supplemented with recombiplastin and the generated thrombin and other cleavage products were evaluated using a SELDI-TOF mass spectrometer method and immunoblotting techniques using anti-bovine thrombin antibodies. To investigate the effects of various thrombin inhibitors, each agent was added to the PCC prior to the recombiplastin activation at graded concentrations of 20, 200 and 2000 ng/mL. Saline was used as a control. The reaction was stopped at 15 min with the addition of EDTA and the mass spectrometeric profile was carried out using the gold chip in the molecular weight range of 3.0–150 kDa. Western blot analysis was carried out to determine the generation of thrombin and activation products.
Results: The native Cofact showed a single distinct band at 71 kDa representing prothrombin whereas upon activation with recombiplastin the 71kDa peak disappeared and a prominent peak at 36 kDa representing thrombin appeared. Parenteral thrombin inhibitors did not inhibit the generation of thrombin at any concentration. Similarly, dabigatran and melagatran failed to inhibit the generation of thrombin, however in addition to the thrombin peak (36 kDa) an additional peak at 50 kDa was generated representing prethrombin. The western blot analysis was consistent with the SELDI-TOF analysis. An additional biomarker peak in activated Cofact at 12.6 kDa was generated and was absent from the native Cofact. This peak was also present in all of the parenteral and oral thrombin inhibitors supplemented mixtures.
Conclusion: These studies demonstrate that upon activation with TF, PCCs such as the Cofact can be activated into thrombin and other activation products. Furthermore, the oral thrombin inhibitors differ from the parenteral thrombin inhibitors and result in the generation of prethrombin along with thrombin. Thus besides being a weaker inhibitor of thrombin generation, dabigatran and melagatran facilitate the formation of prethrombin. This differentiation between the parenteral and oral thrombin inhibitors may impact on the safety and efficacy of these agents in the management of anticoagulation.

PB 1.46-2

Evaluation of edoxaban in Japanese patients with severe renal impairment undergoing lower-limb orthopedic surgery

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Background: There are limited therapeutic options in Japan for prevention of venous thromboembolism (VTE) in patients with severe renal impairment (SRI; creatinine clearance [CL_{CR}] ≥ 15–< 30 mL/min) undergoing lower-limb orthopedic surgery (LLOS) such as total knee arthroplasty (TKA), total hip arthroplasty (THA) and hip fracture surgery (HFS). Edoxaban, an oral direct factor Xa inhibitor, is approved in Japan for VTE prevention in patients undergoing LLOS (TKA, THA, HFS), but contraindicated in those with SRI.
Aims: Compare the safety and pharmacokinetics of edoxaban 15 mg in Japanese patients with SRI with that of edoxaban 30 mg in patients with mild renal impairment (MiRI; CL_{CR} ≥ 50–≤ 80 mL/min) and of fondaparinux 1.5 mg in patients with SRI (CL_{CR} ≥ 20 to < 30 mL/min) undergoing LLOS.
Methods: Patients ≥ 20 years of age undergoing unilateral LLOS with SRI or MiRI were enrolled in this open-label study. Patients undergoing or planning to undergo hemodialysis, at risk for bleeding or VTE, receiving antithrombotic therapy, evidence of hepatic dysfunction or unable to take oral medication were excluded. Patients with CL_{CR} ≥ 20 to < 30 mL/min were randomized to edoxaban 15 mg or fondaparinux 1.5 mg once-daily (QD). All patients with CL_{CR} ≥ 15–< 20 mL/min received edoxaban 15 mg. Patients with MiRI received edoxaban 30 mg. Treatments were administered for 11–14 days. Blood samples for measurement of plasma drug concentrations and determination of prothrombin time (PT) were collected on treatment Day 7 and treatment completion day at pre-dose, 1–3 and 4–8 h post-dose. Safety endpoints included incidence of major bleeding, clinically

relevant non-major (CRNM) bleeding, any bleeding event, and adverse events (AEs).

Results: Of 80 patients enrolled, 29 received edoxaban 15 mg, 21 fondaparinux 1.5 mg, and 30 edoxaban 30 mg; 74 completed the study (45 SRI and 29 MiRI). Most patients were female (91%). Mean age was 88, 86, and 78 years, mean body weight was 43, 46, and 55 kg, and median CL_{CR} was 26.15, 26.17, and 62.71 mL/min, for edoxaban 15 mg, fondaparinux 1.5 mg, and edoxaban 30 mg, respectively. On Day 7, plasma concentrations of edoxaban and PT ratios overlapped between the SRI and MiRI groups. Any bleeding event occurred in 6 (20.7%), 8 (40.0%), and 10 (33.3%) patients in the edoxaban 15 mg, fondaparinux 1.5 mg, and edoxaban 30 mg groups, respectively. No major bleeding events occurred in any treatment group. CRNM bleeding occurred in 1 (3.4%), 1 (5.0%), and 2 (6.7%) patients in the edoxaban 15 mg, fondaparinux 1.5 mg, and edoxaban 30 mg groups, respectively. The incidence and nature of adverse events other than bleeding events was comparable among the treatment groups. No serious AE was considered related to study drug and the majority occurred after completion of treatment.

Summary/Conclusions: These results suggest that edoxaban 15 mg QD appears to be safe and may be a therapeutic option in Japanese patients with SRI undergoing LLOS.

PB 1.46-3

Clinical profile of patients with low quality of oral anticoagulation in regular medical care

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Background: Vitamin-K antagonists (VKA) are frequently used to prevent or treat thromboembolism. Thereby the time in therapeutic range of anticoagulation is a marker of quality of anticoagulation.

Aims: We investigated the clinical profile of patients with low quality of anticoagulation with vitamin-K antagonists.

Methods: In the observational, multi-center thrombEVAL-trial, patients treated with VKA in regular medical care for at least 4 months were eligible. The clinical status was recorded and quality of therapy assessed by the individual time in therapeutic range (TTR), excluding phase of initiation. Data were obtained by a clinical visit, from medical reports and anticoagulation pass. Subjects with adequate documentation of INR measurements were included into analysis.

Results: The sample (N = 892) was split into subgroups with low quality (N = 298) and normal or high quality (N = 594) of OAC by the lowest vs. the two upper tertiles of TTR. The cutoff for TTR was 56.7%. There was no difference in indications for OAC in both groups. Subjects with low quality were older at initiation of therapy (66.03 vs. 64.24 years, P = 0.048) and had shorter measurement intervals (14.8 vs. 17.5 days, P = 0.0033). They had more diabetes and less frequently a positive family history of MI or stroke. Diabetes was independently associated with low quality (OR 1.15 [1.1, 2.0], P = 0.012) in a logistic regression model adjusted for all classical risk factors. The Charlson comorbidity index was significantly higher in patients with low quality than in all others (3.5 ± 2.8 vs. 2.8 ± 2.5, P = 0.00036). Low quality was independently associated with chronic kidney disease (OR 1.5 [1.1, 2.2], P = 0.014), heart failure (OR 1.4 [1.0, 1.9], P = 0.031), and COPD (OR 1.4 [1.0, 2.0], P = 0.047). In this survivor cohort, bleeding events were more often reported in low quality (Rate ratio 1.32 [1.08, 1.61], P = 0.00058), whereas no difference was present in thromboembolic events (P = 0.42).

Conclusion: For clinical practice, lower quality of oral anticoagulation should be considered in patients with diabetes and a larger number of comorbidities, especially chronic kidney disease, heart failure and COPD.

PB 1.46-4

Low intensity and good-controlled anticoagulant therapy can prevent thromboembolism but cannot reduce bleeding in AF patients with both hemodialysis and mechanical valve replacement

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Background: The prevalence of atrial fibrillation (AF) in end-stage renal failure is high, with an increased risk of thromboembolism and bleeding. However, there is little evidence that assess the real risk/benefit of anticoagulation in patients with severe renal impairment. Nevertheless there is an AF population that is required life-long anticoagulation due to mechanical heart-valve replacement. However, patients on hemodialysis (HD) may not derive the same benefit from warfarin as the general population. There might be a prompt answer to anticoagulant therapy in AF patients with end-stage renal failure for now.

Aims: The aim of this study is to clarify whether low intensity anticoagulant therapy and good controlled anticoagulant therapy can prevent thrombotic and reduce bleeding events in AF patients with both hemodialysis and mechanical valve replacement.

Methods: To evaluate the risk of thromboembolic events (TEs) and ISTH major bleeding events (BEs) of Japanese patients on Hemodialysis (HD), 22 consecutive AF patients on HD underwent mechanical valve replacement operations were evaluated. From the same institute mechanical valve replacement of 550 patients, we randomly sampled a control non HD group of 44 patients, frequency matched to the cases by age, sex, other risk factors and calendar year. A nested case-control analysis assessed the risk of these events. Target PT-INR was set 1.5–2.5 according to Japanese guidelines for high risk of bleeding.

Results: Patients were followed up for a mean of 6.6 years. TTR for HD group was 76.8% and non-HD group was 78.2%. There were four TEs and nine BEs (ICH1, GI bleeding 8) in HD group. There was no significant association between TEs (2.0, 95% CI 0.30–13.3) between both groups. However, compared with non HD patients, patients with HD had a significantly increased risk of bleeding (risk ratio 4.0, 95% CI 1.10–14.50). The five-year survival of HD group was 66%. This rate is superior to those of generall hemodialysis patients in Japan.

Conclusion: In this well-controlled and low anticoagulant intensity, AF patients with HD have similar risk for TEs, but have significantly increased risk of BEs compared with non-HD patients. HD group have a good life expectancy compared with general hemodialysis patients without heart valve replacement.

Close and careful anticoagulant control should be needed in order to avoid thromboembolism and bleeding in patients with end-stage renal failure and mechanical valve replacement.

PB 1.46-5

Increased anticoagulant response to drugs targeting thrombin, but not to drugs targeting FXa, in plasma from patients with cirrhosis

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Background: Chronic liver disease was previously considered as the prototype of acquired coagulopathy responsible for bleeding because of a decreased number and function of platelets, decreased synthesis of coagulation factors by the diseased liver, and hyperfibrinolysis. Recently, laboratory studies and clinical observations have provided evidence for a rebalanced hemostatic status in patients with chronic liver disease due to a concomitant decline in pro- and antihemostatic proteins. However, this balance can be easily tipped over to a hypercoagulable or a hypocoagulable state with the risk of both bleeding complications and clinical thrombotic events. In fact, treatment and

prevention for thrombotic complications is frequently required. Nevertheless, patients with liver disease are often withheld from anticoagulant therapy, because of the perceived bleeding diathesis and as a result of the limited clinical experience the anticoagulant of choice for the various indications is still not known. A recent study demonstrated an increased anticoagulant response to low molecular weight heparin in plasma from patients with cirrhosis. This potentially increased response of cirrhotic patients to anticoagulant drugs may require dose adjustments to prevent unwanted bleeding complications.

Aims: Here we evaluated the *in vitro* effect of clinically approved anticoagulant drugs in plasma from cirrhotic patients at different stages of liver disease.

Methods: We studied the anticoagulant potency of antithrombotic drugs by performing thrombin generation tests (Calibrated Automated Thrombinography) in the presence of thrombomodulin, which allows the evaluation of the balance between pro- and anticoagulant factors in plasma. Thirty patients with cirrhosis (10 with mild, 10 with moderate, and 10 with severe disease) and 30 healthy controls were studied. The study was approved by the local medical ethical committee (METc 2012/122 Nl40435.042.12) and informed consent was obtained from all subjects. Thrombin generation was determined before and after addition of unfractionated heparin (0.1 U/mL), low molecular weight heparin (0.2 U/mL), fondaparinux (0.5 μg/mL), dabigatran (300 ng/mL), and rivaroxaban (25 ng/mL).

Results: We observed an increased anticoagulant response in plasma from cirrhotic patients to anticoagulants targeting thrombin. The endogenous thrombin potential was reduced by 85.7 ± 8.5% by heparin in patients, and a 79.6 ± 10.8% reduction was observed in controls ($P = 0.038$). Addition of dabigatran led to a much more pronounced reduction in peak thrombin generation in patients compared to controls (69.6 ± 26.6% reduction in patients vs. −3.3 ± 27.8% reduction in controls, $P < 0.0001$). The enhanced effects of both drugs on thrombin generation were proportional to the severity of disease. In contrast, a similar anticoagulant response to low molecular weight heparin and even a reduced response to fondaparinux and rivaroxaban, which exclusively target factor Xa, was observed in plasma from cirrhotic patients as compared to control plasma.

Conclusions: We observed an increased anticoagulant response to drugs targeting thrombin in plasma from patients with cirrhosis. Furthermore, in contrast to a recently published study, we observed no increased response to drugs targeting factor Xa. These results may imply that drugs targeting factor Xa are the drugs of choice for prevention and treatment of thrombotic complications in cirrhotic patients.

PB 1.46-6

Risk factors for suboptimal efficacy of 3-factor prothrombin complex concentrates in emergency reversal of anticoagulation with vitamin K antagonists in patients with major bleeding

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Background: Three-factor prothrombin complex concentrates (PCC) are commonly used for reversal of international normalized ratio (INR) in patients who are bleeding or require emergency surgery. However, there is little information regarding the optimal dosing strategy for achieving adequate INR reversal.

Aim of the Study: To investigate potential risk factors for suboptimal efficacy of 3-factor prothrombin complex concentrates in emergency reversal of anticoagulation with vitamin K antagonists in patients with major bleeding.

Methods: Patients receiving VKAs and suffering from acute major bleeding were eligible for this study if their international normalized ratio (INR) was higher than or equal to 2.0. Stratified 35–50 IU/kg PCC doses were infused based on initial INR. Patients may also be treated with intravenous infusion of vitamin K. INR was controlled within 30 min from the infusion of PCC. Characteristics of patients who had an adequate INR reversal (INR ≤ 1.5) and inadequate INR reversal were compared.

Results: One hundred and seventy-three patients (mean age 77.46 years, range 34–97 years, 100 males) were included in the study. Main indication for anticoagulation was atrial fibrillation. Mean INR at the time of inclusion was 3.74 (range 2.01–12.80). After PCC administration the mean INR was reduced to 1.51 (range 0.94–3.96), 163 (94.2%) had the INR a < 2.00 and 109 patients (63.0%) had the INR < 1.5. Intravenous vitamin K was used in 147 patients. At the univariate analysis INR value at the time of inclusion, use of intravenous vitamin K, major bleeding site and cause of bleeding were associated with rate of success in the INR reversal. At multivariate analysis only high baseline INR values (> 3.74) (OR 3.78, 95% CI 1.71, 8.32) and the non-use of intravenous vitamin K (OR 2.98, 95% CI 1.22, 7.33) were significantly associated with a suboptimal INR reversal (INR > 1.5).

Conclusion: our study confirms a general efficacy of 3-factors PCC in correcting INR in patients treated with VKAs with a major bleeding complication. Use of intravenous vitamin K increases the efficacy of PCCs Conversely in patients with a high INR 3-factors, PCC seems to have a suboptimal efficacy in correcting supratherapeutic INR. Future studies are necessary to evaluate the best treatment of this subgroup of patients.

PB1.47 – Anticoagulant Agents – IV

PB 1.47-1

Patients with atrial fibrillation undergoing PCI and subsequent triple therapy with aspirin, clopidogrel and VKA – results from a single center retrospective study

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Background and Aims: Patients with atrial fibrillation (AF) are of increased risk for stroke and systemic embolism and often have concomitant coronary artery disease (CAD). In many of these patients bleeding risk correlates with the risk of stroke according to the CHA_2DS_2VASc and HASBLED score systems. Current clinical practice in patients with AF and CAD undergoing percutaneous coronary interventions (PCI) with stent implantation involves dual antiplatelet therapy and the continuation of oral anticoagulation. Although generally perceived as a high bleeding risk population, there is uncertainty about the bleeding rates in these patients. Furthermore the necessity and duration of triple therapy are currently the topic of an intensive debate. We analyzed subsequent patients treated with PCI and stent followed by triple therapy who were admitted to a university hospital medical center (2000 until 2011).

Methods: The fulltext inpatient and cathlab database including discharge summaries and cathlab reports were screened for the key words PCI, Stent, Aspirin, Clopidogrel, and Phenprocouomon (Marcumar). We identified 88 patients that received aspirin, clopidogrel and oral anticoagulation with phenprocoumon for at least 4 weeks following stent implantation and for whom follow up within the inpatient database was available. CHA_2DS_2VASc and HASBLED scores were calculated on the basis of the current patient diagnosis at the time of the index PCI. Major bleeding events were defined as gastrointestinal, pulmonary, retroperitoneal, intracerebral, intraocular or intraarticular bleeding.

Results: Out of the 88 patients, 72% received triple therapy because of AF and 28% received triple therapy for other reasons (including markedly reduced ejection fraction, left ventricular thrombus and pulmonary embolism). Fifty-five percent of all patients had persistent and 17% had intermittent AF at the time of the index PCI. The mean CHA_2DS_2VASc and HASBLED scores of the patients receiving triple

therapy were 3.3 and 2.7 respectively. Forty-four percent of the patients had a reduced renal function (GFR < 60 mL/min). The mean duration of triple therapy was 17.3 weeks. During this period there was no stent thrombosis or target vessel revascularization in the observed population. Major bleeding occurred in 9.1% of all patients, while gastrointestinal bleedings were the most frequent bleeding events (5.7%) of all patients on triple therapy followed by pulmonary bleedings (3.4%). No intracerebral bleedings were observed in the studied population.

Conclusion: Triple therapy consisting of dual antiplatelet therapy with aspirin and clopidogrel and oral anticoagulation with phenprocoumon is associated with an elevated risk for major bleeding events. In a real-world retrospective analysis of patients treated with PCI and stent followed by triple therapy for at least 4 weeks in a university hospital medical center, we observed major bleeding events with a frequency of 9.1% with gastrointestinal (GI) bleedings representing the majority of all bleeding events. These data are in line with reports of other groups and generate the hypothesis that GI-bleedings are the major obstacle associated with triple therapy. Future multicenter clinical studies on triple therapy vs. clopidogrel + oral anticoagulant are needed to define the optimal medical therapy for patients on oral anticoagulants undergoing PCI and stent implantation.

PB 1.47-2

Vitamin K antagonists (VKA) for stroke prevention in atrial fibrillation (AF) in very elderly naïve patients

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Background: Prevalence of atrial fibrillation AF increases with age. Similarly, the incidence of thromboembolism is higher among elderly with AF compared with younger patients. Although VKA strongly reduce stroke in AF, they are underused in the elderly and this mainly depends on the fear of bleeding. In an observational study Hylek et al. found a rate of major bleedings significantly higher in patients ≥ 80 years compared with patients ≤ 80 in the first year of treatment (13.1 vs. 4.7 per 100 patient-years). On the other hand, in a large prospective study with a mean follow up of 2.3 years Poli et al. found a rate of bleeding among patients ≥ 80 years old, followed by a specifically trained centre, of 1.87 per 100 patient-years. Guidelines suggest anticoagulation treatment in elderly patients with AF but they are discontinuously applied by clinicians.

Aims: The aim of this study was to analyse the outcomes in terms of major bleeding, thromboembolic events and deaths in a cohort of very elderly (≥ 85 years) naïve patients starting VKA for stroke prevention in AF.

Methods: This is a retrospective observational study. Electronic records of patients referred to our anticoagulation clinic between 2007 and 2012 were analysed. Major bleedings were considered according to ISTH definition. Ischemic events were classified as stroke or peripheral embolism. Thromboembolic risk profile was ascertained by CHADS2 score. Cardiovascular and non cardiovascular deaths were also registered.

Results: During a mean follow up of 2.25 years, 55 major bleedings occurred in 356 studied patients (7.0%pt-yrs). INR at the time of bleeding was above 3.0 in 20 pts (36.4%). Seven patients were on VKA and antiplatelet therapy (12.7%). Seventeen of 55 major bleedings (2.4%pt-years) were intracranial bleedings and 11 (1.4%pt-years) were fatal. There was no correlation between major bleedings and the CHADS2 score as well as among its single components. Moreover no relation between gender and major bleedings was found. Systemic thromboembolic events were 19 (2.4%pt-years; 16 were stroke and three were peripheral embolism). The INR at the time of thromboembolic events was below 2.0 in 7 pts −36.8% (below 1.5 in 1 pt −5.2%). The mean CHADS2 score in the cohort was 2.67. Seventy-four patients (20.8%) died during follow up and among these 42 (56.7%) were cardiovascular deaths.

Conclusions: According to our data the rate of major bleeding is high over 85 years of age in patients treated with VKA for stroke prevention in AF. There is no relationship between analyzed risk factors and major bleeding. A close monitoring of anticoagulation is suggested.

PB 1.47-3

Determination of dabigatran and rivaroxaban in serum samples from patients on treatment

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Background: Coagulation analysis and determination of the effects of anticoagulants is currently performed from plasma samples. Determinations of the concentration of anticoagulants from serum samples are made using specific and laborious methods such as various chromatographic or mass spectrometry techniques. These methods are not suitable for rapid determinations required in clinical routine for patient care. We have developed specific and sensitive method to determine new oral anticoagulants (NOAC) in serum and urine namely oral thrombin inhibitors (international patent application No. PCT/EP2012/002540) and factor Xa inhibitors (international patent PCT/WO2012/069139A1). Serum samples have several advantages over citrated plasma samples regarding reduced pre-analytical errors. They are taken almost always from patients, can be stored for longer time periods and frozen if clinical conditions may require later testing for specific parameters.

Aim: We have compared the concentration of plasma and serum samples of patients on treatment with 10 mg rivaroxaban od and 110 or 150 mg dabigatran od to identify the feasibility of the determination of the NOACs from serum samples for clinical routine.

Methods: In a prospective study we analysed 120 serum and plasma samples from patients with atrial fibrillation during therapy with 110 or 150 mg dabigatran bid. Samples were taken 2–4 h after intake of the medication. In another prospective study patients (n = 150) received 10 mg rivaroxaban od. Before and 4–6 days after initiation of therapy plasma and serum samples were taken in the morning 12 h after intake of the anticoagulant. Both studies were accepted by the local ethics committee and patients gave written informed consent prior to participation. The methods for determination of the concentration of dabigatran and rivaroxaban used purified human thrombin and factor Xa as enzymes and substrates 2238 for the thrombin assay and S2222 for the factor Xa assay. The methods for detection of dabigatran and rivaroxaban are described in the patents.

Results: Dabigatran and rivaroxaban were purified from commercially available Pradaxa® and Xarelto®, respectively. The purity of the compounds was characterized by analytical methods and was quantified in plasma samples using the thrombin specific S2238 and factor Xa specific S2222 chromogenic substrate assays. The concentrations of dabigatran and rivaroxaban were read from dilution curves from pooled plasma samples spiked with defined amounts of the purified of the NOACs. In controls concentrations of dabigatran in plasma and serum were 15 + 5 ng/mL and 21 + 12 ng/mL, and of rivaroxaban 15 + 12 and 18 + 17 ng/mL, respectively. During therapy concentration of dabigatran were 163 + 116 and 120 + 102 ng/mL, and or rivaroxaban 67 + 41 and 82 + 40 ng/mL, respectively. The correlations between plasma and serum concentrations were r = 0.5 for dabigatran and r = 0.6 for rivaroxaban.

Conclusion: The study demonstrates the feasibility of the determination of NOACs from serum samples. Dabigatran and rivaroxaban can be determined specifically in serum samples as in plasma samples using the described method. This is of advantage in specific clinical situation when plasma samples are not available or if later testing may be required.

PB 1.47-4

Assessment of cross-reactivity in three different fecal occult blood test systems with dabigatran and dabigatran etexilate: identification of useful test methods for gastrointestinal bleeding

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Background: Anticoagulant therapy can elevate the risk of gastrointestinal (GI) bleeding and may affect its management. Fecal occult blood tests (FOBT) are non-invasive, widely-used tests that can aid in detecting early and thus treatable stages of GI lesions or bleeding in patients on these therapies. The literature suggests that use of anticoagulants does not diminish the positive predictive value of FOBT, however a potential limitation and concern of these tests is the possibility for drug interactions with test components which may lead to false positives. Dabigatran is a new oral, reversible, direct thrombin inhibitor currently approved for stroke prevention in patients with atrial fibrillation. Dabigatran etexilate (DE) is the prodrug of the active ingredient dabigatran, and is converted by non-specific esterases in plasma and liver to dabigatran. Approximately 6.5% of DE in Pradaxa® is bioavailable and absorbed in the gut with the remaining 93.5% eliminated in the feces.

Aims: The purpose of this study was to investigate any potential cross-reactivity of dabigatran, DE or Pradaxa® with three commonly-used commercial fecal occult blood tests (FOBTs) with different detection methods.

Methods: Common methods of detection in FOBTs used worldwide include guaiac oxidation in hemoccult tests, immunochromatographic methods (membrane strip precoated with hemoglobin antibody), or the benzamidine method (heme in hemoglobin induces a blue/green color change). Examples of each method were tested. The Hemoccult II® SENSA® (Beckman Coulter Inc.), EZ DETECT (Biomerica Inc.), and BioNexia® Hb/Hp Complex Professionell (FROST Diagnostika GmbH) FOBT were were used for this study and all tests were conducted according to manufacturer's protocol. DE, dabigatran or the contents of a single Pradaxa® capsule were suspended in a 0.5% cellulose solution. Vehicle solution was used as negative control and human blood used as a positive control.

Results: Isolated human blood samples generated positive results in all three FOBTs as expected, whereas vehicle solution did not. Exposure to DE, dabigatran or Pradaxa® suspensions did not result in positive readouts with any detection method of the tested FOBTs.

Conclusions: These studies show that the prodrug DE and its active drug form dabigatran, as well as any further excipients in the Pradaxa® formulation do not cross-react with a set of commonly-used fecal occult blood tests. This supports the usefulness of these three tests in dabigatran etexilate-treated patients.

PB 1.47-5

Low molecular weight heparin monitoring

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Background: Anti-Xa activity (aXa) can be used as biological marker of low molecular weight heparins (LMWHs). LMWH prophylactic and therapeutic ranges have been defined for a large part of molecules and aXa commercial tests are currently available. Because of clinical trials have been conducted without any laboratory monitoring, aXa testing is not routinely recommended and debates are ongoing about clinical utility of laboratory testing. Anyhow, because aXa could be indicated in selected clinical conditions at risk of drug accumulation such as renal disease, over-underweight, prolonged therapy, pregnancy, our local guidelines follow this indication.

Aims: The aim of this study was to describe the prevalence of aXa levels out of range, both in prophylaxis and therapeutic regimens, in patients treated with enoxaparin during hospitalization in two medical and one surgical division of our hospital.

Methods: We prospectively monitored aXa activity in 373 consecutive patients during year 2011. Two hundred and fifty-two determinations were performed on patients treated with therapeutic regimen, while 121 were on patients on prophylaxis. Blood samples were collected after 4 h from the last LMWH injection, in patient treated at least from 2 days. Anti-Xa activity was measured on fresh plasma, by Stago Rotachrom heparin kit chromogenic assay (Diagnostica Stago) on magneto-mechanical instrument (STA-R Roche,Basel). The following protocol of dose adjustement is on use in our hospital: dose/kg standard posology as recommended by international guidelines, but reduced posology by 30% in patients with renal impairement (creatinine clearance < 30 mL/min) and in patients obese, underweight and elderly (age > 80y). We considered the following ranges for enoxaparin: prophylactic range = 0.20–0.49UI/mL, therapeutic range = 0.5–1.0 UI/mL.

Results: aXa out of range were 38% and 34% of the total therapeutic and prophylactic regimen, respectively. Mean creatinine level was = 1.16 ± 0.25 mg/dL in standard group and 2.46 ± 0.42 mg/dL. We analyzed separately results obtained in patients on adjusted doses compared with standard regimen, both in therapeutic and prophylactic regimen. One hundred and seventy-eight patients treated with therapeutic dose in standard regimen and 74 patients in adjusted protocol showed 41.6% and 29.6% out of range respectively. Considering prophylaxis we found 39.6% out of range in 91 patients in standard prophylaxis and 16.7% out of range in 30 patients in adjusted dose.

Conclusions: Confirming recent studies, also our experience show high frequency out of range both in prophylaxis and in therapeutic regimen. LMWH adjusted doses resulted more frequently in recommended range. Anti-Xa activity can easily be performed in a routine coagulation laboratory and could guide LMWH dose adjustment avoiding potentially dangerous out of range in special populations.

PB 1.47-6

Surgical safety threshold in patient under long term Rivaroxaban treatment: is quick PT suitable?

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Aims: The oral direct factor Xa (FXa) inhibitor Rivaroxaban has been developed for treatment and prophylaxis of thrombo-embolic disorders. Most conventional coagulation tests, and especially some Prothrombin Time (PT) assays, are affected by Rivaroxaban. The purpose of this study is to evaluate if the new proposed safety threshold (GIHP, http://site.geht.org/site/Pratiques-Professionnelles/Nouveaux-anticoagulants-oraux/Propositions-du-GIHP-NACO-et-Urgences-decembre-2012_96_810.html) for urgent surgery may be adequately estimated with our thromboplastins (STA-NEOPLASTINER, Stago and Owren's PT, MediRox).

Methods: Rivaroxaban was spiked at increasing concentrations into normal human PPP from five healthy volunteers. Two Prothrombin Times (Quick PT, STA NEOPLASTINE R, Stago and Owren PT, Owren's PT, MediRox), activated partial thromboplastin time (aPTT) (PTT-A, Stago and Actin FS, Siemens), thrombin time (TT – Thrombin10, Stago), were measured. Tests were performed on a STA-R (Stago) and responsiveness to Rivaroxaban was assessed. PT and aPTT were also evaluated on selected Rivaroxaban treated patients and correlated extemporaneously with their Rivaroxaban concentrations (Liquid anti-Xa® Stago).

Results: Rivaroxaban prolonged Quick and Owren PT in a concentration-dependent way with increased inter-variability at high concentra-

tions levels. Our Quick PT assay was more sensitive compared to the Owren PT assay. In addition, Neo-R demonstrated a high sensitivity to Rivaroxaban. aPTT was also prolonged in a concentration-dependent manner but this test seemed less sensitive than PT. Similar results were observed on *ex-vivo* samples. Individual variability did not allow the use of PT to estimate Rivaroxaban concentration. With our reagents, these preliminary results indicate that a PT > 80% (as proposed by GIHP) ensures a Rivaroxaban level lower than 30 ng/mL. However, with this cut off, about eight patients out of 10 will be inadequately postponed.

Conclusions: Despite the use of a high sensitive Quick PT reagent to Rivaroxaban, the individual variability does not allow an estimation of the anticoagulant concentration. Particularly, in front of an urgent surgical procedure, too many patients would be delayed. Therefore, in case of an abnormal PT, a specific test is required for this purpose.

PB1.48 – Anticoagulant Agents – V

PB 1.48-1

Association between CYP2C9, VKORC1 and CYP4F2 genetic variants in anticoagulation related outcomes during initiation period in acenocoumarol therapy

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Background: Acenocoumarol (AC) is a widespread used anticoagulant with a high inter-personal variability in required dose to achieve a stable INR. Thus, it needs careful clinical management to balance the risks of over-anticoagulation and bleeding with those of under-anticoagulation and thrombosis. Such adjustment mainly depends on both clinical and genetic factors (SNPs). There is an abundant evidence of the role of certain SNPs on the pharmacogenetics of AC. The strongest SNPs are *CYP2C9* isoforms and −1639G>A change of *VKORC1*. Recently, the influence of rs2108622 in *CYP4F2* gene in AC dose has been described as slighter but significant. Despite the relevance of these SNPs on the dose required to achieve a stable anticoagulation has been deeply studied, the relationship between these SNPs and other outcomes has not been well defined in the first stages of AC therapy.

Aims: We evaluated the influence of *VKORC1*, *CYP2C9* and *CYP4F2* SNPs in time to over-coagulation and time to achieve stable dosage in our population.

Methods: Characteristics of our primary cohort has been described previously[1]. We performed a retrospective study with patients who had started therapy from 1995 to 2009 and that are being controlled at our Unit. The primary outcomes considered for 2 first months of therapy were: time to stable dosage and time to first INR>4. Statistical analyses were done using SPSS 16.0 (SPSS Inc., Chicago, IL). All statistical tests were two-sided and a $P \leq 0.05$ was considered statistically significant.

Results: A total of 973 patients were enrolled. The relationship between genetic and clinical factors with INR>4 was evaluated by Cox regression model: age, body surface area, antiplatelet therapy, *CYP2C9, VKORC1,* and *CYP4F2* SNPs were statistically significant (univariate analysis, all $P < 0.15$). However, in the multivariate analysis only *CYP2C9, VKORC1,* and *CYP4F2* SNPs remained significant: CYP2C9 3* carriers and *VKORC1* −1639AA patients had a higher risk to over-anticoagulation (HR 1.19, CI95% 1.06–1.34 and HR 1.37, CI95% 1.21–1.54, respectively). Interestingly, in this model *CYP4F2* TT subjects had a low risk to suffer an INR>4 (HR 0.87, CI 95% 0.77–0.99, $P = 0.037$). Time to stable dosage was also evaluated using a Cox regression model where only *VKORC1* SNPs remained significant. Thus, *VKORC1* −1639AA patients spent less days to achieve stable dosage (HR 1.38, CI95% 1.01–1.90, $P = 0.045$).

Summary: Our data show that the main determinants in required dose to achieve a stable INR, *VKORC1* and *CYP2C9* SNPs, have a significant impact on the risk of overdosing in the 8 first weeks of AC therapy. Moreover, we show for first time that *CYP4F2* SNPs, with a slighter weight on AC stable dose, is also influencing the INR overshooting at early stages of therapy. These findings suggest that these four polymorphisms should be included in pharmacogenetic algorithms to guide the first weeks of AC therapy.

Reference:

1. JJ Cerezo-Manchado et al. Thrombosis and Haemostasis 2013.

PB 1.48-2

The effect of dabigatran on the activated partial thromboplastin time and thrombin time as determined by the hemoclot thrombin inhibitor assay in patient plasma samples

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Background: Dabigatran is an oral direct thrombin inhibitor available for stroke prevention in non-valvular atrial fibrillation. Although dabigatran does not require routine laboratory monitoring, an assessment of its anticoagulant effect in certain clinical settings, such as urgent surgery or bleeding is desirable. To date, our understanding of the effect of dabigatran on coagulation assays is based on the analysis of dabigatran-spiked plasma.

Aim: We examined the relationship between dabigatran levels, as determined by the hemoclot thrombin inhibitor assay (HTI) (Hyphen Biomed, France), and the activated partial thromboplastin time (aPTT) and the thrombin time (TT) using patient plasma samples.

Methods: Seventy five samples from 47 patients receiving dabigatran were analysed. Thirty-three patients were assessed for dose-appropriateness, seven patients required urgent surgery/procedure, including three requiring neurosurgery, and seven patients presented with bleeding. These were random plasma samples taken from mainly inpatients and some outpatients. The HTI assay was established to measure dabigatran concentration using three lyophilised dabigatran calibrators (ranging from 30 to 480 ng/mL) to establish a standard curve ($R^2 = 0.995$). aPTTs were performed using our local TriniCLOT aPTT S reagent (Trinity Biotech). We assessed three additional aPTT reagents: aPTT SP (Instrumentation Laboratory), aPTT SS (Instrumentation Laboratory) and Actin FS (Siemens).

Results: Our local aPTT demonstrated a modest correlation with the dabigatran level ($r = 0.80$, $R^2 = 0.645$) with the aPTT range of 46–54 s corresponding to the therapeutic dabigatran level of 90–180 ng/mL. An aPTT of ≥ 64 s correlated with a supra-therapeutic dabigatran level of ≥ 300 ng/mL. The correlation became less reliable at high concentrations. A dabigatran level of 10 ng/mL correlated with an aPTT of 36 s (our reference range 22–32 s). The aPTT reagents differed in their sensitivity to dabigatran with different values obtained across the therapeutic range. The aPTT range for a therapeutic dabigatran level (90–180 ng/mL) and R^2 for additional aPTT reagents was: aPTT SP 61–71 s, 0.505; aPTT SS 51–60 s, 0.663; and aPTT FS 54–64, 0.636. The TT was extremely sensitive to the presence of dabigatran. A dabigatran level of 60 ng/mL resulted in a TT of greater than our reportable limit (> 300 s). The withholding of dabigatran until normalisation of the TT was associated with favourable outcomes in the three patients requiring neurosurgery. Conversely, supra-therapeutic levels were not associated with excessive bleeding in three patients requiring urgent surgery or a procedure. The inability to reverse dabigatran contributed to the one fatality in our series.

Conclusion: The aPTT demonstrated a modest correlation with the dabigatran level, as determined by the HTI assay. In our lab, an aPTT of between 46 and 54 s corresponds to the therapeutic range of dabigatran. aPTT reagents differ in their reactivity to the presence of dabigatran, thus making it difficult to compare values between laboratories

and to make recommendations regarding monitoring. The TT is useful to exclude the presence of dabigatran. The aPTT and TT provide complementary information to assess the presence of dabigatran in guiding peri-procedural management and bleeding scenarios. This data represents our 'real-world' experience of monitoring the anticoagulant effect of dabigatran in patient samples.

PB 1.48-3

Personalised clopidogrel therapy by teststrip-based CYP2C19 genotyping

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Background: Clopidogrel (Plavix[TRADEMARK]) is widely prescribed to reduce recurrent ischaemic complications in patients with acute coronary syndromes and/or post-percutaneous interventions. High interindividual variations in and non-responsiveness to clopidogrel-induced platelet inhibition often complicate the treatment. Genetic polymorphisms in the *CYP2C19* gene, which encodes the principle enzyme responsible for the bioactivation of this prodrug, lead to reduced enzyme activity and reduced levels of clopidogrel's active metabolite. Patients carrying defective *CYP2C19* variants have a higher risk for major adverse cardiovascular events than noncarriers, whereas individuals with increased enzyme activity are more at-risk for developing bleeding.
Methods: A genetic test (StripAssay) was developed for the detection of the *CYP2C19* loss-of-function alleles **2, *3, *4, *5, *6, *7* and **8*, as well as for the gain-of-function allele **17*. The StripAssay is based on multiplex PCR, followed by reverse-hybridisation of biotin-labeled amplification products to a parallel array of allele-specific oligonucleotides immobilised on membrane teststrips.
Results: Genotyping for functionally enhanced (*17) or defective (*2–*8) *CYP2C19* variants allow the classification of patients into ultra-rapid, extensive and poor metabolisers for clopidogrel. Favourable properties, such as the rapid DNA extraction protocol, ready-to-use reagents and teststrips, as well as the potential for automation of the hybridisation/detection step, make the StripAssay convenient and easy to perform within < 6 h.
Conclusion: A simple and reliable diagnostic tool was developed for predicting the response of patients to clopidogrel treatment. The CYP2C19 StripAssay will assist clinicians to achieve a more individualised antiplatelet therapy.

PB 1.48-4

A novel synthetic heparin antagonist neutralizes the anticoagulant actions of branded and generic enoxaparins in the whole blood clotting assay used during interventional cardiology

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Introduction: Heparin and low molecular weight heparins (LMWHs) are commonly used in the management of cardiovascular disorders and interventions. LMWHs, such as enoxaparin, are also used intravenously in the management of cardiovascular patients. Delparentag (PMX 60056, Heptagonist; Polymedix, Radner, PA) is a synthetic LMWH neutralizing agent, which has been shown to also neutralize LMWHs, such as enoxaparin. During interventional cardiology and surgery, the anticoagulant effects of heparin and related drugs are usually monitored using whole blood celite Activated clotting time (ACT) and similar tests. LMWHs produce relatively weaker anticoagulant effects on this test, however, are capable of prolonging this test proportional to the concentration. Recently, generic versions of enoxaparin have also been introduced. In the US there are two generic products available, namely Sandoz enoxaparin (SE) and Watson enoxaparin (WE). The purpose of this study is to compare the neutralization effect of the Delparentag with protamine sulfate (PS) on enoxaparin supplemented whole blood.
Materials and Methods: Four individual batches of SE and three individual batches of WE, along with four batches of branded enoxaparin (BE) were commercially obtained. The whole blood anticoagulant effects of each of these agents were measured using celite ACT at a fixed concentration. Whole blood samples from healthy volunteers were supplemented with each of these drugs at 25 μg/mL. To determine the neutralization profile, equigravimetric amounts of either Delparentag or PS were added to individual tubes (n = 4–7). The results were compiled in terms of cumulative means of the different batches for the native, enoxaparin supplemented and enoxaparin supplemented with both PS and Delparentag.
Results: The baseline ACT values in the native whole blood ranged from 124 to 164 s in the different groups. Supplementation of enoxaparin at 25 μg/mL resulted in a prolongation of celite ACT, resulting in a clotting time of 219–265 s. No significant differences were noted between the generic and BE preparations. Supplementation of Delparentag at 25 μg/mL to each of these different enoxaparin supplemented whole blood resulted in the neutralization of the anticoagulant effects, resulting in a decrease in the clotting times in the range of 137–174 s. While some differences in the batches of BE, SE and WE were noted, these were not significantly different. Overall, the cumulative neutralization of the ACT by Delparentag was comparable for each group of enoxaparins. Similarly, in the neutralization studies with PS, comparable results were observed in all groups. The relative neutralization of the anticoagulant effects of both agents were comparable, resulting in 80–95% neutralization of the anticoagulant effects of enoxaparin.
Conclusions: These studies suggest that both generic and branded versions of commercial enoxaparins produce comparable anticoagulant effects in native whole blood, which can be monitored using celite ACT. At 25 μg/mL, all agents produced a > 200 s prolongation of the celite ACT in comparison to the control (< 140 s). Although minor batch-to-batch differences existed among the generic and branded enoxaparins, the cumulative means were comparable. The relative neutralization of the anticoagulant effects of enoxaparin by both PS and Delparentag was comparable, resulting in > 80% neutralization of these effects.

PB 1.48-5

Antidotal effects of non-specific reversal agents on anticoagulant-induced inhibition of thrombin generation

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Introduction: Reversal of pharmacologic anticoagulation is an issue that arises when an anticoagulated patient has a major bleeding or when an emergency surgery needs immediate correction of coagulation. However, the new oral anticoagulants (NOAC) Rivaroxaban (anti-FXa) and Dabigatran (anti-FIIa) lack specific antidotes and only limited data is available regarding the antidotal effect of non-specific haemostatic agents.
In this *ex vivo* study reversal of anticoagulant activity after the administration of either 20 mg Rivaroxaban or 150 mg Dabigatran was tested *in vitro* using two different PCC (Beriplex, Cofact), aPCC (FEIBA; factor eight inhibitor bypassing activity) or rFVIIa (NovoSeven) at various concentrations.
Method: Ten healthy subjects were first randomized to receive either 20 mg Rivaroxaban or 150 mg Dabigatran in one oral dose.

Citrated venous blood was taken right before (T0) and 2 h after administration (T2) of either Dabigatran or Rivaroxaban. The potential of four haemostatic agents to reverse the anticoagulant effect of the NOACs was evaluated:

Beriplex: 0.25; 0.5; 1; 2 U/mL
Cofact: 0.25; 0.5; 1; 2 U/mL
FEIBA: 0.25; 0.5; 1; 2 U/mL
NovoSeven: 1.25; 2.5; 5; 10 μg/mL

Thrombin generation was the primarily applied assay, along with the parameters aPTT, PT, thrombin time, Rivaroxaban- and Dabigatran levels and Ecarin time. Parameters of interest concerning the thrombin generation assay (TGA) were ETP, *Peak* and Lag time. Thrombin generation in platelet poor plasma was initiated by adding 1 pM tissue factor and 4 μM phospholipids.

Results: Two hours after administration (T2), Rivaroxaban showed remarkable inhibitory effects on the investigated TGA parameters ETP, Peak and Lag time, with a more pronounced inhibitory effect on the Peak. In contrast, Dabigatran at T2 only showed a slight effect on ETP and no effect on the Peak whereas LT was significantly prolonged.

Rivaroxaban-induced inhibition of ETP and Peak were reversed by FEIBA in a concentration dependent manner with an over-correction for the two highest concentrations (1 and 2 U/mL). Cofact and rFVIIa restored ETP dose-dependently and both reached baseline T0 at their highest concentrations. Compared to FEIBA, rFVIIa and Cofact only had a slight but dose-dependent effect on the Peak. Interestingly, the other PCC, Beriplex, did not show any reversal effects on ETP and Peak.

Regarding Rivaroxaban-prolonged LT all concentrations of rFVIIa and FEIBA were responsible for a significant LT-reduction close to baseline, whereas both PCCs did not correct prolonged LT.

Regarding Dabigatran, all doses of rFVIIa, Cofact and FEIBA reduced the LT, with a more pronounced and dose-dependent effect of Cofact and FEIBA. For Beriplex only a slight reduction in LT was observed.

Conclusion: FEIBA and rFVIIa showed significant reversal of anticoagulant activity already at low therapeutic concentrations for both anticoagulants in TGA. Surprisingly, regarding both investigated PCCs, only Cofact showed an antidotal effect on TGA parameters, especially in higher therapeutic concentrations. Considering that the main difference between the two PCCs is that Beriplex contains small amounts of heparin and Cofact does not, it is conceivable that this may explain the observed differences in TGA. Further clinical validation is needed.

PB 1.48-6

D-dimers levels evolution during heparin therapy of patients with cerebral venous thrombosis

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Background: Heparin therapy is widely used as the first line treatment in patients with cerebral vein thrombosis (CVT). However, near 20% of patients presented poor outcome (deaths or major handicaps) during the first weeks of heparin therapy. The aim of this prospective study was to evaluate the evolution of D-dimers levels during the first 2 weeks of anticoagulant treatment.

Methods: All consecutive patients with CVT, admitted in our ICU before heparin treatment initiation, were included in this prospective study. Patients with duration of their symptoms more than 2 weeks before admission, or related to a cancer, an infectious disease or a recent neurosurgery, were not included. All patients were treated with therapeutic doses of UFH for a minimum of 5 days. The heparin dose was adjusted so that the initial APTT was at least doubled. Then, oral anticoagulants (OA) were initiated (INR values between 2 and 3) for a 3–6 month period. Plasma D-dimers levels were evaluated for each patient on admission and then each days during heparin therapy until the biological efficiency of the treatment with oral anticoagulant alone. Outcome assessment: Patients were clinically assessed daily by a clinician unaware of the results of D-dimer test. In case of clinical worsening during the two first weeks of anticoagulant therapy, CT scan or MRI had to be performed. D-Dimers were compared from day 1 to day 14 between the presence or the absence of: a lesion, an aggravation, an infection, more than two sinuses, an hemorrhage, a coma and an epilepsy. The coefficients of equation of the curves of D-dimers evolution were compared between groups using a student's t-test.

Results: Thirty-one patients were included (25 women and six men, mean age: 37.2). The median D-Ddimers levels (Q1-Q3) were, on admission, 1327 ng/mL (836–2410) and at day 417 ng/mL (141–1852). During the two first weeks of anticoagulation therapy, eight patients presented a clinical worsened course related with a thrombosis extension (three patients), a new intracerebral hemorrhage (three patients) or an isolated increase of brain edema (two patients). In patients (n = 17) with initial hemorraghic or ischemic intracerebral lesions, D-dimer levels at day 0 were significantly higher than those (n = 14) without lesions (median: 1885 vs. 955; P = 0.04). The coefficients of equation of the curves of D-dimers evolutions were not statistically different between patients with neurological aggravation and patients without aggravation. No significant difference was also observed between patients with initial coma or epilepsy and patients without coma or epilepsy, between patients with more than two thrombosed sinuses and patients with less two thrombosed sinuses and between patients with brain hemorrhagic complication and patients without hemorrhagic complication.

Conclusion: The evolution of D-dimers levels I patients with CVT is not correlated with the clinical evolution of patients.

PB1.49 – Anticoagulant Agents – VI

PB 1.49-1

Short-term prognosis of intracranial haemorrhage in patients on oral anticoagulant and antiplatelet drug: the VAIP study

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Background: Intracranial hemorrhage (ICH) is the most serious and feared complication of oral vitamin K antagonists (VKAs) and antiplatelet drugs. Despite medical and surgical approach are promptly conducted, a very high rate of mortality and severe disability has been reported in case of VKA-related intracerebral bleeding. Only few data are available on the clinical course of other sites of antithrombotic drug-related ICH, on the efficacy of available treatments and on prognostic risk factors.

Aims: To investigate determinants of short-term prognosis of antithrombotic drug-related ICH.

Methods: The Vka- and Antiplatelet drug-related Ich Prognosis (VAIP) is designed as a retrospective study. Consecutive adult patients with an ICH objectively documented by neuroimaging (cerebral computed tomography or magnetic resonance), occurring during treatment with VKA (warfarin or acenocoumarol), defined by an international normalized ratio (INR) of 1.5 or more, or with an antiplatelet drug (aspirin, ticlopidine, or clopidogrel) at the time of hospital admission, were included. Patients were identified by searching the administrative database of the Cuneo hospital, Piedmont Region, Italy, from 2005 to 2010. As a control group, we randomly selected patients with ICH not on treatment with VKAs or antiplatelet drugs and admitted in the same hospital during the same period.

Results: Overall, 451 patients were included: 75 on VKA, 96 on antiplatelet drug, and 280 as a control group. Patients on VKA were, in comparison with the control group, older (77.7 vs. 72.9 years old; $P < 0.05$), more likely to have a subdural haematoma (49.3% vs. 30%; $P < 0.05$) and less likely an intracerebral haemorrhage (45.3% vs.

66.4%; $P < 0.05$), at higher risk of death during hospital stay (33.3% vs. 22.1%; $P < 0.05$). Patients on antiplatelet drug were, in comparison with the control group, older (77.3 vs. 72.9 years old; $P < 0.05$) with a greater proportion aged more than 80 years (44.8% vs. 32.1%; $P < 0.05$), with a worst Glasgow Come Scale (GCS) at admission (< 8%, 10.4% vs. 26.8%; $P < 0.05$) and a modified Rankin scale at discharged (4–6, 50.0% vs. 37.9%; $P < 0.05$). At the multivariate Cox regression analysis, independent predictors of in-hospital death for ICH were: age > 80 years (HR 2.3, 95% CI 1.5–3.5), GCS < 8 (HR 7.8, 95% CI 5.0–12.1), treatment with VKAs (HR 2.0, 95% CI 1.2–3.4), and treatment with antiplatelet drug (HR 1.8, 95% CI 1.05–3.0); surgical treatment is an independent predictor of survival (HR 0.5, 95% CI 0.3–0.96). Independent predictors of combined outcome in-hospital mortality and disability, defined as modified Rankin scale 4–6, were: age > 80 years (HR 2.1, 95% CI 1.6–2.8), GCS < 8 (HR 3.0, 95% CI 2.2–4.0), and treatment with antiplatelet drug (HR 1.5, 95% CI 1.1–2.1); surgical treatment is an independent predictor of good outcome (HR 0.7, 95% CI 0.5–0.97).

Conclusions: Our data suggest that both VKAs and antiplatelet drugs worsen short-term prognosis of ICH and that a prompt surgical treatment is life saving, in particular for patients with a subdural haematoma.

PB 1.49-2

Risk factors for death and thromboembolic complications in patients with major or clinically relevant non-major bleeding while on oral anticoagulant treatment

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Background: The incidence of major bleeding (MB) complications is about 3% per-year during oral anticoagulant treatment. Optimal management strategies and their effect on thromboembolic complications remain unclear.

Methods: CLIMBING is an Italian, prospective, observational, cohort study including patients on oral anticoagulant therapy admitted to the Emergency Department for MB or clinically relevant non-major bleeding (CRNMB). The aim of the study was to evaluate the clinical management and outcome of MB or CRNMB. MB was defined according to ISTH criteria. The primary endpoint of the study is death or major thromboembolic complications (acute coronary syndrome, ischemic stroke, systemic cardioembolism, pulmonary embolism). The secondary endpoint is bleeding-related or treatment-related adverse events and recurrent bleeding during hospitalization.

Results: As for January 1st 2013, 202 patients were included in the study, 161 with MB (79%). Both previous bleeding (53.6% vs. 13.6%, $P < 0.001$) and HASBLED score ≥ 3 (65.8% vs. 42.8%, $P < 0.001$) were more common in patients referred for CRNMB than for MB. No association was found between clinical presentation as MB or CRNMB respect to age, gender, indication for anticoagulation, INR at admission, ongoing antiplatelet therapy or recent trauma. Fresh frozen plasma (FFP) or prothrombin complex concentrates (PCCs) were used only in patients suffering MB.

Among patients with MB, previous intracranial hemorrhage (ICH) was more common in patients admitted for ICH (7% vs. 0%, $P = 0.05$) while HASBLED score ≥ 3 (53.7% vs. 37.4%, $P < 0.05$) was more common in patients with non-ICH-MB. FFP was used in similar proportions of patients with and without ICH while PCCs was more commonly used in patients with ICH (49.5% vs. 25.9%, P 0.004). In-hospital death occurred in 34 patients with and in seven patients without ICH (31.8% vs. 12.9%, $P = 0.01$) and rebleeding in 12 and eight patients, respectively (16.6% vs. 11.4%, P = ns). ICH (OR 3.09, 95% CI 1.26–7.61, $P = 0.01$) but not HASBLED score ≥ 3 (OR 0.93, 95%CI 0.44–1.97) nor any of the HASBLED components was an independent risk factors for death. During hospitalization, an acute coronary syndrome occurred in four patients (all non-ICH), ischemic stroke in three patients (all ICH), pulmonary embolism in two patients (both ICH). No significant difference was observed in the incidence of thromboembolic complications between patients receiving and those not receiving PCCs (three out of 67, 4.5% vs. six out of 94, 6.4%, P = ns).

Among patients with ICH, age (83 ± 6 vs. 77 ± 9, $P < 0.001$), Glasgow Coma Scale at admission (9 ± 4 vs. 14 ± 2, $P < 0.001$) and parenchymal localization of ICH (44.1% vs. 16.3%, $P = 0.004$) were associated with death, while no association was observed between gender, hypertension, renal failure, previous ICH, INR at admission, the use of PCC or FFP and death.

Conclusions: Among patients with MB while on oral anticoagulant treatment, the risk for death is substantial in those with ICH and is not associated with HASBLED score. The incidence of thromboembolic complications during hospitalization seems not to be associated with PCCs use.

PB 1.49-3

The synthetic pentasaccharide fondaparinux attenuates myocardial ischemia-reperfusion injury in rat via STAT-3

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Background: Acute myocardial infarction is a leading cause of death worldwide. Although highly beneficial, reperfusion of myocardium is associated with reperfusion injury. Pharmacologic inhibition of factor Xa has been shown to attenuate ischemia-reperfusion (I/R) injury but the cellular mechanism is poorly understood.

Aims: To determine the role of blood in fondaparinux (FDX)-induced cardioprotection and the involvement of RISK and SAFE pathways, two major survival signalling cascades commonly associated with protection against I/R injury.

Methods: We investigated the ability of FDX to prevent I/R injury *in vivo*, in a model of transient coronary ligation, and *ex vivo* in a model of crystalloid-perfused isolated-heart. In the *in vivo* model, 40-min of myocardial ischemia was followed by 120-min of reperfusion. FDX (10 mg/kg) was injected intraperitoneally 10-min before reperfusion. In the isolated-heart model, FDX (0.1 mg/mL) was administrated in the recirculating buffer. In both models, infarct size was assessed after 120-min of reperfusion. Myocardial tissues were collected at 15- and 30-min reperfusion for western-blots analysis.

Results: *In vivo*, FDX decreased infarct size by 29% (44.28% vs. 61.98% in FDX-treated rats and controls respectively, $P < 0.05$). FDX induced a significant phosphorylation of STAT-3 and GSK3β as compared with controls. Addition of AG490, an inhibitor of JAK/STAT pathway before I/R prevented the phosphorylation of STAT-3 and GSK3β and abolished the FDX-induced cardioprotection. On the contrary, FDX had no effect on infarct size and hemodynamic parameters in the isolated-heart model.

Conclusion: FDX decreased I/R injury *in vivo* but not in a crystalloid-perfused isolated-heart. The protective effect was mediated through the phosphorylation of STAT-3. In our experimental conditions, FDX needed whole blood to be protective but its beneficial effect was not directly related to anticoagulant effect but to the activation of the SAFE pathway.

PB 1.49-4

A pharmacodynamic comparison of otamixaban and bivalirudin in primates

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Background: Preventing blood clots is important in the treatment of myocardial infarction and unstable angina. Research on new anticoagulants continues to develop safer and/or more effective drugs. It is not yet clear whether there is an advantage in terms of safety (less bleeding) or effectiveness (less clotting) to inhibiting factor (F) Xa rather than thrombin in such patients.
Aims: The purpose of this study was to compare the pharmacodynamic relationships for bivalirudin (direct thrombin inhibitor; The Medicines Company) and otamixaban (direct FXa inhibitor; Sanofi-Aventis Paris) following iv administration to primates.
Materials and Methods: Following anesthesia, primates (*Macaca mulatta*; $n = 8$) were administered bivalirudin (0.185, 0.375 or 0.75 mg/kg) or otamixaban (0.05, 0.1 and 0.2 mg/kg) intravenously with a 1 week washout period between treatments. Blood samples were collected over a 60 min period. Celite ACT (Hemochron) and tissue factor activated thromboelastography (Haemonetics TEG) were assessed using fresh whole blood. Plasma aliquots were used for clotting (PT, aPTT, Heptest), amidolytic (anti-Xa, anti-IIa) and fluorogenic (thrombin generation; Technoclone) assays. Area under the curve values were determined by trapezoidal approximation (SigmaPlot 12; Systat Software). Differences between treatment regimens (drug/dose) for all study endpoints were tested for significance by two-way analysis of variance (SigmaPlot 12); P-values ≤ 0.05 were considered significant.
Results: Using anticoagulant monitoring tests, otamixaban and bivalirudin were both cleared rapidly from the circulation, with the drug effect in most assays gone by the 30 min. Comparable prolongations of the ACT could be achieved 5 min following administration of otamixaban (278.1 $\pm$ 19.1 s) or bivalirudin (270.3 $\pm$ 8.2 s), although otamixaban was more potent, producing this effect at a dose of 0.2 vs. 0.75 mg/kg for bivalirudin. Dose-dependent increases in clotting time could be measured by aPTT and Heptest for both drugs. PT, however, was only prolonged by bivalirudin. Using assays to analyze the mechanism of the anticoagulant action of these two types of drugs, in the tissue factor supplemented TEG assay, bivalirudin prolonged the clotting time (R-time), reduced the rate of clot formation (angle) and decreased clot strength (maximal amplitude). In contrast, otamixaban produced different effects on these parameters suggesting differential mechanisms for modulating clot formation dynamics. In a fibrinokinetic clot formation assay, bivalirudin completely inhibited clot formation, while otamixaban did not. Furthermore, otamixaban more effectively inhibited thrombin generation than did bivalirudin, and otamixaban more effectively inhibited thromboplastin-induced platelet activation.
Conclusions: The anticoagulant effects of otamixaban and bivalirudin exhibit comparable plasma time courses which can be effectively monitored by the ACT suggesting that otamixaban may be useful in surgical or interventional indications. However, the observed differences in the effect of these two types of drugs on the dynamics of clot formation, thrombin generation inhibition and platelet activation inhibition reveals a different balance among the inhibitory effects on the various hemostatic parameters, suggesting that otamixaban may have superior safety and/or efficacy advantages over bivalirudin. The mechanistic differences between the thrombin inhibitor and the FXa inhibitor warrant further investigation to determine how they translate into clinical outcomes in patients undergoing interventional procedures.

PB 1.49-5

Evaluation of the new DG-Chrom Anti-Xa kit from Grifols for detection and quantification of new oral anti-Xa anticoagulants

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Background: A new chromogenic kit for the detection and quantification of anti-Xa activity (DG-Chrom Anti-Xa) was evaluated by Grifols. This kit will offer an assay capable to detect and quantify anti-Xa activity of the new oral anticoagulants.
Aims: The aim of this study is to evaluate the preliminary performance of this new kit with Rivaroxaban in a wide range of concentrations.
Methods: The study was performed in the optical coagulometer Q Hemostasis Analyser (Grifols).
Linearity of the calibration curve was analyzed using two different sets of commercial Rivaroxaban calibrators. A precision study was performed using three commercial controls containing three different concentrations of Rivaroxaban during five different days. The accuracy of the concentration determination was also tested.
Results: Linearity was found acceptable in the range of 0.0–433.3 ng/mL showing correlation coefficients higher than 0.99 using log-lin axis transformations. Linearity was found acceptable in the range of 0.0–145.6 ng/mL showing correlation coefficients higher than 0.99 using lin-lin axis transformations. Precision results showed appropriate coefficients of variation (CV) of 5.6% for the high control, 7.9% for the medium control and 10.2% for the low control Accuracy was found acceptable showing the following% of bias, 2.5% for high control, 4.3% for medium control and 1.3% for low control.
Conclusion: DG-Chrom Anti-Xa, a new chromogenic kit for detection and quantification of new oral anti-Xa anticoagulants, was found acceptable in terms of linearity, precision and accuracy with a wide range of concentrations of Rivaroxaban tested in Q Hemostasis Analyzer.

PB 1.49-6

Can high quality vitamin K-antagonist treatment be further improved?

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Background: The safety and efficacy of vitamin K-antagonist treatment depend on the clinical setting, and specialized anticoagulant clinics using computer-assisted dosage have been shown to provide treatment of high quality. To achieve further improvements, a focus on specific groups of patients seems necessary. In example, patients with venous thrombotic disorders (VTE) have been shown to be particularly vulnerable to the thrombotic complications of under-anticoagulation in a large multicenter study.
Aims: We investigated whether three groups of potentially vulnerable patients were prone to unstable responses to anticoagulant treatment. These were patients with VTE, patients with variant alleles of the *CYP2C9* and *VKORC1* genes and young patients.
Methods: Consecutive patients ($n = 308$, 67% men, median age 67 years) from a nurse-managed anticoagulant clinic at a Danish hospital were interviewed about health-related behaviour and socio-demographics at baseline and followed for 1 year. The majority of the patients had received anticoagulant therapy for more than 1 year, and the most common indications were atrial fibrillation ($n = 180$) and VTE ($n = 83$). Most of the patients were on warfarin ($n = 300$). The patients received standard care provided by the clinic, except that the longest accepted interval between INR measurements was 4 weeks (usually, intervals of 6 weeks are accepted). Information about clinical variables was found in DAWN, the dosage-assisting software used in the clinic. Time in therapeutic range of INR (TTR) was calculated by Rosendaals method.

Results: The mean TTR during the follow-up period (307 patient-years) was 73.9% and seven patients experienced a total of eight clinical events; two thrombotic (one stroke, one transient ischemic attack) and six hemorrhagic, none of which were fatal. There were no differences in TTR or time spent below therapeutic range among the different indications for anticoagulant therapy. Patients heterozygous for the common *VKORC1* rs9934438 polymorphism had a slightly, but significantly reduced TTR of 71.8% ($n = 154$, $P = 0.04$), while the six patients with two variant alleles of *CYP2C9* (*2/*3 or *3/*3) had a substantially reduced TTR of 60.5% ($P = 0.03$). The 34 patients below 50 years had a significantly lower TTR compared with the remaining patients (67.4% vs. 74.7%, $P = 0.01$). The young patients had a higher score on the perceived stress scale at baseline (mean score 15.5 vs. 8, $P < 0.01$) and were more likely to report their general health as 'poor' or 'fair' than the older patients (46% vs. 24%, $P = 0.01$).
Conclusions: Since the number of clinical events was too low for statistical analysis, the surrogate marker TTR was used to assess quality of treatment among different groups of patients. The results did not indicate that patients with VTE were at particular risk of unstable anticoagulation, and only a small minority of patients had a clinically relevant reduction in TTR due to genetic polymorphisms. Interestingly, the youngest patients had both lower TTR and higher levels of perceived stress, and therefore constitute a relevant and easily identifiable target group for increased attention and support from the clinical staff at anticoagulant clinics.

PB1.50 – Blood Coagulation System – I

PB 1.50-1

Association of D-dimer levels with all-cause mortality in a healthy adult population: findings from the MOLI-SANI study

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Background: Elevated D-dimer levels are reportedly associated with higher risk of total mortality in patients with different diseases. We investigated if a similar association could be found in a large apparently healthy population.
Methods: Seventeen thousand three hundred and fifty-nine (47% men, age ≥ 35) individuals free of clinically recognized cardiovascular and cancer disease, for whom baseline D-dimer was available, were studied within the MOLI-SANI cohort, randomly recruited from the general adult population of the Molise region, Italy. The cohort was followed for a median of 4.2 years (73,807 person-years). D-dimer was measured on fresh citrated plasma by an automated latex-enhanced immunoassay (HemosIL-IL, Milan). Hazard ratios (HRs) were calculated using three Cox-proportional hazard models (Table 1).
Results: Two hundred and eighty deaths could be recorded. When modeled as a continuous variable, D-dimer at baseline showed a non-linear association with mortality, whose incidence increased only in the upper quartile of the distribution (D-dimer ≥ 221 ng/mL). Thus, the group of individuals with D-dimer < 221 ng/mL (75% of the population) acted as the reference group, while the remaining individuals were subdivided in tertiles that were compared with the former group. Multivariable HRs for mortality were 1.06, 1.45 and 1.97, respectively (P for trend < 0.0001) across the three categories of increasing D-dimer. The association was slightly attenuated, but still highly significant (P for trend 0.0002), after further adjustment for white blood cell count and C-reactive protein.
Conclusions: Elevated D-dimer levels were independently associated with increased risk of death for any cause in an apparently healthy adult population.

PB 1.50-2

Phosphatidylserine exposure on platelets'surface upon binding to rigid fibrin scaffold

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Background: An adequate surface exposure of phosphatidylserine (PS) on the platelet outer membrane leaflet promotes thrombin formation by providing a catalytic membrane surface for vitamin-K dependent coagulation factors. Difficulties to evoke PS exposure on platelet surface in fluid-phase suggest the requirement of additional stimuli. Recently, by employing intra-vital confocal microscopy, we demonstrated that platelets expose PS and fibrin accumulate only in the center of the thrombus but not in its periphery.
Aim: To address the question how exposure of platelet anionic phospholipids is regulated within the thrombus, an *in-vitro* experiment has been designed.
Methods: Confocal Laser Scanning Microscopy was employed to study the PS exposure on the platelet surface incorporated into fibrin network.
Results: Almost all platelets exposed PS after treatment with tissue factor, thrombin or ionomycin, and platelet PS exposure was strictly dependent on the sustained intracellular calcium concentration $[Ca^{2+}]_i$. Argatroban abrogated fibrin network formation in all samples, however, platelet PS exposure was inhibited only in tissue factor- and thrombin-treated samples but not in ionomycin-treated samples. FK633, an $\alpha_{IIb}\beta_3$ antagonist, and cytochalasin B impaired platelet binding to the fibrin scaffold and significantly reduced PS exposure evoked by thrombin. Gly-Pro-Arg-Pro amide abrogated not only fibrin network formation, but also PS exposure on platelets without suppressing platelet binding to fibrin/fibrinogen.
Conclusions: Our data confirm that PS exposure evoked by TF or thrombin is $[Ca^{2+}]_i$ dependent and precisely regulated by time- and space-dependent mechanisms. Integrin outside-in signals generated by platelet binding to a rigid fibrin network, and resulting in generation of mechanical foci through this binding, appear to be essential for modulation of anionic phospholipids' exposure. Such regulation of platelet PS exposure may play an important physiological role in the control of hemostasis.

PB 1.50-3

The effect of tissue factor pathway inhibitor (TFPI) on thrombin generation and post-operative bleeding in patients undergoing surgery requiring cardiopulmonary bypass

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Background: TFPI is an important inhibitor of the initiation phase of coagulation. Its release is stimulated by heparin. Therefore it may play a role in determining bleeding in patients undergoing surgery requiring the use of cardiopulmonary bypass
Aim: To measure TFPI concentrations before and after cardiopulmonary bypass, correlate these with thrombin generation and investigate the effect of inhibiting TFPI as a potential therapeutic target.
Methods: One hundred and two patients undergoing valve surgery, surgery on the thoracic aorta or undergoing coronary bypass grafting plus another procedure, were recruited. Informed consent was obtained and the study received approval from the local research ethics committee. Blood samples were collected into 3.2% citrate (for

TFPI measurement) and 3.2% citrate with 20 μg/mL Corn Trypsin Inhibitor (for thrombin generation experiments), in the anaesthetic room before heparin was given, and then in the operating theatre 30 min after reversal of heparin. An ELISA was used to quantify full-length and total TFPI using an anti-c-terminus and anti-KD2 antibody respectively. Thrombin generation experiments were performed by calibrated automated thrombography using Thrombinoscope PPP Low trigger reagents. Thrombin generation was measured in the presence and absence of a polyclonal anti-TFPI antibody (AF2974, R&D Systems, Abingdon, UK) at a concentration of 100 nM. Post-operatively patients were observed for evidence of bleeding for 24 h. Abnormal bleeding was defined as drain loss of more than 1 L at 24 h, more than 200 mL/h for two consecutive hours, more than 2 mL/kg/h for two consecutive hours in the first 6 h, the need for haemostatic treatment or the need to return to theatre for bleeding. SPSS software was used to analyse the data.

Results: Compared to pre-cardiopulmonary bypass samples the median full-length TFPI concentration was lower in the post-cardiopulmonary bypass samples (20.4 vs. 16.6 ng/mL, $P < 0.001$). In contrast, median total TFPI levels increased (55.7 vs. 112.4 ng/mL, $P < 0.001$). There was no observed difference in either full-length or total TFPI levels in those who bled excessively compared to those who did not. Thrombin generation was also reduced in the post-cardiopulmonary bypass samples compared to those pre-surgery (median ETP 530.3 nM/min pre vs. 108.5 nM/min post, $P < 0.001$; peak thrombin 42.7 nM pre vs. 5.4 nM post, $P < 0.001$). There was a weak inverse correlation between full-length TFPI concentration and thrombin generation. Addition of AF2974 resulted in a significant increase in thrombin generation parameters in both pre and post-cardiopulmonary bypass samples (ETP 1352.3 nM pre, peak thrombin 245.5 nM pre $P < 0.001$; ETP 1045 nM post, peak thrombin 223.8 nM post, $P < 0.001$) the change being greatest in the post-surgery samples.

Conclusions: Post-cardiopulmonary bypass full-length TFPI is lower than pre-surgery whilst total TFPI is increased. Measurement of TFPI at the time points chosen does not correlate with observed bleeding. Inhibition of TFPI significantly enhances thrombin generation and may be a therapeutic target in patients suffering from excess bleeding.

PB 1.50-4

Safety and effectiveness of anti inhibitor coagulation complex (AICC) in routine clinical management: a post-authorization safety study (PASS)

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Background: Hemophilia patients with inhibitors present a treatment challenge due to an increased risk of difficult-to-treat bleeds. Activated prothrombin complex concentrate, AICC [FEIBA NF], has been a key therapeutic option for the prevention and management of bleedings in patients with inhibitors for over three decades. FEIBA NF has been approved in more than 60 countries with a prophylaxis indication in more than 40 countries. Launched in 2008, FEIBA NF is essentially the same as the previous formulation of FEIBA (VH) but with an additional viral removal step (35 nm nano-filtration) added during the final stages of manufacturing.

Aims: FEIBA NF PASS is a prospective open-label, observational surveillance program that documents the adverse events (AEs) and hemostatic effectiveness associated with FEIBA NF use in routine practice. Data collection includes dosage of FEIBA NF (total dose, number of infusions) required for prophylaxis or bleed resolution, infusion rates, physician rated efficacy assessment, bleed type and location, incidence of non-serious and serious adverse events related and unrelated to FEIBA NF, and anamnestic response.

Methods: An electronic data collection system is utilized to monitor the safety and effectiveness of FEIBA NF in subjects for 12 ± 2 months. Standard quality of life assessment tools (SF-36, Peds-QL and EQ-5D) are used at time of enrollment and at the end of the observation period. Furthermore, the surveillance will attempt to identify best practices in managing hemophiliacs with inhibitors on regular FEIBA prophylaxis. All patients gave informed consent and the study was conducted according to the Declaration of Helsinki and its amendments.

Results: As of January 2013, 44 study sites in 10 countries have been initiated and eighty five patients have been enrolled. Sixty two patients have completed a 1 year study period. Enrollment has closed in the UK, France, Germany, Spain, Belgium, Sweden, Poland, Italy, and the United States but is still ongoing in Canada. At enrollment, 86% of patients have been diagnosed with congenital hemophilia A, 13% with acquired hemophilia A and 1% with congenital hemophilia B. At screening, 41 subjects were prescribed regular prophylaxis and 39 were prescribed on-demand treatment for bleeding episodes. Subject profiles highlighting bleeding episodes and treatments over the study period were created for every patient and representative graphs were generated. Three related serious adverse events (haemarthrosis, catheter related infection, and thrombophlebitis superficial) have been reported, all associated with on demand treatment.

Summary: The development of inhibitors is the most serious complications in the treatment of hemophilia and their management continues to challenge health care professionals. FEIBA NF PASS provides an opportunity to collect data on hemophilic patients with inhibitors during routine care, and serves as an invaluable tool for documenting the safety and effectiveness of FEIBA NF in a variety of clinical settings including prophylaxis, surgery and bleed management during ITI.

PB 1.50-5

Thrombin generation profile of dehydrated solvent/detergent treated plasma and FFP

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Background: The risk of infection during transfusion of plasma was significantly reduced with introduction of solvent/detergent (S/D) treatment. Also the S/D plasma delivers reduced antibody titers, reduced lipids and microparticles, reduced non-hemolytic transfusion reactions and more consistent content of clotting factors as compared to standard single donor FFP. S/D plasma, like banked FFP, remains dependent on frozen shipment and storage. Dehydrated plasma on the other hand, has been shown to possess stability at higher temperatures, and quicker availability due to elimination of the thawing step. Dehydrated plasma has been shown to function *in vitro* and *in vivo* in a similar manner to banked frozen FFP. Dehydration methods currently in development are lyophilization, and spray drying. However, there have been no reports in the literature of comparisons of these.

Methods: Effect on thrombin generation and inhibitions.

Aims: To compare the effect of dehydration methods on thrombin generation coagulation factor recovery and hemostatic function of S/D plasma as compared to banked FFP.

Methods: For the current comparison, spray dry and lyophilized plasmas were prepared in-house using S/D plasma from Kedrion (Lucca, Italy) or blood bank FFP (UNC Hospital, NC). All dry plasmas were reconstituted with 5 mM Citric/Phosphate pH 3.5 buffer, producing fully rehydrated plasma of the same pH and protein concentration as the original plasma before drying. We used calibrated automated thrombography (CAT) to evaluate hemostatic function of plasma. Using Thrombinoscope (Stago), we measured thrombin generation in S/D plasma or FFP and in their reconstituted spray-dried and lyophilized forms. In addition, we measured concentration of major clotting factors using STA-Compact Analyzer (Stago) and assessed plasma coagulation function with rotational thrombelastography (ROTEM, TEM Systems, NC).

Results: We found that the lyophilization of S/D plasma generated small but significant changes in thrombin generation profile but that spray-drying had a negligible effect: at 1pM TF, the ETP = 1354 nM*min for SDSDP vs. 1301 for S/D plasma and 1430 for lyophilized S/D, at 5pM TF the ETP = 1258 for SDSDP vs. 1286 for S/D plasma and 1372 for lyophilized S/D. The results with FFP products were more variable with ETPs 10–30% less than their S/D counterparts. Despite faster thrombin generation in S/D plasma and its derivatives, thrombin inhibition was equally fast, suggesting of intact thrombin inhibition pathways. ROTEM did not detect these same differences. There were variations in clotting factor levels between spray-dried and lyophilized preparations but not consistently across the S/D plasma vs. FFP groups, except for a reduced level of vWF activity in spray-dried plasmas.
Summary: In general, the thrombin generation profile of SDSDP was remarkably similar to the S/D plasma from which it was prepared. Both S/D plasma and SDSDP have shorter thrombin generation times and more rapid thrombin inhibition than FFP or its dried forms. Additional investigation is needed to understand the significance of the differences between lyophilized and spray-dried techniques on hemostatic function.

PB 1.50-6

Comparision of recombinant coagulation factor VII (ARYOSEVEN®) with NOVOSEVEN® in patients with FVIII & IX deficiency with an inhibitor

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Introduction: Coagulation factor replacement therapy in severe hemophilia prevents disabling arthropathy and improves life expectancy and quality of life. This treatment is ineffective in patients with neutralizing antibodies to the deficient coagulation factor (inhibitors), in which case treatment with FVIIa is an option. The aim of this study was to compare the efficacy of a new biosimilar recombinant factor VIIa (Aryoseven®) with Novoseven® in controlling bleeding episodes in patients with hemophilia A or B with inhibitors.
Methods: A double blind clinical trial was conducted in comprehensive hemophilia care centers in Iran. Inclusion criteria were: 1-congenital hemophilia A and B and an inhibitor titer > 5 Bethesda units (BU); 2-age > 2 years; 3- intravenous access. Exclusion criteria were: 1- any other coagulation disease; 2- immune tolerance induction treatment during the last month with Novoseven®; 3- platelet count < 50,000/mL; 4-presence of anti-FVII antibodies; 5. history of advanced arthrosclerosis due thormbogenic of rFVIIa. We randomized 61 consenting male patients into two groups, with four consecutive block randomization, for treatment of one hemorrhage. Group A (28 patients, 45.9%) received Aryoseven® and group B (33 patients, 54.1%) received Novoseven®. Factor VII dosage was 90 μg/kg intravenously. Primary outcome was self-reported joint pain and joint movement with the Kavakli scoring system 9 h after infusion of FVIIa
Results: Mean age was 21 year (range: 2–53) and similar in both groups. Mean plasma level of Factor VII: C at inclusion was 104.3 (range 78–106) IU/dL in group A and 98.5 (range 76.2–119) IU/dL in group B. Mean inhibitor level was 36.9 BU (SD 45.7) in group A and 28.9 (SD 35.7) BU in group B. Time from self-reported start of bleeding symptoms to FVIIa infusion was similar in both groups (group A:1377 ± 1082, group B:2333 ± 1795 min). There were 28 bleeds treated with Aryoseven® and 33 bleeds with Novoseven®. Median Kavakli score for post-infusion pain and joint movement was similar in the two groups, at 7.00 (range 6.00–8.00). Response to treatment was positive in 27 (96.4%) in group A, and 29 (87.9%) in group B by self-reporting joint pain severity.
Discussion: We tested a biosimilar FVIIa concentrate in comparison with the standard concentrate in patients with neutralizing antibodies to FVIII or FIX, in the treatment of acute joint bleeding. The response to concentrate infusion was the same for both concentrates.

PB 1.51-1

Control of vitamin-K antagonist treatment by measuring thrombin generation in whole blood – effect of thrombomodulin

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Background: Whole blood thrombin generation (TG) testing has been recently introduced. If it can be used for evaluating the effect of antithrombotic treatment this would be a starting point for the development of point of care and home control of vitamin K antagonist (VKA) treatment. The antithrombotic action of VKA treatment results from the fact that its effect on factors II, VII, IX and X overrules the concomitant decreases of the natural anticoagulants protein C and S. In clotting tests the effect on anticoagulant factors goes unobserved, but not in TG experiments. We therefore also investigated the effect of addition of soluble thrombomodulin (TM).
Aims: To compare, in samples from patients taking VKA, TG in whole blood to TG in plasma and to investigate the effect of activation of the APC system with TM in both types of test and to relate the results to the degree of anticoagulation as assessed by the International Normalized Ratio (INR).
Methods: In blood samples from 105 consenting patients on VKA treatment (mainly because of atrial fibrillation) TG was measured with calibrated automated thrombinography (CAT) in the absence and presence of exogenous TM. This was performed in whole blood, platelet rich and platelet poor plasma (PRP, PPP). The INR was determined in PPP of the same patients.
Results: Endogenous thrombin potential (ETP), peak height, lag time and time to peak (ttpeak) measured in whole blood were all significantly correlated with the ones determined in plasma (P-value < 0.01). The concentration dependent parameters of TG (ETP and peak) from whole blood, PRP and PPP of the patients correlated significantly to the inverse of the INR (P-value < 0.01). The time dependent TG parameters (lag and ttpeak) correlated linearly with the INR (P-value < 0.01).
In the majority of the patients, addition of TM (20 nM) to a level that reduced the ETP to around 50% in normal plasma, caused less inhibition in plasma from the patients: 29.8 ± 12.8% in whole blood, 28.5 ± 19.8% in PRP) and 39.6 ± 12.0% in PPP. This effect was virtually independent of the INR (1.1–5.9).
Conclusion: This study demonstrates that whole blood thrombin generation can be considered as a reliable tool for determining the anticoagulant activity of vitamin K antagonists. VKA therapy induces TM - resistance when assessed by TG in whole blood as well as in plasma. This effect appears to be independent of the level of anticoagulation.

PB 1.51-2

False-positive results for a lupus anticoagulants occur in patients on low molecular weight heparin but not fondaparinux, by dilute activated partial thromboplastin time

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Background: Recent International Society for Thrombosis and Haemostasis guidelines on the detection of lupus anticoagulants (LA) state that screening for LA in the presence of low molecular weight heparin (LMWH) is possible, and that the thrombin time (TT) should be used to identify the presence of heparin. We describe how reliance on the TT may lead to the mis-diagnosis of LA by dilute activated partial thromboplastin time (APTT) in patients on LMWH (prophylactic/therapuetic), and how the diagnosis of LA is not affected by fondaparinux.
Aims: To show that the dilute APTT cannot be used to detect LA in patients on LMWH; that TT cannot be used to rule out the presence of LMWH; and that fondaparinux does not affect LA results.
Methods: Plasma samples sent for routine LA testing from non-anticoagulated patients previously found to be negative by dilute Russell's viper venom time and dilute APTT were spiked with either enoxaparin, tinzaparin or fondaparinux. All samples were tested for LA by dilute APTT and TT.
Results: Twenty-five samples were spiked with enoxaparin. At 0.20 IU/mL, 9 (36%) of these gave results above the locally-derived cut-off for the dilute APTT assay, and of these 2 (8%) showed correction sufficient to make a false diagnosis of LA. At 0.80 IU/mL, all results were above the assay cut-off, with 5 (20%) giving a false positive diagnosis of LA.
Sixteen samples were spiked with tinzaparin. At 0.20 IU/mL, 11 (69%) of these gave results above the locally-derived cut-off for the assay, and of these 9 (56%) showed correction sufficient to make a false diagnosis of LA. At 0.80 IU/mL, all results were above the assay cut-off, with 3 (19%) giving a false positive diagnosis of LA.
Twenty samples were spiked with fondaparinux. At 0.20 μg/mL, 3 (15%) of these gave results above the locally-derived cut-off for the assay, but none of these showed correction sufficient to make a false diagnosis of LA. At 0.80 μg/mL, 5 (25%) gave results above the assay cut-off, but none of these showed correction sufficient to make a false diagnosis of LA.
When measuring TT on samples spiked with enoxaparin or tinzaparin, TT was raised only when anti-Xa activity exceeded 0.25 IU/mL. This is above the level of LMWH observed to affect LA assays by dilute APTT. TT measurements were not affected by fondaparinux.
Conclusions: The results of this study suggest that LA cannot be measured accurately in the presence of LMWH, and that prophylactic levels of LMWH will not affect the thrombin time. Fondaparinux did not interefere with the detection of LA by dilute APTT in our study.

PB 1.51-3

Monitoring of parenteral anticoagulant drugs with the prothrombinase induced clotting time (PICT)

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Introduction: Several parenteral direct thrombin inhibitors have been approved for the anticoagulation in heparin compromised patients. These agents include hirudin (Refludan, BayerHealthcare, Germany), argatroban (Acova, Mitsubishi, Japan) and angiomax (Bivalirudin, Medicines, Co, Parsippany, NJ). The parenteral thrombin inhibitors do not require antithrombin as a cofactor and have a short half life. They are used in the anticoagulation of patients undergoing percutaneous coronary angioplasty (PTCA). These agents do not require monitoring however, in some patients such as those with liver or kidney disease, monitoring may be necessary.
Materials: Citrated blood was drawn from five donors and spun at 3000 rpm to obtain platelet poor plasma (PPP). The PPP was supplemented with hirudin, argatroban or angiomax in a concentration range of 0–10 μg/mL. The plasma samples were analyzed using three PT/INR reagents (Innovin, Dade-Behring, Germany; Recombiplastin, Instrumentation Laboratories, Bedford, MA; Neoplastin, Stago, Parsippany, NJ), two APTT reagents (Platelin, TCoag, Ireland; Actin FSL, Instrumentation Laboratories, Bedford, MA), Heptest and the two stage PICT (Pentapharm, Basal, Switzerland). The PICT assay is a new clot-based assay that was developed for the monitoring of anticoagulant drugs. In the two step assay, FXa, phospholipid and RVV-V are incubated with the sample. The formed thrombin is inhibited by the presence of the thrombin inhibitor. Following recalcification the clotting time is measured. All assays were performed on the ACL 300 Plus (Instrumentation Laboratories, Bedford, MA). The PICT was also run on the ST4 (Stago, Parsippany, NJ).
Results: In the citrated plasma the hirudin, angiomax and argatroban demonstrated assay dependent differences in the clotting times. In the PT/INR, angiomax showed the strongest response with Innovin, however using Neoplastin or Recombiplastin both angiomax and argatroban gave similar results. Hirudin showed the weakest activity. In the APTT assay, angiomax was strongest, but similar results were observed for both hirudin and argatroban. In the Heptest, argatroban was the strongest. In the two stage PICT assays all three agents demonstrated a concentrated dependent response. Hirudin showed the strongest prolongation of the PICT at 5 μg/mL followed by angiomax and argatroban (Hirudin, 190.7 + 17.8 s; Angiomax, 131.6 + 15.3 s; Argatroban, 95.3 + 9.7 s),.
Conclusion: These results demonstrated the different reagents, within the same assay, show differential responses to the parenteral antithrombin agents. Therefore depending on the type of reagent used different response can be measured using the same test. The two stage PICT is a simple, fast assay that can be adapted to any coagulation instrument and is sensitive to all types of anticoagulants including the parenteral antithrombin agents. Therefore the PICT assay may be a universal clot-based method that can be used to monitor all types of anticoagulant drugs.

PB 1.51-4

Evaluation of in-house normal pool plasma for activated partial thromboplastin time mixing test as a part of diagnosting protocol for lupus anticoagulant detection

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Background: Mixing test is commonly used as screening assay for laboratory investigation of inhibitors, thus respresenting an integral part of routine diagnostic protocol for lupus anticoagulant (LA) detection. The first criterion in identifying LA is prolongation of at least one laboratory test dependent of phospholipids, such as the activated partial thromboplastin time (APTT). This prolonged test must then be repeated on a mixture of patient¢s plasma and normal plasma (APTT mixing test, APTTmt) to distinguish between inhibitor such as LA and a single factor deficiency.
Aims: The aim of this study was to evaluate in-house pool normal plasma (NP) for APTTmt compared with commercial NP that is routinely used in our laboratory in the diagnosting protocol for LA detection.
Methods: A total of 158 subjects were evaluated for LA, of which 11 subjects were LA positive. In-house pooled NP was obtained from 40 subjects directed to routine screening for prothrombin time (PT) and APTT, without any known coagulation disorder and with PT and APTT results within reference interval. Immediately after collection

and centrifugation (3000 rpm 10 min), in-house pooled NP was divided into aliquots and frozen at −20 °C until analysis. Immediately before use, aliquoted in-house NP was thawed at 37 °C for 15 min. Commercial NP (Control plasma N, Siemens, Germany) was routinely used for APTTmt (Actin FSL, Siemens, Germany). Patient¢s samples were mixed in the equal volume (1 + 1) with commercial NP as well as with in-house pool NP and evaluated in parallel for APTTmt. For in-house prepared pool NP we determined within-run precision by 10 consecutive measurements of APTTmt and between-run precision by measuring of APTT in 10 consecutive days after freezing and thawing of aliquoted in-house NP. Comparison of APTTmt results with commercial NP and in-house NP was evaluated by Passing-Bablok regression and Bland and Altman difference plot.

Results: Within-run coefficient of variation (CV) was 1.5% for APTTmt determined with in-house pool NP. Between run CV was 2.4% for APTT determined in aliquoted pooled NP. The results of APTTmt (seconds) comparison expressed as median (range) were as follows: in-house NP 27.0 (22.0–32.0); commercial NP 27.0 (23.0–33.0). Passing and Bablok regression analysis showed that there were no significant constant or proportional differences between the two methods regression line equation y = 0.000 + 1.000x; 95% confidence interval (CI) for intercept 0.000–5.200 and 95%CI for slope 0.800–1.000, thus indicating good agreement, linear relationship and statistically unbiased results between in-house and commercial NP used for APTTmt. These results were also confirmed by Bland and Altman difference plot. Commercial NP and in-house NP gave the same number of positive screening LA results (11/11) and positives were detected in the same specimens.

Conclusions: Comparison between in-house pool NP and commercial NP for APTTmt indicated that a careful selection of donors allows use of in-house pooled NP that fits the quality demands for routine APTTmt. Our results confirmed that use of in-house prepared NP could be implemented in routine diagnostic protocol for LA detection.

PB 1.51-5

Prothrombin time with recombiplastin 2G correlates strongly with plasma rivaroxaban levels

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Background: The new oral anticoagulants are now being increasingly used in clinical practice. Although routine monitoring of their anticoagulant activity or plasma level is not indicated there is a realisation that, in certain situations (e.g. acute haemorrhage or emergency surgery), the ability to estimate residual anticoagulant effect may be clinically useful. Therefore, it has been suggested that haemostasis laboratories develop suitable quantitative assays as well as determining the sensitivity of their routine coagulation assays, prothrombin time (PT) and activated partial thromboplastin time (APTT), to these agents.

Aims: To calibrate an anti-Xa chromogenic assay for rivaroxaban plasma concentration, and assess the sensitivity of our local PT and APTT assays to rivaroxaban levels.

Methods: The Liquid Anti-Xa chromogenic assay (IL, Instrumentation Laboratories) run on an IL TOP 700 was calibrated using lyophylised standard rivaroxaban plasmas (Hyphen Biomed, France) to create a rivaroxaban assay linear between 0 and 400 ng/mL. Lyophylised plasmas from Diagnostica Stago (France) were used for internal quality control. Citrated plasma samples were obtained at routine clinic visits from patients receiving therapeutic dose rivaroxaban for treatment, or secondary prevention of DVT. All patients had been receiving rivaroxaban, and had avoided other anticoagulant therapy (low molecular weight heparin or warfarin), for at least 7 days (median 3 weeks). PT (Recombiplastin 2G, laboratory reference range 9–13 s) and APTT (Synthasil, laboratory reference range 27–38 s) were determined on IL TOP 700.

Results: Samples from 20 patients receiving rivaroxaban (six during 15 mg bd dosing and 14 during 20 mg od dosing) were obtained either around peak (1–4 h, $n = 10$) or trough (12–30 h, $n = 10$) time points. Anti-Xa rivaroxaban levels around peak were generally higher than expected (median 378 ng/mL, range 204–707 ng/mL) while trough levels were as expected (median 45 ng/mL, range 11–215 ng/mL). One patient with a 2.5 h 'peak' level of only 35 ng/mL was suspected of medication non-compliance. Corresponding PT results (range 10–29 s) showed a strong linear correlation with anti-Xa rivaroxaban levels (correlation co-efficient 0.971) whereas APTT results (range 30–52 s) were less well correlated (correlation co-efficient 0.461). All eight patients with PT ≤ 13 s had an anti-Xa rivaroxaban level < 60 ng/mL.

Conclusions: We have demonstrated that the IL Liquid Anti-Xa chromogenic assay can be easily calibrated to measure plasma rivaroxaban levels. Routine PT (using Recombiplastin 2G on an IL TOP 700), more so than APTT, may provide an approximate estimate of rivaroxaban anticoagulant activity. A PT ≤ 13 s appears to imply a low rivaroxaban level of < 60 ng/mL, perhaps inferring suitability for surgery or other invasive procedure. However, these findings will require validation in a larger cohort (including rivaroxaban-treated patients scheduled for invasive procedures).

PB 1.51-6

A global hemostasis assays in laboratory monitoring of low molecular weight heparin treatment in patients after surgery

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Background: Low molecular weight heparin (LMWH) treatment can be complicated by such adverse events like bleedings or thrombosis, so the laboratory control of the LMWH effect can be useful.

Aims: To investigate the efficacy of the global hemostasis assays to monitor the LMWH treatment in patients after surgery.

Methods: Twenty one patients after surgery were enrolled in the study. Thrombosis risk was scored by Caprini risk assessment model: two patients had low risk (but coagulation tests showed hypercoagulation state direct after surgery), six patients had medium risk and 13 patients had high risk. Five patients also had thrombocytopenia ($< 150 \times 10^9$ platelets/mL). The thromboelastography (TEG), Thrombodynamics, D-dimers and anti-Xa activity were determined before LMWH treatment (daily bemiparin injection 2500 or 3500 IU), then before and 3 h after each injection during 3 days. Thrombodynamics is a new global assay based on a spatial fibrin clot growth registration; the stationary clot growth rate (Vst) and spontaneous clotting (SC, indicator of prothrombotic risk) are registered.

Results: These global tests showed procoagulant deviation from normal state in patients with high thrombosis risk. Before LMWH injection, TEG parameters R, K, Angle and MA demonstrated the hypercoagulation state in 38% of measurements, but the average values were normal: 14.2 ± 5.5 min, 4.6 ± 2.8 min, 46.6 ± 14.5° and 64.2 ± 10.9 mm, respectively (the normal ranges were 9–27 min, 2–9 min, 22–58° and 44–64 mm, respectively). The Thrombodynamics parameter Vst was increased in 70% of measurements and the average value was 33 ± 6 μm/min (the normal range was 20–30 μm/min). SC was observed in eight patients. D-dimers were increased in 85% of measurements.

After LMWH injection, all parameters of global assays except MA returned back to normal or even mild hypocoagulation state. R and K increased in 1.6 ± 0.8 and 2.7 ± 1.8 times respectively, Angle and Vst decreased in 2.0 ± 1.1 and 2.0 ± 0.5 times respectively. No SC in Thrombodynamisc was observed. Anti-Xa activity was 0.88 ± 0.39;

yet it is known that this parameter indicates only LMWH activity in blood plasma but not overall hemostasis state of patients.
All parameters of laboratory tests performed before LMWH injection did not show any dynamics within the first 3 days after the surgery; the same picture was observed for the tests performed after LMWH injection. The one exception was observed: SC in Thrombodynamics registered in six patients on 1st day, three patients on 2nd day and two patients on 3rd day. There was no difference between patients with or without thrombocytopenia ($P > 0.05$).
Conclusion: Thrombodynamics and thromboelastography assays are sensitive to hypercoagulation in patients with high thrombosis risk and can be effective in monitoring LMWH treatment.

PB1.52 – Blood Coagulation Tests – III

PB 1.52-1

Comparison of calibrated automated thrombogram and chronometric or chromogenic assays for the monitoring of DOACs in patients with non-valvular atrial fibrillation

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Background: Direct oral anticoagulants (DOACs) include anti-IIa agent (dabigatran etexilate: DE) and anti-Xa agents (rivaroxaban, apixaban and edoxaban). DOACs do not require monitoring nor frequent dose adjustment. However, searching for the optimal dose in the individual patient may be useful in some situations. Recent studies have shown that activated partial thromboplastin time (aPTT), Hemoclot Thrombin Inhibitor® (HTI) and ecarin clotting time could be used to monitor dabigatran whereas PT and anti-Xa chromogenic assays are preferable to monitor anti-Xa agents. HTI, aPTT and PT only measure the initiation phase of the coagulation cascade. Calibrated Automated Thrombogram (CAT) which measures the entire thrombin generation process could be used to better discriminate the inhibitory profile of DOACs in patients.
Aims: The aim of this study is to assess the impact of DE and rivaroxaban in patients suffering from non-valvular atrial fibrillation by thrombin generation assay. Results will be compared to other traditional chronometric and chromogenic assays.
Methods: Five patients under DE (150 mg *bid*) and five patients under rivaroxaban (20 mg *od*) for atrial fibrillation were included in this study. Blood samples were taken at 2 and 3 h after drug administration, and just before the next scheduled intake of the drug (C_{trough}: 12 h for DE and 24 h for rivaroxaban). The following tests were performed at each time-point.
Calibrated Automated Thrombogram: The most sensitive reagents were used based on results from previous in house *in vitro* studies. PPP-Reagent and PPP-Reagent High were used for rivaroxaban (Peak and mVRI). For DE, PPP-Reagent Low and PPP-reagent were used (Lag Time and T_{max}).
Chronometric and chromogenic assays: For rivaroxaban, PT was assessed using Triniclot PT Excel S® and Innovin®. Biophen Direct FXa Inhibitors was used to estimate the plasma drug concentrations,
For DE, aPTT was assessed using CK-Prest® and Synthasil®. The HTI was also performed to estimate the plasma drug concentrations.
Results and Discussions: The correlation between rivaroxaban plasma concentration and the Peak showed an exponential decay relation ($r^2 = 0.98$ and 0.94 for PPP-Reagent and PPP-Reagent high respectively). The same relation was observed for the mVRI ($r^2 = 0.98$ and 0.95). The correlation between PT and CAT parameters is less pronounced than those with the estimated plasma concentration ($r^2 < 0.90$).
The correlation between dabigatran plasma concentration and the Lag Time showed no specific relation (r^2 for linear correlation = 0.45 and 0.25 for PPP-Reagent and PPP-Reagent Low, respectively). The same dispersion in the results is observed with other parameters. There is no good correlation between CAT parameters and the aPTT ($r^2 < 0.35$).
Conclusion: In this pilot study, CAT results obtained in patients treated with DE are inconclusive. On the other hand, the good correlation results obtained between CAT parameters (Peak and mVRI with PPP-Reagent) and plasma drug concentrations in patients treated with rivaroxaban is encouraging. Such data should be confirmed in a larger cohort study with the aim of providing cut-offs CAT parameters reflecting a therapeutic level of anticoagulation.

PB 1.52-2

Minimizing the impact of preanalytical variables on intrinsic coagulation during blood collection improves sample quality for thromboelastography and the calibrated automated thrombogram

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Background: Thrombelastography (TEG) is useful in testing coagulation efficiency of whole blood (WB) and has found important applications during surgery and anesthesiology. The calibrated automated thrombogram (CAT) assay performed in plasma is used to investigate patients with hypo- or hypercoagulopathies. These assays are highly sensitive relative to traditional coagulation tests and vulnerable to contact activation where accumulated FXIIa in citrated specimens can markedly augment down-stream thrombin generation (TG). Accordingly, we examined the effect of blood collection tubes comprised of different polymeric containment materials and the select use of targeted intrinsic pathway inhibitors on select outputs of the TEG and CAT assays.
Methods: Citrated human WB was transferred from a blood collection bag into coated (siliconized) glass or plastic blood collection tubes, or uncoated glass, polypropylene (PP), polystyrene (PS), or polyethylene terephthalate (PET) conical bottom tubes, either alone or in the presence of inhibitors targeting kallikrien or FXIa. After 15 min incubation, the TEG *R* value was obtained immediately after addition of 10 mM $CaCl_2$. Matched plasma specimens were analyzed by the CAT in the presence and absence of 1pM Tissue Factor (TF) and by APTT (Stago Compact). Data were analyzed by ANOVA with Tukey's post-test and by linear regression.
Results: Plastic blood collection tubes delivered significantly higher WB clotting 'R' times (CT) (15.0 ± 1.02 min) than either uncoated glass (6.3 ± 0.73) or coated glass tubes (9.9 ± 0.58) $P < 0.01$ while providing equivalent results to all other plastic containers which ranged from 15 ± 1.0 to 17.7 ± 1.9 min, $P > 0.05$ APTT assays were insensitive to differences between uncoated glass (29.5 ± 1.6 s) and PP (29.8 ± 0.6 s) tubes. Moreover, CAT peak thrombin levels were significantly lower in plastic collection tubes relative to coated glass both in the absence (22.2 ± 4.4 vs. 167.7 ± 2 nM, $P < 0.05$) and presence (16.7 ± 3.4 vs. 127.3 ± 9 nM ($P < 0.05$) of TF. TEG WB CT correlated well with TF-initiated CAT lag time ($R^2 = 0.8116$), time to peak TG ($R^2 = 0.8308$) and peak TG, the latter in the absence of intrinsic inhibitors ($R^2 = 0.8401$). Targeted inhibition of kallikrien also increased WB CT in uncoated glass samples from 4.9 ± 0.30 to 27.5 ± 8.30, which was significantly higher than both coated glass tubes (9.4 ± 0.40) or plastic tubes (14.3 ± 2.60) in the absence of inhibitor ($P < 0.001$). Similarly, targeted inhibition of FXIa increased WB TEG CT in coated glass and plastic tubes above 18 min and abrogated TG in the absence of TF.
Conclusions: Plastic blood collection tubes offered advantages over coated glass for the CAT and TEG while the APTT was insensitive to these polymeric differences. Inhibition of kallikrein, even in uncoated glass, elevated WB CT beyond that of plastic suggesting additional

benefits of contact pathway inhibition beyond those polymer-mediated. Inhibiting FXIa abolished TG in the absence of TF, suggesting quantitative intrinsic pathway blockade may require inhibition downstream of FXIIa.

PB 1.52-3

Spatial fibrin clot growth dynamics: interlaboratory evaluation of the assay reproducibility and standardization

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Background: A new global clinical test of blood coagulation, Thrombodynamics, based on the *in vitro* spatial clot growth imaging has recently become available. Thrombodynamics mimics the *in vivo* blood plasma coagulation processes occurring during the vessel damage.
Aims: To develop and test a stable and user-friendly assay protocol, consumables and controls.
Method: The standard operating procedure of the assay includes putting the recalcified platelet free plasma sample into a special plastic cuvette and contacting it with the activator insert containing surface-immobilized tissue factor. Plasma clotting process propagates from the activation surface into the bulk of plasma. Fibrin is detected by light scattering videomicroscopy and numerical parameters characterizing clot formation are calculated with a specially designed software[1].
Results: A new kit including lyophilized reagents with long term shelf life was developed. Aliquots of reagents allowed quick, comfortable and unified test performing. Besides, lyophilized form removed additional dilution of the sample and precisely dosed amount of reagents. Specially developed lyophilized plasma control allowed to verify all test measurements. To study the test reproducibility using the new kit, there were four analytical series of measurements performed, each one with a new lot of reagents (not less than $n_1 = 400$ tests each, $n_2 = 3226$ tests total). Each analytical series was performed by different operators (3÷5) at four different laboratories using four pools of fresh blood plasma (from at least five healthy donors each one), respectively. The coefficient of variation measured for the spatial velocity of fibrin clot growth did not exceed 6% in each analytical series and was equal 5% between analytical series, respectively. For the test verification research using plasma control ($n_3 = 572$ tests total), the coefficient of variation measured for the parameters of spatial fibrin clot growth did not exceed 10%.
Conclusion: The obtained data confirm sufficient reproducibility of Thrombodynamics both within and between the reagent lots, and both within and between laboratories. The developed reagent kit and control plasma meet the laboratory requirements of usability and standardization.
Reference:
1. Thromb Haemost 2005;3:321–31; J Thromb Haemost 2011;9:1825–34.

PB 1.52-4

Assessment of rotational thromboelastometry in cardiac surgery: correct prediction of clinically relevant thrombocytopenia and hypofibrinogenaemia after 5 min

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Background: Rotational thromboelastometry (ROTEM) has proven to be an important diagnostic tool in cardiac surgery partiallydue to its short turn around time (TAT) compared to conventional laboratory tests. Multiple guidelines still recommend the conventional laboratory measurements in situations of major blood loss but appoint that thromboelastometry might be an important diagnostic tool to assist in characterizing the coagulopathy and in guiding haemostatic therapy and therefore need to be studied further.
Aims: In the present study we have prospectively investigated whether ROTEM could predict a clinically relevant thrombocytopenia and hypofibrinogenaemia in cardiac surgery using the clot amplitude after 5 min (A5) compared with the established A10 and maximal clot formation (MCF). A new parameter, PLTEM, in which the contribution of fibrinogen is eliminated by subtracting FIBTEM from EXTEM, was investigated. TAT for ROTEM tests and conventional laboratory tests were determined in the central laboratory.
Methods: In a prospective study including 97 cardiac surgical patients, the correlation between EXTEM/FIBTEM A5 and A10/MCF values, EXTEM A5/A10/MCF and platelet count, and FIBTEM A5/A10/MCF and fibrinogen was evaluated using the Pearson's correlation coefficient. Moreover, predictive values of ROTEM tests for clinically relevant thrombocytopenia and hypofibrinogenaemia were evaluated using receiver operating characteristic (ROC) curves and corresponding areas under the curve (AUCs). The study was approved by the medical ethical committee of the Maastricht University Medical Center (MUMC+).
Results: EXTEM A5 and FIBTEM A5 showed an excellent linear correlation with A10 (R:0.99, 0.99) and MCF values (R:0.97, 0.99). The correlation between EXTEM A5 and platelet count (R:0.75) was comparable with A10 (R:0.74) and MCF (R:0.70), and FIBTEM A5 predicted fibrinogen levels (R:0.88) as well as A10 (R:0.88) and MCF (R:0.88). PLTEM A5/A10 (R:0.86/0.85) correlated better with platelet count than EXTEM A5/A10 (R:0.75/0.74) and showed significantly better AUC values in ROC-curves than EXTEM for predicting clinically relevant thrombocytopenia (A5 $P = 0.025$; A10 $P = 0.019$). Turnaround time of emergency requests for platelet count and fibrinogen level were 13 and 26 min respectively and TAT for ROTEM tests was approximately 12 min.
Conclusion: ROTEM is able to detect hypofibrinogenaemia and thrombocytopenia during cardiac surgery using A5, instead of the frequently used A10. PLTEM predicts platelet count and thrombocytopenia more accurately than EXTEM. Implementation of these parameters in ROTEM guided-transfusion protocols might reduce the use of blood products. Further studies are needed to evaluate whether implementation of EXTEM A5, FIBTEM A5 and PLTEM A5 in ROTEM-guided transfusion protocols will result in improved transfusion management.

PB 1.52-5

Calibrated automated thrombography in identification of patients with high bleeding risk on vitamin K antagonists treatment

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It is suggested that an individual's potential to generate thrombin better correlate with patients phenotype compared to traditional coagulation tests and so it may allow individualized treatment with vitamin K antagonists (VKA), particularly in patients with thrombophilia. VKA treatment affects the protein C system as well as the procoagulant system and this aspect can be assessed if thrombin generation (TG) is evaluated in the presence of thrombomodulin (TM). Aim of our study was to evaluate relationship between TG parameters both in the presence and in the absence of thrombomodulin (TM) and international normalized ratio (INR) – standardized method of wide clinical utility. Methods. The study involved 28 controls and 48 patients with venous thromboembolism (VTE) in the extended-treatment phase (M/F 24/24, mean age 54.6 ± 15.8 years). Duration of VKA was of 1.5–4 years, and INR range of 0.9–3.3. Thrombin generation in platelet poor plasma was measured using the Calibrated Automated Thrombogram (CAT), developed by Hemker et al. The concentrations of tissue factor and phospholipids in the test system were 4 pm 5 μm respectively. The procedure was carried out with an automated fluorometer (Fluoroscan Ascent, Thermolab system, Finland). Thrombin generation curves were calculated using the Thrombinoscope software (Thrombinoscope BV, The Netherlands). Four parameters were derived from the thrombin generation curves: Lag time (min), endogenous thrombin potential ETP (nMmin), peak height for thrombin (nM), time to peak (min). STATISTICA 6.1 was used, data are given as mean ± SD. Results. Lag time, ETP, peak height for thrombin, time to peak obtained with and without TM demonstrated strong positive correlation (R between 0.93 and 0.98, $P < 0.05$). Both in the absence and in the presence of TM strong inverse correlation with INR was found for ETP ($R = -0.85$ and $R = -0.79$, respectively, $P < 0.05$) and peak height ($R = -0.84$ and $R = -0.79$, respectively, $P < 0.05$). Lag times and times to peak showed a positive correlation with INR both in the absence of TM ($R = 0.61$ and $R = 0.57$, respectively, $P < 0.05$) and in the presence of TM ($R = 0.60$ and $R = 0.59$, respectively, $P < 0.05$). ETP and peak height for thrombin measured in the absence of TM were higher than those measured in the presence of TM. In patients ($n = 15$) with INR 2-3, which is considered optimal, ETP was 358 ± 210 nMmin in the absence of TM and 293 ± 167 nMmin in the presence of TM. Importantly in one patient with INR 2.8 having no bleedings at the time of observation ETP and peak thrombin either in the absence or presence of TM were markedly decreased (73 and 71 nMmin; 4.6 and 4.9 nM respectively), lag-time and time to peak were markedly prolonged (31.0 and 24.3 min; 41.6 and 34.3 min respectively). Summary. A strong correlation between all TG parameters and INR was observed both in the absence and in the presence of TM. We suggest that in certain patients with INR within therapeutic range high risk of bleeding can be assessed by CAT and these patients could benefit from dose adaptation under CAT control.

PB 1.52-6

The value of epistaxis as a predictor of a bleeding disorder: a prospective study in an Egyptian cohort of children

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Background: Epistaxis is a common problem in children with 40–56% presenting by at least one episode by age of 5–10 years. Some studies showed that one third of children referred for recurrent epistaxis have a diagnosable coagulopathy.

Aim: To identify prevalence of previously undiagnosed bleeding disorder in Egyptian children with significant epistaxis and determine predictors of such an underlying disorder.

Patients and Methods: A prospective study recruiting all children 2–18 years old presenting to the Pediatric Ear Nose and Throat (ENT) and Hematology outpatient clinics, emergency department and inpatient wards over a 6 months period with the following inclusion criteria, not having a known bleeding disorder with recurrent epistaxis lasting longer than 10 min and/or requiring intervention. All were subjected to the questionnaire of a bleeding disorder including personal, family and bleeding history as well as physical examination including ENT. Severity of epistaxis was graded using epistaxis scoring system by Katsanis et al,1988. All patients underwent testing for haemostasis including complete blood count, full coagulation screen and specific tests accordingly.

Results: The study included 63 children (84.1% ≤ 10 years, 52.4% males) with 38.1% of consanguineous marriage and 47.6% having similar condition. Epistaxis was severe in 41.3% of patients leading to anemia in 38%, bilateral in 65.1% precipitated by hot weather in 43% and post-traumatic in 19% of children. Other bleeding symptoms were reported in 30 (47.6%) children though a diagnosis of a bleeding diathesis was only reached in 18 (28.6%). This included eight with thrombocytopenia, eight with inherited disorders; platelet dysfunction (4Glanzmann's thrombasthenia and 4Bernard Soulier syndrome), one mild hemophilia A and one von Willebrand Disease type 1. Interestingly, 39.7% had ENT causes and 31.7% were idiopathic. Clinical predictive factors for having a bleeding diathesis were explored and included young age, consanguinity, epistaxis severity, the presence of other bleeding symptoms and decreased hemoglobin level (P 0.004, < 0.001, < 0.001, < 0.001, < 0.001) respectively.

Conclusion: Children with recurrent significant epistaxis should be screened for an underlying bleeding tendency especially if having any predictor factors.

PB1.53 – Blood Coagulation Tests – IV

PB 1.53-2

The impact of reference ranges in platelet aggregation study

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Background: Platelets play an important role in primary hemostasis, however may have deleterious effects, participating in the atherothrombotic process and in the pathogenesis of Acute Coronary Syndromes. More than 30 years have been studying the use of antiplatelet drugs in the prevention of thromboembolic events. However, some patients, even under antiplatelet therapy may have thrombotic events that may be related to an ineffective inhibition of platelet function. Studies show that factors such as patient's clinical condition, risk factors and genetic polymorphisms may be related to different degrees of reactivity and responsiveness to antiplatelet therapy. There is no consensus in establishing laboratory criteria which may be used to describe a poor response to antiplatelet agents. However, some laboratory methods have been described and evaluated, such as the platelet aggregation test by Light Transmittance Aggregation (LTA) or Impedance Method.

Aims: This study aimed to compare the results of platelet aggregation in patients in use of antiplatelet drugs (aspirin and clopidogrel) obtained by impedance method and LTA applying local reference ranges.

Methods: For this comparative study, platelet aggregation was performed in 53 patients. The reference range (RR) for the impedance method was performed in 41 normal subjects.

The LTA was performed in platelet rich plasma (PACKS-4 – Helena Laboratories) and impedance method in whole blood (Multiplate Platelet Function Analyer- Dynabyte). The agonists used in comparative studies were: Adenosine diphosphate – ADP (final concentration – LTA: 2.5 μM; impedance method: 6.5 μM) and Arachidonic Acid – AA (final concentration – LTA: 50 μg/mL; impedance method: 0.5 mM). For LTA the response was considered normal when the final percent final aggregation was higher than 60% (for AA and ADP). The impedance RR recommended by the manufacturer is 38–85 AUC for ADP and for 16–71 AUC for AA. The RR obtained from 41 normal subjects were 45–89 AUC for ADP and 39–83 AUC for AA.

Results: Comparing the two methods, we realized that using the reference values recommended by the manufacturer we had a lower concordance between both methods (69.3% and 57.7%) than when we used the calculated RR (75% and 77%) for ADP and AA respectively.

When evaluating results of patients in use of aspirin, we found that 88% were poor responsive for AA in LTA. We noted lower rates in impedance method (29% with AA) when using RR method recommended by the manufacturer. By using the RR calculated rates rise 76.5% with AA.

Regarding patients in use of clopidogrel, 100% of patients were poor responsive with ADP in LTA method. We noted lower rates in impedance method (40% with ADP) when using reference range method recommended by the manufacturer. The rates increase to 80% with ADP when we use the RR calculated for impedance method.

Conclusions: Consistent data are observed when using the impedance methodology based on calculated reference values. We conclude that it is essential to define RR specific to each lab, obtained from a local normal population to interpret platelet aggregation by the impedance method.

PB 1.53-1

A new clotting biomarker for predicting sepsis severity

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Background: It is well known that the regulation of the coagulation cascade is abnormal in sepsis. A procoagulant state is induced due to exposed tissue factor and inflammatory modulation of the coagulation cascade [1]. Fibrinolysis is also inhibitied due to an increased activity of PAI-1, impaired thrombomodulin activity and reduced active protein C [2]. A procoagulant state and inhibited fibrinolysis can lead to formation of microthrombi throughout the vasculature known as Disseminated Intravascular Coagulation (DIC). It is hypothesised that these microthrombi contribute to multi-organ failure in sepsis by impairment of circulation and perfusion into the major organs [3]. Formation of thrombi systemically can eventually lead to consumption coagulopathy, in which bleeding is prevalent. We hypothesise that the progression and severity of sepsis can be detected from observation of the clot formation process in these patients. This project aims to assess the potential of a novel new clotting biomarker of clot structure, D_f [4], in predicting the severity of sepsis by comparison with organ failure assessment scores.

Methods: This study was undertaken with full ethical approval from the local research ethics committee. Informed written consent was given by all patients that had capacity to do so. Informed written ascent was given in cases where consent was not possible. Patients with a diagnosis of sepsis were recruited on admission to the Intensive Therapy Unit in a large teaching hospital in Wales. Blood was taken for routine testing, ROTEM thromboelastometry, and rheological analysis (New biomarkers T_{GP} and D_f) on admission, at 2–6, 24 h and 3–7 days to assess progression. Disease Severity was assessed by APACHEII Score on admission and by SOFA score at each sampling point. Eighteen patients were recruited: nine with Severe Sepsis and nine Septic Shock. Eighteen Healthy volunteers were recruited as a matched control.

Results: Mean D_f in the control group was 1.74 ± 0.04. Mean D_f in the severe sepsis and septic shock groups was 1.67 ± 0.09 and 1.64 ± 0.07 respectively. A moderate but non-significant negative correlation was observed between D_f and APACHEII at baseline (Pearson correlation coefficient = −0.36, $P = 0.1$). A weak correlation between D_f and SOFA was observed at baseline (Pearson correlation coefficient = −0.26), however when compared at sequential data points, a moderate significant correlation between D_f and SOFA was observed (Pearson = −0.36, $P < 0.05$).

Summary: D_f correlates with severity in sepsis patients on admission to ITU and, furthermore, significantly correlates with organ failure in these patients when observed at sequential data points.

It is unclear if a weakened clot structure in the more severe patients is due to disease progression or more aggressive treatment that could be received by these patients i.e. fluid resuscitation.

Further analysis is required to assess the impact of treatment on D_f.

If sensitive to dilution, D_f could be a useful biomarker of adequate fluid resuscitation. This will have to be investigated further.

References:

1. Satran and Almog: IMAJ 2003;5:516–520.
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PB 1.53-3

Robustness of the thrombin generation assay in factor VIII-supplemented hemophilic plasma

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Background: Fluorogenic thrombin generation assay (TGA) is a popular global hemostasis assay extensively used in preclinical and clinical evaluation of hemophilia A treatments. Some researchers believe that TGA has advantages over current methods of treatment monitoring, e.g., because TGA may better reflect clinical endpoints. However, published evidence on the clinical utility of the TGA in hemophilia is inconsistent and controversial despite several standardization exercises and a decade of studies that utilized a fairly harmonized protocol.

Aims: We hypothesized that inconsistent predictive value of the TGA may be related to poor robustness of the existing method implementation. Therefore, we evaluated the effects of analytical variables, instrumentation and software algorithms on the fundamental property needed for treatment monitoring: the ability to discriminate between Factor VIII (FVIII)-deficient plasma samples before and after FVIII supplementation.

Methods: We used several fluorescent microplate readers as well as manual and automated pipetting techniques. In house and commercial software were used for evaluation of thrombin generation curves using a variety of published algorithms. The potential effects of small variations of analytical variables on the TGA outcomes were evaluated using commercial congenital FVIII-deficient human plasma supplemented with FVIII.

Results: Thrombin peak height (TPH) was chosen as the most robust and sensitive measurement of procoagulant activity in hemophilic plasma. The following parameters were found to affect the absolute value of the TPH: tissue factor (TF) concentration and specific activity, lipid concentration and specific activity, fluorogenic substrate and calcium concentrations, ratio of human plasma volume to the TF and calcium concentrations. Mathematical algorithms, e.g., correction of substrate consumption, instrument noise and non-linearity (inner filter effect) were non-consequential under typical conditions. However, the relative difference between the FVIII-deficient and FVIII-supplemented plasmas and the low limit of FVIII detection depended almost

entirely on the plasma source and the presence of exogenously added or contact-activated intrinsic factors IXa and XIa.
Conclusions: Stability and lot-to-lot variability of reagents used for the TGA may negatively affect the robustness of the method. Use of external plasma calibrator, e.g., reference plasma sample, may potentially correct for these deficiencies. These studies may assist in establishing the consensus pre-analytical and analytical test conditions by which TGA can be standardized across laboratories.
Disclosure: This is an informal communication and it represents authors' own best judgment. These comments do not bind or obligate FDA.

PB 1.53-4
Results of performance assessment of CoaguChek XS INR monitors by external quality control

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Introduction: External quality assessment (EQA) of test results is a manner to establish whether a laboratory is able to produce accurate results as well as to assess the between-laboratory variation for a particular test. EQA is nowadays a well-known and accepted procedure for the assessment of the quality of regular laboratory testing. However, EQA for point-of-care testing (POCT) has not yet been widely introduced. One of the problems is the availability of suitable control material. POC monitors are designed for the use of whole blood. Unfortunately it is difficult to distribute to participants stable whole blood samples suitable for measurement of the International Normalised Ratio (INR).
Aim: Fortunately with the CoaguChek XS monitor, the most widely used monitor inEurope, it is also possible to use citrated plasma. The ECAT Foundation therefore designed an EQA programme for CoaguChek XS INR monitors using lyophilised plasma pools of patients on anticoagulants. Because of the direct relationship between the measured INR value and the treatment of the patient it is important that a POCT monitor measure the correct INR value. Therefore in our EQA approach for the CoaguChek XS we focus on the trueness of measurement (accuracy).
Methods: The quality set consists of four different plasmas, covering the whole therapeutic range (INR 2–4.5). The results of all four measurements are evaluated in an integrated linear regression model. With this model it can be assessed whether it is possible to measure accurate results over the entire therapeutic range. The assigned values are established by the Dutch Reference Laboratory for Anticoagulation (RELAC) using different lot numbers of test strips as well as different monitors and are traceable to the international human thromboplastin reference preparation. The quality control (QC) samples are stable for up to 6 h after reconstitution. Acceptance criteria are based on the deviation from the target value (target value $\pm$ 15%), slope ($0.77 <$ slope < 1.79), intercept ($-1.78 <$ intercept < 0.72) and correlation coefficient (> 0.900). The acceptance criteria are established by modelling the regression analysis.
Results: Up to now 150 different CoaguChek monitors have been evaluated. The established acceptance criteria were met by 136 monitors. Thrombosis Service Centers may test monitors batch-wise with one set of QC samples. In the case of 10 batch-wise-tested monitors a systematic error in one of the QC samples could be observed, which may indicate a pre-analytical error. This means that only four out 150 monitors did not met the acceptance criteria for accurate testing. The between-monitor variation varied between 2.3% and 6.6%. No differences were observed between different lot numbers of test strips.
Conclusion: Current experience with this novel EQA programme for CoaguChek XS monitors shows that it is a valuable tool for quality control of POC INR testing. In the first experience with this EQA programme < 3% of the monitors fail to met the acceptance criteria which indicates a good performance of the CoaguChek XS monitors.

PB 1.53-5
Comparison of Biophen DiXaI®, prothrombin time with a reference HPLC-MS/MS method to monitor patients receiving rivaroxaban

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Background: Pharmacokinetic properties of rivaroxaban seemed to be relatively predictable. However, dependence of renal or hepatic function could result in considerable variation in drug plasma concentrations. Moreover, according to the recent update of the EU-SmPC of rivaroxaban, a reduction of the dose from 20 mg once daily to 15 mg once daily should be considered if the patient's assessed risk for bleeding outweighs the risk for recurrent DVT and PE in patients with moderate or severe renal impairment. This may suggest that monitoring could be used to assess the response of an individual patient. Furthermore, it may also be useful in patients with hepatic impairment, drug interaction, severe bleeding, recurrence of thrombosis, in case of anticoagulation bridging, before urgent or elective surgery, in infants or pregnant women or in patients with extreme body weights. PT or chromogenic anti-Xa assays such as Biophen Direct Factor Xa Inhibitor (DiXaI)® have been proposed to estimate plasma drug concentration but these findings are based on *in vitro* analysis.
Aims: To compare the measurement of calibrated PT and Biophen DiXaI® with the reference High Performance Liquid Chromatography coupled with tandem mass spectrometry (HPLC-MS/MS) in patient's plasma samples.
Methods: Fifty-two plasmas from patients treated with rivaroxaban were included in this study. Calibrated PT was performed using the most sensitive reagent (Triniclot PT Excel S®) and home-made calibrators to give results expressed in ng/mL. Biophen DiXaI® has been performed according to the instructions of the manufacturer on a STA-R Evolution® coagulometer. For the HPLC-MS/MS, after preparation of the sample, separation of the analytes was achieved on a Phenomenex Kinetex® column shield RP18 and the detection was performed using a MICROMASS QUATTRO Micro® mass spectrometer operating in positive electrospray ionization mode.
Results: The plasma-concentration range was from 0 to 485 ng/mL as measured by the HPLC-MS/MS method.
The Biophen DiXaI® and HPLC-MS/MS are highly correlated (Pearson correlation coefficient [r^2] of 0.95 [95% confidence interval: 0.91 to 0.97; *P*-value: < 0.0001]). The Bland-Altman analysis showed a mean difference of −16 ng/mL (standard deviation (SD): 25 ng/mL; 95% limits of agreement: −65 to 32 ng/mL). The limits of detection were 9 and 3 ng/mL for Biophen DiXaI® and HPLC-MS/MS, respectively.
The calibrated PT and HPLC-MS/MS were poorly correlated (Pearson correlation coefficient [r^2] of 0.53 [95% confidence interval: 0.33–0.70; *P*-value: < 0.0001]). The Bland-Altman analysis showed a mean difference of −24 ng/mL (SD: 79 ng/mL; 95% limits of agreement: −179 to 132 ng/mL).
Conclusions: There is an important inter-individual variability with Triniclot PT Excel S® and even calibrated, it poorly correlates with rivaroxaban plasma concentrations in patients measured by HPLC-MS/MS. Therefore, PT should not be used to estimate plasma drug concentrations. On the contrary, Biophen DiXaI® is highly correlated with HPLC-MS/MS and is poorly influenced by inter-individual variability. Biophen DiXaI® should therefore be recommended to estimate rivaroxaban plasma concentration.

PB 1.53-6

Relationship between thromboelastometry measurements, hemostasis factors and two global hemostasis tests: thrombin generation and thromboplastin-thrombomodulin-mediated time

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Background: Global hemostasis assays have been used for decades as the first step to study the hemorrhagic tendency of patients. In the last years the interest has focused on global assays that generate information regarding thrombophilia tendency. Here we describe the correlation between Thromboelastometry (TEM) and hemostasis factors such as fibrinogen and coagulation factors as well as Thrombin generation (TG) and Thromboplastin-Thrombomodulin- mediated time (Tp-TM.T).

Material and Methods: *Subjects:* Blood samples were examined from 137 individuals unrelated to each other (69 women and 68 men) with a mean age of 52 years (range 27–85).

Hemostasis assays: TEM Rotem®: device (Pentapharm GmbH, Munich, Germany) was used for thromboelastometric analysis determined in citrated blood with the reactive intem (with ellagic acid). We studied the Maximal Clot Firmness (MCF) and the maximal Velocity (maxVel)

Thrombin generation (TG) was studied with the Calibrated Automated Thrombograpy method and the Fluoroskan Ascent instrument. It was determined in platelet poor plasma with 5 pM tissue factor and 4 μM phospholipids. We studied the endogenous thrombin potential (ETP) and the maximal thrombin generation (Peak height)

Tp-TM.T: a modified prothrombin time (1) in the presence and absence of thrombomodulin. The results are expressed as R1: ratio of the patient's clotting time vs. control clotting time using the reactive with thrombomodulin and R2: the same ratio using the reactive without thrombomodulin.

Fibrinogen, platelet count, FVII, FVIII, FIX, FXI, FXII was determined by standard methods.

Statistical analysis: Relationship among TEM parameters and the other tests were studied with the Pearson correlation coefficient. A value of $P < 0.05$* or $P < 0.01$** was considered statistically significant.

Results: The *MCF* showed a significant correlation with the following parameters: Fg ($r = 0.59$**), platelet count ($r = 0.53$**), FVII ($r = 0.27$**), FIX ($r = 0.26$**), FXI ($r = 0.36$**).

Also it showed a significant correlation with Tp-TMT R1 ($r = -0.32$**), Tp-TMT R2 ($r = -0.43$**) and TG ETP ($r = 0.30$**), TG Peak ($r = 0.28$**). No correlation was found with levels of FVIII and FXII.

- The *MaxVel* showed a significant correlation with the same parameters with the following coefficients: Fg ($r = 0.43$**), platelet count ($r = 0.52$**), FVII ($r = 0.2$*), FIX ($r = 0.17$*), FXI ($r = 0.34$**), Tp-TM.T R1 ($r = -0.26$**), Tp-TM.T R2 ($r = -0.29$**), TG ETP ($r = 0.32$**), TG Peak ($r = 0.28$**). Again no correlation was found with FVIII and FXII.

Conclusions:

- TEM activated with ellagic acid (*MCF* and *MaxVel*) is sensitive to levels of the studied parameters except for FVIII and FXII. TEM also has a good correlation with Tp-TM.T (R1 and R2) and TG (ETP and Peak)
- MCF and MaxVel are good parameters to evaluate thromboelastometry as indicators of coagulation physiology.

Reference:

1. Haematologica 2002; 87:415–419.

PB1.54 – Blood Coagulation Tests – V

PB 1.54-1

Normalized ratio for silica clotting time and dilute Russell venom time is an excellent tool for diagnosis of lupus anticoagulant

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Background: The laboratory criteria for the lupus anticoagulant (LA) diagnosis have been updated (JTH, 2009;10:1737–40). The report recommends a% correction (%CORR), as calculated by the equation [(screen result – confirm result)/screen result] × 100%, greater than the locally determined cut-off value be considered as positive. The package inserts for the Hemosil Silica Clotting Time (SCT) and the Hemosil LAC Screen (LAC-S)/LAC Confirm (LAC-C) tests (Instrumentation Laboratory), however, recommend the use of a Normalized Ratio (NR) to determine positivity for LA. The NR is calculated by dividing the Screen Ratio (patient screen result/mean of screen normal range) by the Confirm Ratio (patient confirm result/mean of confirm normal range). The normalization using the mean normal value for each individual test is intended to compensate for any day-to-day variation associated with the test as well as the differences in the normal ranges of the individual tests.

Aim: This laboratory sought to compare the final interpretation of laboratory results using the %CORR vs. the NR for consecutive samples submitted for LA testing.

Methods: Blood collection and processing of specimens submitted for LA testing were done according to the LA Scientific Standardisation Committee (LA-SCC) recommendations. Using a single aliquot, the SCT and the LAC-S/LAC-C tests were performed simultaneously on an ACL TOP CTS 500 (Instrumentation Laboratory) with the Hemosil reagents. The PT/INR and Thrombin Time (TT) were measured on all specimens to detect significant anticoagulant therapy. The results for LA testing on 1556 consecutive samples, with INR < 1.5 and TT < 80 s, were analyzed using both the calculated % CORR and the NR. The normal value cut-off for each test was set at the 99th percentile as obtained from 50 normal donors (SCT-%CORR < 35%, SCT-NR < 1.33, LAC-%CORR < 33% and LAC-NR < 1.33).

Results: Positivity for %CORR vs. NR for SCT [6.3% vs. 5.8%) and for LAC (15.2% vs. 14.5%) showed 96% and 91% concordance, respectively. The mean ± 1SD of positive results were 50.8 ± 13.7% (SCT-%CORR), 2.07 ± 0.82 (SCT-NR), 60.5 ± 3.7% (LAC-% CORR) and 1.61 ± 0.36 (LAC-NR). All discordant samples were in the lower range of the %CORR for both SCT (mean = 35.5, range 35.5–36.0) and LAC (mean = 33.7, range 33.5–33.8%). Regression analysis of %CORR vs. NR demonstrated excellent correlation for both SCT (semi-log relationship, $r^2 = 0.9416$, $P < 0.001$) and LAC (linear relationship, $r^2 = 0.9907$, $P < 0.001$).

Conclusions: The NR for the determination of the presence of LA shows excellent agreement by concordance and regression analysis with the %CORR, the method currently recommended by the LA-SCC. The small percentage of discordance seen here may be accounted for by the determination of the cut-off values and/or by the natural variation seen in the performance of these tests. In conclusion, the use of NR for the determination of the presence of LA is a viable alternative to the %CORR.

PB 1.54-2

Dynamic APTT characteristics in a case–control study of venous thrombosis

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Background and Aims: High levels of clotting factors (VIII, IX, XI) and short Activated Partial Thromboplastin Time (APTT) have been related with venous thrombosis risk. Recently, dynamic APTT parameters have been assessed in venous thrombosis patients (*B. Sørensen and J. Ingerslev, J Thromb Haemostas 2012;10:244–50*). We explored this relation among a sub-group of patients in the framework of an ongoing case-control study of venous thromboembolism (RETROVE project: Riesgo de Enfermedad TROmboembólica VEnosa). The aim of RETROVE project is to investigate algorithms for predicting the individual risk of venous thrombosis.

Methods: An initial group of 37 individuals of RETROVE project where included in this analysis. The data set contained 27 subjects (14 females and 13 males, aged 22–88 years old) and 10 controls (four females and six males, aged 20–87 years old).

The APTT was measured in a ACL-TOP analyzer using SynthAsil as a reagent, both from Instrumentation Laboratory, Spain. The clotting time digital signals were recorded by the equipment, which had an output profile consisting of the normalized signal and its first derivative. Both were cleared of high-frequency noise artifacts. The digital signals allowed us to extract two static and two dynamic parameters: (i) maximum clot firmness or APTT-MCF, (ii) time to maximum clot firmness or APTT-t-MCF; (iii) maximum velocity or APTT-MaxVel, and (iv) time to maximum velocity or APTT-t-MaxVel. We applied the Welch student's t-test with a significance level less than $P = 0.1$ to measure the statistical difference between the patients and the controls.

Results: Both the APTT-t-MaxVel and the APTT-t-MCF were shorter in the patients than in the controls (P-values 0.0881 and 0.0774 respectively). However, we have not found any statistically significant difference in the other two parameters (APTT-MCF and APTT-MaxVel).

Conclusions: We have confirmed the previous results concerning the relation of dynamic APTT characteristics and thrombosis. Our analyses are preliminary, due to the small sample size, although the results could be considered as promising. In the future we plan to test a larger set of dynamic APTT phenotypes and on a larger sample of individuals. Also, we will attempt to apply multi-variate and time-series methods for a better description of the dynamics of APTT signals.

PB 1.54-3

Comparison between different point of care systems for PT INR testing

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Background: Portable coagulation systems are used to perform PT-INR test on capillary blood, to monitor antivitamin K antagonists (AVK). Differences in PT INR determination depend on reagents, coagulometers and calibrations. Agreement between portable monitors and reference systems should be evaluated.

Aims: Evaluate microINR and Coagucheck XS, two commercial portable monitors, in comparison with PT-INR provided by central laboratory reference system, using two different thromboplastins

Methods: PT INR was performed on two portable monitors: Coagucheck XS (Roche Diagnostics, Basel, Switzerland) using freeze-dried rabbit thromboplastin with ISI of 1.84 and microINR (IL Instrumentation Laboratory – Werfer Group) using human thromboplastin with ISI of 1.00. As reference system for PT-INR determination from venous blood specimens a magneto-mechanical coagulation analyzer was used (STA-R, Stago France). All venous samples were tested with two different thromboplastins: a rabbit thromboplastin with an ISI=1.28 (Neoplastin Plus Stago Paris, France) and a recombinant thromboplastin (Hemosil IL International Laboratory) with an ISI = 0.99.

One hundred and fifty-three AVK patients, with PT INR ranging from 2 to 4 (INR range 2–4), were evaluated. In the same daily session venous blood samples *were collected, centrifuged and frozen at* −80 °C and capillary PT-INR test was performed on the two portable monitors. Statistical analysis was performed by linear regression and Bland Altman method.

Results: The correlation among different systems was good showing: Coaguchek XS vs. neoplastin plus = 0.953 and vs. Hemosil = 0.965; microINR vs. Neoplastin Plus = 0.883 and vs. Hemosil 0.910. Differences in PT INR $> \pm 0.5$ were observed in about 5% of the total patient population, except for the comparison between Coaguchek XS and Hemosil, that showed a higher disagreement (PT INR $> \pm 0.5 = 14.4\%$). Bias calculated by Bland Altman are the following: Coaguchek XS vs. Neoplastin Plus = 7.8; Coaguchek XS vs. Hemosil = 13.3; microINR vs. Neoplastin Plus =1.9; microINR vs. Hemosil = 3.1.

Conclusion: Results showed good correlation between the different methods. MicroINR ratio system is simple to use and sufficiently accurate. The disagreement showed by Bland Altman method probably depends on thromboplastin calibrations and their different sensitivity. The correlation among portable monitors and reference methods is good and improves comparing systems with homogeneous reagents. Before use a portable monitor we suggest to evaluate the agreement with reference system. Companies should communicate ISI value of thromboplastin used in portable monitors and calibration model

PB 1.54-4

Local verification and assignment of ISI values: recent mayo clinic experience and outcome

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Background: Warfarin dosing relies on an accurate international normalized ratio (INR) which is calculated from the prothrombin time (PT), International Sensitivity Index (ISI) of the PT reagent (thromboplastin), and geometric mean (GM) of PT. FDA approved certified plasma's and ISI assignments are not available for all instruments. ISI varies with reagent/instrument combinations and change with each lot of thromboplastin. Failure to harmonize INR results may result in the same patient to receive varying warfarin dosing instructions when tested at different anticoagulation clinics utilizing different instruments and/or thromboplastins. CLSI guidelines encourage laboratories to verify/establish local ISI and GM, however, the process is challenging.

Aims: To develop a standardized process that will verify/establish ISI and GM assignments of a single thromboplastin across multiple instruments.

Methods: Over a 2 year period, two lots of thromboplastin (RecombiPlasTin 2G, R2G, Instrumentation Laboratories, Medford, MA) were compared at 4 (lot# 1) and 6 (lot# 2) sites on four different instruments: ACL TOP (Instrumentation Laboratory, Bedford, MA) and the STA-R Evolution, Compact, and Satellite (Diagnostica Stago, Parsippany, NJ). For lot# 1, the ISI for the thromboplastin on the ACL TOP (established by Instrumentation Laboratory) was verified on site by orthogonal regression analysis using 31 stable warfarin patients and 20 random normal donors; establishing the TOP as the reference method. Using the same 51 samples, PT results from the reference method were plotted against PT results from the other three sites and analyzed by orthogonal regression to assign individual ISI's. Forty random healthy donor samples were recruited to verify GM at each testing site. For lot# 2, 13 sets of pooled plasma samples (INR range: 1.3–4.0) were created from 142 stable warfarin patients. The manufac-

turer's assigned ISI was first verified on the reference method, then at the additional five sites using orthogonal regression analysis. GM at each site was verified or established by both small scale normal range verification procedure (using 25 random healthy donor samples) and the intercept of INR linear regression analyses as described by Favaloro et al (2012). Finally, both local ISI and GM were further verified by three sets of WHO certified plasma samples.

Results: Lot# 1: the manufacturer assigned ISI (1.05) and laboratory established GM (11.0) were verified on the TOP. Locally assigned ISI and GM values were derived as follows, respectively: Evolution, 1.14, 12.3; Compact, 1.12, 10.9; and Satellite, 1.11, 10.6. Linear correlations of the INR results from all four laboratories demonstrated an r^2 of > 0.99. Post implementation internal proficiency tests among all four laboratories demonstrated excellent agreement. Lot#2: (manufacturer assigned ISI of 0.95), locally derived ISI and GM values as follows, respectively: TOP, 0.95, 11.0; Evolution, 1.04, 11.6; Compact, 1.02, 11.5; and Satellite, 0.99, 10.9. Linear correlations of the INR results from all six laboratories demonstrated an $r^2 > 0.98$.

Summary/Conclusion: This method for ISI and GM verification/assignment across instruments provides a simple, robust and reproducible approach. The data confirm the change in ISI on different instruments with different lots of thromboplastin reaffirming the need for this exercise.

PB 1.54-5

Thrombin generation and post-operative bleeding in patients undergoing surgery requiring cardiopulmonary bypass

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Background: Bleeding following surgery requiring cardiopulmonary bypass is a major cause of morbidity. Evidence has suggested thrombin generation (TG) assays may identify patients at risk of excessive post-operative bleeding. However, TG lacks standardisation and optimal conditions remain to be finalised. Altering the conditions may improve the ability of TG to identify an increased risk of bleeding.

Aim: To measure TG in the presence and absence of corn trypsin inhibitor (CTI), at different concentrations of tissue factor (TF) and correlate the results with post-operative bleeding.

Methods: After ethical approval and informed consent, 102 patients were recruited who were undergoing elective valve surgery, surgery on the thoracic aorta or undergoing coronary bypass grafting plus another procedure. Blood samples were collected into 3.2% citrate with or without 20 μg/mL CTI before heparin, and 30 min after heparin reversal. TG was performed using the method of Hemker. Trigger solutions contained 0.5, 1, 5 and 10 pM of TF and 4 μM phospholipids (20% PE, 20% PS, 60% PC). Commercially available trigger solutions (PPP low and PPP, a gift from Stago) were also used. Before TG assays, the 96-well plate was warmed to 37 °C for 30 min.

Abnormal post-operative bleeding was defined as drain loss of more than 1 L at 24 h, more than 200 mL/h for two consecutive hours, more than 2 mL/kg/h for two consecutive hours in the first 6 h, the need for haemostatic treatment or the need to return to theatre for bleeding.

Results: For samples collected into CTI, the median Endogenous Thrombin Potential (ETP) with 0.5 pM TF pre-surgery was 59.33 vs. 5.25 post ($P < 0.0001$); 1 pM TF 133 nM/min pre, 77.67 nM/min post ($P = 0.337$); 5 pM TF 605 nM/min pre, 530.33 nM/min post ($P = 0.013$); 10 pM TF pre 756.67 nM/min, 614.33 nM/min post ($P < 0.0001$); PPP low 339 nM/min pre, 191.67 nM/min post ($P < 0.0001$); PPP 987.67 nM/min pre, 777.50 nM/min post ($P < 0.0001$).

When collected into citrate alone the median ETP with 0.5pM TF was 710 nM/min pre vs. 197.67 nM/min post ($P < 0.0001$); 1 pM TF 640 nM/min pre, 228 nM/min post ($P < 0.0001$); 5 pM TF 754.67 nM/min pre, 543.33 nM/min post ($P < 0.0001$); 10 pM TF 806.33 nM/min pre, 639.50 nM/min post ($P < 0.0001$); PPP low 841.67 nM/min pre, 345.67 nM/min post ($P < 0.0001$); PPP 1087 nM/min pre, 806.33 nM/min post ($P < 0.0001$). The ETP was lower for samples collected into CTI, regardless of the trigger used ($P < 0.0001$ except for 5 pM TF post where $P = 0.003$, and PPP where $P = 0.460$). There was a significant increase in the ETP with increasing concentrations of TF ($P < 0.0001$).

The only statistical difference in the ETP between those who did and did not bleed excessively was seen in pre-operative samples using 0.5 and 1 pM TF and blood loss > 200 mL/h as an end-point ($P = 0.029$ and 0.023 respectively).

Conclusion: TG assays performed in the manner and time points selected in this study do not identify those at increased risk of post-operative bleeding. CTI and different TF concentrations produce different results but do not change sensitivity to predict bleeding.

PB 1.54-6

The overall haemostasis potential; A novel global coagulation assay for use in canine plasma

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Background: The overall haemostasis potential (OHP) is a turbidimetric global coagulation assay, dynamically measuring fibrin generation (overall coagulation potentialcurve, OCP) and lysis (OHP curve). By subtracting the OHP from the OCP, we can calculate the overall fibrinolytic potential (OFP). Thromboembolism and hypercoagulability is increasingly being recognised in canine patients, though cost and limited availability of global assays impede our ability to pre-empt and recognise these conditions. The OHP is cost effective and can be run with common absorbance spectrophotometry equipment.

Aims: The purpose of this study was to optimise the OHP assay for use with canine platelet poor plasma, determine normal ranges, and compare OHP results with those for a thrombin generation assay (CAT) and thromboelastography (TEG).

Methods: Citrated plasma was collected from 40 clinically healthy dogs. The OHP assay and standard coagulation assays (prothrombin time [PT], activated partial thromboplastin time [APTT], and fibrinogen [Fg]) were performed for each sample. TEG and OHP was performed in nine dogs, and CAT and OHP in 23 dogs.

Results: Modifications to the published methodology for the OHP assay were required, with less coagulation activator (thrombin) and more fibrinolytic agent (tPA). The canine fibrin-time curve was generated faster and had a lower maximal optical density (MaxOD) than the human curve. Males had a higher OHP than females ($P = 0.04$). Stress during sampling was associated with increase in speed of clot generation ($P = 0.02$). High fibrinogen levels were associated with increased Max OD and OCP ($P \leq 0.05$), and a reduced PT was associated with increases in Max OD, OCP, OHP & Maximum slope ($P \leq 0.05$). The OHP parameter correlated with TEG parameters K (speed of clot generation), angle, maximum amplitude and G (clot strength) ($P < 0.05$). The OHP parameter correlated with the peak thrombin generation in the CAT assay ($P = 0.02$).

Summary: These results support the use of the OHP assay as an accessible, cost-effective global coagulation assay for use in canine plasma as an alternative to TEG andCAT.

PB1.55 – Coagulation Factor VIII – II

PB 1.55-1

Amino acid sequence epitope mapping of four factor VIII monoclonal antibodies

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Background: Inhibitory antibodies (inhibitors) form the greatest challenge in the care of haemophilia A. The majority of inhibitors in congenital haemophilia A are polyclonal (IgG) directed primarily against the A2 and C2 domains of Factor VIII (FVIII). There is as yet no high throughput technology to define amino acid specificity of inhibitors. We describe the first application in haemophilia of a novel, ELISA based re-usable microarray (Pepscan®) to describe the amino acid sequence epitope of a series of domain characterised anti-FVIII monoclonal antibodies. This platform uses overlapping 20-mer linear peptide or 15-mer overlapping looped peptide libraries. Usage of looped peptides aims to mimic secondary structure, creating a conformational epitope that may enhance antibody binding. This technology has previously been used to describe the binding epitope of two anti-CD20 monoclonal antibodies (rituximab and GA101).
Aims: To assess the amino acid binding sequence epitopes of six anti-FVIII monoclonal antibodies and correlate these with their known domain specificities.
Methods: Six anti-FVIII monoclonal antibodies (mAb): 58.12 (anti-A1), C5 (anti-A1), R8B12 (anti-A2), 2D2 (anti-A3), GMA-8011 (anti-A3) and B02C11 (anti-C2) were tested on both linear and looped arrays. A negative control (buffer media) was tested prior to and in between each monoclonal antibody tested. R8B12 was tested in two separate experiments using the linear and looped FVIII arrays to demonstrate reproducibility of its identified epitope. This antibody was also tested using a FIX looped and linear array to demonstrate FVIII specificity.
Results: Of the six monoclonal antibodies, binding peaks were seen for four antibodies (58.12, C5, R8B12 and 2D2) on both linear and looped arrays (shared epitopes). All epitopes demonstrated solvent accessibility as modelled on PyMOL. All four of these antibodies showed binding within their previously described domains. Of the two non-binding antibodies (GMA-8011 and B02C11), B02C11 has been documented as having a complex discontinuous epitope. The amino acid sequence epitopes were as follows: mAb 58.12 YLGAVELSWD (A1 domain positions 6–15); mAb C5: TDSEMDVVRF (a1 domain positions 351–360); mAb R8B12: ISAYLLSKNN (a2 domain positions 726–735) and mAb 2D2: EPRKN (A3 domain positions 1801–1805). The binding epitope of R8B12 was reproducible on repeat testing and no binding was seen on the negative control array (FIX linear or looped arrays). The R8B12 epitope we describe is discrete from the previously published discontinuous epitope. No demonstrable carry-over of binding was seen between testing of monoclonal antibodies on this re-usable platform.
Conclusions: This approach offers a simple novel technique for amino acid epitope mapping of FVIII monoclonal antibodies. Data from our experiments has demonstrated solvent accessible binding within the domains previously described. We describe candidate amino acid binding sequences for three monoclonal antibodies not previously characterised to this level. We continue to optimise this technology for wider applications.

PB 1.55-2

Excretion and pharmacokinetics of glycopegylated rFVIII (N8-GP) after single intravenous dose administration to rats

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Background: N8-GP is a FVIII (turoctocog alfa) which is O-glycoPE-Gylated in the B-domain and currently in clinical development for prophylaxis and on-demand treatment of bleeding in haemophilia A. The B-domain contains a single 40 kDa branched PEG.
Aims: Excretion data for N8-GP with focus on the fate of the [^{3}H]-labelled PEG moiety will be presented together with PK parameters of total plasma radioactivity. The results will be compared with data obtained from rats dosed with the [^{3}H]-labelled 40 kDa PEG moiety only.
Methods: Han Wistar rats (n = 3) were dosed with a single intravenous dose of either [^{3}H]-labelled N8-GP (0.23 mg/kg, ~1900 U/kg, and a PEG load of 0.05 mg/kg) or 40 kDa [^{3}H]PEG (1, 12, 100 or 200 mg/kg). Animals were kept in metabolism cages continuously for the first 3 days of the study period and thereafter for 24 h intervals on a weekly basis up to 12 weeks. Recoveries of radioactivity were extrapolated to obtain accumulated recovery data. Plasma samples were collected for up to 12 weeks at all dose levels and analysed by liquid scintillation counting (LSC) and HPLC coupled to a radiochemical detector. Selected urine samples were also analysed by HPLC. All pharmacokinetic parameters were calculated using a standard two-compartment model.
Results: For [^{3}H]N8-GP, an estimated mean recovery of total radioactivity of 103% was found over the 12 week period, with 60% excreted renally and 33% recovered in faeces. Low levels of radioactivity were still detected in excreta and plasma 12 weeks post-dose. Terminal plasma half-life ($t_{½}$) of radioactivity was 12.5 days. The total volume of distribution (V_{ss}) was estimated to be 325 mL/kg – the volume of the central compartment (V_c) being 50 mL/kg and the peripheral (V_p) 280 mL/kg. The total plasma clearance (CL) was 1.9 mL/h/kg. For [^{3}H]PEG, an estimated mean recovery was found in the range of 88–93% for the different dose levels with 58–68% excreted renally and 12–13% in faeces. Dose linearity was confirmed between the four dose levels, but the $t_{½}$, V_p and therefore also V_{ss} were found to vary with dose. The $t_{½}$ increased with dose (range: 25–54 days), which appeared to be driven by an increase in V_p (range: 310–680 mL/kg), while V_c (80–90 mL/kg) and CL (range: 0.8–1.1 mL/h/kg) were found to be constant.
Summary/Conclusions: [^{3}H]N8-GP and the 40 kDa [^{3}H]PEG moiety were shown to be excreted both via urine and faeces – urine being the major excretion route for radioactivity pertinent to both compounds. [^{3}H]N8-GP was excreted to a higher degree in faeces compared to 40 kDa [^{3}H]PEG which may be associated with N8-GP being cleared by the liver.
This observation is supported by a 2-fold higher plasma CL of [^{3}H] N8-GP compared with [^{3}H]PEG. Further, terminal half-life of [^{3}H]N8-GP was 12.5 days compared to 25 days for [^{3}H]-PEG at 1 mg/kg. In conclusion, 40 kDa [^{3}H]PEG has been shown to be excreted both via urine and faeces. The clearance of [^{3}H]N8-GP was twice as high as for [^{3}H]PEG, probably due to liver-specific clearance of N8-GP.

PB 1.55-3

Accessibility to thrombin cleavage does not limit the circulating half-life of longer-acting factor VIII molecules

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Background: A number of strategies, including Fc-fusion and PEGylation, have been used to prolong the circulating half-life ($t_{½}$) of factor VIII (FVIII). Strikingly, all of these strategies attain the same upper limit of 1.5- to 2-fold prolongation of $t_{½}$ in preclinical and clinical testing. Thus, it is possible that there is a common limiting factor. Because thrombin cleavage is an essential step in the activation of FVIII, all longer-acting FVIII molecules have been designed to maintain the ability to be cleaved by thrombin. Once cleaved by thrombin, activated FVIII (FVIIIa) is subject to numerous clearance pathways. Cleavage releases von Willebrand factor, which has been shown to protect FVIII from receptor-mediated clearance by low-density lipoprotein receptor-related protein (LRP) and from binding to membranes where it is subject to further degradation by activated protein C. Furthermore, thrombin cleavage is hypothesized to change the conformation of the A2 domain, exposing an additional LRP binding site. Finally, FVIIIa is highly unstable because of rapid A2 dissociation. It is therefore possible that low-level basal activation of FVIII by thrombin may play an important role in the clearance of FVIII and variants of FVIII that are designed to be longer acting.
Aim: To explore the possibility that accessibility to thrombin cleavage is the common limiting factor in FVIII $t_{½}$ prolongation.
Methods: To test this hypothesis we expressed and purified variants of FVIII, including a PEGylated FVIII from which the thrombin cleavage sites were removed (R373Q and R1690Q). The pharmacokinetics of the thrombin-resistant FVIII and thrombin-sensitive FVIII were identical when tested in FVIII knockout hemophilia A (HemA) mice.
Results: Combination of thrombin-site mutants with site-specific PEGylation, which has been previously shown to increase $t_{½}$ by approximately 2-fold in HemA mice, did not lead to a significant improvement beyond this 2-fold threshold.
Conclusion: In this study we have demonstrated that susceptibility to cleavage by thrombin does not limit the circulating $t_{½}$ of longer-acting FVIII.

PB 1.55-4

Evaluation of recombinant canine FVIII production from three cell types transduced with either a B domain deleted or codon optimized and truncated B domain cFVIII transgene

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Background: Historically, canine cryoprecipitate has been used to maintain the severe hemophilia A dog colony at Queen's University. Recombinant canine FVIII (rcFVIII) provides a substitute consistent with animal care principles of replacement, refinement, and reduction. However, expression of FVIII is significantly lower than other recombinant proteins of similar size.
Aims: To develop an expression system for colony-scale rcFVIII production and characterize the effect of vector engineering and host cell type on productivity and efficacy in the canine hemophilia A model.
Methods: A codon-optimized (CO) transgene was developed from canine B-domain-deleted (BDD) FVIII with the addition of a Kozak consensus sequence, the re-introduction of a portion of the 5′ B-domain sequence containing six N-linked glycosylation sites flanked by SQ-linkers, and 24% base pair substitution to optimize translation efficiency with respect to canine codon bias. Both transgenes (BDD and CO) are regulated by the ubiquitous EF1α promoter with the addition of a strong endothelial enhancer element (EPCR). VSV-G pseudotyped lentiviral vectors containing either transgene were used to transduce canine blood outgrowth endothelial cells (cBOEC), Madin-Darby canine kidney (MDCK), and human endothelial kidney (HEK) 293T cells at MOIs between 5 and 250. FVIII activity was measured using a 1-stage assay against a canine pooled-plasma standard. Integrated transgene copy numbers were determined with qPCR using human or canine β-actin as an endogenous reference. rcFVIII was purified with anion and cation exchange chromatography. Candidates for larger-scale production were expanded in triple-flasks or on collagen-coated microcarrier beads to produce test lots for pharmacokinetic (PK) evaluation in three hemophilia A dogs. Treatment-scale lots were tested for endotoxin and dogs were administered 20–29 U/kg. FVIII levels were determined at 0.5, 1, 2, 4, 8, 12, 24, and 48 h post-infusion.
Results: Regardless of cell type, the CO transgene showed significantly greater ($P < 0.001$) FVIII productivity per transgene copy than the BDD version, representing a 3.4-, 6.4-, and 6.5-fold increase for HEK 293T, MDCK, and cBOECs respectively. Additionally, CO cBOECs showed the highest specific productivity at 2.0 U/10^6 cells/24 h. However, this protein was also the least stable, as rcFVIII activity was reduced to 79% following a 2 h incubation in hemophilic plasma at physiological temperature. Inefficient expression following culture polarization in MDCK cells procluded the use of these cells for larger scale production. Pharmacokinetic evaluation in three hemophilia A dogs showed that the CO cBOEC protein achieved less than half the dose-normalized peak recovery of the BDD cBOEC protein, and also demonstrated significantly reduced ($P = 0.037$) bioavailability measured by area-under-the-curve (AUC).
Summary/Conclusions: Six cell lines were developed to express either CO or BDD cFVIII transgenes. Although the CO transgene showed markedly improved productivity, the resulting rcFVIII had reduced performance in procoagulant stability and efficacy. HEK 293T products were more cost-effective per activity unit due to high cell densities and simple media requirements. Production efficiencies will be further developed from a process engineering perspective.

PB 1.55-5

Instability of the His118Arg mutation in both factor VIII and activated factor VIII causing discrepancy in FVIII assays

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Background: A wide range of factor VIII activity (FVIII:C) levels is presented in different haemophilia A (HA) patients with an identical missense mutation in the F8 gene, which usually causes a mild or moderate HA. Studies to date, however, have not elucidated the reasons clearly for discrepant FVIII:C levels based on genetic factors and molecular mechanisms.
Objective: To assess the effect of the FVIII His118Arg mutation on FVIII:C assays.
Patients and Methods: Two patients from the same family with severe laboratory phenotype but mild bleeding expression were investigated. To evaluate the stability of FVIII in the patients, FVIII:C was determined with one-stage assay kept at 25 and 4 °C for designed times during 2 h after samples collecting, as well as after storing at −20 or −80 °C for indicated days. The amount of FVIII heterodimer was assayed with ELISA using an anti-FVIII light chain monoclonal antibody as a capture antibody and a radish peroxidase-conjugated heavy chain monoclonal antibody as a detection antibody. To evaluate the stability of the patients' FVIIIa, FVIII:C was detected by chromogenic assay after indicated incubation times. Genetic analysis of the F8 gene was carried out by directly sequencing.
Results: Plasma FVIII:C levels were dramatically reduced over the indicated times from 18.9 to 1.6 IU/dL (at 25 °C) and to 0.8 IU/dL

(4 °C) for 2 h in patient 1, from 14.7 to 0.9 IU/dL and to 0.9 IU/dL in Patient 2. After storage at −20 and −80 °C for 2, 14 and 28 days, plasma levels of FVIII:C in both patients were dramatically reduced to the range of 2.6–4.6 IU/dL at 2 days and to ≤ 1 IU/dL at 14 days with a similar reduction pattern between −20 and −80 °C. The intramolecular stability of FVIII was calculated as the residual amounts of FVIII heterodimer relative to initial values (%). The data showed that the amount of FVIII heterodimer decreased rapidly to < 2% in plasma of the two patients. Plasma FVIII:C levels decreased significantly during incubation from 3 to 5 min, and continued to decrease up to 10 min in both patients using chromogenic assay, indicating that the stability of FVIIIa was reduced. His118Arg mutation in the F8 gene was identified in the patients. These data revealed that the instability exhibited by the His118Arg mutant FVIII in both the FVIII and FVIIIa forms caused discrepancies observed in FVIII assays.
Conclusion: Our results indicate that FVIII genotypes causing destabilization of both FVIII and FVIIIa may be associated with discrepant FVIII:C values and misdiagnosis of the severity of HA.

PB 1.55-6
Functional characterization of rVIII-SingleChain and comparison with commercially available recombinant FVIII products

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CSL Behring is developing a novel recombinant human coagulation factor VIII product for the prophylaxis and treatment of acute bleeding episodes in haemophilia A patients. It is expressed in Chinese hamster ovary cells as a single-chain factor VIII molecule (rVIII-SingleChain) with a molecular weight of approximately 170 kDa. The harvests containing rVIII-SingleChain are subjected to a sequence of chromatographic and virus inactivation/removal process steps, formulated and lyophilized. The aim of the present study was the functional characterization of rVIII-SingleChain, also in comparison to commercially available, recombinant factor VIII products.
The activation of rVIII-SingleChain using thrombin was analyzed by SDS-PAGE and Coomassie Blue staining. rVIII-SingleChain fully activated by thrombin resulted in a band pattern consisting of light chain, A1-domain, and A2-domain. The pattern was comparable to activated commercially available rFVIII products.
The thrombin generation potential of rVIII-SingleChain in FVIII depleted plasma initiated by either intrinsic or extrinsic activators was investigated by a thrombin generation assay. With increasing activity rVIII-SingleChain showed a decline of the time-to-peak values and an increase of the peak height, respectively. The results were in good agreement with other commercially available rFVIII products.
The activated FVIII (FVIIIa) dependent activation of factor X by activated factor IX was analyzed by a chromogenic, FXa-specific substrate. rVIII-SingleChain revealed a time dependent increase in absorbance comparable to that of the rFVIII comparators that was leveling off at the maximum incubation period.
The inactivation of activated FVIIIa in absence or presence of activated protein C (APC)/protein S was analyzed by a one-stage FVIII clotting assay. In absence of APC/protein S the activated rVIII-SingleChain and rFVIII comparator products showed a comparable decline of the activity down to 10–20% of the initial value within a period of about 20 min. As expected, in presence of APC and protein S, an accelerated activity decrease down to 1–3% within 20 min was observed for all products investigated.
In summary, this study demonstrated the characteristics of rVIII-SingleChain concerning the activation by thrombin, the thrombin generation assay, the co-factor function of FVIIIa, and the inactivation of FVIIIa in absence or presence of APC and protein S. The comparison of rVIII-SingleChain with commercially available rFVIII products demonstrated a comparable behavior on the basis of the methods used.

PB1.56 – Tissue Factor – I

PB 1.56-1
Platelets do not express the oxidized or reduced forms of tissue factor

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Background: Tissue factor (TF) expression and activity on the platelet surface is controversial and dependent upon the laboratory and reagents used. Due to the recent characterization of two forms of TF, it can be hypothesized that these discrepant results may be due to the expression of oxidized (active) vs. reduced (inactive) TF on the platelet surface. Previous observations by our laboratory, based on flow cytometric analyses using a well-characterized monoclonal antibody (anti-TF-5) that recognizes the oxidized form of TF and functional assays, demonstrate a lack of active TF expression by unactivated or activated platelets.
Aims: The goal of this study was to extend our observations by assessing platelet TF expression using a specific polyclonal antibody and biotinylated active site-blocked factor VIIa, both of which recognize the oxidized and reduced forms of TF.
Methods: Expression of TF by platelets activated under different conditions was determined by flow cytometry. In additional experiments, active TF expression was measured using functional and immunoassays that can detect picomolar concentrations of factor Xa and factor IXa.
Results: Western blotting analyses of full-length, recombinant and natural placental TF under reducing and non-reducing conditions demonstrated that, in contrast to anti-TF-5, a sheep polyclonal antibody directed against human TF (sheep anti-hTF) recognizes both reduced and oxidized forms of TF. As we previously have shown using anti-TF-5, there is no expression of oxidized or active TF on the platelet surface. Sheep anti-hTF was used to confirm these observations and determine whether platelets express the reduced or inactive form of TF on their membrane surface. Unactivated platelets or platelets activated for 15 or 120 min with thrombin receptor agonist peptide (SFLLRN-NH_2) (100 μM) were used. Platelet activation was confirmed by flow cytometry using a specific fluorescently-labeled monoclonal antibody directed against the activation-dependent marker, P-selectin. Additional flow cytometric analyses demonstrated that fluorescently-labeled sheep anti-hTF did not react with the surface of unactivated platelets or platelets activated under either condition. This observation was confirmed using 10 nM biotinylated, active site-blocked factor VIIa: no binding was observed by flow cytometry following its detection with fluorescently-labeled streptavidin. Similarly, and consistent with these observations, no factor Xa or factor IXa generation was observed on the surface of these platelets in the presence of 10 nM active factor VIIa using highly sensitive functional assays. The specificity of these reagents and assays was confirmed in parallel experiments using lipopolysaccharide-treated monocytes as a positive control.
Summary/Conclusions: Platelets do not express either the active or inactive forms of TF.

PB 1.56-2
Inflammation and tissue factor expression by acute promyelocytic leukemia cells

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Background: Acute promyelocytic leukemia (APL) is a subtype of acute myeloid leukemia, mostly characterized by a chromosomal translocations t(15;17) resulting in creation of PML- RARα fusion protein that inhibits cell differentiation. APL cells show a high expression of tissue factor (TF) and of annexin 2, a receptor for tissue-type plasminogen activator (t-PA), which strongly increases the activity of

t-PA. The combined action of these prothrombotic and pro-fibrinolytic activities contributes to the severe bleeding complications that are characteristic for APL. Of these activities TF is the triggering factor for the complications. APL used to be invariably fatal, but introduction of treatment with all-trans retinoic acid (ATRA) and chemotherapy has considerably reduced mortality. Still, severe hemorrhage is responsible for death in 5% of the patients. APL cells express besides the inflammatory cytokines, tumor necrosis factor (TNF) and interleukin 1b, which are capable of increasing TF expression in many cell types.

Aim: The study the role of inflammation in expression of TF by APL cells.

Methods: Two cell lines were used: NB4 cells, an APL cell line containing the t(15;17) translocation; treatment with high ATRA concentrations counteracts the effect of the PML- RARα fusion protein and leads to differentiation. U937/PR9 is a myeloid cell line stably transfected with DNA encoding PML- RARα under control of the methallothionin promoter. U937/PR9 cells express PML- RARα only when treated with Zn^{2+}. We used quantitative PCR to investigate the effect on TF and TNF expression of treatment with ATRA, with inhibitors of TNF (adalimumab) of IL-1b (ANAKINRA) or with drugs that inhibit the inflammatory signaling proteins NFkB (inhibitor BAY11-7085) or p38 (SB203580, SB202190 or Birb796)

Results: Treatment of U937/PR9 cells with Zn^{2+} for 24 h induced an increase in TF (3.3 ± 1.0 – fold, mean ± SD) and in TNF (4.8 ± 1.1 – fold). Treatment of NB4 cells with ATRA reduced TF and TNF mRNA (by 95 ± 2% and 86 ± 3%, respectively), but increased IL1b mRNA. Inhibition of TNF reduced TF expression by 50%, whereas inhibition of IL1b had only a modest effect (< 20% reduction). Inhibition of the inflammatory signaling protein NFkB reduced TF mRNA and TNF mRNA (by 65 ± 10% and 78 ± 14%, respectively), whereas inhibition of p38 reduced TF mRNA (at a maximum by 38 ± 11%) and rather increased TNF mRNA. The maximal inhibitory effect of NFkB inhibition was attained within 30 min.

Conclusions: The PML- RARα fusion protein is responsible for an increased expression of TNF and TF by APL cells. ATRA-mediated differentiation of APL cells strongly reduces expression of TNF and TF. At least part of the elevated TF in APL cells seems to be due to an autocrine inflammatory stimulation by TNF, but not by IL1b. Inhibition of the signaling intermediate NFkB rapidly reduces TF and TNF expression. Clinically, inhibition of inflammatory signal transduction should be investigated as an adjunctive therapy to further reduce hemorrhagic complication in APL in the early treatment phase.

PB 1.56-3

Disulfide reduction abolishes tissue factor cofactor function

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Background: Tissue factor (TF), an *in vivo* initiator of blood coagulation, is a transmembrane protein and has two disulfides in the extracellular domain. The integrity of one cysteine pair, Cys186-Cys209, has been hypothesized to be essential for an allosteric 'decryption' phenomenon, presumably regulating TF procoagulant function, which has been the subject of a lengthy debate. The conclusions of published studies on this subject are based on indirect evidences obtained by the use of reagents with potentially oxidizing/reducing properties.

Aim: To evaluate the influence of disulfides on the TF co-factor function.

Methods: The status of disulfides in recombinant TF_{1-263} and natural placental TF in their non-reduced native and reduced forms (with or without alkylation) was determined by mass-spectrometry. Activity-based assays were performed to assess TF cofactor function and immunoassays were used to evaluate TF binding to factor VIIa.

Results: In native proteins, all four cysteines of the extracellular domain of TF are oxidized. Upon reduction, TF retains factor VIIa binding capacity but completely loses the cofactor function in both a membrane-independent fluorogenic assay using a low molecular weight synthetic substrate and a membrane-dependent extrinsic factor Xase.

Conclusion: The reduction of TF disulfides (with or without alkylation) eliminates TF regulation of factor VIIa catalytic function.

PB 1.56-4

Perioperative plasma tissue factor levels in patients with advanced coronary heart disease and heart failure subjected to off-pump coronary bypass grafting

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Background: Disturbances in the coagulation system after coronary bypass grafting (CBG) surgery is a major cause of perioperative mortality. In circulation, tissue factor (TF), a major initiator of blood coagulation, is found on the surface of activated monocytes and as a soluble protein in plasma. To date, levels of soluble TF in patients with advanced coronary heart disease (CHD) subjected to off-pump CBG has never been assessed.

Aims: We wanted to investigate plasma TF before and after CBG surgery.

Methods: The study has been approved by the institutional committee on the research ethics. Twenty eight patients with established three-vessel CHD were initially enrolled in the prospective study after the informed consent has been obtained. Twelve patients withdrew there participation, two patients were lost to follow up. Patients did not receive anticoagulants or platelet inhibitors for a minimum of 3 days prior to surgery. Blood samples were collected 2 days prior to, 1 and 17 weeks after surgery. Plasma TF levels were measured using ELISA kit (Hyphen BioMed, France), as well as plasma pro-BNP, NYHA heart failure functional class, left ventricle ejection fraction (LVEF), end diastolic volume (LVEDV), end systolic volume (LVESV) were assessed at the same times. Multiple regression analyses were performed between variables, group means were compared using t-test, two-sided $P < 0.05$ were considered significant. Data are presented as percentages, or mean ± SEM.

Results: The study included 11 males and three females aged 56.0 ± 7.9 years, with following distribution of NYHA classes (I-IV): 21.4%, 64.3%, 7.1%, 7.1%, respectively, prior to surgery (2.0 ± 0.2 on average), and 71.4%, 21.4%, 7.1%, and 0%, respectively, at 17 weeks postop (1.4 ± 0.2 on average). Average preop levels of plasma TF were 102.1 ± 8.5 pg/mL and were 133.3 ± 27.5 pg/mL in NYHA I patients, 96.9 ± 7.1 pg/mL in NYHA II, and 78.5 ± 20.7 pg/mL in NYHA III/IV, with a strong negative correlation between preop NYHA class and plasma TF levels ($R = -0.568$, $P = 0.034$). Preop LVEF, LVESV, LVEDV and pro-BMP levels correlated well with NYHA class. After surgery, TF plasma levels temporarily decreased to 87.0 + 5.2 pg/mL ($P > 0.05$) after 1 week and came back to the preop levels after 17 weeks postop. Noteworthy, we found strong positive correlation between plasma TF levels prior to surgery with plasma TF levels 1 week postop ($R = 0.671$, $P = 0.009$) and 17 weeks postop ($R = 0.737$, $P = 0.003$).

Conclusions: Preoperative values of plasma TF correlates well with short-term and long-term postoperative TF values as well as clinical parameters of heart failure in patients with advanced CHD subjected to CBG surgery.

PB 1.56-5

Eicosapentaenoic acid and docosahexaenoic acid suppress the release of tissue factor as cell-derived microparticles, from cancer cell lines

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Background: Recent studies suggest that treatment of cancer patients with the polyunsaturated omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) may have beneficial influence. EPA and DHA have been reported to downregulate pro-inflammatory and pro-angiogeneic factors and have been shown to inhibit the growth of cancer cells *in vitro*. Cell derived microparticles have been implicated in the pathogenesis of a number of chronic diseases and in particular, tissue factor (TF)-bearing microparticles have been implicated in the promotion of cancer-related venous thromboembolism. However, the beneficial potential of EPA and DHA treatment on the release of TF as cell derived microparticles has not been investigated.

Aim: In this study, we have examined the influence of EPA and DHA supplementation on the release of TF-microparticles and the associated procoagulant activity in cancer cell lines from five different tissues.

Methods: Five cells lines representing pancreatic (AsPC-1), breast (MDA-MB-231), colocarcinoma (Caco-2), ovarian (SKOV-3) and melanoma (A375) cancers were cultured in the recommended complete media and were supplemented with EPA (0–90 mg/mL) or DHA (0–60 mg/mL). The conditioned media were harvested at intervals for up to 7 days. Microparticles were isolated from the conditioned media by ultracentrifugation and the density of microparticles determined using the Zymuphen microparticle assay. Microparticles were resuspended in PBS and the microparticle-associated TF antigen and activities were measured using an ELISA and a thrombin generation assay, respectively. Cell numbers were determined by crystal violet staining. Total cell surface TF antigen was analysed by flow cytometry and ELISA. Analysis of the incorporation of EPA and DHA into the cells and microparticle membranes was carried out by tandem gas chromatography-mass spectroscopy.

Results: Incubation of cells with a low dose of EPA (30 μg/mL) or DHA (10 μg/mL) resulted in increased release of microparticles from all the cells examined. In contrast, both the total concentration of the released TF and the adjusted values, calculated as the ratio against the respective microparticle densities, were reduced in the majority of the cell line examined. Treatment of cells with EPA or DHA also resulted in the suppression of cell proliferation in an EPA/DHA concentration-dependent manner, in all the cell lines tested. Furthermore, the reduction in cell numbers correlated with the amount of EPA and DHA incorporated into cell membrane but was cell-type dependent. However, increased levels of cell death, together with augmented TF release were observed at higher concentrations of omega-3 fatty acids.

Conclusions: Our studies suggest that omega-3 fatty acids may have anti-inflammatory properties which may in turn alter the procoagulant potential of cancer cell-derived microparticles. Our findings agree with previous data suggesting that these fatty acids may also be anti-proliferative, and at higher concentrations they may induce cell death. Throughout this study, we have used concentrations of fatty acids which encompassed those that may be detected as a consequence of an omega-3-rich diet. Therefore, omega-3 fatty acids may have an anti-inflammatory influence which may be helpful in the prevention of thromboembolism in susceptible individual including cancer patients.

PB 1.56-6

Plasma tissue levels and risk factors for cardiovascular disease in the cancer (EPIC)-Italy cohort

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Background: Tissue factor activity has been associated with risk factors for cardiovascular disease. However few studies have evaluated such an association for circulating levels of TF. We evaluated the association between plasma TF and risk factors for cardiovascular disease in a random subcohort of four out of five of the (EPIC)-Italy cohort.

Materials and Method: We measured TF in 834 men and women, in citrate plasma samples collected at baseline (1993–1997) and stored in liquid nitrogen.

TF levels was measured by ELISA (IMUBIND, TF ELISA, Instrumentation Laboratories, Milan, Italy). The association between TF levels and cardiovascular risk factors was evaluated by linear regression and expressed as β and 95% CI.

Results: The levels of TF in our cohort of apparently healthy subjects ranged from 0.070 to 2318.0 pg/mL (median: 310.7, interquartile range: 192.2–491.2 pg/mL). TF levels were significantly lower in women (β −131.62; 95%CI −174.36, −88.88) and they were inversely associated with HDL-cholesterol (β −3.19; 95%CI −4.91, −1.47)) and positively associated with triglycerides (β 0.76; 95%CI 0.43, 1.10), insulin (β 5.63; 95%CI 2.52, 8.73) and waist circumference (β 3.19; 95%CI 1.39, 5.00).

Conclusions: Our data further support the evidence for a link between TF levels and risk factors for cardiovascular disease in the population. High TF levels could represent a link between metabolic risk and cardiovascular disease.

PB1.57 – Fibrinogen/Fibrin – II

PB 1.57-1

Dysfibrinogenemia associated with obstetric complications in four unrelated female patients

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Background: Women with dysfibrinogenemia are at risk of vaginal bleeding, recurrent miscarriages, placental abruption, fetal death, postpartum haemorrhage and so on, although the incident rate is relatively lower than that in women with afibrinogenemia or hypofibrinogenemia.

Aims: Our study is to investigate the obstetric complications occurred in four unrelated female patients with dysfibrinogenemia.

Methods: The antigen level (Fg:Ag) and activity level (Fg:C) of fibrinogen in plasma of the patients were detected by immunoturbidimetric assay and clauss assays, respectively. Thromboelastogram (TEG) test was performed to evaluate the comprehensive coagulation status of patients. PCR amplification and direct sequencing technology were used to identify the mutations in these patients. Aα, Bβ and γ chains of fibrinogen were detected by Western blot. Fibrinogen polymerization and fibrinolysis measurement were applied to evaluate the function of fibrinogen in patients.

Results: Proband A experienced vaginal bleeding twice during her pregnancy, while proband B had indication of threatened premature labor. Proband C, gravida 2 para 1, suffered fetal death after her seven-month pregnancy and proband D, gravida 4 para 1, underwent

fetal death and abnormal embryo growth throughout her life. Fg:C in them were 0.57, 0.84, 0.22 and 0.46 g/L, respectively. The ratio of Fg:C and Fg:Ag was < 0.5 in all four probands, so they were diagnosed as dysfibrinogenemia. Since Fg:Ag in proband C was < 1.5 g/L, so she was diagnosed as hypodysfibrinogenemia. CI value in TEG test in proband A and B were both within the normal reference range, while that in proband C and D were lower than the low limit of normal reference range, with the value of −13.1 and −6.3, respectively, which was consistent with their severe clinical manifestations. Heterozygous AαArg16Cys, AαPro18Leu, γAla327Thr and γTrp208Leu were identified in four probands, respectively. Western blot showed normal molecular weight of three chains in probands except proband C, who had abnormal glycosylated γ chain caused by γAla327Thr mutation. In the fibrinogen polymerization measurement, the take-off time of proband A, B and D was prolonged compared to that of normal pooled plasma, suggesting impaired fibrinogen polymerization caused by these mutations. Fibrinolysis rate in these four probands was much slower than that in normal pooled plasma. At the end of fibrinolysis test, only the fibrin formed by proband D can be dissolved completely with the addition of plasminogen and uPA, with the dissolving rate of 100%, while it was 20.8% and 26.7% in proband A and B respectively, and 0% in proband C, indicating the fibrinolysis defects in them.

Conclusion: Dysfibrinogenemia patients caused by heterozygous AαArg16Cys, AαPro18Leu, and γTrp208Leu mutations may have obstetric complications due to impaired fibrinogen polymerization, while γAla327Thr mutation may lead to the generation of insoluble embolus, causing fetal death during pregnancy in affected women. TEG was applied in our study, first highlighting its sensitivity and accuracy in predicting the severity of obstetric complications and its vital role in guiding the replacement therapy in pregnant women with dysfibrinogenemia.

PB 1.57-2

Optimisation of fibrin clot microstructure: mechanical and microstructural properties of fibrin clots, from incipiency to the acquisition of haemostatic functionality

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Background: Fractal analysis of the mechanical properties of whole-blood clots provides a method of quantifying clot microstructure of the incipient clot in terms of a fractal dimension, *df*, which can be used as a functional biomarker of haemostasis [1]. A recent haemorheological study of healthy volunteers (n = 52) showed a narrow range of d_f for healthy blood (= 1.74 ± 0.04) and significant correlations of d_f ($P < 0.005$) with fibrinogen concentration [2].

Aims: The aims of the present study were to investigate the possibility that the narrow range *df* encountered in healthy volunteers represents a highly efficient mechanism of clot assembly, in terms of the amount of polymerised mass required to establish a functional network capable of achieving haemostatic functionality.

Methods: Concurrent rheological measurements and Confocal Laser Scanning Microscopy (CLSM) were performed on fibrin gels formed by polymerisation of Fibrinogen (10 mg/mL) in the presence of a range of thrombin (0.02–0.2 NIH Units/mL). The latter technique provides the high speed imaging during fibrin network formation necessary for real time visualization of microstructure during the early stages of polymerization and provides assessments of the instant at which the fibrin networks acquire sufficient mechanical rigidity to withstand motion (i.e. become static).

Results: The incipient clot formed at 0.02 NIH/mL is characterized by the lowest value of *df* (1.82), indicating that its fibrin network is relatively open and tenuous whereby the incipient gel formed at 0.2 NIH/mL is characterized by a higher value of *df* (2.03), indicating the formation of a far tighter, more densely structured fibrin network. Assessment of the CLSM images showed that, in the case of high thrombin levels, approximately 95% of the mass required to perform haemostatic functionality is present at incipiency. This is in stark contrast to clots formed at low thrombin levels, where < 20% of the mass required to perform haemostatic functionality is present at incipiency. In the latter, it is evident that a significant part of the incipient clots rapid growth is attributable to its ability to scavenge additional polymerized mass by incorporating adjacent, pre-existing clusters.

Conclusions: The clots formed at relatively low levels of thrombin may represent the optimal mechanism for the efficient construction of a functional clot insofar as (i) the incipient clot can be established in the most parsimonious manner in terms of the polymerized mass required and (ii) the clot is capable of rapid enhancement to attain sufficient elasticity to withstand motion. Large levels of thrombin result in incipient clots which are relatively inefficient, in the sense that their production involves the incorporation of extravagant amounts of mass and results in extravagant levels of elasticity. The consequences of such clots forming must be assessed in terms of their resistance to lysis and fluid mechanical stresses.

References:

1. Weisel (2010); Blood: 116(17) 3123–3214.
2. Evans et al (2010); Blood: 116(17) 3341–3346.

PB 1.57-3

Investigation of exogenous fibrinogen substitution on fibrinogen synthesis in a chronic pig model of blunt liver injury

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Background: The early application of fibrinogen concentrate has been shown to be effective to reduce blood loss in trauma-induced coagulopathy. As fibrinogen is an acute phase protein with proinflammatory properties, a potential influence of exogenous fibrinogen substitution on endogenous fibrinogen synthesis may have important consequences for immune response.

Aims: In a coagulopathic pig model with blunt liver injury we investigated the effects of fibrinogen substition on endogenous fibrinogen synthesis over 24 h.

Methods: After local ethical approval this study was performed in 20 anaesthetised male pigs. After cannulation, haemorrhagic shock and grade III blunt liver injury were induced. A 10 min shock phase was followed by re-transfusion of washed red blood cells to avoid early death from severe anaemia. Subsequently animals were randomized to receive either fibrinogen (100 mg/kg; F-group, n = 10) or normal saline (NF-group, n = 10). Plasma fibrinogen (both Clauss method and porcine specific ELISA), synthesis of fibrinogen mRNA (rtPCR from liver tissue), thromboelastometry (ROTEM), and thrombin generation were monitored for 24 h and blood loss was measured. Statistical analysis was performed using ANOVA with Tukey *post hoc*. A $P < 0.05$ value was considered as statistically significant.

Results: Haemorrhagic shock and trauma caused a significant alteration of coagulation parameters, as indicated by a prolongation of ROTEM clot formation (baseline: 45 ± 9 s; post-injury: 130 ± 21 s) and prothrombin time (baseline: 9 ± 1 s; post-injury: 18 ± 2 s). Correspondingly, clot strength (baseline: 72 ± 4 mm; post-injury: 48 ± 4 mm) and concentrations of fibrinogen (baseline: 150 ± 28 mg/dL; post-injury: 43 ± 5 mg/dL) were significantly reduced ($P < 0.05$ for all). The substitution with fibrinogen restored clot strength (63 ± 3 mm), shortend clot formation time (70 ± 11 s) and was associated with reduced blood bloss (1062 ± 216 mL) compared to

non-treated animals (1643 ± 244 mL, $P < 0.05$). Although fibrinogen supplementation initially increased plasma fibrinogen levels (Clauss-method) as expected, a significant decline in plasma fibrinogen was observed in the 360 min following fibrinogen administration, although major blood loss was already terminated 120 min post-injury. Correspondingly, the mean difference in fibrinogen levels between treated and non-treated animals decreased over time. In contrast, both ELISA and rtPCR analysis revealed an increase of fibrinogen (starting 6 h post-inury) and mRNA levels (starting 2 h post-injury), respectively. The levels of the proinflammatory cytokines, interleukin-6, and tumor necrosis factor-α showed a modest increase over time, but did not significantly differ between the groups. Aside, only small effects of fibrinogen on thrombin generation were detected.

Conclusions: The substitution of exogenous fibrinogen has no impact on endogenous synthesis of fibrinogen nor on the inflammatory response, and is not associated with adverse events. Despite termination of major bleeding the constant gradual reduction in plasma concentrations of fibrinogen over time suggests that exogenously provided fibrinogen might be metabolised. The clinical importance and the pathway of clearance need further investigations.

PB 1.57-4

Effects of homocysteine-thiolactone on fibrin networks

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Background: Hiperhomocysteinemia has been proposed as an independent risk factor for vascular disease. Non enzymatic chemical reactions between biological molecules and homocysteine or homocysteine-thiolactone (HTL) are named S or N-homocysteinylation. HTL reacts with proteins by acylation of free basic amino groups; in particular, the epsilon-amino group of lysine residues form adducts and induces structural and functional changes in plasma proteins.

Aim: To study the effects of HTL on plasma fibrin networks.

Methods: Normal human plasma was incubated with HTL solutions (final concentrations: 0 – 100 – 500 and 1000 μM); 18 h, 37 °C. Saline solution was used as control. Determinations of protrombin time (PT), activated partial tromboplastin time (APTT) and thrombin time (TT) were assayed according to established methods. In order to perform fibrinoformation studies, clots were generated by adding thrombin (0.05 IU/mL) and calcium chloride (20 mM) to the preincubated plasma. The kinetics was evaluated measuring optical density (OD) at 405 nm every 1 min up to constant values. Assays were carried out in polystyrene strips. The sigmoid curves obtained (OD vs. time) were characterized by three parameters: lag phase, maximum velocity achieved and final network OD. All assays were performed in quadruplicate. Suitable fibrin networks were evaluated by electronic microscopy. Each network was characterized measuring number of fibers per field, network percentage (ratio between total surface of the fibers and the total field area x 100), and width and length of the fibers.

Results: HTL significantly prolonged global coagulation tests in concentration dependent manner respect to control. For HTL (1 mM) the increases were 7.3%, 14.5% and 7.2% for PT, APTT and TT respectively. Fibrinformation kinetic parameters showed statiscally significant differences between HTL treated plasma respect to control in concentration dependent manner. In particular, with HTL 1 mM, lag phase: 19.30 ± 0.88 vs. 11.58 ± 0.22 min; V_{max}: 0.044 ± 0.005 vs. 0.087 ± 0.004/min and final network OD: 0.884 ± 0.009 vs. 0.939 ± 0.011; in all cases, $P < 0.01$. Electronic microscopy analysis showed that HTL related networks were more compact (network%: 81 ± 7 vs. 63 ± 8; number of fibers/field: 61 ± 7 vs. 39 ± 4) and shorter than control (length: 0.82 ± 0.20 μm vs. 2.87 ± 0.83 μm). No significant differences in fibers width were observed.

Conclusions: N-homocysteinylation process modifies the structure of fibrin networks and alters the kinetics of fibrin formation.

PB 1.57-5

The clinically asymptomatic dysfibrinogenemia (FGG H103N) is associated with abnormal polymerization but no thrombin generation impairment

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Background: Dysfibrinogenemia is a rare disease characterized by an abnormal fibrinogen present in plasma with a normal amount but an abnormal function. It is due to occurrence of a mutation of one of the three coding genes, FGA, FGB and FGG. Moreover fibrinogen has been shown to potentiate thrombin generation when using a fluorogenic thrombin substrate (Hemker HC and coll., Pathophysiol Haemost Thromb 2003).

Aims: To look for potential implication of fibrin polymerization in the facilitating effect of fibrinogen on thrombin generation in a new case of dysfibrinogen.

Methods: Plasma fibrinogen was measured by Clauss functional and immunonephelometric assays. Fibrinogen was extracted from plasma by Kazal precipitation and further studied by SDS PAGE, fibrinopeptide liberation by thrombin, repolymerization of fibrin monomers, facilitating effect on thrombin generation. For this last technique, the thrombin generation was evaluated by calibrated automated thrombinography in presence of fibrinogen depleted normal plasma, 1 g/L patient fibrinogen, 4 μM phospholipids and 5 pM tissue factor.

Results: A case of familial dysfibrinogenemia was discovered in a 9-year old patient presenting on emergency ward with a Human Herpes Virus 6 infection. Symptoms (fever and lymphopenia, late cutaneous eruption) were spontaneously regressive. A clear discrepancy was found between decreased plasma coagulable fibrinogen concentration (0.60 g/L, normal values = 1.8–4.1 g/L) and antigenic concentration (2.9 g/L, 1.80–3.5 g/L). These values were confirmed several months after initial diagnosis and found also in the mother. APTT and PT were slightly prolonged; reptilase time was prolonged over 120 s. None of both affected family members presented any thrombotic or hemorrhagic features, even following several dental extractions for the child and a breast surgery for the mother. Sequence analysis revealed a heterozygous C2587>A mutation of the gene coding for the gamma chain (FGG) in both affected family members, leading to a p.H129N mutation in the native protein (His103Asn in the circulating protein). Gamma chain appeared as a duplicate on electrophoresis under reducing conditions, with similar amounts for each band. Fibrinopeptide liberation was found normal. However patient's fibrin monomers were not able at all to repolymerize as compared to controls. The effect of extracted fibrinogen on thrombin generation was similar to controls.

Summary/Conclusions: A case of gamma chain mutation has been found in an asymptomatic patient and his asymptomatic mother. It is associated to the expression of two gamma chains found in plasma with similar amounts. The heterozygous mutation is awaited to change a His by an Asn in the coiled coil portion and give rise to a NXS sequence, a potential new glycosylation site (asparagine-X-serine). Such a glycosylation could explain the clear electrophoretical mobility difference. The only noticed functional impairment is a deficient monomer repolymerization, as often found in case of gamma chain impairment. It could be due to abnormal steric interactions of monomers with each other. The normal capacity of dysfibrinogen to enhance thrombin generation is not in favour of a role of monomer polymerization in facilitating thrombin generation.

PB 1.57-6

A new automated D-Dimer assay optimized for minimizing interferences to heterophilic antibodies

Esteve F

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D-Dimers are the terminal products of stabilized fibrin degradation. D-Dimer assays represent an important aid in the diagnosis of the exclusion of venous thromboembolic disease. Immunoassays, which are commonly used in diagnostic testing, may be prone to interferences with heterophilic antibodies when present in patient plasma.

The prevalence of these interferences with D-Dimer assays in the diagnosis of VTE is low but may cause an overestimation of the result.

The new STA® – Liatest® D-Di PLUS has been developed, based on the same formulation as the well-known STA® – Liatest® D-Di with an improvement aimed at minimizing these interferences.

The dosage buffer includes a blocking agent targeting heterophilic antibodies, therefore minimizing the interference toHAMAand eliminating the interferences to Rheumatoid Factor (RF) up to 1000 IU/mL.

The level of RF which does not interfere has been determined with plasma RF levels to be < 1000 IU/mL. This limit has been confirmed based on a regression study using serial dilutions of plasma with RF using a plasma pool with a D-Dimer concentration of 1.0 µg/mL FEU. Four patient plasmas of known RF concentration were also analyzed and correlated using an ELISA assay (which is insensitive to RF) and STA®-Liatest® D-Di Plus.

In addition, a correlation study on 200 plasmas with D-Dimer levels ranging from 0.28 to 18.21 µg/mL FEU has been carried out with STA®-Liatest® D-Di PLUS and STA®-Liatest® D-Di. Results obtained are as follow: $r = 0.998$, slope = 1.006 and intercept = −0.057.

In conclusion, the STA® – Liatest® D-Di PLUS performs as well as STA® – Liatest® D-Di with additional benefits as it is insensitive to rheumatoid factor levels up to 1000 IU/mL.

PB1.58 – Other Coagulation Factors – I

PB 1.58-1

A platelet-targeted factor VIIa. XTEN fusion protein with increased circulating half-life and improved clotting activity

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Background: Recombinant activated factor VII (rFVIIa) is used for treatment of bleeding in hemophilia A and B patients with inhibitors. Because of its short circulating half-life of 2–3 h and low binding affinity to activated platelets, rFVIIa is primarily limited to on-demand treatment and often requires repeated dosing for efficacy. A long-lasting and more potent form of rFVIIa will significantly improve the clinical management of hemophilia patients with inhibitors and the prevention and treatment of bleeding episodes.

Aims: To develop a rFVIIa variant using a strategy that combines half-life extension and activity improvement. The approach is to extend the circulating half-life of rFVIIa by fusing with XTEN, a hydrophilic and unstructured polypeptide that increases the hydrodynamic radius of proteins, while enhancing the coagulation activity by targeting it to platelets with a single chain fragment variable region (scFv) that binds to receptor αIIbβ3 with high affinity.

Methods: rFVIIa variants were expressed in HEK293 cells by transient transfection and purified from conditioned media. The clotting activity of purified rFVIIa variants was measured by soluble tissue factor-dependent prothrombin time (sTF-PT) assay, thrombin generation assay (TGA), and rotational thromboelastometry (ROTEM). The pharmacokinetics in hemophilia A mice was determined using sTF-PT assay. Effect of rFVIIa variants on human platelet clearance was evaluated in human platelet transfused NOD/scid/gamma (NSG) mice.

Results: rFVIIa-$XTEN_{288}$, which was produced by recombinantly fusing the XTEN sequence of 288 amino acids into rFVIIa, was found to have a terminal half-life of about 9 h in hemophilia A mice, representing an 8-fold increase in half-life compared with rFVIIa. However, the activity of rFVIIa-$XTEN_{288}$, based on the whole blood ROTEM assay, was lower than that of rFVIIa. To improve its activity, we incorporated a platelet-targeting scFv into rFVIIa-$XTEN_{288}$. This scFv was derived from a monoclonal antibody that specifically recognizes the human platelet receptor αIIbβ3. When fused to rFVIIa, the scFv increased binding of the protein to platelets with no detectable effect on platelet activation and clearance *in vitro* and *in vivo*. Furthermore, we identified an optimal configuration that allows recombinantly fusing both the $XTEN_{288}$ and the platelet-targeting scFv into rFVIIa to obtain a platelet-targeted rFVIIa–XTEN fusion protein with enhanced binding to platelets, and more importantly, with a significant increase in clotting activity compared to rFVIIa, as measured by TGA and ROTEM assays.

Conclusions: Combining XTEN and platelet-targeting is a novel approach that results in marked improvements in both the circulating half-life and clotting activity of rFVIIa. Our results demonstrate the potential for developing a long-lasting, platelet-targeted rFVIIa suitable for both on–demand and prophylactic treatments of hemophilia A and B patients with inhibitors.

PB 1.58-2

Hepatocyte growth factor down-regulates protein C inhibitor expression in hepatocytes via MEK pathway

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Background: Protein C inhibitor (PCI), a member of the SERPIN family, inhibits an anticoagulant activated protein C (APC), which inactivates blood coagulation cofactors, factors Va and VIIIa in plasma. We have shown that PCI is expressed in various human tissues, including liver and kidneys, and suppresses the tumor infiltration by inhibiting urinary plasminogen activator (uPA), and tumor metastasis by inhibiting angiogenesis. Recently, we demonstrated that PCI also plays a role as an inhibitor for hepatocyte growth factor activator (HGFA), a factor XIIa-like serine protease, which is an activator of HGF precursor to HGF. HGF plays an important role in tissue repair and regeneration, and the precursor of HGFA (proHGFA) is activated by thrombin generated at the sites of tissue injury. Thus, PCI may control liver regeneration by regulating the activation of HGF precursor by means of the HGFA inhibition.

Aim: In this study, we investigated the effect of HGF on expression of proHGFA and PCI in hepatocytes.

Methods: Concentrations of proHGFA and PCI in culture medium of primary hepatocytes or HepG2 cells were measured using specific ELISA for each protein. PCI mRNA expression in HepG2 cells was evaluated by real-time PCR analysis. Binding of HGF to c-Met, a receptor of HGF on hepatocytes, was determined by Western blot analysis using antibody to phosphorylated c-Met. The signal transduction pathway related to the HGF-induced change of PCI expression in HepG2 cells was analyzed by measuring the PCI production using specific ELISA in the presence of HGF and inhibitors of signal transduction factors.

Results: HGF showed no effect on the proHGFA production in both primary hepatocytes and HepG2 cells, but it dose-dependently reduced

the PCI production in both cells by reducing the mRNA expression of PCI. Western blot analysis showed that HGF induces c-Met phosphorylation in HepG2 cells. The HGF-induced decreased expression of PCI in HepG2 cells was restored by MEK inhibitor, but not restored by NFκB inhibitor, nor by inhibitors of various signal transduction factors such as JUN kinase, and p38 MAP kinase.
Conclusion: The present study suggests that, in liver regeneration, HGF down-regulates the expression of PCI, an inhibitor of HGFA, by means of the phosphorylation of c-Met followed by the activation of MEK, to enhance the HGFA-mediated activation of HGF precursor, which may lead to further promotion of the hepatocyte regeneration.

PB 1.58-3

Quantitative whole body autoradiography (QWBA) study on the biodistribution of a recombinant factor rVIIa linked to human albumin

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Background: The recombinant fusion protein linking the human coagulation factor VIIa to recombnant human albumin, rVIIa-FP (CSL Behring GmbH), is currently undergoing clinical investigation in the clinical trial program PROLONG-7FP.
Aim: The present study was conducted to evaluate the effects of albumin fusion on the biodistribution of rVIIa-FP.
Methods: [^{3}H]-rVIIa-FP, [^{3}H]-rFVIIa or [^{3}H]-albumin, labeled using the N-Succinimidyl [2,3,-^{3}H] propionate (NSP) method, were administered intravenously to male rats at a single radioactive dose of approximately 400 μCi/kg. Using whole-body autoradiography (QWBA), tissue radioactivity was determined up to 24 ([^{3}H]-rFVIIa) or 240 ([^{3}H]-rVIIa-FP, [^{3}H]-albumin) hours (h). In addition to full body sections, the hind limbs were separately subjected to QWBA to obtain more detailed information on the products' distribution within the bone marrow and knee joint region. In parallel, plasma, urine and feces were collected at several time points throughout the observation period to calculate excretion balance and assess physiological elimination pathways. The radioactivity associated with the [^{3}H]-labelled proteins was determined by quantitative radiochemical analysis (QRA) and high performance liquid chromatography (HPLC). The radioactivity associated with plasma, urine and feces samples was also determined by QRA. Biological activity of rFVIIa and rVIIa-FP after [^{3}H]-labeling was confirmed measuring FVII activity by means of a chromogenic assay.
Results: For both proteins, [^{3}H]-rVIIa-FP and [^{3}H]-rFVIIa, the major route of elimination was urinary excretion accounting for approx. 80% of total. While 144 h were required for the urinary excretion of 72% of [^{3}H]-rVIIa-FP associated radioactivity, equal proportions of [^{3}H]-rFVIIa radioactivity could be recovered within 24 h, indicating accelerated clearance of [^{3}H]-rFVIIa compared to [^{3}H]-rVIIa-FP. However, the biodistribution profile of [^{3}H]-rVIIa-FP and [^{3}H]-rFVIIa were comparable, both distributing predominantly into well vascularized tissues and major metabolic organs with both proteins being rapidly present within the bone marrow. Analysis of the knee joint region also confirmed distribution of [^{3}H]-rVIIa-FP and [^{3}H]-rFVIIa to synovial and mineralized joint regions where they seemed to mostly localize to the zone of calcified cartilage within the growth plate regions of long bones. The longest retention time was observed in the bone marrow and endosteum of long bones. In contrast, the biodistribution of [^{3}H]-albumin appeared to be different with only very low initial penetration of bone marrow and liver but rapid, homogeneous distribution throughout the whole body including muscle, skin and connective tissue. While both [^{3}H]-rVIIa-FP and [^{3}H]-albumin were well detectable over 72 h after administration, comparable [^{3}H]-rFVIIa derived signals could only be observed up to 24 h further supporting the extended tissue half-life of [^{3}H]-rVIIa-FP due to albumin fusion.
Summary/Conclusion: Consequently, this study shows that rVIIa-FP exhibits biodistribution characteristics comparable to competitor products but clearly distinguishes itself by its extended plasma half-life and tissue retention potentially allowing a reduction in dosing frequency leading to increased convenience and compliance in haemophilia patients with inhibitors.

PB 1.58-4

Utilization of FXIII concentrates by FXIII deficiency patients in Canadian hemophilia treatments centers: 10 years national data from the canadian hemophilia assessment and resource management system

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Background: Factor XIII (FXIII) deficiency is a rare inherited bleeding disorder affecting both females and males. It is associated with a risk of life-threatening bleeds (intracranial hemorrhage); hence, infusions of FXIII concentrate for both prophylaxis and treatment of bleeds is recommended. The Canadian Hemophilia Assessment and Resource Management System (CHARMS) tracks the use of factor concentrates (FC) by patients in hospitals and at home.
Aim: To identify the number of patients with FXIII deficiency, FXIII concentrate usage and indications over a 10 years period.
Methods: All 26 Canadian Hemophilia Treatment Centres (HTCs) received infusion data from hospitals and patients' bleed diaries. HTCs export their local CHARMS data to the national CHARMS database (CentrePoint) which is validated and analyzed.
Results: From 2001 to 2010, data for 50 patients with FXIII deficiency (mild, moderate and severe) were sent to CHARMS CentrePoint. More than half (58%) were female and 21 patients (42%) had 10 years infusions data available. The number of patients increased from 26 in 2001 to 40 in 2010. Overall, 92% of patients (46/50) were registered in the Canadian Hemophilia Registry (CHR) at the time of the infusion; that number was at 98% by 2010. Over 10 years, these patients infused a total of 7,614,500 units of FXIII concentrates (*Fibrogammin P*®). The yearly total of units infused ranged from 992,750 in 2001 to 1,234,500 in 2005 (in large part due to immune tolerance induction in a single patient) then dropped to 435,750 in 2010. At 250 units per vial, the 2010 amount corresponds to 1743 vials for 40 patients. On average, pediatric patients used two vials per infusion for prophylaxis and one for bleeding whereas adults used respectively four and two. Infusions were mostly done for prophylaxis: 56–81% total units infused yearly; 80% in 2010. Only 1–4% of FXIII concentrates was infused for bleeding (maximum 7 patients a year). One patient had infusions for immune-tolerance in 2004, 2005 and 2006; the units infused corresponded respectively to 35%, 37% and 19% overall units infused by all patients.
Conclusion: This analysis showed that these FXIII deficiency patients were mostly infused with FXIII concentrates for prophylaxis as recommended. The single digit proportions of amount infused for bleeding seems to indicate that few patients have bleeding episodes each year. The longitudinal follow up of FC utilization by these rare bleeding disorder patients was possible because of the combination of the national registry (CHR) and FC tracking system (CHARMS).

PB 1.58-5

Ex vivo factor XIII supplementation dose-dependently improves clot stability in blood samples from cardiac and scoliosis surgery patients

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Background: Excessive blood loss remains a significant problem during and after major surgical procedures. Factor XIII (FXIII) contributes to clot stability and it has therefore been suggested that supplementation with FXIII concentrate may be used clinically to improve perioperative hemostasis. However, little is known about the functional effects of FXIII supplementation in surgical patients and the relationship between dose and response. Furthermore it is not known if potential effects are maintained when FXIII is combined with other prohemostatic blood products.

Aims: We assessed the effects of increasing doses of FXIII on clot formation, – alone or in combination with fibrinogen or platelets, in blood samples from two distinctly different surgical populations, cardiac and scoliosis surgery patients.

Methods: Whole blood samples were collected immediately after surgery in cardiac surgery patients (n = 6) and scoliosis surgery patients (n = 6). All blood samples were supplemented with three increasing, clinically relevant doses (+20, +40 and +60%) of plasma-derived FXIII concentrate (Fibrogammin^{O}, CSL Behring, Marburg, Germany) alone or in combination with a fixed dose of fibrinogen concentrate (+1.0 g/L) (Riastap^{O}, CSL Behring) or fresh apheresis platelets (+92 $\times$ 10^9/L) from the institutional blood bank. Clot formation was assessed with modified rotational thromboelastometry (ROTEM®) with FIBTEM maximum clot firmness (MCF) and EXTEM clotting time (CT).

Results: Cardiac surgery patients were markedly older and had higher BMI than scoliosis patients. Postoperative platelet count was lower in cardiac surgery patients (136 $\pm$ 32 vs. 233 $\pm$ 54 $\times$ 10^9/L, P = 0.004), while fibrinogen levels (2.8 $\pm$ 0.5 vs. 2.0 $\pm$ 0.4 g/L, P = 0.019) and FXIII activity was higher (82 $\pm$ 13% vs. 62 $\pm$ 9%, P = 0.013).

Despite differences in preoperative and postoperative variables, supplementation with FXIII had comparable effects on clot formation in postoperative samples from cardiac and scoliosis patients. FXIII alone caused a dose-dependent increase in FIBTEM-MCF in both cardiac surgery patients (+17% (10–25) with the highest dose (median and interquartile range), P = 0.028) and in scoliosis patients (+24% (14–33), P = 0.028) (P = 0.71 between groups). EXTEM-CT tended to decrease in both cardiac and scoliosis surgery patients when FXIII was added (P = 0.07 both).

The effect on FIBTEM-MCF was markedly more pronounced when fibrinogen was added to FXIII (+133% (88–150) for cardiac patients and +150% (140–166) for scoliosis patients, P = 0.028 both, P = 0.69 between groups). The dose-response effect of FXIII on FIBTEM-MCF was sustained also when fibrinogen was added. As expected, addition of platelets to FXIII did not influence FIBTEM-MCF. EXTEM-CT was shorter when fibrinogen or platelets was added to FXIII compared to supplementation with only FXIII. No dose-response effect of FXIII on EXTEM-CT could be detected in the presence of fibrinogen or platelet concentrates.

Conclusions: Ex vivo supplementation with clinically relevant doses of FXIII dose-dependently improved clot stability in blood samples from different groups of surgical patients, both when given as monotherapy and in combination with fibrinogen. The effect of FXIII on clot stability and clot initiation was however markedly less pronounced than the effect of fibrinogen.

PB 1.58-6

Compositional differences in commercially available prothrombin complex concentrates

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Introduction: Prothrombin complex concentrates (PCCs) are used to manage bleeding complications with warfarin. Since PCC activation generates factor Xa and IIa, these agents may neutralize Xa and thrombin inhibitors. This study compared the composition of currently available PCC's.

Material and Method: Protein content of PCCs was measured using Lowry's method. SDS-PAGE and Western blot analysis was performed to determine the presence of prothrombin, prethrombin, and thrombin. Native and tissue factor activated prothrombin complexes were characterized using surface-enhanced laser desorption/ionization (SELDI) mass spectrometry. Tissue factor mediated thrombin generation by each PCC was studied using a fluorometric method (Technoclone, Vienna, Austria).

Results: The protein content of the PCCs ranged from 18 to 106 mg/100 U. SDS-PAGE showed multiple protein bands ranging from 15 to 250 kDa. Other PCCs contained bands ranging from 15 to 66 kDa representing albumin and other degradation products. SELDI analysis was consistent with the SDS-PAGE profile. The immunoblotting studies showed a major band 70–75 kDa (prothrombin) along with a 50 kDa band (prethrombin). Feiba® exhibited a distinct additional 37 kDa band (thrombin). SELDI analysis indicated varying amounts of prothrombin peaks. Thrombin was generated upon activation of all PCCs. Functional thrombin generation by each prothrombin complex was concentration dependant and ranged from 103 to 1044 nM/1.25 units/mL. Octaplex and Cofact produced the strongest responses whereas Beriplex and Prothromplex produced the weakest effects.

Conclusion: This study showed that at equivalent Factor IX unit potency, PCCs widely vary in their composition. Beriplex® and profilnine® were relatively purer preparations. Upon activation by tissue factor each PCC is capable of generating different amounts of thrombin. Thus, each of these products should be considered as a distinct drug and their efficacy individually determined in a given indication.

PB1.59 – Coagulation: Miscellaneous – I

PB 1.59-1

Absence of variation in tissue factor and procoagulant phospholipid activity after plasmapheresis in lung transplantation patients

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Background: Plasmapheresis (PPR) is commonly used to prevent humoral rejection in lung transplantation. The replacement of patient plasma by a human serum albumin solution is frequently used but causes transient deficiencies of coagulation factors, which could increase the hemorrhagic risk. Despite a marked decrease of coagulation factors in some cases [prothrombin time (PT) < 10% and fibrinogen < 0.1 g/L], none of the patients exhibited a hemorrhagic syndrome after PPR.

Aims: We postulated that procoagulant activity could counteract the decrease in coagulation factors. To test this hypothesis we measured tissue factor activity (TFa), the main trigger of coagulation, and procoagulant phospholipids (PPL) before and after PPR, along with

classical hemostasis tests – PT, activated partial prothrombin time (APTT), fibrinogen and D-dimers.
Methods: In 20 cases of PPR, PPL and TFa were measured. PPL activity was measured using a factor Xa-based coagulation assay in which shortened clotting times are associated with increased levels of PPL. TFa was measured in a one-stage kinetic chromogenic method. This assay measures the ability of TF-FVIIa to activate factor X to factor Xa. D-Dimers were quantified using an latex immunoassay.
Results: The median PPL level of 31 healthy volunteers was 68.4 s (range 51.2–84.2) and 0.25 pM/L for TFa (range 0.08–0.45). Before PPR, TFa and PPL activity was significantly higher ($P < 0.001$) than in healthy controls (0.76 pM/L and 44.4 s, respectively). PT, fibrinogen and D-dimer were significantly decreased after PPR and the APTT were prolonged, due to the plasma dilution. Median PT before PPR was 99%, post PPR 43%, $P < 0.001$. The median APTT ratio before PPR was 1.1, post PPR 1.78 ($P < 0.01$), median fibrinogen before PPR was 2.2 g/L, post PPR 0.74 g/L ($P < 0.001$) and median D-dimer before PPR was 0.7 μg/L, post PPR 0.4 ($P < 0.01$). The variations in TFa and PPL were not significant (TFa after PPR 0.4 pM/L, $P = 0.13$, PPL after PPR 45.1 s, $P = 0.84$).
Conclusion: The persistence of elevated levels of TFa and PPL post PPR could indicate injury of the vessel wall or stimulation of TF synthesis by the endothelium or the leukocytes. This could induce a procoagulant state which counteracts the absence of any hemorrhagic state despite the decrease in coagulation factors

PB 1.59-2
Activation of FXII-dependent coagulation pathway by hand heating

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Background: In many studies of metabolic variables, 'arterialized' venous blood is used as a proxy for arterial blood. The arterialization is usually induced by heating of the hand, which opens arterio-venous shunts. The considerable overlap between metabolic disorders, inflammation and cardiovascular disease emphasizes the potential scientific and clinical interest to determine biomarkers of metabolism, inflammation and cardiovascular risk in the same setting. Nevertheless, the possible effect(s) of the arterialization process on cardiovascular biomarkers is still unknown.
Aims: We investigated whether arterialization of venous blood by hand heating affects selected biomarkers of inflammation, coagulation, fibrinolysis and endothelial function.
Methods: Venous blood samples were drawn from the cubital vein of 10 healthy, non-fasting volunteers after the subjects rested in the supine position for 20 min. Thereafter, the contralateral hand and wrist joint were covered for 10 min. with a heating pad, in order to generate arterialized venous blood, which was drawn from a dorsal hand vein. Concentrations of albumin, C-reactive protein (CRP), tissue-type plasminogen activator (t-PA), plasminogen activator inhibitor type 1 (PAI-1), and von Willebrand factor antigen (vWF) were measured in platelet poor plasma (2000 g at 4◦) by validated assays. Thrombin generation was determined by the calibrated automated thrombogram method.
Results: The average sO_2 in blood drawn from the heated hand into a preheparinized syringe was 91.8% (SD: 3.9%) and pO_2 64.5 mmHg (SD: 16.4 mmHg), demonstrating that an arterialization of venous blood took place. The concentrations of the liver-synthesized proteins albumin and CRP were slightly but significantly lower in arterialized than in venous blood (albumin: 43.8 and 44.8 g/L, $P = 0.02$), indicating that normal haemoconcentration was reduced by the arterialization process. Concentrations of t-PA and PAI-1 did not differ between venous and arterialized blood with or without adjustment for albumin concentration. The concentration of vWF was lower in arterialized than in venous blood, but the difference was no longer statistically different after adjustment for albumin concentration. The endogenous thrombin potential (ETP, area under the thrombin generation curve) was significantly higher in arterialized blood (1929 vs. 1872 nM*min, $P = 0.02$), and the difference became even more pronounced after adjusting for albumin concentration. When the FXIIa inhibitor Corn Trypsin Inhibitor (CTI) was added before the thrombin generation test, no differences in ETP were detected between arterialized and venous blood.
Conclusions: The present results suggest that arterialization of venous blood by hand heating induces less haemoconcentration compared to normal capillary perfusion. Thrombin generation was enhanced by the arterialization process, reflected as an increased ETP. Since this effect was eliminated in the presence of CTI, we suggest that hand heating activates the FXII-dependent coagulation pathway.

PB 1.59-3
Severe factor XII deficiency presenting as over-heparinisation prior to urgent cardiac surgery for a right atrial myxoma

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Background and Aims: FXII (Hageman Factor), the zymogen form of FXIIa, is a plasma serine protease considered to play no active role in physiological haemostasis. Recent literature suggests a potential role of FXII in pathological thrombosis. Here we present the case of a patient with an incidental finding of severe FXII deficiency and its implications in the setting of cardiopulmonary bypass surgery.
Methods and Results: A 51 year old female presented with dyspnoea, cough and impaired exercise tolerance. Echocardiogram demonstrated a right atrial myxoma. The patient was admitted to a local hospital prior to transfer for urgent cardiac surgery. She was commenced on a heparin infusion and her initial coagulation screen demonstrated a markedly prolonged APTT. This was initially thought to be due to over-heparinisation as a baseline APTT had not been conducted. However, repeated testing demonstrated a persistently prolonged APTT despite heparin cessation. She was then referred to our facility where further investigations were conducted. The APTT was > 150 s (reference interval 25–35 s). The thrombin time was normal which excluded heparin contamination. Mixing studies with pooled normal plasma demonstrated a correction of the APTT to 34s, suggesting the presence of a factor deficiency. Factor assays demonstrated a FXII level of < 5% (RI 60–150%) indicating a severe deficiency. A review of the literature suggested the use of the activated clotting time (ACT) to manage the heparinisation required for cardiopulmonary bypass (CPB). Baseline ACT was markedly prolonged at 906s. Two units of fresh frozen plasma (FFP) were administered, resulting in a correction of the ACT to 204s prior to a bolus of 70,000 units of heparin being administered for CPB. ACT was measured at 30 min intervals and was 999 s until the cessation of CPB. On the completion of CPB, 800 mg of protamine was administered resulting in an ACT of 204 s. In view of ongoing mild bleeding, a further 200 mg of protamine and 2 units of FFP were administered with a resulting ACT of 190s. The patient was warfarinised post operatively and discharged when stable. Six days following discharge she represented with a syncopal episode (INR 2.7) and was found to have a pericardial effusion with compression of the left ventricle. This was drained and a decision to cease warfarin at that time was made. Two months later she presented to her cardiologist and was found to have a pulmonary embolism.
Conclusion: This case demonstrates the complexity of managing intravenous heparin in patients with FXII deficiency and highlights the importance of performing baseline coagulation studies prior to anticoagulation, as well as the challenges in managing such a patient for CPB. The administration of FFP prior to surgery helped to normalise the ACT to a measurable level initially. This case further illustrates that there may be an increased risk of pulmonary embolism in patients with Factor XII deficiency.

PB 1.59-4

Spectrum of the factor XI mutations in Chinese population and TEG apply to patients with factor XI deficiency

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Background: Factor XI deficiency is a mild tendency bleeding disorder with an autosomal heredity. There were found several mutations occur in particular ethnic groups.

Aims: Our research is to investigate the mutation spectrum of FXI in Chinese patients with FXI deficiency and analyze the TEG use in evulate the FXI deficiency patients' coagulation.

Methods: Thirty-seven unrelated patients from different places of China were enrolled in this study. We analyzed their clinical and coagulation features, and used direct sequencing of the whole FXI gene to figure out the mutations in these patients. Two patients were analyzed by TEG to evaluate their coagulation condition comprehensively. One, in particular, was monitored by TEG before and after a surgery under the infusion of FFP.

Results: Twenty-eight different sequence variants were found in 37 patients from non-consanguineous families with FXI deficiency, including 14 missense mutations、six nonsense mutations、six splice site mutations and two small deletions. These mutations concentrate on exons 7, 8 and 11,12,13. Four mutations, W228X (p.W246X)、G400V (p.G418V)、C.1136-4 delGTTG and Q263X (p.Q281X),make up more than half of all. Out of the four mutations, W263X (p.W281X) and C.1136-4delGTTG have not been reported in other population except Chinese. In the use of coagulation condition evaluate, TEG showed a sensitive result of the coagulation condition evaluation.

Conclusion: Factor XI gene mutations remain highly heterogeneous at molecular level. Chinese FXI deficiency gene mutations have its special characteristic which is different from other races. Furthermore, the TEG may work as a useful measurement in patient with FXI deficiency.

PB 1.59-5

Understanding platelet-virus interaction

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Background: Although the direct interaction between viruses and platelets was described many years ago, it is not yet clear whether platelets, by taking up viruses, contribute to the dissemination of infection, to the host antiviral defense or both. The ssRNA Coxsackievirus B (CVB), classified as an Enterovirus of the *Picornaviridae* family, represent an excellent candidate to study the platelet-virus interaction as the coxsackie adenovirus receptor (CAR) is expressed on the platelet surface, the CVB replication cycle is short (< 12 h) and platelets have the required machinery to translate constitutive mRNAs into proteins and hence, to potentially allow CVB replication.

Aims: To understand the interaction between platelets and virus and the role of platelets in viral infections.

Methods: Human blood platelets depleted of leukocytes were infected with CVB1 and CVB3 strains at 0.1–10 MOI for 1 h at 37 °C and then exhaustive washed to ensure viral removal. Supernatants and pellets were collected at different hours post-infection (hpi) in order to measure infectivity and analyze viral replication at a molecular levels. Results are expressed as mean ± SEM, $n = 5–7$, $*P = 0.05$ by ANOVA.

Results: We found that platelets interact with CVB1 and three as both RNA and a CVB1 capsid protein were detected at 24 hpi by RT-PCR and immunoblotting/immunofluorescence, respectively. Surprisingly, while CVB binding to HeLa cells was inhibited by a CAR-blocking antibody, this inhibitory effect was not observed in platelets, suggesting that CVB-platelet interaction might be mediated by platelet surface glycoproteins. Although RT-PCR amplification of intermediate negative strand was negative, implicating that platelet were not susceptible to a replicative infectivity cycle, titration assays with HeLa cells showed that infectious viral particles were detected in supernatants and pellets from CVB-infected platelets at 6, 24, 48 and 72 hpi. Flow cytometry analysis indicated that CVB binding to platelets resulted in an increased basal P-selectin expression (crucial mediator of platelet-leukocyte interaction) and phosphatidylserine exposure (phagocytosis signal for monocytes) after 1 hpi ($2.2 \pm 0.2*$ and $2.1 \pm 0.6*$ fold change vs. control, respectively) but not after 4, 24 or 48 hpi, suggesting that the augmented platelet reactivity was associated with the initial contact of platelets with CVB.

Conclusions: Our data suggest that platelets play an ambivalent role in CVB infection: on one hand, platelets might contribute to viral dissemination as they can harbor infectious CVB by several days and on the other hand, CVB enhances the exposure of surface proteins that mediate interaction with leukocytes, probably increasing platelet phagocytosis and subsequent destruction of the virus.

PB 1.59-6

Regulation of blood coagulation by poly-phosphate

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Background: Poly-Phosphate (Poly-P) is a large inorganic polyphosphate molecule localized in the dense and alpha granules of platelets. Recent studies have described initiation of the blood coagulation cascade by poly-P via activation of factor XII and pre-kallikrein.

Aims and Methods: Here, we determined the influence of poly-P on activation of blood coagulation by studying endogenous thrombin potential (ETP). To this end, we used $n = 45$ poly-P, which approximates platelet poly-P *in vivo*. Interestingly, the area under the curve (AUC) of the ETP was significantly reduced when increasing concentrations of poly-P were added to the standard plasma (unical). To further analyze this finding, we investigated the effect of poly-P in factor II-, V-, VII- and X-dependent coagulation, by engaging one-stage factor clotting assays.

Results: Factor V-dependent coagulation was prolonged in the presence of poly-P in a dose-dependent manner, whereas factor II-, VII- or X-dependent coagulation was unaffected by poly-P.

Summary: In conclusion, poly-P inhibits coagulation factor V-mediated thrombin generation. Given the storage of both factor V and poly-P in platelet granula1) and their release upon platelet activation, our present findings suggests a new potential regulatory role of platelet-derived poly-P in factor V function.

Reference:

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PB1.60 – Cancer and Thrombosis – II

PB 1.60-1

Role of Gas6 in cancer-induced venous thrombosis

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Background: Venous thrombosis is the most common morbid complication related to cancer and its treatments. The onset of cancer predisposes patients to an increased risk of developing venous thrombosis.

Growth arrest specific 6 (gas6) is a vitamin K dependent secreted protein that is expressed by different cell types and is involved in several cellular processes such as cell proliferation, adhesion and survival. Gas6 null mice are protected against life threatening thrombosis, and develop smaller thrombi in venous thrombosis models as compared to wild type mice.

Aims: The objective of the present study is to elucidate the role of gas6 in cancer-induced venous thrombosis.

Methods: To test this hypothesis, wild type (WT) and Gas6 null (−/−) mice received an intravenous injection of M27 murine lung cancer cells. After 2 weeks, WT and $Gas6^{-/-}$ mice developed end stage cancer. Venous thrombosis was induced using 10% ferric chloride. Thrombus size was measured by (i) ultrasonography measuring surface area and (ii) pathologically using clot weight. For mechanistic *in vitro* studies we co-cultured murine endothelial cells, prepared from lungs of both WT and $Gas6^{-/-}$mice, with M27 cells. RNA was extracted from endothelial cells and whole genome microarray analysis was used to identify differential gene expression induced by cancer in the presence or absence of gas6.Microarray results were confirmed using real-time qPCR, western blot analysis and ELISA.

Results: We found that thrombi induced in WT mice with cancer were larger than those induced in healthy mice by both ultrasound ($P < 0.05$) and pathologically ($P < 0.05$). Furthermore, $Gas6^{-/-}$ mice were protected against the larger thrombi induced by cancer cells. Microarray analysis revealed that 28 genes were differentially expressed in WT or $Gas6^{-/-}$ cells in the presence of cancer. Of interest, when exposed to cancer, we found that thrombospondin 2 was downregulated in WT endothelial cells and even further decreased in $Gas6^{-/-}$ cells. We confirmed these results using real-time qPCR and western blot analysis ($P < 0.05$). Preliminary results also confirm that plasma levels of thrombospondin 2 levels is lower in $Gas6^{-/-}$ mice with cancer as compared to WT mice with cancer.

Summary/Conclusions: These results suggest the possibility that gas6, through the downregulation of thrombospondin 2, has a pathophysiologic role in cancer-induced thrombosis.

PB 1.60-2

Acceleration of lung metastasis of melanoma cells in mice fed a high-fat diet: changes in circadian expressions of thrombotic factors and cell-adhesion molecules through day-night reversal feeding

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Background: Circadian clocks, which primarily comprise transcriptional/translational feedback loops involving set of clock genes, and a number of studies in mammals have indicated a connection between circadian clocks and feeding behavior, and peripheral oscillators can be strongly affected by daily feeding cycles. It is also known that there is a definite relationship between diabetes and increased risk of colon and rectal cancer, and recent studies have shown that metabolic syndrome may play a role in several cancers. Further, epidemiologic studies on large numbers of night and rotating shift workers have suggested an increase in the incidence of breast and colon cancer in these populations.

Aims: In the present study, we measured the number of metastatic colonies and expression levels of various factors including invasion, coagulation/fibrinolysis in lung of mice fed a high-fat diet under the day-night reversal schedule after administration of B16BL6 melanoma cells, to understand whether or not fluctuations of these factors might affect on cancer metastasis under the bad dietary habit.

Methods: Six-week-old wild and *clock* mutant mice were divided into each four groups and were fed a normal (5.2% fat) or a high-fat (24% fat) diet under the condition of daytime or mid-night feeding for 8–10 weeks. At 2 weeks before end of the feeding, BL16B6 melanoma cells were injected via tail vein and mice were sacrificed under anesthesia 2 weeks later, and then the number of colonies was determined. In the same time, plasma, lung, liver were recovered and, in addition to lipid level, antigens of adipocytokines, PAI-1, thrombomodulin, and gene expressions of molecular clocks (per2, bmal-1), adhesion molecules and MMP-2, were measured.

Results: The values of glucose tolerance test, fasted insulin, cholesterol, leptin in plasma from high-fat diet fed wild mice were higher than those from normal diet fed mice. The number of metastatic colonies in the lung was increased in the group of high-fat fed mice by 40% compared with that of control, regardless of its eating time. The colonies were also increased with significance in wild mice fed normal diet during daytime compared with the nighttime feeding. No difference was observed in *clock* mutant mice under the same condition. Comparing values from mice fed normal diet, those fed high-fat diet increased MCP-1 and PAI-1 in plasma, and reduced thrombomodulin in lung, and these changes were associated with disturbed expressions of per2 and bmal-1. Expressions of per2 and bmal-1 in mice fed the diet in daytime were phase advanced and those of PAI-1 and thrombomodulin were also phase shifted. Expressions of p-selectin and vcam-1 in the lung of mice increased under the same condition, though that of mmp-2 did not change.

Conclusion: These observations demonstrate that not only excess eating of high-fat diets but also irregular eating habit is objectionable to prevent cancer cell invasion, since tumor cell metastasis was accelerated by disturbed fluctuations of circadian clocks accompanied by changes in diurnal variation of thrombotic activities.

PB 1.60-3

Microvesicles bearing tissue-factor: a new potential biomarker for thrombosis in acute promyelocytic leukemia

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Introduction: Patients with hematological malignancy have a 28-fold increased risk of venous thromboembolism (VTE). Among patients with acute myelogenous leukemia (AML), the 2-year cumulative incidence of VTE is 5.2%. The induction mechanism of a hypercoagulable state is not fully understood. Multifactorial aspects such as patients' immobility, chemotherapy adverse effects or the overexpression of several procoagulant substances (i.e. tissue factor (TF)) by cancer cells are often evoked. Several studies strongly suggest that microvesicles (MVs) harboring TF may have a procoagulant role in promoting deep vein thrombosis and possibly disseminated intravascular coagulation (DIC) commonly seen in acute promyelocytic leukemia (APL)

Objectives: The aim of this study is to assess the capacity of untreated (APL) cells to shed procoagulant MVs.

Methods: APL cell lines (NB4 and HL-60) were cultured for 48 h in liquid medium at 600,000 cells/mL. Cells and MVs were separated by filtrations (Millipore 0.1-0.22-0.45-0.65 μm). The Pro-Coagulant Activity (PCA) was assessed by thrombin generation assay. Alternatively, MVs were incubated with anti-TF antibodies (10 μg/mL), with annexin V (0.5 μM) to assess the contribution of TF and phospholipids to the PCA. Alternatively, the cells were incubated with with $HgCl_2$ (an activator of TF).

Results and Discussion: NB4 cells have a high PCA mainly triggered by MVs of size under 0.45 μm. Thus, NB4 cells spontaneously release MVs of various size, which can augment TGA. By using an anti-TF antibody (HTF-1) and annexin V, we confirm that the PCA of MVs is related to the expression of active TF and PL. Interestingly, we show

that HL-60 cells have a weaker PCA since TF is mostly present in an inactive form. Moreover HL-60, do not produce MVs < 0.65 μm associated with PCA.
Conclusions: Microvesicles could have a predicting value for venous thromboembolism and DIC in patients with acute promyelocytic leukemia and could inform hematologists for the thrombosis prophylaxis.

PB 1.60-4
Pro-metastatic human breast cancer cell line, MDA-MB-231 (MDA) and platelet interacctions in an '*in vitro*' aggregation assay

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Background: Metastasis remains the major cause of cancer treatment failure. Platelets play a key role in hematogenous metastasis and contribute to this process by both thrombin-dependent and -independent mechanisms.
Aims: Our aim was to characterize interactions between pro-metastatic human breast cancer cell MDA and platelets, and compare the effectiveness of antiplatelet therapies to define strategies to study the platelet role in metastasis.
Methods: MDA were cultured at 37ºCand 5%CO_2 in DMEM containing glucose and supplemented with 1% glutamine, 1% penicilin/streptomycin and 10% heat-inactivated fetal bovine serum. Cells were scraped, washed and suspended in Ca^{2+} Mg^{2+} Tyrode's buffer 3 days after being passed.
Blood was collected from healthy volunteers who had not taken any drugs known to affect platelet function for at least 14 days prior to the study. Washed platelets (WP) in Ca^{2+} Mg^{2+} Tyrode's buffer were prepared at 2.5×10^8 platelets/mL.
The interactions between WP and MDA were measured by light aggregometry. WP were placed in a lumi-aggregometer (Chronolog), incubated for 15 min at 37 °C, stirring at 1000 rpm, with increasing MDA cells with or without citrated plasma (1/800 final dilution). The effects of heparin (1 UI/mL) and three antiplatelet drugs (100 mM aspirin, 1 nM PGE1 and NO donnor 10 μM sodium nitroprusside) were assayed. The time to produce 50% aggregation of WP was registered (T50%).
Results: MDA cells were tested for their ability to induce WP aggregation. MDA cells ($1.3\ 10^5$–$6.5\ 10^5$ cells/mL) did not produce WP aggregation when they were incubated in the aggregometer for 15 min at 37 °C in absence of citrated plasma. However when citrated plasma was added, in a very low dilution (1/800) to avoid fibrin formation, MDA cells induced WP aggregation in a concentration-dependent manner ($1.3\ 10^2$; $1.3\ 10^3$; $-1.3\ 10^4$ cells/mL). A linear relationship was obtained when T50% was plotted vs. MDA cell concentration in linear-log mode. Heparin at 1 UI/mL completely inhibited WP aggregation suggesting a mechanism mediated by thrombin generation.
When WP were treated with asprin or PGE1, T50% increased a 45% (n: 4, $P = 0.03$) and a 76% (n: 4, $P = 0.03$) respectively for $1.3\ 10^4$ MDA cells/mL, keeping the same linear-log relationship for lower MDA cell concentration. When NO donor sodium nitroprusside was assayed, WP aggregation was completely inhibited at $1.3\ 10^2$ and $1.3\ 10^3$ cells/mL and T50% was increased a 280% (n: 4, $P = 0.01$) at $1.3\ 10^4$ cells/mL.
Conclusion: In our '*in vitro*'study, MDA-MB-231 (distinguished by their invasive phenotype) were capable of producing WP aggregation in a thrombin-dependent manner at very low concentrations of clotting factors and tumour cells, revealing their thrombogenic power.
Only the antiplatelet therapy with NO donor sodium nitroprusside was effective, this finding may help to define strategies to study the platelet role in metastasis in '*in vivo*' models. Impairment of platelet function within the tumour microenvironment may provide a clinically useful approach to inhibit metastasis.

PB 1.60-5
Activation of coagulation by lenalidomide-based regimens for multiple myeloma

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Background: Lenalidomide (Len) is a structural analogue of thalidomide. Combining Len with steroids led to improved response rates in the treatment of multiple myeloma. However, deep vein thrombosis (DVT) has been noted as one of the most serious side effects with this regimen, and the mechanism by which Len-based regimens cause DVT is unclear.
Aims and Methods: We investigated the procoagulant effects of Len-based regimens, focusing on tissue factor (TF) and phosphatidylserine (PS). Because we have previously shown that thalidomide- or Len-based regimens did not induce procoagulant activity (PCA) on myeloma cells, we further examined the effects of a pharmacological concentration of Len with or without the steroid dexamethasone (Dex) and the proteasome inhibitor bortezomib (Bor), using the human vascular endothelial cell line EAhy926 and the monocytic cell lines THP-1 and U937.
Results: Cell surface PCA was induced by Dex-including regimens on EAhy926, THP-1 and U937 cells. Expression of TF antigen on the cell surface and of TF mRNA was markedly increased by Dex-including regimens in all cell lines compared to control and Len alone. Exposure of PS was modestly increased by the Len-based regimen in all cell lines. Exposure of PS was modestly increased in EAhy926 cells, and markedly increased in THP-1 and U937 cells by the Bor-including treatment. An anti-TF monoclonal antibody almost completely blocked the induced PCA.
Summary/Conclusions: When Len is given in combination with Dex, PCA may be induced on endothelial cells and monocytes through TF expression and PS exposure. In blood vessels, Len, with its antiangiogenic activity, may disturb the repair of injured endothelium by other anti-tumor agents, and make other drugs' effects stronger and prolong the induced PCA. Therefore, monitoring procoagulant markers, such as D-dimers, and including prophylactic anticoagulant strategies should be considered in Len-based combination regimens.

PB 1.60-6
Development of the overall haemostatic potential assay for murine plasma and identification of elevated fibrin and thrombin generation in tumour bearing mice

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Background: Patients with advanced malignancies have an increased risk of thromboembolism which may be linked to elevated inflammation. While mouse tumour models provide opportunities to study the underlying mechanisms, there is a need for functional assays to assess coagulation potential in mice.
Aims: We aimed to optimise the overall haemostatic potential (OHP) assay for use with mouse plasma, and to validate the assay by investigating a murine model that exhibits features of enhanced clotting associated with an inflammatory state and cachexia. We assessed changes in *ex vivo* coagulation function in Colon 26 tumour-bearing mice using fibrin and thrombin generation and related parameters.
Methods: The OHP assay was validated in mice as follows. Conditions were optimised using plasma from non-tumour bearing mice. Four groups of immuno-competent mice were then studied: i) Mice engrafted subcutaneously with murine Colon 26 (C26) carcinoma cells ($n = 16$), with 16% weight loss at 14 days post tumour implant; ii) Mice engrafted with a variant C26 tumour that does not elicit cachexia ($n = 9$); iii) Non-tumour bearing controls ($n = 14$); iv) Non-tumour bearing mice pair-fed with food intake matched to the cachectic C26 tumour mice ($n = 10$). Plasma was prepared from citrated blood

collected after 14 days. The OHP assay was used to measure overall coagulation potential (OCP), delay to fibrin clot formation and clot lysis time (CLT) in a subset of each group. A modified calibrated automated thrombogram (CAT) assay was also performed to measure mean endogenous thrombin potential (ETP) and lagtime. Plasma fibrinogen was measured by Western blot, while tissue factor pathway inhibitor (TFPI) and IL-6 levels were measured by ELISA.

Results: The OHP assay was successfully adapted for murine plasma, and tumour bearing mice were found to have elevated fibrin generation (OCP) compared with non-tumour bearing mice (0.1 arbitrary units (AU)), with a trend towards even higher OCP in cachectic (3.7 AU) than non-cachectic mice (2.8 AU). There was no significant difference in CLT between any group. Cachectic mice had an ETP of 494.3 AU which was significantly higher than non-cachectic C26 tumour bearing mice (188.7 AU). Surprisingly, delay to fibrin formation and lag-time to thrombin generation were both significantly increased in tumour bearing mice, which may be explained by significantly higher levels of TFPI. Plasma fibrinogen was higher in tumour-bearing mice, and more so in cachectic than non-cachectic mice. Circulating IL-6 was below detection in non-tumour bearing mice and just detectable in non-cachectic mice, while markedly elevated in cachectic mice at 216 pg/mL.

Summary/Conclusion: We demonstrate a novel development of the OHP assay in murine plasma and show increased fibrin generation in C26 tumour variants, while fibrinolysis was not significantly altered. The elevated coagulation potential persisted despite increased TFPI in tumour bearing mice. The cachectic C26 variant had dramatically higher levels of circulating IL-6 and demonstrated significantly elevated thrombin generation and a trend towards higher fibrin generation.

PB1.61 – Cancer and Thrombosis – III

PB 1.61-1

Pathogenesis of hemostatic abnormalities due to L-asparaginase in children with acute leukemia

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Background: L-asparaginase (L-asp) is a key drug for treating acute lymphoblastic leukemia in children. Injection of L-asp often causes hemostatic abnormalities, which are critical issues for continuing chemotherapy in these patients. L-asp-induced hemostatic abnormalities are often associated with thrombotic rather than hemorrhagic events. Therefore, prophylactic replacement of anti-thrombin (AT) is generally performed. However, this method is not supported by scientific evidence, because the mechanism underlying the hemostatic abnormalities caused by L-asp remains unclear.

Aims: In order to clarify the mechanism underlying the hemostatic abnormalities caused by L-asp, we investigated hemostatic changes in children with acute leukemia during chemotherapy including L-asp.

Methods: Eleven children with acute leukemia, who received a total of 15 courses of chemotherapy including L-asp, were evaluated for thrombin generation potential (TGP) before and after L-asp injection. To neutralize the effect of heparin contamination in blood samples obtained via central venous catheter, heparinase was added to the samples just before coagulation tests including the thrombin generation assay. The heparinase used was hep-TEM®Lyo from Finggal Link Co. Ltd. To evaluate thrombin generation, Thrombogram® and PPP reagent (phospholipid, 4 mM, tissue factor, 5pM), both from Thermo Electron Corp, were used. Endogenous thrombin potential (ETP) served as the main parameter of TGP.

Results: In general, ETP was significantly increased after L-asp injection (1410 ± 341 nM*min) as compared with before (1164 ± 180 nM*min) ($P = 0.018$), indicating that L-asp induces a thrombotic tendency. This hemostatic change was suggested to be strongly associated with decreases in both AT and protein C (PC). We examined the effects of L-asp on hemostatic abnormalities during each treatment phase. ETP was increased in three cases and decreased in 2, after L-asp injection in the induction phase ($n = 5$), indicating that contradictory hemostatic changes involving thrombosis or hemorrhage are likely to occur with L-asp in this phase. ETP was increased in nine cases after L-asp injection in the re-induction or consolidation phase ($n = 10$), indicating that thrombosis is likely to occur with L-asp in these phases. There was a negative correlation between ETP and AT ($r = -0.66$, $P < 0.001$), while ETP correlated positively with CRP ($r = 0.45$, $P = 0.02$), on multiple regression analysis. Thrombotic change was related to decreased AT and the presence of infection.

Summary/Conclusion: In children with acute leukemia, the hemostatic abnormalities caused by L-asp involved mainly a thrombotic tendency, reflecting decreased AT and PC. Infection during chemotherapy exacerbated this thrombotic tendency. Therefore, we must keep in mind a high risk of thrombosis during the re-induction or consolidation phase, especially when infection is present.

PB 1.61-2

Simple laboratory variables are prognostic factors in hospitalized cancer patients with acute pulmonary embolism

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Background: The Pulmonary Embolism (PE) Severity Index has a poor prognostic value in hospitalized oncological patients with PE, because cancer is already included as a predictor. Only limited evidences are available on the clinical impact of laboratory variables on mortality rate.

Aims: The aim of this analysis was to assess the prognostic value of the most common laboratory tests performed in oncological patients with PE.

Methods: Consecutive patients admitted to the tertiary hospital of Varese (Italy) with an objectively diagnosed PE between January 2005 and December 2009 were included. Information on clinical presentation, risk factors, blood tests, treatment and mortality rate at 1-month follow-up was collected. This sub-analysis examines the 150 oncological patients extracted from the entire population.

Results: Mean age was 69.6 (± SD 11.6) years and 84 (56%) patients were male. Most common primary sites of cancer were lung (25.2%), colon-rectal (13.3%) and bladder (8.4%). Active cancer was present in 121 patients, of whom 47 had a metastatic disease. Overall mortality rate was 20.7% (95% CI, 15.0–27.8%) at 1-month.

In the subgroup of patients with active cancer, clinical predictors and laboratory test results, significantly associated with mortality at the univariate analysis, were: age > 80 years old (29.2% vs. 11.3%, $P = 0.049$), systolic blood pressure below 100 mmHg (37.5% vs. 7.2%, $P = 0.001$), altered mental status (16.7% vs. 0%, $P = 0.001$), leukocytes > 11,000/mm^3 (60.9% vs. 31.9%, $P = 0.010$) and creatinine clearance < 30 mL/min according to MDRD formula (20.8% vs. 6.3%, $P = 0.044$); while concomitant deep vein thrombosis was a protective factor (25.0% vs. 58.8%, $P = 0.003$).

These factors were therefore tested in a multivariate Cox regression analysis. Apart from well-established clinical variables (low blood pressure and altered mental status), also leucocytosis (HR 3.01, 95% CI 1.19–7.63, $P = 0.020$) and severe renal insufficiency (HR 2.91, 95%

CI 1.04–8.19, $P = 0.043$) emerged as independent risk factors for mortality.

Conclusion: The results of this analysis suggest that laboratory parameters should be merged with clinical variables in the creation of a new prediction rule, in order to redefine the risk of death in cancer PE patients.

PB 1.61-3
Bleeding rates and thrombotic complications in patients with hematological malignancies admitted to hospital

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Background: Thromboprophylaxis is recommended for cancer patients admitted to hospital. But for patients receiving high dose chemotherapy or undergoing allogeneic stem cell transplantation (SCT) for hematological malignancies, the risk of bleeding might outweigh the risk of thrombotic events. The incidence, management and outcomes of these complications are poorly studied in this patient population.

Aims: The objectives of this study are to determine (i) the bleeding rates and severity in the hospitalized leukemia/bone marrow transplant (LBMT) population; (ii) the rates and clinical outcomes of patients with symptomatic venous thrombotic events (VTE), including catheter-related thrombosis (CRT); and (iii) the prevalence of anticoagulant thromboprophylaxis.

Methods: A retrospective review of all admissions to the Vancouver General Hospital LBMT unit from January 1, 2010 to June 30, 2010 was performed. Only the first admission for each patient during the study period was included. Admissions to other hospital units or those lasting < 48 h were excluded. Data were extracted from chart review using standardized forms. Only objectively confirmed VTE were included. All bleeding events were graded according to ISTH criteria. Each patient's records were evaluated independently by two study members.

Results: One hundred and sixty-six patients with one or more admission during the study period were included. Median age was 52 years (range 18–76 years), 61.4% were male, and the median length of hospital stay was 19 days (range 2–182 days). Underlying diagnosis was acute leukemia in 51.8%, lymphoma in 28.3%, multiple myeloma in 9.0%, chronic myeloid leukemia 2.4%, myelodysplastic syndrome 2.4% and other 6.0%. VTE risk factors included prior VTE in 16 patients (9.6%), and indwelling central venous catheter in 145 patients (87.4%). Reason for admission was administration of high-dose chemotherapy in 39.2%, allogeneic SCT in 16.3%, autologous SCT in 16.9%, immunosuppressive therapy in 1.2% and acute medical management in 26.5%. At least one dose of VTE prophylaxis was prescribed during 11 (6.6%) admissions (seven received unfractionated heparin, four received low molecular weight heparin) and veno-occlusive disease prophylaxis with intravenous unfractionated heparin was prescribed in 29.5% of admissions. Bleeding events occurred in 31 patients (19%); 12 had major bleeding episodes and 13 had clinically relevant non-major bleeding episodes. Bleeding was associated with organ dysfunction in five patients and was fatal in two patients. 9/72 patients (12.5%) who received any anticoagulant compared with 23/98 patients (23%) without anticoagulation experienced bleeding. Thrombotic complications occurred in six patients (4%), including DVT and/or PE in three patients, CRT and PE in one patient, splenic infarct in one patient and superficial thrombophlebitis in one patient; only one patient received thromboprophylaxis prior to VTE diagnosis. Four patients were started on therapeutic anticoagulation during admission for documented (4/6) thrombotic events. Therapeutic anticoagulation was associated with a major bleeding episode requiring anticoagulant discontinuation in two patients.

Conclusions: Patients undergoing aggressive chemotherapy or SCT for treatment of hematological malignancies have high rates of clinically significant bleeding, even in the absence of anticoagulants. Further studies evaluating the risks and benefits of thromboprophylaxis in this patient population are needed.

PB 1.61-4
Management and outcomes of venous thromboembolic events in patients with concomitant cancer-associated thrombocytopenia: a retrospective cohort study

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Background: Hospitalized patients with active malignancy have an increased incidence of both venous thromboembolic events (VTE) and thrombocytopenia. Thrombocytopenia increases the risk of hemorrhage but does not confer protection from VTE. The optimal management of VTE in patients with thrombocytopenia is not established, and outcomes in such patients are largely unknown.

Aims: Our Objectives were to (i) describe a cohort of hospitalized cancer patients diagnosed with VTE in the setting of concomitant thrombocytopenia, (ii) describe management strategies implemented in this cohort (iii) determine incidences of and factors associated with subsequent thrombotic and hemorrhagic outcomes.

Methods: Retrospective cohort study of all inpatients admitted to the University of Alberta Hospital's hematology service, or the Cross Cancer Institute (Edmonton, AB) between 2006 and 2011. All adult patients (> 17 years) with imaging-confirmed VTE (excluding superficial venous thrombosis) and concomitant thrombocytopenia (plt < 100) were included. Primary outcome of interest was the composite of hemorrhagic and thrombotic events within 3 months of the index VTE.

Results: Seventy-four patient charts were reviewed. Mean (SD) age was 59 (15) years, 35 (47.3%) were male, 56 (75.7%) had hematologic malignancy, and 48 (64.9%) were undergoing chemotherapy at the time of the event. Median [IQR] platelet count at the time of VTE was 49[28,78] with median[IQR] nadir platelet count of 15 [8,46]. Thrombocytopenia was attributed to cytotoxic chemotherapy in 26 (35.1%) patients, marrow involvement with cancer in 15 (20.3%), a combination of marrow involvement and chemotherapy in 19 (25.7%), immune-mediated mechanisms in 4 (5.4%) and an unknown or other etiology in 10 (13.5%). Prolonged thrombocytopenia (> 1 month duration) was observed in 50 (67.6%) patients. Fifty-two patients (70.3%) had proximal limb deep vein thromboses, of which 21 were lower extremity, 31 were upper extremity, and 28 were catheter-associated. Twenty-seven patients (36.5%) had pulmonary embolism, and 3 (4.1%) patients had VTE at other sites. Patients were divided into treatment categories: 17 (23.0%) patients did not receive any anti-thrombotic therapy, 30 (40.5%) completed a minimum 3-month course of full-dose antithrombotic therapy, and 27 (36.5%) received a modified course of antithrombotic therapy. Over the 3-month period following the index VTE, 29 (39.2%) patients experienced a total of 36 events; 23 (31.1%) patients experienced a second symptomatic thrombotic event, and 13 (17.6%) suffered a clinically significant bleeding event (WHO class ≥ 2). Compared to those not suffering a hemorrhagic or recurrent thrombotic event, those who experienced an event were less likely to have received a full course of antithrombotic therapy (13.8% vs. 57.8%, $P < 0.001$), and were more likely to have received a partial course of therapy (55.2% vs. 24.4%, $P = 0.002$). Other factors associated with reaching the primary endpoint were duration of thrombocytopenia > 1 m (82.8% vs. 57.8%, $P = 0.04$), and hematologic malignancy (96.6% vs. 62.2%, $P < 0.001$). There were a total of 23 deaths; none were attributed to hemorrhage, and two were due to pulmonary embolism precipitating respiratory failure.

Conclusions: There remains considerable variability in the management of VTE in the setting of thrombocytopenia. This population carries a high risk of both recurrent thrombosis and hemorrhage. Future studies should aim to determine the optimal management of this complex clinical situation.

PB 1.61-5

Patients with advanced stage germ cell tumors have a high risk of thromboembolic events

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Background: Venous and arterial thromboembolism (TE) accounts for significant morbidity and mortality in cancer patients[1][2]. Rates of TE have been reported ranging from 0.9% to 28.0%, depending on the population and malignancy[3]. In patients with germ cell tumors, studies have reported a 4.0–8.4% risk of TE immediately following administration of chemotherapy with cisplatin-containing regimens[4][5].
Aims: Our population of germ cell tumor patients comprises a higher-risk population with advanced disease, many of whom receive high-dose chemotherapy and peripheral blood stem cell rescue. To more completely understand the role played by advanced germ cell tumors in causing TE events in addition to the risk presented by administration of chemotherapy, we sought to evaluate these events in patients with long-term follow-up.
Methods: Data were collected through a retrospective chart review of germ cell tumor patients with regard to patient, disease and treatment variables, as well as clinical TE events, from diagnosis until the occurrence of a TE event (ranging from TE on presentation to almost 20 years after diagnosis). A logistic regression model was fitted to determine variables that predispose patients to TE.
Results: Forty-four consecutive patients visiting our germ cell tumor clinic between 11/05/2012 and 1/22/2013 were selected for the study. Seven patients (15.9%) were identified as having had venous thromboembolic events whereas none had arterial events. Five of seven patients (11.4%) had TE events within 16 weeks of chemotherapy, two had TE events 10 and 19 years after diagnosis, respectively. Two had bilateral pulmonary emboli (PE) (4.5%), three had upper or lower extremity DVTs, or both, and one patient had both bilateral PE and multiple DVTs. Five of seven patients with TEs had non-seminomatous germ cell tumors, two had non-testis primaries, four had relapsed disease, two with late relapse (> 7 years after initial diagnosis), six of seven had metastatic disease, three had retroperitoneal lymph node dissection, and all seven received platin-based chemotherapy. In logistic regression analysis, significant risk factors for TE included relapse ($P = 0.016$), bulky retroperitoneal lymphadenopathy ($P = 0.006$), alpha-fetoprotein > 10,000 ($P = 0.047$) beta-HCG > 1000 ($P = 0.020$), chemotherapy ($P = 0.031$), and platin-refractory disease ($P = 0.055$).
Conclusions: Germ cell tumor patients have a high risk of venous TE. Those with relapsed disease, bulky retroperitoneal lymphadenopathy, platin-based chemotherapy, and platin-refractory patients are at increased risk. Our risk estimates are higher than quoted in the literature and do not include any arterial events. To confirm these findings, we plan to extend this study to include 100 consecutive patients, most of whom have advanced disease states. If confirmed, these unique features of TE events may reflect advanced staging of disease in our patients and long-term follow-up.
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PB 1.61-6

Venous thromboembolism-related mortality in cancer patients

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Background: Thrombotic risk is increased in patients with cancer. Venous thromboembolism (VTE) is the second most common cause of death in this population. In this sense, it is important to provide information on predictors of mortality in cancer patients with VTE based in clinical data.
Aim: To determine the mortality and risk factors related to VTE among patients with cancer.
Methods: In a retrospective study, the survival of cancer patients admitted as a consequence of VTE between 2006 and 2010 was analyzed. The etiology of death was registered. Covariables included in the study were age, sex, functional status, comorbidities as Charlson index, and blood parameters such as hemoglobin, platelets, leukocytes and INR levels at the time of the event. Factors related cancer were the location, date of diagnosis and presence of metastasis. Cancer treatment and previous admissions were also included. All patients were followed until death or the date of the end of study on 31st December 2011.
Results: One hundred and eighty patients were included in the study. Of them, 130 (72%) died during the follow-up. The causes of death were: 36 (28%) due to VTE and 68 (52%) secondary to advanced cancer. Primary cancer included lung, gastrointestinal and breast in 23%, 18%, 12%, respectively. One hundred and four (58%) subjects were admitted as a consequence of pulmonary thromboembolism. Metastasis and cancer treatment was present in a 68% and 58%, respectively. Factors related associated with VTE-mortality were: previous admission, leukocytes > 13,000/μL, hemoglobin < 9 g/dL, advanced age, the presence of cancer metastasis and the ausence of anticoagulant therapy. No differences were found between low molecular weight heparine and oral anticoagulants.
Conclusions: Venous thromboembolism (VTE) is the cause of death in a high proportion of cancer patients. Cancer patients with older age, recent admission and metastasis are in high risk to fatal event after VTE. In these subjects, secondary prophylaxis should not be discontinuated.

PB1.62 – Antiphospholipid – II

PB 1.62-1

Autoantibodies against component of complement 1 contribute to the complement activation and to the manifestations of refractory antiphospholipid syndrome (APS)

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Backgrounds: In the pathogenic mechanisms of antiphospholipid syndrome (APS), it is recognized that pathogenicity of antiphospholipid antibodies (aPL) has dominant effects. Complement is part of the innate immune system and is one of the main effector mechanisms of antibody-mediated immunity. We have previously reported that complement activation prevalently coexists in sera of APS patients and functions as source of procoagulant cells activation. Recently, autoantibodies against C1q, the component of complement 1, were reported to correlate with complement activation in patients with systemic lupus erythematosus (SLE). They are not neutralizing antibodies but target the neoepitopes of deformed C1q bound to various molecules such as anionic phospholipids. The binding of anti-C1q antibodies to C1q induces accelerated activation of complement pathway. There are

no previous studies discussing the involvement of anti-C1q antibodies in APS patients.
Aim: To investigate the existence and significance of anti-C1q antibodies in APS patients.
Methods: This study was comprised of 54 consecutive primary APS patients that visited Hokkaido University Hospital rheumatology clinic from 2002 to 2011. Informed consent was obtained from every patients and the study was approved by the ethics committee of the Hokkaido University Hospital. All the patients were retrospectively analyzed of their clinical manifestations and laboratory data. Refractory APS was defined as a clinical status of relapsing thrombosis or pregnancy morbidity during adequate secondary prophylaxis. Twenty patients with non-SLE connective tissue disease and 20 healthy control subjects were also included. An enzyme-linked immunosorbent assay (ELISA) was used to measure serum levels of anti-C1q antibody titers and anaphylatoxins (C3a,C4a). Association between anti-C1q antibody titers and the clinical or laboratory data were statistically analyzed.
Results: Anti-C1q antibodies were more frequently detected in primary APS patients (18/54) than in non-SLE connective tissue disease patients (2/20) or healthy control (0/20) ($P < 0.01$ for each). In APS patients, anti-C1q antibody titers were significantly correlated with serum C4a level ($P = 0.007$) and inversely correlated with serum C4 level ($P = 0.01$). Serum C3a and C3 levels showed positive and inverse association with anti-C1q antibody titer, respectively, but failed to arrive statistical significance ($P = 0.09$ and 0.11, respectively).
The prevalence or the titers of anti-C1q antibodies were not associated with any of the specific clinical manifestations of APS such as arterial/venous thrombosis, pregnancy morbidity or thrombocytopenia. Also, the prevalence of anti-C1q did not associate with specific aPL and the titer of anti-C1q antibody did not correlate with aPL score. However, all of the refractory APS patients had positive anti-C1q antibodies (7/7) while APS patients without flare had low prevalence (11/47). The titer of anti-C1q antibodies was significantly higher in the refractory APS patients compared with APS patients without flare. (refractory APS vs. APS without flare: 32.5 ± 4.72 vs. 9.91 ± 1.58 U/mL, $P = 0.0000016$). With cut off level of anti-C1q antibodies 20 units/mL, odds ratio of refractory APS was 61.5 (5.85–646 95% CI, $P = 0.00007$).
Summary/Conclusions: These findings indicate that anti-C1q antibodies are associated with complement activation in APS and may contribute to the manifestation especially in the refractory cases.

PB 1.62-2
Exploring the diagnostic opportunities of the measurement of circulating procoagulant phospholipids by a coagulation based assay in lupus anticoagulant positive samples

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Background/Aims: Cellular microparticles (MP) are associated with thromboembolic diseases, including the antiphospholipid syndrome (APS). In plasma the procoagulant phospholipids (PPL) mainly come from MP. We evaluated whether the presence of increased activity of PPL measured through STA-Procoag-PPL is applicable in patients with a lupus anticoagulant (LAC). LAC, one of the laboratory criteria of APS, has an *in vitro* anticoagulant effect resulting in prolongation of phospholipid-dependent coagulation assays and interference with the clotting time (CT) measured by STA-Procoag-PPL is expected.
Methods: Shortening of the CT (in seconds, s) of the STA-Procoag-PPL of the patient plasma (PP) means an increase in PPL. The CT is dependent of the patient's PPL only since in the test procedure, the PP is diluted 1:1 in PPL-depleted normal pooled plasma (MPfree NPP). The STA-Procoag-PPL was performed on citrated plasma in duplicate on STAR Evolution (both from Stago BNL B.V., Brussels, Belgium). Normal ($n = 25$), factor deficient ($n = 10$), LAC positive ($n = 60$) and LAC negative samples ($n = 22$) were analysed. To evaluate the sensitivity of the assay the CT was compared with the number of MP in a serial dilution of NPP spiked with MPs. Semi-quantitative MP analysis was performed by flow cytometry (CD41-Annexin V derived MP). Predilutions of LAC positive samples ($n = 7$) were made to dilute the LAC effect and to evaluate the effect on the CT in function of the number of MP. Equally, the change in CT was evaluated on prediluted (1/20 and 1/40) MPfree NPP spiked with MP.
Results: CT was comparable between LAC negative samples, normal and factor deficient samples. LAC positive samples showed a significantly prolonged CT compared to the normal samples.
NPP spiked with MP showed a clear correlation between the CT and the number of MP. A decrease in MP amount (e.g. $50*10^3/\mu L$, $20*10^3/\mu L$, $2.5*10^3/\mu L$ or $0.5*10^3/\mu L$) resulted in a prolongation of the CT (14s, 3s, 1.2s, 1.0s, respectively).
The effect of LAC on the CT in undiluted LAC positive samples was variable. The STA-Procoag-PPL in prediluted samples up to 1/80 with MPfree NPP indicated that a 1/20 or 1/40 predilution may neutralize the LAC effect, but inherently results in dilution of the amount of MP. Therefore, MPfree NPP was spiked with MP and diluted up to 1/80 with MPfree NPP. The dilution series showed a good correlation between the change in CT and the change in number of MP: a decrease of $10*10^3 MP/\mu L$ caused an increase of 12 s CT; a decrease of 200 MP/μL still showed an increase of 6 s CT.
Summary: The presence of increased activity of PPL in APS patients measured through STA-Procoag-PPL might be a good alternative for more labour intensive assays for MP analysis. The presence of LAC interfering with the STA-Procoag-PPL CT can be overcome by predilution of the samples. We demonstrated that in highly prediluted samples theSTA-Procoag-PPL CT still responds with the number of MP. This opens options to analyse LAC positive samples. Further study is needed to evaluate the clinical relevance of measuring the STA-Procoag-PPL in patients with APS.

PB 1.62-3
Beta2-glycoprotein I plasma levels are in relation to antiphospholipid antintibody profile in Antiphospholipid Syndrome

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Background: Antiphospholipid Syndrome (APS) is characterized by the presence of antiphospholipid (aPL) antibodies associated with thrombosis or pregnancy morbidity. The antibodies mainly involved in these disorders are directed against β2-Glycoprotein I (β2GPI).
Aim: The purpose of this study was to evaluate the plasma concentration of β2GPI in patients with different aPL antibody profiles.
Methods: It was set up a 'sandwich' enzyme-linked immunoadsorbent assay (ELISA) using a rabbit polyclonal anti-human β2GPI coated into plate, and a mouse monoclonal anti-human β2GPI to detect the plasma concentration of bound β2GPI. The system was developed by using a phosphatase-labeled goat anti mouse antibody. Increasing concentrations of human purified β2GPI were used to create a calibration reference curve. Plasma of 23 patients with APS and triple positivity (LAC+, IgG aCL+, IgG aβ2GPI+), eight with double positivity (IgG aCL+, IgG aβ2GPI+, LAC negative), five with single positivity (IgG aβ2GPI+, LAC and aCL negative) and 20 controls were evaluated. Median values between groups were compared by Mann-Whitney test.
Results: A linear dose-dependent optical density was obtained by using Increasing concentrations of purified β_2GPI (from 0.05 to 0.8 mg/mL). Mean β_2GPI concentration in control plasmas was 0.175 mg/mL. Plasma from patients with triple positivity (LAC+, IgG aCL+, IgG aβ2GPI+) showed a significantly higher mean β2GPI concentration than that obtained in control subjects (0.4 vs. 0.175 mg/mL,

$P = 0.009$). Plasma from double (IgG aCL+, IgG aβ2GPI+) and single (IgG aβ2GPI+) positive patients showed mean β_2GPI levels not different from those obtained in control subjects: 0.240 and 0.260 mg/mL, respectively.

Conclusions: Our results demonstrate for the first time that patients with antiphospholipid syndrome (APS) and high risk aPL profile (triple positivity) have significantly higher β_2GPI plasma concentration compared to controls. On the other hand no difference from controls in double and single positivity was found. These data may be of help in understanding the pathogenic mechanism underlying the clinical manifestations of APS.

PB 1.62-4

The influence of anti-tissue factor pathway inhibitor (TFPI) antibodies on thrombin generation (TG) in thrombotic patients with and without antiphospholipid syndrome

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Background: Anti-tissue factor pathway inhibitor antibodies (αTFPI) have been reported in the antiphospholipid syndrome (APS). It has been suggested that these antibodies may impair TFPI activity, contributing to the observed hypercoagulability in these patients. TFPI acts as the principal inhibitor of the tissue factor (TF)-induced activation of the coagulation cascade, by forming a quaternary inhibitory complex with TF-FVIIa, and FXa.

Aim: To investigate the influence of αTFPI on thrombin generation (TG) in thrombotic patients with and without APS.

Methods: Ninety-one patients with arterial and/or venous thromboembolism, 46 with APS and 45 non-APS receiving warfarin anticoagulation, and 74 healthy normal subjects were studied. IgG αTFPI was measured using recombinant human TFPI (Chiron Corp, California, USA) by an optimised in-house ELISA. Results were expressed in arbitrary units (U/mL) with reference to a plasma sample from an index high titre αTFPI APS patient arbitrarily considered as 100 U/mL. A cut-off value of 60.8 U/mL was determined to be the 99th centile in the 74 normal subjects. TG was assessed using the automated calibrated thrombogram with PPP-reagent (5 pM TF, 4 μM phospholipid; Diagnostica Stago). Endogenous thrombin potential (ETP), peak, and lag time were assessed. Unless otherwise indicated, results are expressed as median and observed range.

Results: αTFPI levels were detected in 29.7% of the thrombotic patients, both APS and non-APS (45.7, 2.3–213 U/mL). Subgroup analysis revealed a higher prevalence of αTFPI in APS compared to non-APS patients, with αTFPI present in 37.0% (17/46) of the APS and 22% (10/45) of the non-APS group respectively ($P = 0.0413$). There was also a trend towards higher αTFPI values in the APS (52.2, 17.1–213.3 U/mL) compared with the non-APS (34.3, 2.3–167.0 U/mL) patients. Twelve of the 46 APS patients (26%) and only 4/45 (8.7%) of the non-APS patients had markedly raised αTFPI (> 100 U/mL) ($P < 0.001$). INR values were comparable in both APS (2.5, 1.0–4.4) and non-APS (2.2, 1.0–4.8) patients. However, evaluation of TG revealed longer lag times in both APS (9.8, 5.3–55.7 min) and non-APS patients (6.7, 3.7–15.6) when compared to normal subjects (4.3, 3.7–5.3). No association was observed between prolonged lag times and either αTFPI or LA positivity in either APS or non-APS patients. APS patients however, exhibited lower ETP and peak values compared to non-APS patients (median ETP 20.4 and 29.9 and median peak 26.5 and 38.5, respectively). No significant differences were observed between αTFPI positive and αTFPI negative APS patients in terms of lag time, ETP, and peak.

Conclusion: Our results suggest that the presence of αTFPI does not appear to have an effect on any of the parameters of TG in either APS or non-APS thrombotic patients. Possible influences on the protein C/S coagulation pathway that may modulate TG are under investigation. Despite comparable INR values and the observed prolongation of lag time in both thrombotic patient groups, APS patients tended to exhibit a greater prolongation of lag time, lower ETP and peak values compared to non-APS patients.

PB 1.62-5

Lack of association of serum mannose binding lectin or ficolins with complement activation in patients with antiphospholipid antibodies

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Background: Complement activation is proposed to play a role in the pathogenesis of thrombotic and obstetric complications of APS with most evidence for this demonstrated in murine models of APS. Recently, we demonstrated increased levels of complement activation products, Factor Bb and C3aDesArg, in a large group of patients with primary APS (PAPS) or isolated antiphospholipid antibodies (aPL), confirming previous smaller studies but it is unclear how complement activation is occurring in these patients.

Complement activation occurs via the classical, alternative or lectin pathway. Mannose/mannan binding lectin (MBL), L-, H- and M-ficolins are components of the lectin pathway involved in recognition of pathogen-associated molecular patterns on microbial surfaces, leading to activation of complement by stimulation of MBL-associated serine proteases (MASPs). Most studies of the association of lectins with disease have been in patients with infection, although the lectin pathway has been implicated in development of autoimmune diseases such as systemic lupus erythematosus (SLE), but have not been studied in patients with aPL.

Aims: We hypothesised complement activation in patients with APS might be occurring through activation of the lectin pathway and sought to measure lectin pathway components in patients with PAPS or isolated aPL.

Method: Following approval from our local ethics committee, we obtained samples from patients at our institution who had PAPS according to International Consensus statement criteria, or had persistent aPL without associated complications. Patients with PAPS included 100 patients (median age 44, range 20–73 years) of whom 42 had previous thrombotic complications (venous and/or arterial), 27 had obstetric complications and 31 had isolated aPL. Thirteen healthy controls (median age 35, range 20–60 years) were also recruited. ELISA assays measuring MBL, L- and H-ficolins were performed. Statistical analysis of results was performed using Graphpad Prism statistical software. A P-value of < 0.05 was considered statistically significant.

Results: There were no significant differences in levels of MBL (mean 1.1 μg/mL, SD 1.0, $P = 0.6$), L- or H-ficolin levels (mean L-ficolin 1.9 μg/mL, SD 0.9, $P = 0.3$, H-ficolin 19.9 μg/mL, SD 8.1, $P = 0.09$) in patients with aPL/PAPS compared to healthy controls (mean MBL 1.1 μg/mL, SD 1.0, L-ficolin 1.7 μg/mL, SD 0.7, H-ficolin 21.6 μg/mL, SD 7.6), or when patients were analysed according to clinical phenotypes of aPL (thrombotic PAPS, obstetric PAPS or isolated aPL). MBL, L- and H-ficolin levels observed in this study were similar to previously reported human serum concentrations.

Conclusion: Low levels of C3 and C4 have been demonstrated previously in patients with aPL in addition to increased levels of complement activation products, C3a-desArg and Factor Bb so complement activation rather than deficiency has been proposed as a potential mechanism. Our findings of normal levels of potential initiating factors MBL, L- and H-ficolins provide no support for activation of the lectin pathway.

In conclusion, we have demonstrated normal levels of lectins, MBL, L- and H-ficolin in patients with aPL. Further studies of the mechanism of complement activation in patients with aPL are required.

PB 1.62-6

Resistance to anticoagulant activity of annexin A5 in patients with antiphospholipid syndrome with and without systemic lupus erythematosus receiving hydroxychloroquine

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Background: Disruption of the annexin A5 (AnxA5) anticoagulant shield has been proposed to play a role in the pathogenesis of thrombosis and obstetric complications in patients with antiphospholipid antibodies (aPL)[1]. Hydroxychloroquine has been reported to decrease thrombotic events in patients with antiphospholipid syndrome (APS) and systemic lupus erythematosus (SLE)[2–4] and *in vitro* has been shown to reverse binding of aPL to syncytiotrophoblasts and restore AnxA5 expression[5]. To date there have been no human studies of the effects of hydroxychloroquine on the laboratory parameters of patients with aPL.

Aims: The aim of our study was to assess resistance to AnxA5 anticoagulant activity in patients with aPL before and after commencing hydroxychloroquine.

Methods: Following approval from our local ethics committee, we obtained samples from 18 patients with primary and secondary APS (16 females, two males, median age 55 (range 18–70) years). Eleven patients had primary APS and seven had APS in association with SLE. Of the patients with primary APS, 10 had previous thrombotic events and one patient had previous apl related obstetric complications. Of the patients with aPL associated with SLE, five had previous thrombosis, and two had previous apl related obstetric complications. Twelve patients were receiving Vitamin K antagonists and four were receiving aspirin. AnxA5 anticoagulant activity of patient plasma was assessed before, and 3 months after starting hydroxychloroquine 200 mg daily. The anticoagulant activity of AnxA5 was calculated as follows: AnxA5 anticoagulant ratio = (coagulation time in the presence of AnxA5/coagulation time in the absence of AnxA5) × 100%.

Results: No significant differences in AnxA5 anticoagulant activity ratio were observed in patients 3 months after commencing hydroxychloroquine (mean 193% SD 31.0). compared to before commencement (mean 187.3% SD 29.4)

Conclusions: In conclusion, no changes in resistance to AnxA5 anticoagulant activity were found in this small patient cohort with APS after commencing hydroxychloroquine.

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PB1.63 – Arterial Vascular Disorders – I

PB 1.63-1

Effects of exercise stress testing on blood coagulation and fibrinolysis in asymptomatic aortic stenosis

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Background: A role of haemostasis in aortic valve stenosis (AVS) is still unclear. Enhanced thrombin formation combined with platelet activation in circulating blood and fibrin deposition on the aortic valve were observed in AVS patients. Recently, we have reported that hypofibrinolysis is more common in these patients than in controls. Acute physical exercise is known to increase blood coagulation and fibrinolysis in healthy subjects.

Aim: We sought to investigate changes in coagulation and fibrinolysis parameters in AVS caused by physical strain.

Methods: Exercise stress echocardiography was performed by 32 consecutive asymptomatic moderate to severe AVS patients. We measured peak thrombin generated [C_{max}] using the calibrated automated thrombogram, clot lysis time [CLT], thrombin activatable fibrinolysis inhibitor [TAFI], plasminogen activator inhibitor-1 [PAI-1], tissue plasminogen activator [tPA], and plasminogen four times: at rest, at peak exercise, 1 and 24 h after exercise. Thirty-two age-and sex-matched individuals served as controls.

Results: The duration of echocardiography stress test was shorter in AVS group ($P = 0.008$), and mean workload reached 81.3 Watt. C_{max} at peak exercise was 25% higher (median, 309.8 vs. 253.7 pg/mL, $P < 0.001$) in AVS group, and reached its highest level 24 h from exercise, while it gradually decreased with time post exercise in controls. CLT was 13% longer in AVS patients at baseline (median, 104.5 vs. 92.3 min, $P = 0.006$) and further increased at peak exercise in AVS group, while decreased in controls; the intergroup difference was 28% ($P < 0.001$). CLT decreased in AVS patients by 41% 1 h after exercise reaching a level similar to the control group. Twenty hours after physical strain CLT became longer with its 15% higher value than in controls ($P < 0.001$). TAFI activity was higher in AVS group than in controls in all time points (all $P < 0.001$). TAFI tended to increase at peak exercise, and after 1 h its activity decreased ($P < 0.001$), and after following 24 h reached a higher value than prior to the exercise ($P = 0.003$). No such changes in TAFI activity were observed in the control group. Plasminogen was higher in AVS group at peak exercise ($P = 0.023$), and then one ($P < 0.001$) and 24 h after its termination ($P = 0.019$). Under exertion no changes in PAI-1 and tPA antigens were observed in AVS patients. There were no associations of coagulation and fibrinolysis markers with disease severity and exercise induced changes in echocardiographic parameters.

Conclusion: This study is the first to show that asymptomatic moderate or severe AVS patients respond to physical activity with a more pronounced increase in thrombin activation and impaired fibrinolysis compared with age and sex-matched controls. The prothrombotic in AVS patients following exercise may promote fibrin deposition on aortic valve leaflets, thus enhancing the progression of this heart disease.

PB 1.63-2

Fibrin clot formation and fibrinolysis in patients with coronary stent thrombosis

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Background: Coronary stent thrombosis (ST) is a feared complication of percutaneous coronary intervention (PCI) leading to high morbidity and mortality. The incidence of ST is approximately 1–5%, despite dual antiplatelet therapy with aspirin and clopidogrel. Multiple factors underlie the pathophysiological mechanisms of ST, such as stent underexpansion and high residual platelet reactivity. Fibrin clot formation and fibrinolysis may be different in ST patients compared to patients not suffering from ST and thus play a role in the occurrence of ST. A single study by Undas et al. (ATVB, 2010) showed that, compared to control patients, ST patients formed denser fibrin clots which were more resistant to lysis, suggesting that alterations in fibrin clot formation and lysis could indeed contribute to the pathophysiology of ST.

Aim: The present study aimed to assess the plasma fibrin clot formation and fibrinolysis of ST patients compared to control patients.

Methods: A single-center case-control study was performed. Cases had a history of an angiographically confirmed ST (Academic Research Consortium criteria). Controls had a stent implantation without ST during follow-up (minimal 12 months) after index PCI. Controls were matched based on indication and time of the index PCI of cases. Subjects using oral anticoagulants at the time of blood collection were excluded. Fibrin clot formation was measured with a turbidity assay for the parameters lag time and maximal absorbance. Platelet poor plasma (PPP) samples were diluted 1:1 with Hepes buffer (25 mM Hepes, 150 mM NaCl, pH = 7.5) and 0.75 nM human thrombin, 16 mM $CaCl_2$, and 10 μM phospholipids were added to initiate clot formation. All concentrations were final concentrations. Fibrinolysis was measured with a clot lysis assay for the parameter clot lysis time (defined as time from 50% clot formation to 50% clot lysis). PPP samples were diluted similar to the turbidity assay and 50 ng/mL tissue plasminogen activator (tPA) was added to start fibrinolysis. Fibrinogen levels were determined using the von Claus method. All subjects provided written informed consent. The study was approved by the local institutional review board and was conducted according to the principles of the Declaration of Helsinki.

Results: A total of 27 cases and 27 controls were included. From the cases, 12 patients had experienced an early ST (≤ 30 days after PCI) and 15 patients a late ST (> 30 days after PCI). Fibrin clot formation was not significantly different between cases and controls; lag time 173 ± 47 vs. 161 ± 27 s ($P = 0.26$), maximal absorbance 0.78 ± 0.16 vs. 0.83 ± 0.21 ($P = 0.36$). In addition, no difference in clot lysis time was observed for cases compared to controls; clot lysis time 69.2 ± 20.1 vs. 71.3 ± 25.3 min ($P = 0.75$). Fibrinogen levels were comparable in cases vs. controls (3.50 ± 0.73 vs. 3.60 ± 0.74, $P = 0.65$).

Summary/Conclusion: Plasma fibrin clot formation and fibrin clot lysis for ST patients are comparable to control patients. Based on the results of these ex vivo tests, a role for fibrin clot formation and lysis in the process of stent thrombosis is less likely. The reasons for this apparent discrepancy with previous data warrants further exploration.

PB 1.63-3

Risk of myocardial infarction and ischaemic stroke and the impact of hypercoagulability – the RATIO case control study

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Background: Myocardial infarction (MI) and ischaemic stroke (IS) are both acute forms of arterial thrombosis. Some risk factors are shared, but others are not, indicating possible different pathophysiological mechanisms.

Aims: This study aims to determine whether measures of hypercoagulability have a comparable impact on the risk of MI and IS.

Methods: The RATIO study is a case control study involving young women (< 50 years) with MI, non-cardioembolic IS and healthy controls. To compare the impact of prothrombotic factors we calculated relative odds ratios (OR_{IS}/OR_{MI}) and their corresponding confidence intervals based on published results. Additionally, we calculated population attributable fractions (prevalence in cases *(OR-1/OR)), which reflect the fraction of cases prevented if the exposure were to be eliminated from the general population. Informed consent was obtained from all participants and the study protocol was approved by the medical ethic committees from al participating hospitals.

Results: In total, 30 prothrombotic risk factors were identified as measures of hypercoagulability (see Figure 1). The population attributable fractions show that measures of hypercoagulability have a bigger impact on IS risk than for MI. Twenty-one of these risk factors have a relative odds ratios > 1, 14 > 2, and 6 > 2.75 being high levels of activated FXI and FXII, combination of oral contraceptive use and factor V Leiden trait, the presence of lupus anticoagulans, and a single nucleotide polymorphism in the gene coding for coagulation factor XIII.

Conclusion: Overall, prothrombotic factors have a bigger impact on the risk of IS that than of MI, suggesting a different role of hypercoagulability in the underlying mechanism of these two diseases. The results from the RATIO study are subject to potential sources of these biases, such as reverse causation and residual confounding. However, because we compare results within a single study, the impact of this bias is similar for the MI and IS analyses and therefore cannot explain the observed contrast.

PB 1.63-4

A pig model of primary angioplasty of acute myocardial infarction in human like conditions

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Background: The principal challenge in primary angioplasty is not to achieve culprit vessel reopening (which is regularly obtained) but to prevent the myocardial microvascular obstruction: the no-reflow phenomenon which determines initial and long term prognosis of patients. The main factors of the no-reflow are the micro-thrombotic distal embolizations from the main thrombus and the ischemia reperfusion which includes endothelial reactivity induced by numerous cellular reactions. Although pig is a suitable animal to test human materials and conditions, as it is close to human thrombotic and vascular reactivity, the experimentally used animal model is usually a very young animal (to fit the size) with optimal endothelial reactivity. Besides the animal, the model of coronary occlusion is usually achieved mechanically, thus the existing models are poorly predictive of the human pathological conditions.

Aim: Thereby, we set up a new model of thrombotic coronary occlusion for primary angioplasty in elderly downsized naturally hypercholesterolemic pigs characterized by a major endothelial dysfunction.
Materials and methods: Coronary artery thrombotic occlusion was achieved by placing a commercially available coil (for reproducibility) into the right coronary artery (RCA) (size adapted to animal coronary size) via a guiding catheter in 13 elderly downsized swines genetically selected to be spontaneously hypercholesterolemic (FBM pigs). The RCA was chosen in order to limit the risk of irreversible ventricular fibrillation. Repeated angiographic imaging confirmed the progressive (mean time 20 min, from 1 to 60 min) RCA total thrombotic occlusion. After 1 h of waiting (aiming to mimic human conditions) the animal was treated with the usual antiplatelet and anticoagulant pharmacological preparation so as to allow to proceed to recanalization with a guidewire pre-dilatation with a semi compliant balloon. Thrombus was visualized and quantified with Optical Coherence Tomography (OCT) imaging and, finally, deployment of a stent (of a proportional to the coil length and diameter) and repeated OCT. Total occlusion time 90 min, mean recanalization time 15 min and post reperfusion waiting time till sacrifice 180 min. Myocardial infarction was confirmed by ECG, biochemical, histological, macroscopic examination and TTC (triphenyltetrazolium chloride) staining in all animals.
Results: Thrombotic occlusion has been acutely achieved in all 13 pigs. Eight of them presented ventricular tachycardia (VT) or/and ventricular fibrillation (VF) episodes in the early phase of revascularization· transthoracic direct current (DC) electrical cardioversion was succeeded in all these situations. MI was confirmed in all animals. Besides, all animals survived until the previously selected time for sacrifice.
Conclusion: The generation of thrombotic occlusion in atherosclerotic pigs is feasible by percutaneous intracoronary placement of a coil. The above described model is suitable for the purpose of experiments in materials and treatments efficacy. The model of elderly genetically selected to be hypercholesterolemic pigs allows short term evaluation as well as long term evolution of these animals. The whole experimental model is highly representative of usual AMI patients.

PB 1.63-5

Increased arterial thrombo-embolic events and major bleeding in patients with atrial fibrillation and chronic kidney disease on vitamin K-antagonist treatment

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Aim: To analyze the risk of arterial thrombotic events (ATEs), major bleeding and its predictors in patients with chronic kidney disease (CKD) compared with non-CKD patients, treated with vitamin K-antagonists (VKAs) for atrial fibrillation (AF).
Methods: Medical records of 417 CKD and 300 non-CKD (GFR > 60 mL/min) patients starting VKA therapy between 1997 and 2005 were searched for ATEs and major bleeding. ATE was defined by myocardial infarction, stroke/transient ischemic attack, claudicatio intermittens, unstable angina, carotid or peripheral bypass graft/angioplasty. Major bleeding was defined by bleeding being fatal, causing a drop in Hb level $\geq$ 1.24 mM, requiring transfusion of $\geq$ 2 units of whole blood/red cells, or being symptomatic in a critical area.
Results: ATEs occurred in 108/737 (14.7%, 95%CI 12.3–17.4) patients. Patients with a GFR < 30 mL/min were at increased risk of ATE compared with non-CKD patients (hazard ratio (HR) 3.4 (95% CI 2.1–5.6). ATEs occurred as frequent in patients with GFR 30–60 mL/min as in non-CKD patients (HR 1.1, 95%CI 0.7–1.7). Major bleeding occurred in 115/737 patients (15.6%, 95%CI 13.2–18.4). The risk of major bleeding increased in patients with a GFR < 30 mL/min compared with non-CKD patients (HR 1.8, 95%CI 1.1–3.0). Major bleeding risk was not increased in patients with a GFR 30–60 mL/min compared with non-CKD patients (HR 0.9, 95%CI 0.6–1.4). Low GFR levels were associated with high variability within a patients INR values (correlation term −0.17, $P \leq 0.001$). INR variability > 0.5 (HR 1.5, 95% 1.0–2.4) increased the major bleeding risk.
Conclusion: Patients with a GFR < 30 mL/min are at increased risk of ATEs and major bleeding compared with non-CKD patients. The increased bleeding risk may be due to high INR variability, a marker of suboptimal VKA therapy.

PB 1.63-6

Thromboembolic complications or intracerebral haemorrhages in patients with atrial fibrillation in the emergency departments. Complications of atrial fibrillation in Bologna: the CAF-BO study

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Background/Aims: To establish the incidence of thromboembolic and cerebral haemorrhagic events in subjects with non valvular atrial fibrillation in the area of Bologna, Italy. Design and setting: prospective study in the Emergency Departments in the area of Bologna, Italy.
Patients and Methods: In the area of Bologna a web based network of anticoagulation clinics allows the surveillance of approximately 8000 (57%) patients on vitamin K antagonists out of estimated 14 000 patients with atrial fibrillation in our area. We evaluated consecutive patients with atrial fibrillation referred to the Emergency Departements with either thromboembolic complications or spontaneous intracerebral haemorrhages from January to June 2012. All patients underwent computed tomography and on the day of admission demographic data were collected along with the presence of atrial fibrillation, use of vitamin K antagonists or antiplatelet agents.
Results: We recorded 178 patients with cardioembolic events and 20 patients with spontaneous intracerebral haemorrages. The majority of patients with events were in the age range 70–90 and females (66%). Atrial fibrillation was persistent in 97 (49%) patients, paroxysmal in 65 (33%) and diagnosed during hospitalization in 12 (6%). Thromboembolic events were cerebral in 159 (89%) and peripheral in 19 cases (11%). Patients with cardioembolic events were receiving vitamin K antagonists in 17% of cases, antiplatelet agents in 58% of cases and no anti-thrombotic treatment in 22% of cases. In patients treated with vitamin K antagonists the INR was in the therapeutic range in 42% of cases and subtherapeutic in 52% of cases. Among patients with spontaneous intracerebral haemorrhages, 12 were on vitamin K antagonists and the INR was in theraputic range in 85% of cases. The inhospital mortality rate was 19% (34 cases) in subjects with thromboembolic events and 45% (9) cases in patients with intracerebral haemorrhages. We can extrapolate that in patients with non valvular atrial fibrillation older than 70 year the incidence of thromboembolic events is 0.56% patients years while on vitamin K antagonists and 4.54% patients years when on antiplatelet agents or no treatment (relative risk reduction: 88%). The estimated incidence of intracerebral haemorrhages is 0.27% patients years on vitamin K antagonists and 0.26% patient-years when on antiplatelet agents or no treatment.
Conclusions: A relevant number of subjects with atrial fibrillation do not receive vitamin K antagonists and the estimated incidence of intracerebral haemorrhages is similar in patients treated with vitamin K antagonists and in patients treated with antiplatelet agents or not receiving any treatment.

PB1.64 – Diagnosis of VTE – I

PB 1.64-1

Clinical validity of a quantitative point of care d-dimer assay in an acute ambulatory DVT service

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Background: University Hospitals of Leicester acute ambulatory DVT service assesses 3000 patients a year. All patients are initially assessed using the modified Wells 2 level score. D dimer testing is carried out in patients with DVT unlikely scores and those with positive d-dimers have whole leg compression ultrasound (CUS) scans. Calf DVTs are treated. Turnaround time for laboratory d-dimer results is several hours. In an attempt to reduce patient waiting times and decrease the time to scan a pilot of a quantitative point of care (POC) d-dimer assay (Cardiac d-dimer, Roche) using the cobas h232 POC instrument was carried out. The initial pilot included 49 patients with the POC d-dimer with parallel laboratory d-dimer testing. Patients with a positive d-dimer by either method underwent whole leg CUS. Two patients (4%) with negative POC test but positive laboratory d-dimers had calf DVTs on CUS and this prompted a larger clinical validation study.
Aims: To clinically validate the performance of the POC d-dimer test in our acute ambulatory DVT service.
Methods: Five hundred patients with DVT unlikely Wells scores underwent parallel d-dimer testing with the POC d-dimer test and in the laboratory using the rapid STA Liatest method on the STA-R analyser. Patients with a positive d-dimer by either method had whole leg CUS. Those with negative d-dimers or CUS were followed up at 90 days.
Results: Two hundred and five of the 500 patients (41%) were discharged without a CUS scan as the POC and laboratory d-dimers were negative. Of the 295 scans performed 248 were negative for DVT. Forty-seven of the 295 (16%) of CUS scans demonstrated DVTs; 27 proximal (9%) and 20 distal (7%). There were 63 discordant d-dimer results (13%). 62/63 had negative POC results and positive laboratory results. Using the POC test alone with a cut off of 0.5 μg/L would have lead to three small calf DVTs being missed. The sensitivity and negative predictive value for the POC d-dimer for proximal DVTs was 100% using a cut off of 0.5 μg/mL. At this d-dimer cut off the sensitivity of the POC d-dimer for calf vein thrombosis was 86.9% and a negative predictive value (NPV) of 98.9%. Using the laboratory d-dimer test alone 59 of 62 positive POC d-dimer results (20% of CUS scans) would have been CUS scan negative and could have been avoided if the POC test had been used alone. Using a POC d-dimer cut off of 0.4 μg/mL there were 29 discordant results all with positive laboratory results. The sensitivity and negative predictive value for proximal and distal DVT was 100% with the POC d-dimer and would still have reduced the scans required by 29 (10%).
Conclusion: POC d-dimer testing in the initial screening of patients with suspected DVTs would reduce the number of CUS scans for patients with DVT unlikely Wells scores by 20% compared with our very sensitive laboratory method using a cut off of 0.5 μg/mL. Lowering the POC d-dimer threshold to 0.4 μg/mL is not justified for the minimal improvement in NPV from 98.9% to 100%.

PB 1.64-2

A review of computed tomography pulmonary angiograms in three teaching hospitals: the rise of subsegmental pulmonary emboli and their management

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Background: The availability of Computed Tomography Pulmonary Angiography (CTPA) has led to an increase in the number of investigations for Pulmonary Embolism (PE). With more widespread use of these high resolution scans, the frequency of identification of isolated Small Sub-segmental Emboli (SSPE), which are of undetermined clinical significance, is also expected to increase.
Aims: To review the frequency of Pulmonary Embolism and Sub-segmental Pulmonary Embolism identified through CTPA as well as their management
Methods: Retrospective review of 1525 patient charts who underwent CTPA in three Hamilton teaching hospitals from 2009 to 2011. In depth chart review of patients with SSPE was undertaken to determine the indications for, frequency and complications of anticoagulation.
Results: Our patient population (mean age 64) consisted of 758 medical inpatients (50%), 512 surgical inpatients (34%) and 256 (17%) emergency department patients.
The frequency of PE was 26% (n = 392). Of these, the largest pulmonary filling defects were SSPEs in 58 patients (4% of total scans and 15% of identified PEs). In 40 of these 58 SSPEs, an alternative diagnosis to PE was identified on CT to explain the patients' symptoms. Isolated SSPE were present in 18 patients.
Approximately 44% (n = 26) received anticoagulation for SSPE.
Of the 1133 CTPAs that did not identify PE, an alternative diagnosis to account for the patient's symptoms was identified on CT in 737 (65%) and no alternative cause was found in 361 (35%).
Summary/Conclusions: Our study demonstrated a much lower frequency of pulmonary embolism in comparison to the 50% rate of PE reported in CTPA studies in the literature. SSPEs account for 15% of all PEs found in our study population – and were present in 4% of all patients undergoing CTPA. A substantial proportion of patients were anti-coagulated SSPE, though the safety and efficacy of this practice is unknown.

PB 1.64-3

Diagnostic accuracy of lung ultrasound for pulmonary embolism: a systematic review and meta-analysis

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Background: Computed tomographic pulmonary angiography (CTPA) has simplified the diagnostic approach to patients with suspected pulmonary embolism (PE). However, PE diagnosis is still probabilistic and CTPA should be used with caution in some patient groups, such as patients with severe renal insufficiency and pregnant women. Among alternative imaging tests, lung ultrasound is the most promising technique.
Aims: We aimed to systematically assess the diagnostic accuracy of lung ultrasound for PE diagnosis.
Methods: Studies evaluating the diagnostic accuracy of lung ultrasound for the diagnosis of PE were systematically searched in the MEDLINE and EMBASE databases (up to June 2012). QUADAS – 2 tool was used for the quality assessment of the primary studies. A bivariate random-effects regression approach was used for summary estimates of both sensitivity and specificity.
Results: Ten studies, for a total 887 patients, were included. A composite reference test was used in six studies, with single-row detector CTPA as the principal imaging test in four studies. Overall, seven studies used a proper reference test. Lung ultrasound bivariate weighted mean sensitivity was 87.0% (95% confidence interval [CI] 79.5, 92.0%), whereas bivariate weighted mean specificity was 81.8% (95% CI 71.0, 89.3%).
Conclusion: Our findings suggest that lung ultrasound may be a useful diagnostic tool in the management of patients with suspected PE. However, several methodological drawbacks of the primary studies limit any definite conclusion. Further well-designed accuracy studies are necessary before planning diagnostic management studies, in particular in those with a contraindication for CTPA

PB 1.64-4

Diagnosis, management and outcome of non-catheter related proximal upper extremity deep vein thrombosis

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Background: Upper extremity deep vein thrombosis (UEDVT) occurs less commonly than leg DVT but is increasing in incidence given the use of central catheters. Studies often do not differentiate between catheter and non-catheter related UEDVT. There is a paucity of data regarding the diagnosis, management and prognosis of non-catheter related proximal UEDVT.

Aim: To explore the diagnosis, risk factors, management and outcome of proximal UEDVT in patients without central venous catheter.

Method: A retrospective case series of patients with objectively diagnosed proximal (axillary/subclavian) UEDVT identified through the radiology database at Monash Medical Centre (AUS) and through a pre-existing database at North Shore hospital (NZ) in the period of 01/2007 to 03/2012. Central venous catheter (CVC), or pacemaker associated UEDVT were excluded.

Results: We identified 65 patients (M:F = 36:29, mean age 51.6 years) with non-CVC UEDVT, categorised at diagnosis as: 32 (49.2%) primary, 8 (12.3%) minimally provoked, and 25 (38.5%) secondary UEDVTs. Twenty four (36.9%) patients had a history of active cancer at the time of diagnosis, and a further three out of 41 patients were diagnosed with cancer following UEDVT at a median age of 61 years, giving an incidence rate of 4.5 cancer diagnoses per 100 person-years for those categorised as primary UEDVT. In particular, the incidence rate of newly diagnosed cancer was 11.4 per 100 person-years if the over 50 years old were considered. Four (6.2%) patients suffered from concurrent symptomatic PE at presentation. All patients started anticoagulation with LMWH (95.4%) or heparin (4.6%). None underwent thrombolysis. Subsequently, 28 (43.1%) and 47 (56.9%) patients continued anticoagulation with LMWH (median duration = 3 months) and warfarin (median duration = 7 months) respectively. One patient underwent rib resection because of marked PTS. The patients with cancer related proximal UEDVT were older than the non-cancer UEDVT (mean age 63.8 vs. 44.5 years, $P < 0.001$). Cancer related UEDVT patients had a short median survival of 11.1 months, and higher estimated cumulative incidence of VTE recurrence at 12 months compared to the non-cancer group: 9.2% (95% CI: 2.5–34.6%) vs. 2.6% (95%CI: 0.4–18.2%). All VTE recurrences in the cancer associated UEDVT were pulmonary embolism. Overall major bleeding rate was 2/65 (3.1%, 95%CI: 0.2–11.2%). Repeat ultrasound showed residual defects in 29 (61%) patients in the 47 patients who were rescanned. Dynamic venography were performed in 12 patients with primary UEDVT. Evidence of a significant thoracic outlet obstruction defined by occlusion to flow > 90% on abduction was observed in five patients (41.7%, 95% CI: 19.4–68.1%). Quality of life and PTS score surveys are currently pending.

Conclusion: Age identified patients at higher risk of cancer associated UEDVT which has poorer prognosis both in terms of VTE recurrence (occurring as primarily PE) and mortality. Younger patients typically presented with primary UEDVT, with a diagnosis of thoracic outlet syndrome commonly observed in those assessed for it. Anticoagulation therapy alone appeared sucessful in managing most patients in our cohort with few undergoing lytic therapy or first rib resection.

PB 1.64-5

Development and accreditation of a standardized training program in thrombosis and vascular medicine – a Canadian initiative

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Background: Having a policy statement on the educational mandate of a training program – a formal curriculum – is imperative in pursuing the goals of developing appropriate learning and evaluation strategies for trainees and providing quality care for patients on the receiving end of the products of clinical and research training initiatives. While the field of Thrombosis Medicine has rapidly evolved over the last few decades with respect to clinical and scientific advancements that continue to change the way consultants practice, there has been a distinct lack of progress in the development of standardized and accredited training programs in Canada and abroad that are accountable to licensing and government bodies. Canada has been a leader in the training of thrombosis consultants producing globally recognized prolific research and clinical careers. However, all thrombosis programs currently in existence are based on informal, locally developed curricula. The benefits of a formal and nationally accredited curriculum, which not only holds up an explicit standard to which teaching and learning take place, but also provides stakeholders with a measuring stick against which educational activities can be considered in view of practice requirements, cannot be ignored in this era of competency based education reforms and calls for greater accountability by the stakeholders in health care.

Aims: To develop and implement a standardized and nationally accredited training program in Thrombosis and Vascular Medicine through the Royal College of Physicians and Surgeons of Canada (RCPSC – the main licensing body for specialist physicians).

Methods: A multi-phase needs assessment was completed, and subsequently a committee was recruited and a curriculum document created based on the CanMEDS (RCPSC) framework of essential competencies for physician specialists. An application for formal national recognition and accreditation of a program in Thrombosis and Vascular Medicine was submitted to the RCPSC in the new area of accreditation designation, an 'Area of Focused Competence'.

Results: The application detailing the benefits of standardization and accreditation of training in Thrombosis and Vascular Medicine along with the extensive and practice-based Competency Training Requirements document have received favourable recognition and have been accepted for further review with the RCPSC. The proposed curriculum contains competency-based training requirements in areas of both venous and arterial thrombosis.

Conclusion: Canadian thrombosis training programs, accessed by national and international physicians, are on the route to obtaining formal, national, accreditation status. Given the ongoing growth in clinical care and research in Thrombosis and Vascular Medicine, establishing a standardized, validated curriculum is necessary to promote the highest standards of training and thus patient care. Similar training programs in other countries may find guidance in the needs assessment and formal curriculum to adapt them to their own population of trainees and clinical practice requirements. Future efforts will focus on the development of specific practice modules and assessment for this curriculum.

PB 1.64-6

An improved screening system for protein S type II deficiency: expanded versatility of total protein S activity assay

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Background: We have developed a total protein S-assay system to screen protein S type II deficiency (Tsuda, T. *et al*., *Blood Coagul Fibrinolysis* 23: 56–63, 2012). This assay system measures both the activity and quantity of total protein S at a high precision by using a commonly-used general-purpose automated analyzer and is proved to be a useful tool to screen molecular abnormalities of protein S.
Aims: The activity assay part of the system, a three-reagent system, comprises three reagents (R1, R2 and R3) and a dilution buffer (DilB) and requires 22 min for one assay. Because of these facts, not all analyzers can accommodate the assay. In the present study, we modified it to increase its applicability to a greater variety of instrument types in laboratories.
Methods: We modified the chemical compositions and the assay protocol of the total protein S activity assay to fit into a two-reagent system (R1, R2 and DilB), a more popular and less time consuming configuration, as opposed to the original three-reagent system (R1, R2, R3 and DilB). In the new assay, R1 contains activated factor V, R2 activated factor X, prothrombin, and a thrombin substrate S-2238 and DilB activated protein C. These chemical compositions were adjusted to obtain similar time course profiles to that of the original three-reagent assay. All data were collected using a Hitachi 7170S automated analyzer.
Results: The new two-reagent assay has fewer components which reduced reaction steps and assay time (10 min) compared with the original 22 min assay. The data of plasma samples (n = 33) obtained with the new two-reagent assay were well correlated with those with the three-reagent system ($y = 0.94x + 2.0$, $r = 0.93$), while intra- and inter-assay reproducibilities are better in the new one. The intra-assay C.V. improved to 0.9–2.0% from 1.4% to 4.5% of that of the three-reagent assay. Likewise, the inter-assay C.V. improved to 1.1–3.2% from 2.8% to 5.6%.
Conclusions: The modification presented here not only expanded the versatility of the total protein S activity assay but also shorten the assay time and made the reproducibility better. This enables us to screen molecular abnormality of protein S more efficiently and easily, and the screening system of protein S type II deficiency is now more practical.

PB1.65 – Diagnosis of VTE – II

PB 1.65-1

Health-related quality of life after pulmonary embolism – a case–control study

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Background: Pulmonary embolism (PE) is a common acute medical emergency. Research and clinical practice mainly focuses upon the medical treatment and clinical outcomes of PE. Surprisingly the psychological aspects and the impact of acute PE on quality of life (QoL) have not been studied properly.
Aims: The study aimed to determine Health-Related Quality of Life (HRQoL) in patients surviving acute pulmonary embolism compared to age- and sex-matched population having no previous history of venous thromboembolism (VTE).
Methods: This case-control study evaluated HRQoL using the generic QoL questionnaire EQ-5D among patients who had objectively verified PE between 2002 and 2011 in Oestfold Hospital Fredrikstad – Norway. Each patient was asked to hand over the same questionnaire to two age- and sex-matched (± 5 years) relatives or friends having no previous history of VTE, so called Buddy Controls. The questionnaire consists of the following five dimensions: mobility, self-care, usual activities, pain/discomfort and anxiety/depression. It also includes a vertical self-rated grading scale for total health-condition from 0 to 100, called EQ-VAS scale. Lower scores represent worse QoL. The EQ-5D dimensions can be converted to a single summary index value obtained from the general population. The study was approved by the Regional Ethics Committee; written and informed consent was obtained from all patients.
Results: A total of 208 patients (mean age 61 years; range 23–86 years) and 114 controls completed the questionnaire. When the five dimensions of health profile (mobility, self-care, activity, pain and anxiety) were dichotomized into no problems and problems, a statistically significant difference was found between cases and controls throughout all dimensions. The mean index value of EQ-5D was 0.81 (SD 0.22) among patients and 0.92 (SD 0.16) among controls. The difference between cases and controls in mean index value was 0.11. This difference was statistically significant ($P < 0.001$, Mann-Whitney) and also likely to represent a clinically meaningful difference (≥ 0.08). On the EQ-VAS patients reported a significantly lower mean score than controls, 67.2 (SD 21.3) and 81.3 (SD 16.9), respectively ($P < 0.001$, t-test).
Summary/Conclusions: This study indicates that the quality of life is reduced in patients who have sustained an acute pulmonary embolism as compared to age- and sex-matched population without previous history of VTE. These results confirms previous reports by Klok et al showing reduced QoL after PE in comparison to Dutch population norms, using the generic questionnaire SF-36. To our knowledge this is the first study in which health-related quality of life (HR-QoL) was compared to age- and sex-matched VTE – free controls. This is a novel research-field concerning PE, which should be further investigated specifically regarding the factors affecting HR-QoL and the socio-economic consequences after PE.

PB 1.65-2

Treatment patterns of venous thromboembolism in a real world population: the Q-VTE study cohort

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Background: Describing treatment patterns of venous thromboembolism (VTE) in a real world population may identify remedial gaps in patient care.
Aim: We aimed to characterize anticoagulant use following incident VTE in a real world setting.
Methods: We used the linked administrative healthcare databases of the province of Québec, Canada, including the hospitalization, universal healthcare services, and out-patient prescription databases. We identified all beneficiaries with an incident DVT or PE between 2000 and 2009, which we classified as definite or probable VTE using *a priori* determined diagnostic algorithms based on ICD-9-CM or ICD-10-CA diagnosis codes. We formed two patient cohorts, one with definite and the other including definite or probable first-time VTE, that were followed until death or end of study (December 31, 2009). Anticoagulant out-patient prescription patterns were analyzed for both patient cohorts.
Results: From 245,452 Québec residents between 2000 and 2009 with at least one VTE diagnosis in RAMQ or MED-ÉCHO, we formed the definite VTE cohort including 40,776 definite cases and the any VTE cohort consisting of 54,803 definite or probable cases. In the definite cohort, 13,858 (34%) cases required hospitalization for VTE, 24,203

(59%) developed VTE during hospitalization, and 2715 (7%) were diagnosed and managed in the out-patient setting. In the any VTE cohort, 13,214 (24%) cases were hospitalized for VTE, 23,152 (42%) developed VTE in hospital, and 18,437 (34%) were diagnosed in the out-patient setting. In the definite cohort, 74% of cases filled an out-patient prescription for an anticoagulant, and most received a vitamin K antagonist (VKA) either alone or in combination with low molecular weight heparin (LMWH) (54% VKA alone, 33% VKA and LMWH, and 10% LMWH alone).

In the any VTE cohort, 71% were dispensed an anticoagulant. In the definite cohort, out-patient prescription dispensation varied according to the setting of diagnosis (91% for cases requiring hospitalization for VTE; 66% for cases diagnosed with VTE while hospitalized; and 55% for cases diagnosed and managed in the out-patient setting). Similar findings were found in the any VTE cohort, although a greater proportion of cases (63%) in the out-patient setting filled a prescription for an anticoagulant. Among definite cases who were dispensed a VKA and filled more than one VKA prescription (n = 26,684), 64% discontinued VKA within 3 months, 13% between 3 and 6 months, and 9% after 6 months of initiating therapy. In all, 50% of cases discontinued VKA after 64 days of use. The mean duration of VKA therapy was 109 days (Standard Deviation 164). Similar findings were found in the any VTE cohort.

Conclusions: Our study provides useful information on VTE management in a real world population. Our results suggest that there may be important treatment gaps since only 74% of patients with VTE were dispensed a prescription for an anticoagulant, those managed in the out-patient setting were less likely to fill a prescription for an anticoagulant than patients who had been hospitalized, and a significant proportion of patients discontinued VKA therapy early.

PB 1.65-3

Single whole-leg compression ultrasound for exclusion of deep vein thrombosis in symptomatic ambulatory patients: a prospective observational cohort study

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Background: United Kingdom guidance has recently recommended serial proximal compression ultrasound (CUS) as first line imaging for suspected deep vein thrombosis (DVT). Single whole-leg CUS is a routine alternative diagnostic strategy that can reduce repeat attendance and identify alternative pathology.

Aims: We sought to assess the performance characteristics of an established emergency department ambulatory protocol incorporating whole-leg CUS by non-physicians for exclusion of DVT.

Methods: A prospective observational cohort study. Consecutive, ambulatory, adult patients with suspected DVT and negative or inconclusive whole-leg CUS had anticoagulation initially withheld and were followed up after 3 months.

The primary outcome was a predefined clinically relevant adverse event rate: a subsequent diagnosis of symptomatic venous thromboembolism (VTE) or VTE related death during 3 month follow up. Secondary outcomes included alternative diagnoses, technical failure rate and patient characteristics associated with failure.

Results: Two hundred and twelve patients agreed to participate and were followed for 3 months. One patient was subsequently diagnosed with a calf DVT. The adverse event rate was thus 1/212, 0.47% (95% Confidence Interval [CI] 0.08–2.62%). For patients deemed at high clinical risk by Wells scoring prior to ultrasound (n = 47), the adverse event rate was 2.1% (95% CI 0.4–11.1). One patient died during the follow up period. This death was deemed unrelated to VTE by the central adjudication committee.

150/212 patients were provided with a clear documented alternative diagnosis. CUS directly contributed to or confirmed the alternate diagnosis in 55/150 patients, of which the top three diagnostic labels were musculoskeletal injury, Bakers cyst and venous incompetence. Technical imaging failure occurred in 11.3% of cases (95% CI 7.7–16.3). Several potential predictors of an inconclusive result were identified on multivariate analysis: obesity (odds ratio 4.15, 95% CI 1.5–11.2), recent immobilzation (odds ratio 4.9, 95% CI 1.5–16.2), active cancer (odds ratio 7.9, 95% CI 1.5–41.7) and acute infection (odds ratio 2.9, 95% CI 1.1–8.0).

Conclusion: Patients who have anticoagulation withheld following a negative or inconclusive whole leg CUS for suspected DVT have a low rate of adverse events at 3 months. In addition, whole leg CUS provided additional diagnostic information and confirmed an alternative diagnosis in over a quarter of patients within this study.

Technical failure remains an issue: several factors were significantly associated with an inconclusive result in our cohort and their presence may warrant an alternative diagnostic approach.

PB 1.65-4

Retrospective audit of incidental VTE events over 3 years in a large teaching hospital

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Background: Studies previously reported the prevalence of incidental pulmonary embolism (PE) in patients having CT scans for reasons other than PE investigation. A subsequent meta-analysis of these studies found a mean prevalence of incidental PE of 2.6% (CI 1.9–3.4). Further retrospective review has elucidated the mean prevalence of abdominal vein Deep vein thrombosis as 1.74% (CI 1.29–2.34). We recently reported incidental VTE events from a 900 bed teaching hospital, with numerous tertiary services, as 1.64% (55 of 3358 scans) over 2011. The three most common VTE risk factors were Age, > 60 year (85%), active cancer (76%) and significant medical co morbidity (36%). Other associated information was the prevalence of Hospital Acquired Thrombosis (HAT) and the reason for CT scanning. The mortality associated with these diagnoses was 39 of 55 (71%), occurring a mean of 120 days after diagnosis, with 12 month mortality of 36 of 55 (65%).

Aims: Data has been collected for the last 3 years on incidental VTE events, to see if the prevalence, risk factors and association with HAT changed over that time. Also, to compare incidental findings pick-up rate with those scans that were targeted to investigation for VTE and to compare mortality.

Methods: The hospital radiology reporting system (CRIS) has been reviewed retrospectively, to determine incidental VTE diagnoses in CT scans of chest, abdomen and pelvis (or combination there of). This information was cross-checked with the hospital patient administration system (PIMS) to identify whether the patient event qualified as HAT.

Results: The incidental VTE prevalence rates for 2010, 2011 and 2012 were 76 of 6079 (1.25%), 55/3358 (1.64%) and 57/4914 (1.16%), for non-VTE targeted CT scans. HAT events were 28%, 20% and 33% respectively, yielding prevalence for HAT incidental findings of 0.34%, 0.33% and 0.39%. The pick-up rates for all VTE targeted investigations for the same periods were: 640/3819 (16.8%), 751/4660 (16.1%) and 738/4818 (15.3%). For HAT events, the targeted CT scan pick-up rates were: 31%, 31% and 27%.

The most prevalent risk factors for incidental findings in 2010, 2011 & 2012 are: age > 60 years: 78%, 85% and 91% respectively, Cancer: 68%, 76%, 67% and serious medical comorbidity: 32%, 36%, 30%.

The overall mortality for incidental VTE events is: 57/76 (75%) from 2010, 39/55 (71%) from 2011 and 29/57 (51%) for 2012, giving 12 month mortality rates of 63% for 2010 and 65% for 2011.

The three most common reasons for CT scanning, in order were: staging for cancer, further investigation for abnormal findings on previous scans and unexplained weight loss, for all 3 years.

Summary: As expected, this radiology outcome data identifies incidental VTE to be relatively low prevalence, when compared to targeted scans for VTE. Twelve month mortality associated with incidental VTE (63% and 65%) is significantly greater than published data related to all cause VTE events (15%). This is not surprising, acknowledging the high mortality associated with cancer the second most common risk factor.

PB 1.65-5

Provision of an external quality assessment programme for D-dimer point of care testing kits

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Background: There is a drive towards Point of Care Testing (POCT) in areas of haemostasis other than INR monitoring and recent developments have led to the availability of POCT devices for D-dimer testing. These devices are portable desktop analysers that are well suited to non laboratory areas but still require quality control to ensure reliable test results. External Quality Assessment (EQA) is already available to laboratories with 681 centres registered in the UKNEQAS BC laboratory programme for D dimer testing.

Aims: To develop a specific programme for D-dimer testing to meet the needs of POC users.

Methods: In-house studies had previously been performed which validated the use of lyophilised plasma for EQA testing with the quantitative methods -Cobas h232 (Roche) and Triage (Alere) and with qualitative- Clearview Simplify kits (Alere). The precision of results on this material and the accuracy of results compared to the UK NEQAS BC laboratory programme medians for these samples were assessed. Interested POCT centres were then approached to take part in a series of pilot studies to evaluate the use of lyophilised material for POC D-dimer EQA testing. Samples were distributed over a period of 3 years with Cobas users receiving 14 samples and Clearview users receiving 7. Patient scenarios were introduced in pilot 4–7 (four samples for Clearview and eight for Cobas users). Users were asked to indicate whether deep vein thrombosis (DVT) could or could not be excluded using their result and the patient scenario provided. Seven pilot studies have taken place over a period of 3 years with up to 42 Clearview users and 35 Cobas users. Users of the Cobas device received two samples per pilot study with sample medians ranging from 0.17 to 1.2 μg/mL FEU. Users of the Clearview device received one sample per study and were asked to state if it was positive or negative.

Results: Precision for the Cobas device was similar to that observed in theUKNEQASBC laboratory programme for D dimer testing with coefficients of variance between 8% and 25% (outliers excluded – some results being clear outliers) from the median value. Of the seven samples circulated to the Clearview users only two samples had results on which all centres agreed. For those samples with patient scenarios provided the clinical interpretations (DVT excluded or not excluded) showed full agreement in 4/8 of the samples for Cobas users and 1/4 samples for the Clearview users. An exercise will take place for the Triage users in early 2013.

Conclusion: EQA is required for POC D-dimer tests and lyophilised UKNEQAS BC samples are suitable for this purpose. Our data shows that there is not only variance in both the results achieved and in their interpretation.

PB 1.65-6

Modified strain gauge plethysmography as an additional screening test for patients with suspected deep vein thrombosis

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Background: Only 15% of patients with suspected DVTs have Doppler scan confirmed thrombi. The use of clinical decision rules (e.g Wells score) and d-dimer testing can exclude DVT in 25–50% of patients presenting with suspected DVTs thus decreasing the need for confirmatory Doppler scanning. The introduction of point-of-care (POC) d-dimer testing has enabled DVT assessment to be carried out in the community. In 2009 at Brentwood Community Hospital we established a nurse led, haematologist supported primary care DVT screening service using the modified Wells score and a quantitative POC d-dimer test (cardiac d-dimer, Roche). Doppler scanning was carried out on site by vascular sonographers on patients who either had a Wells score of > 1 (DVT likely) or a Wells score of < 2 (DVT unlikely) but a raised d-dimer (cut off 0.5 μg/mL). Over the first 2 years 80% of referred patients required Doppler scans, but only 20% were positive.

Aims: To assess the potential usefulness of computerised Strain Gauge Plethysmography (SGP; Venometer V3, Amtec), utilising new software with a modified algorithm, as an additional screening test, to see if this might improve DVT screening effectiveness and decrease the number of Doppler scans necessary.

Methods: All patients continued to have modified Wells scores and POC d-dimer testing performed as above and the results continued to be the main determinant for Doppler scanning. In addition SGP scans were carried out by the trained nursing staff on patients with their verbal agreement but were not used to determine further management.

Results: One hundred and eighty-seven patients had all three screening tests carried out, Wells score, d-dimer and SGP, and also had Doppler scans performed. One hundred and eighteen patients had negative SGP scans and in only three were Doppler scans positive (2.5%; all being distal calf DVTs). Thus the sensitivity and negative predictive value (NPV) of a negative SGP scan for excluding proximal DVT was 100%. If all DVT positive Doppler scans were considered, the sensitivity and NPV of a negative SGP scan for excluding any DVT was 89% and 97% respectively. If all three screening tests were positive, 65% of patients had Doppler scan confirmed DVTs. If two out of three tests were positive, 11% had positive Doppler scans. If only one out of three tests were positive, only 5% of Doppler scans were positive. Selective use of SGP in our patients with discordant Wells scores and d-dimer results would have decreased the need for Doppler scans from 65% to 35%. Two distal calf DVTs would have been missed.

Conclusion: The addition of modified SGP to the Wells score and a d-dimer in the initial screening of patients with a suspected DVT improved the efficiency of DVT screening and could make more cost-effective use of Doppler scanning. This is a potential advantage when screening takes place in a primary care setting where Doppler scanning might not be locally or readily available. It is suggested that further prospective studies are undertaken to validate this approach.

PB1.66 – Hormones, Pregnancy, Women's Issues – I

PB 1.66-1

The pregnancy health-care program: a model for the prevention of venous thromboembolism in pregnancy

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Background: Venous thromboembolism (VTE) is the leading cause of maternal mortality and morbidity in developed countries, with an annual incidence 7–10 times higher than outside pregnancy. There is a great variability of individual risk profiles, and recommendations on antithrombotic prophylaxis are based on case-control studies or experts' opinions that do not entirely satisfy the individual optimal management of pregnant women.

Aims: The aim of this study was to develop and use a 'Pregnancy Health-care Program' (PHP) that allows clinicians to tailor antithrombotic prophylaxis in pregnant women applying uniform criteria for evaluation of the individual risk of VTE.

Methods: The PHP was created inCremona, Italy, by a working group of haematologists who trained gynaecologists, obstetricians and medical practitioners. A number of risk factors, including personal and family history of VTE, several medical conditions, age, parity, BMI, and thrombophilia (when available), were expressed as odds ratios obtained from published studies and converted in a severity risk score ranging from 0.5 to 3. On the basis of different score ranges, women were stratified in three different categories and received clinical observation, compression stockings, LMWH or their combination during pregnancy. Visits were scheduled at the end of each trimester and if a woman changed risk category, antithrombotic prophylaxis changed accordingly. Physicians who did not join the PHP followed their pregnant women according to their clinical practice. Data on pregnancy outcome of the non-PHP women were obtained after delivery through hospital discharge records.

Results: From January 2008 to December 2010, 3715 deliveries occurred in Cremona hospital, of which 3697 were live births and 18 were intrauterine fetal deaths. The PHP reached 1787 pregnant women, whereas the remaining 1996 were managed without specific risk assessment. LMWH prophylaxis or therapy was prescribed to 85 women. In the PHP group only a pudenda vein thrombosis occurred. In the non-PHP group, eight events were recorded (0.4%), five VTE and three lower limb superficial vein thrombosis. The risk of VTE was higher in the non-PHP than in PHP group (c square test 4.49, $P < 0.03$). Thrombophilia abnormalities were searched in the nine symptomatic women and found in four of them (two homozygous and one heterozygous factor V Leiden, one heterozygous G20210A prothrombin mutation). Four women in the non-PHP group had positive family history of VTE in one 1st degree relative at an age < 50 years. No major bleeding was observed in LMWH users.

Conclusion: Management of pregnant women with our PHP based on stratification of individual risk of VTE resulted in a significant reduction of VTE events.

PB 1.66-2

Influence of maternal thrombophilia status on the outcome of assisted reproduction

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The chances of pregnancy after a first attempt of assisted reproduction technologies (ART) are about 30%. Pregnancy rate progressing to term and resulting in live birth of a child is somewhat lower, 25%. Repeated cycles of ART increase success rate. However, a substantial number of couples fail to achieve pregnancy after three cycles. Thrombophilia has been suggested to play a role in failure of ART by interfering with embryo implantation and placentation but available evidence remains controversial. Large prospective studies to confirm this hypothesis are lacking.

The objective of our study is to investigate the association of thrombophilia with outcome of ART in women with recurrent, unexplained failure of assisted reproduction.

We set up a prospective study including women with a history of at least three failures of fresh ART cycles with replacement of one or two good quality embryos.

Blood samples are taken before the start of a next treatment cycle to avoid influence of ovarian stimulation on coagulation parameters. All women are tested for inherited thrombophilia (antithrombin activity, protein C activity, free protein S antigen, factor V Leiden and prothrombin G20210A mutation) and evaluated for the presence of antiphospholipid antibodies (lupus anticoagulant, anticardiolipin and anti-b_2GPIb antibodies).

Study end point is reached after live birth or failure of ART after six treatment cycles. Follow-up information is obtained from the patients' medical records.

Implantation rate, clinical pregnancy rate and live birth rate are defined according to the ICMART glossary of ART terminology.

Stimulation cycles where aspirin, low molecular weight heparin and/or corticosteroids were used, were excluded as these drugs could influence outcome.

This study was approved by the medical ethics committee of the University Hospital of Brussels. All patients signed informed consent.

Between 2008 and 2012, 136 women were enrolled. Today, sixty-one women have reached study end point. Twenty-one women had a live-born baby while 40 did not. There were no significant differences in prevalence of thrombophilia between the two groups. Positivity for antiphospholipid antibodies was the most prevalent parameter among the two study groups, 5% (1/21) in the live birth group and 22% (9/40) in the non-live birth group ($P = 0.13$).

After inclusion, these 61 women underwent a total of 111 ART cycles. Cycles with interfering drug use were excluded, leaving 77 cycles for investigation. Implantation rate and clinical pregnancy rate were not statistically different in women with inherited and/or acquired thrombophilia compared to women without thrombophilia. There is, however, a trend towards lower live birth rate in the patient group with thrombophilia compared to the group without, 4.8% (1/21) and 18.5% (10/54) respectively ($P = 0.13$).

These results suggest that there is no significant association between maternal thrombophilia and failed embryo implantation. A slight trend towards lower live birth rate in women with thrombophilia was observed. The results presented here are the preliminary data of a large, prospective study that is still ongoing and thus need to be considered with caution. Failure to demonstrate statistical significance may be due to the limited sample size of this interim analysis.

PB 1.66-3

Pelvic vein thrombosis after cesarian-section delivery

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Background: Venous thrombosis (VT) constitutes the leading cause of maternal mortality in the developed world. Pelvic vein thrombosis (PVT) is an important cause of VT during pregnancy and post-partum. Few studies have evaluated the occurrence and natural history of PVT after cesarian C-section delivery in the postpartum period.

Aims: Evaluate the incidence and natural history of PVT after cesarian C-section delivery.

Methods: Informed consent was obtained of all subjects. This study comply with the Declaration of Helsinki. We prospectively studied a cohort of 50 women after C-section delivery. Women underwent magnetic resonance venography (MRV) in the early postpartum period (1–7 days) and were followed for 3 months. Analysis considered two groups classified as low- and high-risk for venous thrombosis.

High-risk subjects had one or more of following characteristics: age above 35 years; obesity defined as body mass index above 35 kg/m^2 before pregnancy; immobility above 3 days; personal history of VT; hypertension grade 2 ($\geq$ 160 mmHg systolic blood pressure and/or $\geq$ 100 mmHg diastolic blood pressure) or higher; congestive heart failure class III or IV; preeclampsia or eclampsia; current infection; multiparity, i.e., above four previous deliveries; current gemelar pregnancy; current smoking and need for urgent modification of delivery mode (vaginal to C-section). Low-risk subjects had none of the characteristics cited above.

Results: We identified definite asymptomatic PVT in four women (8%), of whom one out of 25 (4%) in the low risk group and three out of 25 (12%) in the high risk group (RR 3.27; 95% CI [confidence interval], 0.34–31.46). Duplex scan of both legs was performed in all four women and proximal VT was detected in one. Anticoagulation was not instituted.

All four patients underwent a new MRV after 3 months, which showed complete disappearance of the intra-luminal filling defects. No symptomatic thrombosis developed in the remaining 46 women after 3 months' follow-up.

Conclusion: PVT occurs in the early postpartum period as an asymptomatic event in women undergoing C-section. The risk of developing PVT seems to be higher in women with high risk factors for VT. There was no complication of the PVT within 90 days' follow-up.

PB 1.66-4

The coagulation profile of preterm delivery

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Background: The trigger initiating preterm birth is, in most cases, elusive and unidentified. Not all premature uterine contractions cause preterm birth, challenging to predict in which cases contractions will lead to preterm delivery. Coagulation anomalies were suggested to be etiologically involved in the development of preterm birth, however the coagulation state of preterm parturients is still incompletely revealed.

Aims: The aim of this study was to evaluate the haemostatic system of pregnant women with premature uterine contractions, and to observe the coagulation state of their infants.

Methods: This cohort study population consisted of healthy pregnant women admitted with regular uterine contractions before 37 weeks of gestation. Blood samples were collected from the recruited women, the clinical outcome and the week of gestation at delivery were recorded. The study group consisted of 38 women who experienced preterm birth, 14 of which were with preterm premature rupture of membranes (PPROM). The control group included 38 women who had premature contractions but eventually had normal term deliveries. Both groups were matched for maternal age, number of births and gestational age at admission. Blood samples were tested for parameters of the haemostatic system and coagulation activation markers. Furthermore, samples of umbilical cord blood were collected at delivery for coagulation studies from 43 patients with premature uterine contractions.

Results: We documented significantly shorter activated partial thromboplastin time (aPTT) in study than in the control group (25.7 $\pm$ 2 vs. 27.4 $\pm$ 2.7 s, $P = 0.003$). Prothrombin time (PT) was also shorter in the study group (9.96 $\pm$ 0.5 vs. 10.1 $\pm$ 0.4 s, $P = 0.05$). There was no significant difference between the two groups in the level of other haemostatic markers: Fibrinogen, D-Dimer, Protein C-Global, Free Protein S antigen (fPS), Factor VIII activity, von willebrand factor antigen, Plasminogen activator inhibitor-1, Prothrombin Fragment F1 + 2 (PT F1 + 2), and Tissue factor.

Within the study group, PT F1 + 2 levels were significantly lower in association with PPROM than with preterm deliveries without rupture of membranes (351 $\pm$ 99 vs. 561 $\pm$ 242 pM, $P = 0.003$).

All the haemostasis markers mentioned above were evaluated also in 43 umbilical cord blood samples collected at delivery. aPTT was found to be significantly longer in neonates that were delivered prematurely compared to neonates that were delivered at term (38.8 $\pm$ 7.5 vs. 34.4 $\pm$ 3.1 s, $P = 0.024$). fPS levels were remarkably lower in cord blood samples of prematurely delivered neonates than in normal term neonates samples (38.3 $\pm$ 11.6% vs. 48.7 $\pm$ 10.2%, $P = 0.004$).

Conclusions: Our findings suggest that shortened aPTT and PT time in women with premature contractions might be associated with preterm birth. Furthermore, low levels of PT F1 + 2 were found with PPROM preterm delivery. In addition, longer aPTT and decreased fPS levels at the neonate may be associated with premature delivery. These findings document increased thrombotic activity affecting both mothers and newborns in cases of 'real' preterm delivery, suggesting that a shortened aPTT and PT may be a sign that premature contractions will eventually lead to preterm labor.

PB 1.66-6

The effect of thrombotic markers on severity of bleeding symptoms in women with inherited bleeding disorders

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Background: Women with inherited bleeding disorders demonstrate heterogenicity in bleeding symptoms that does not always correlate to underlying clotting factor levels. This is problematic when predicting the bleeding risk, especially when haemostatic challenge is anticipated. The bleeding tendency is influenced by haemostatic factors and thrombotic variables that have the potential to counteract one another. The effect of thrombotic variables in reducing the overall bleeding tendency, have demonstrated a protective effect on bleeding disorders such as haemophilia. The correlation of clotting factor levels, presence of thrombotic variables, and bleeding score in women with inherited bleeding disorders have not been thoroughly investigated.

Aims: To assess the correlation between bleeding tendency, haemostatic and thrombotic variables in women with inherited bleeding disorders.

Method: Quantitative measurement of the bleeding tendency was assessed using the standardized ISTH Bleeding Assessment Tool. Fifty women with inherited bleeding disorders (42 with von Willebrand disease and eight carriers of haemophilia) and 20 women without any bleeding disorders were included. Blood samples were then obtained from the individuals for assessment of clotting factors (factor VIII and von Willebrand factor) and thrombophilia screen (lupus anticoagulant, antithrombin, protein C and protein S, factor V Leiden mutation and Prothrombin G20210A mutation).

Results: Bleeding score was inversely related to von Willebrand factor antigen (VWF:Ag), ristocetin cofactor (VWF:RCo) levels and factor VIII activity. So far no correlation has been demonstrated between bleeding score and protein C, protein S and antithrombin activity. Two women tested positive for prothrombotic mutation, one with lupus anticoagulant (bleeding score of 5) and another with factor V Leiden mutation (bleeding score of 7).
Conclusion: Although bleeding score is not influenced by levels of antithrombin, protein C and protein S activities this study is still in progress and further data is required for sufficient statistical analysis. It is useful to consider the history (bleeding score) as well as the possibility of underlying thrombotic markers, when performing a bleeding risk assessment.

PB1.67 – Inflammation: Basic – I

PB 1.67-1

PI3Kinase is a key regulator of CD40L surface expression and release from activated platelets

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Background: Beside their crucial role in haemostasis, platelets are considered as important modulators of the immuno-inflammatory reaction. Among the inflammatory molecules expressed by platelets, CD40 Ligand (CD40L, CD154) is of interest since it is directly involved in the modulation of the immune response and inflammation. Activated platelets release large amounts of soluble CD40L (sCD40L). Platelets have been postulated to be the major source of circulating sCD40L.
Aims: Mechanisms regulating platelet CD40L availability are not fully understood. We aim to determine signaling pathways leading to CD40L expression and release by human activated platelets.
Methods: Flow Cytometry; CD40L ELISA; Gelatin Zymography; Western blot.
Results: Here, we show that PI3K pharmacological inhibition diminishes by 50% the release of sCD40L from human activated platelets with thrombin and PAR1 agonist, whereas the inhibition reaches 100% upon PAR4 stimulation. sCD40L release requires the $\alpha II_b\beta 3$ integrin activation that is controlled by PI3K. $MnCl_2$ that forces integrins to take an active conformation independently of intracellular signal, did not restore normal sCD40L release in presence of PI3K inhibitors. Thus, PI3K independently regulates sCD40L release and $\alpha II_b\beta 3$ activation. Then, we asked whether the PI3K involvement in sCD40L release could be a consequence of CD40L surface expression modulation. Inhibition of PI3K fully blocked CD40L surface expression when activated by PAR4 (500 μM AYPGKF), whereas it was not affected when induced via PAR1 (10 and 50 μM SFLLRN). The pharmacological inhibition of PI3K isoforms indicates the only contribution of PI3K-beta in CD40L surface expression during PAR4 activation. These results argue in favor of a differential implication of PI3K in the regulation of CD40L surface expression and release regarding the PAR engaged.
The observation that PI3K inhibitor decreases sCD40L release but has no effect on surface CD40L upon PAR1 stimulation prompted us to explore the involvement of PI3K in MMP2 release and activation. Indeed, MMP2 was shown to cleave CD40L from platelet surface. Zymography and western blot analysis of PAR1-activated platelet releasates did not show any implication of PI3K in MMP2 release and activity. Ongoing experiments of MMP2 activity measurement on platelet surface will help us to unravel the role of PI3K in sCD40L release through MMP2 activity.
Conclusion: PI3K regulates platelet CD40L bioavailability. However, its implication differs regarding the PAR engaged. Nevertheless, PI3K represents a key element of the platelet-mediated inflammatory process.

PB 1.67-2

T cells, inflammation and tissue factor in gastric malignancy

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Background: Thrombosis significantly contribute to the morbidity and mortality of patients with malignancy.
Aims: We investigated the role of T-cell subsets in the induction of tissue factor (TF) production by human monocytes in patients with gastric cancer. Helicobacter pylori is type I oncogenic factor for gastric adenocarcinoma and H. pylori-related inflammation contributes to gastric oncogenesis.
Materials and Methods: We derived T-cell clones from seven patients with gastric adenocarcinoma and we studied the ability to induce TF production of 10 T helper (Th) 1, 10 Th2 and 10 Th17 clones. T-cell blasts of antigen-specific clones (8×10^5/mL) were cocultured for 16 h with autologous monocytes (4×10^5/mL) in the presence of medium, mitogen or the specific antigen. TF was quantitated by a specific enzyme-linked immunosorbent assay kit (American Diagnostica Inc, Greenwich, CT).
Results: After activation, all T-cell clones with Th1 and Th17 cytokine profile, but not Th2 clones, induced TF production. T-cell blasts from Th1 and Th17 activated clones were fixed with paraformaldehyde and added to monocytes in the presence of medium alone or their supernatants. Addition of Th1/Th17 cells or supernatants induced low TF production (1.3 ng/mL + 0.13), whereas addition of cells/supernatants resulted in higher TF synthesis (2.9 ng/mL + 0.5). No TF synthesis was induced by Th2 cells, whereas addition of Th2 cells and Th1 or Th17 induced TF production. The addition of anti-IFN-gamma antibody, anti-IL-17 or Th2 supernatants to monocytes stimulated with Th1/Th17 cells plus supernatant resulted in inhibition of TF synthesis.
Summary: $CD8^+$ and $CD4^+$ T cells, Th1/Th17, but not Th2, T cells can help TF production. Both cell-to-cell contact with activated T cells, Th1-type and Th17 cytokines, in particular IFN-gamma and IL-17, are crucial for optimal TF synthesis, whereas Th2-derived cytokines (IL-4, IL-13, and IL-10) are inhibitory.
Conclusion: The results obtained so far are of potential interest for the development of novel future preventive or therapeutic strategies of thrombosis in patients with malignancy.

PB 1.67-3

Protease activated receptor 4 contributes to host defense in streptococcus pneumoniae induced pneumonia

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Background: *Streptococcus (S.) pneumoniae* is a common causative pathogen of pneumonia and sepsis. Pneumonia and sepsis are associated with enhanced activation of coagulation, resulting in the production of several host derived proteases at the primary site of infection and in the circulation. Serine proteases cleave protease activated receptors (PARs), which form a molecular link between coagulation and inflammation. PAR4 is one of four subtypes of PARs and is widely expressed by multiple cell types in the respiratory tract implicated in pulmonary inflammation, by immune cells and by platelets. In mice, PAR4 is the only thrombin receptor expressed by platelets.
Aims: To determine the contribution of PAR4 to the host response during pneumococcal pneumonia.
Methods: Pneumonia was induced by intranasal inoculation with *S. pneumoniae* in PAR4-deficient (*par4*−/−) and wild-type mice. Mice

were sacrificed after 6, 24 or 48 h. Blood, lungs, liver and spleen were collected for analyses.
Results: After 48 h of infection, higher bacterial loads were found in the lungs of *par4*−/− mice ($P < 0.05$), accompanied by higher histopathology scores and increased cytokine levels ($P < 0.05$). At 24 h post infection *par4*−/− mice displayed lower plasma and lung thrombin-antithrombin complex (TATc) levels, whereas they showed higher TATc concentrations at 48 h. Of interest, *par4*−/− mice displayed a trend towards lower plasma platelet factor 4 levels at this latter time point, indicative of decreased platelet activation in spite of the apparently increased thrombin formation.
Conclusion: Our findings suggest that PAR4 contributes to antibacterial defence during murine pneumococcal pneumonia.

PB 1.67-4

Increased systemic interleukin-1β accelerates the onset of stroke in stroke-prone spontaneously hypertensive rats

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***Background*:** Epidemiological data suggest that interleukin (IL)-1β is a potential cause of stroke. However, there is no direct evidence to suggest that IL-1β accelerates stroke onset.
***Aims*:** We therefore examined the effect of IL-1β on stroke onset in stroke-prone spontaneously hypertensive rats (SHRSP).
***Methods*:** We measured plasma IL-1β levels in male Wistar Kyoto rats (WKY), spontaneously hypertensive rats (SHR), and SHRSP. IL-1β signal-related gene expression was measured in cerebrovascular endothelial cells (CVECs) from each strain. To determine the direct effects of IL-1β on stroke onset, IL-1β or goat anti-rat IL-1β polyclonal antibody were administered continuously to SHR or SHRSP using an osmotic pump.
***Results*:** At 10 weeks of age, plasma IL-1β levels were significantly higher in SHRSP than in WKY ($P < 0.001$ vs. SHRSP) and SHR ($P < 0.001$ vs. SHRSP). Expression levels of IL-1β signal-related genes in CVECs, including IL-1β, IL-1 receptor (IL-1R)-1, IL-1R-2, caspase-1, monocyte chemoattractant protein-1 (MCP-1), and intercellular adhesion molecule-1 (ICAM-1), were significantly higher in SHRSP than in WKY or SHR. Furthermore, IL-1β (10 ng/mL) treatment significantly increased MCP-1 and ICAM-1 mRNA expression levels in CVECs. Continuous administration of IL-1β (2 μg/day) using an osmotic pump slightly increased the incidence of stroke in SHR ($P = 0.046$) and significantly accelerated the onset of stroke in SHRSP ($P = 0.006$) compared with vehicle controls. In addition, continuous administration of anti-rat IL-1β antibody (600 μg/day) to SHRSP slightly, but not significantly, delayed the onset of stroke compared with control. One rat in the control group, none in the IL-1β group, and three in the anti- IL-1β antibody group showed no signs of stroke at the end of the 4-week administration period.
***Conclusions*:** SHRSP had increased plasma IL-1β levels compared with WKY and SHR prior to the onset of stroke. Further, exogenous IL-1β accelerated stroke onset. These results suggest that a stimulated IL-1β signal might be a cause of stroke onset under conditions of concomitant severe hypertension, and suppression of the IL-1β signal might thus be a target for stroke prevention.

PB 1.67-5

Diverse roles of alphaMbeta2 integrin in lipopolysaccharide-mediated inflammation

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Background: Several lines of evidence indicate that αMβ2 integrin mainly expresses on leukocytes and plays a crucial role in the differentiation of monocyte and macrophage.
Aim: Here we investigated the individual role of CD11b and CD18, the two major subunits of αMβ2 integrin, in the lipopolysaccharide (LPS)-induced inflammatory responses in macrophages.
Methods/Results: Our data showed that LPS treatment significantly increased the levels of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-10 and nitric oxide (NO) production. Preincubation with CD18 neutralizing antibody completely abrogated the LPS-induced IL-10 expression, but CD18 neutralizing antibody administration did not affect the expressions of TNF-α, IL-1β and nitric oxide (NO) production. In contrast, CD11b neutralizing antibody up-regulated the LPS-induced IL-10 expression with partial inhibition of TNF-α, IL-1β and NO production. In addition, inhibition of CD18 abrogated the LPS-induced activation of signal transducer and activator of transcription-3 (STAT-3), whereas blockade of CD11b had no such effect on STAT-3 activation. Moreover, inhibition of CD11b, but not CD18, attenuated the LPS-induced NF-κB activation. We also found that LPS increased the interaction of Toll-like receptor 4 and CD11b/CD18 complex in macrophages.
Summary: We proposed CD11b and CD18 may display differential regulatory roles during LPS-mediated inflammatory responses. These results will provide new insights into the diverse roles of CD11b/CD18 in LPS-mediated inflammation, which is important in the therapy of sepsis in the future.

PB 1.67-6

Plasmin induces *in vivo* monocyte recruitment through protease-activated receptor-1, MEK/ERK and CCR2 mediated signaling

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Background: The Plasminogen/Plasmin (Plg/Pla) system is associated with a variety of biological activities beyond the classical dissolution of fibrin clots, including cell migration, tissue repair and inflammation. Although the capacity of Plg to induce cell migration is well defined, the mechanism *in vivo* underlying this process is elusive.
Aims: To investigate the effect of Pla on cell migration *in vitro* and *in vivo* and the role of mitogen-activated protein kinase (MAPK) ERK1/2, protease activated receptor-1 (PAR-1) and CCL2/CCR2 axis in this process.
Methods: We performed *in vitro* migration assay (wound healing assay) in culture of MEFs (Mouse Embryonic Fibroblasts) or mouse leukemia monocyte macrophage (RAW 264.7). The cells were treated with Pla (2 μg/mL) at different times, or pretreated with the MEK1/2 inhibitor U0126 (15 μM), a serine protease inhibitor Leupetin (25 μg/mL) or the lysine analog tranexamic acid (0.01 M) 60 min. prior to and throughout Pla treatment and processed for microscopic counting of migrating cells or western blot analysis for phosphorylated ERK1/2. BALB/C mice were challenged by i.pl. (intrapleural) injection of Pla (2 μg/cavity) and the cells present in the pleural cavity harvested at dif-

ferent times or 48 h after pre-treatment with specific inhibitors (U0126 60 μg/cavity, i.pl.; leupeptin 100 μg/mouse, i.p.; SCH79797, 5 mg/kg, i.p.) 1 hour before Pla. Cells were processed for total and differential leukocyte counts and western blot analysis for P-ERK1/2 and IkB-a. Pleural levels of cytokines IL-1β, IL-6 and TNF-α and the chemokine monocyte chemoattractant protein 1 (MCP-1/CCL2) were analyzed by ELISA. CCR2 knockout mice and wild-type littermates were injected with plasmin and the cells present in the pleural cavity were harvested at 48 h after and processed for total and mononuclear cell count. All procedures described here had prior approval from the Animal Ethics Committee of Universidade Federal de Minas Gerais (CETEA/UFMG, *Protocol number:* 19/2011).

Results: Pla induced *in vitro* migration of murine fibroblast and macrophages dependent on MEK/ERK pathway and by requiring its proteolytic activity and lysine binding sites on cell surfaces. Plasmin injection into the pleural cavity of mice induced a time-dependent influx of mononuclear cells that was associated with augmented ERK1/2 and IkB-α phosphorylation and increased levels of CCL2 and IL-6 in pleural exudates. *In vivo* inhibition of protease activity by using leupeptin or a PAR-1 antagonist (SCH79797) prior to Pla injection abolished Pla-induced mononuclear recruitment and ERK1/2 and IkB-α phosphorylation. Interestingly, inhibition of MEK/ERK pathway abolished Pla-induced CCL2 upregulation and mononuclear cell influx. In agreement with a requirement for CCL2 to Pla-induced cell trafficking, $CCR2^{-/-}$ mice were not responsive to Pla-induced mononuclear recruitment.

Conclusion: Pla-induced mononuclear cell recruitment *in vivo* was dependent on PAR-1 activation of the MEK/ERK/NF-kB pathway which led to the release of CCL2 and activation of CCR2.

Financial Support: FAPEMIG, CNPq and PRPq-UFMG.

PB1.68 – Inherited Risk Factors Venous Thrombosis: Basic – I

PB 1.68-1

Identification of rare variants in ADAMTS13 through next generation sequencing implicated in pediatric stroke

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Background: Recently we reported a gene network of ADAMTS genes implicated in pediatric stroke through a genome-wide association study (GWAS)[1]. However, the causative variants underlying the association remain elusive.

Aim: To identify rare variants in ADAMTS13 contributing to stroke risk in children using next generation sequencing (NGS) in 48 affected children and 48 unaffected siblings.

Methods: Genomic regions implicated through our GWAS [1] were enriched using the Nimblegen EZ choice target enrichment kit yielding a target region for NGS of ~6.8 MB genomic sequence comprising total 48 genes from 15 chromosomes, among them ADAMTS13. The enriched paired-end libraries from 96 children were subsequently sequenced on 2 flow cells and 2 × 100 cycles using an Illumina HiScan-SQ instrument yielding a total of ~180 Gb sequence data at an average coverage of < 50X. Sequence mapping was performed using the BWA algorithm followed by bioinformatic analysis using the GATK software for variant detection, and Annovar and SNPEff for functional annotation. Statistical significance of found variants in 46 siblings was determined using the Sibship Disequilibrium Test (SDT). A collapsing test (CALPHA) was used to calculate the cumulative effect of rare variants in the ADAMTS13 gene on stroke risk. Promising genetic variants were subsequently verified using capillary sequencing and an ABI 3730 automated sequencer, followed by TaqMan genotyping of the full cohort comprising 248 nuclear families.

Results: A total of 194 SNPs were identified in the ADAMTS13 gene and its proximity (5Kb upstream and downstream), 23 of which are not present in dbSNP135. In addition, 16 indels were found, six of which are novel. One hundred and forty-six SNPs reside in intronic regions, 40 SNPS are located upstream and 10 downstream of the ADAMTS13 gene. Eight SNPs lead to synonymous, and seven SNPs leads to non-synonymous changes in the ADAMTS13 coding region. Four SNPs reside in the 5′untranslated region (UTR). One intronic SNP, rs4962153, was previously reported in a GWAS on liver enzyme levels, 10 SNPs affect transcription factor binding sites and two SNPs fall within a conserved genomic region. All exonic SNPs are involved in splicing regulation and alter splicing enhancer binding sites or transcription factor binding sites. Of the 194 SNPs, 65 have a minor allele frequency of < 0.02, 22 of which are novel. The CALPHA test for cumulative association of these SNPs shows a *P*-value of 6.3×10^{-5} and an association with pediatric stroke.

Summary and Conclusion: Our data support the presence of novel variants in the ADAMTS13 of functional relevance, which may shed light on the genetic architecture underlying the association between ADAMTS13 and pediatric stroke. Further studies are underway to characterize the functional consequences of these genetic variants on ADAMTS13 expression, splicing patterns as well as activity.

Reference:

1. Arning et al., Blood 2012.

PB 1.68-2

Identification of mutations in the protein C gene in a panel of 83 Spanish families with protein C deficiency

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Background: There are no data on characteristics and molecular basis of the hereditary protein C (PC) deficiency in Spain.

Aims: To analyze a panel of probands with PC deficiency from symptomatic Spanish families.

Methods: We sequenced the nine exons (E) and their flanking intron (I) regions of the PROC in 83 unrelated probands with venous thromboembolism (VTE) and 174 relatives of 52 probands. Informed consent was obtained from all subjects of the study and it was approved by the ethics committee of our Institution.

Results: In 71 probands (86%) we found 35 different mutations in heterozygosis associated with the PC deficiency, 20 of them not yet reported. One mutation was localized in I1, four in E3, one in I3, three in E5, three in I5, three in E6, one in I6, six in E7, three in E8, one in I8 and nine in E9. Twenty-eight mutations resulted in the change of one nucleotide (nt), mainly missense mutations, but five caused a premature stop codon. Three mutations with one nt, four nt and 16 nt deletion caused a frameshit. One mutation resulted in a three nt deletion (p.Ile321del) and one in a six nt insertion (p.Lys241_Glu242 insLeuAsp). About 40% of the point mutations were localized in CpG dinucleotides. Three mutations were localized in two unrelated families, three in three families, four in four families and one in eight families. Three caused type II mutations, one in E3 (identified in four unrelated families) resulting in a p.Glu16Lys change in the PC Gla domain, one also in E3, resulting in the change p.Arg-1His, and one in exon 8 (p.Lys193Glu). The mean minus 2 SD PC level in 436 healthy individuals and 325 VTE patients without PC deficiency was 67% and 63%, respectively. In 10 probands with PC levels from 49% to 70%

and in two probands on coumarin therapy we did not find any mutation. Twenty-four relatives with PC levels ranging from 51% to 73% from 16 PC deficiency families did not carry the mutation found in the proband.

Conclusion: This is the first Spanish registry of PC deficiencies and confirms the considerable heterogeneity in the genetic abnormalities. The data suggest that the Glu16 is essential for the *in vivo* expression of the anticoagulant PC function, possibly by allowing a correct folding of the Gla domain. The study has provided genetic counseling to those patients and relatives whose PC levels were near lower normal limit. Thus, by adding a genetic test it is possible to assign a subject to either the normal or the deficiency group, and estimate precisely the correlation between the PC deficiency and thrombosis. The spectrum of recurrent mutations in the Spanish population is completely different from that found in the Netherland, where the most frequent mutations were Gln132 and Arg230, and is more similar to that of one French study where the most frequent were Arg178 and Pro168. In the present study, Val 297 (eight families) and Tyr124, Pro168, Arg178 and Lys16 (four families) were the most prevalent.

PB 1.68-3

What is the origin of factor V Leiden mutation in East Mediterranean Arabs and has it occurred there first?

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Background: Studies reported high prevalence of factor V Leiden mutation (FVL; G1691A) in Caucasian populations (1–15%) and patients with venous thrombosis (15–65%), but FVL was rare or absent in non-Caucasians like Africans and Asians. Molecular studies on Caucasians of European origin reported FVL to be associated with one haplotype consisting of nine single nucleotides polymorphisms (SNPs) in the Factor V gene. These data suggested that FVL should have occurred as a single event in the far past in a single European Caucasian ancestor whose offspring are the current carriers of FVL. However, more recent studies found a relatively higher prevalence of FVL in Arab populations living in Eastern Mediterranean countries (5–27%), who are not generally considered as Caucasians. This questioned the origin of FVL in Arabs: is it the same ancestor for Caucasians or a different one? Also, where did this mutation occur first?

Aims: This study was conducted focusing on Arab carriers of FVL to find what alleles they have at the nine SNPs positions associated with FVL, and whether Arabs have the same or a different haplotype than the one present in Caucasian carriers. Results should provide answers for the above mentioned questions.

Subjects/Methods: A total of 300 Arab subjects were studied: 100 cases known to have FVL from previous studies (18 homozygous and 82 heterozygous) and 200 cases without it. Real-time PCR was performed on DNA samples of all cases to confirm FVL (wild-type G or mutant A alleles) and determine nine SNPs in the Factor V gene: 327A/G, 495G/A, 1470C/T, 1806G/A, 2298T/C, 2325C/T, 2391G/A, 2833A/T and 5380A/G.

Results: The haplotype of nine SNPs reported to be associated with FVL in Caucasians [G, A, C, G, C, T, A, A, A] was found in our Arab cases. First and foremost it was present in all the 18 homozygous cases (100%) indicating complete association with FVL mutation. Also it was present in 17 of the heterozygous cases with FVL (20.7%), which was significantly more than its presence in non-carriers of the mutation (15 out of 200; 7.5%).

Conclusions: The results of this study argue that Arab and Caucasian carriers of FVL had most probably descended from the same ancestor, which is especially reflected in the homozygous cases. The study also combines the molecular results obtained here with the epidemiological and anthropological facts to strongly and systematically suggest that FVL should have occurred first in the East Mediterranean area and then moved to Europe.

PB 1.68-4

Molecular characterization of PCE29K missense mutation

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Background: We have detected compound heterozygous missense mutations of protein C (PC) E29K and PC R147W in a young male patient with hereditary PC deficiency and pulmonary embolism. The novel PC E29K missense mutation was also detected in his father with PC deficiency. The proband's father had experienced portal venous thrombosis in his forties.

Aims: To investigate the molecular pathogenesis of PC E29K gene missense mutation in patients with PC deficiency.

Methods: Expression vetor of PC E29K mutation was constructed by site-directed mutation method using wild-type PC plasmid as template. The plasmids were transfected into COS-7 cells by lipofectin transfection method and cultured. PC antigen levels of the supernatant and cell lysis medium of the COS-7 cells transfected with PC E29K plasmid were measured by ELISA method. Laser confocal microscope was used to detect the cellular immunofluorecent localization of the cultured cells.

Results: PC antigen level of the supernatant of COS-7 transfected with PC E29K plasmid was 23.7% of that of wild-type ones. The PC antigen level in the cell lysis medium of COS-7 cells transfected with mutant plasmid was 81.1% of that of the wild-type. PC E29K mutated protein was mostly trapped in the endoplasmic reticulum and its concentration was decreased in Golgi bodies.

Conclusion: Only part of PC E29K mutated protein was secreted from the cells and part of it was degraded inside the cells. Impaired secretion and partial degradation is the cause of PC deficiency resulting from PC E29K missense mutation.

PB 1.68-5

Venous thrombosis in adolescent girls on oral contraceptives – association with estrogen gene polymorphisms

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Background: Adolescent girls on oral contraceptive pills (OCPs) are at a high risk for venous thromboembolism (VTE). OCP induced changes on coagulation and fibrinolytic pathways are complex with high inter-individual variability. An increased risk of thrombosis related to the estrogen receptor a polymorphism (ESR-ap, c.454-397T>C) has been reported in post-menopausal women. Data in the adolescent age group are lacking.

Aims: Our study aims were to: (i) Determine the prevalence of ESR–ap in patients with OCP induced VTE (Cases) (ii) Correlate the prevalence of ESR–ap with markers of hypercoagulability as determined by global coagulation assays in healthy adolescent girls who started OCPs (Prospective cohort)

Methods: *Cases:* On 14 girls with OCP induced VTE, data were collected for age, ethnicity, sites of thrombosis, presence of other thrombophilic markers and the time interval between OCP initiation and occurrence of VTE. Archived DNA was available on 10 of the 14 cases and was tested for ESR-αp.

Prospective Cohort: In this ongoing study, 45 healthy non-pregnant adolescent girls starting OCPs were enrolled after IRB approval. Samples were obtained prior to and after 3 months of initiating OCPs and were analyzed for clotting and anticoagulant factor assays, tissue factor initiated thromboelastography (TF-TEG), with t-PA (tissue plasminogen activator) modification for detecting fibrinolysis (t-PA-TEG), thrombin generation +/− thrombomodulin (TM)(TG+/− TM) and

estrone levels. All participants were genotyped for FV Leiden, F2 G20210, plasminogen activator inhibitor-1 (PAI-1) promoter polymorphism and ESR-αp. SNP genotypes were compared to TG and TEG parameters and differences pre and on OCPs were examined.

Results: *Cases:* The median age of cases was 15 years with eight Caucasian, five African American and one Hispanic girls. The median time interval between the initiation of OCPs and occurrence of the thrombotic event was 3 months (range: 1–6 months). The sites of thrombosis were: lower extremity DVT (n = 7), PE (n = 2), both PE and DVT (n = 2), cortical sinus thrombosis (n = 2) and mesenteric thrombosis (n = 1). The ESR-ap was present in the heterozygous state in 55% and homozygous state in 33% of cases.

Prospective Cohort: Median age of participants was 17 years (range 13–20). All were African American except one Caucasian participant. The ESR-αp was present in the heterozygous state in 51% and homozygous state in 15% of the participants respectively. Although, CT and CC genotype was associated with shorter lag time at baseline with TG-TM (2.7 ± 0.25 vs. 1.7 ± 0.14 min, P = 0.026) and not with TF-TEG, no correlation was found with TG-TM or TF-TEG *after* initiation of OCPs. The ESR-ap polymorphisms did not correlate with estrone levels or other coagulation parameters in participants.

Conclusions: ESR-αp were highly prevalent in adolescent girls with OCP induced thrombosis but did not correlate with markers of hypercoagulability as tested by global coagulation assays in healthy girls who started OCPs. OCP induced VTE may be caused by mechanisms other than ESR-αp and larger studies are needed to further elucidate this mechanism.

PB 1.68-6

A common PROS1 variant and its association with reduced free Protein S levels in patients of African ancestry; a risk factor for thrombosis?

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Protein S is a vitamin K dependent plasma glycoprotein which is an important anticoagulant. It functions in both an activated Protein C-dependent manner, acting as a non-enzymatic co-factor of activated Protein C in the degradation of FVa and FVIIIa, and in an APC- independent anticoagulant pathway. Protein S circulates in the plasma as 2 fractions, a bound fraction complexed to the complement C4b binding protein and an unbound free fraction. Only free Protein S, which comprises approximately 40%, has anticoagulant activity.

Protein S deficiency is an autosomal dominant disorder associated with an increased risk of venous thrombosis. Patients with hereditary Protein S deficiency usually present with free Protein S levels in the 35–60% range. The laboratory diagnosis of Protein S deficiency is fraught with difficulty as free Protein S levels are influenced by a number of factors, including age, gender, use of oral contraceptives, use of anticoagulants etc. Recently it has been observed that free Protein S levels also vary between different ethnic groups, with Black Africans having significantly lower free Protein S levels than age matched Whites. There is an increased risk of thrombosis in Africans and Afro-Caribbean's and reduced free Protein S levels may be a contributing factor.

Over the last few years there has been a significant increase in the number of African patients sent for *PROS1* analysis in our laboratory following a phenotypic diagnosis of Protein S deficiency. Causative mutations have been identified in only a subset of these patients. In 1994 a variant in exon two was described (p.Arg40Leu; c.119G>T/Arg-2Leu; rs7614835) which was found only to occur in individuals of African descent with a minor allele frequency of 0.007/15. This variant is located close to the propeptide cleavage site and has been shown to be associated with abnormal carboxylation of the Gla domains of the mature peptide. The status of this variant as either a polymorphism or mutation has not yet been fully elucidated. The initial identification of the variant suggested that it was associated with Type IIb Protein S deficiency so it was classified as a mutation, however a 2010 study suggested that it be classified as a polymorphism based on its high frequency in African populations; the heterozygous genotype is seen in approximately 6% of Yoruba and Luhya individuals.

We have observed this polymorphism at a higher than expected frequency in African patients who presented with consistently low free Protein S levels and in whom no other *PROS1* mutation was been identified. The polymorphism was not observed in a cohort of African patients with thrombosis but normal free Protein S levels. We suggest that the p.Arg40Leu variant is pathogenic and may be sufficient to explain the phenotype of reduced free Protein S in these patients.

PB1.69 – Non-Inherited Risk Factors VT – I

PB 1.69-1

Obesity measures and future risk of venous thromboembolism and myocardial infarction

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Background: The prevalence of obesity has increased dramatically in populations of developed countries during the last decades. Obesity is recognized as a major risk factor for both venous thromboembolism (VTE) and myocardial infarction (MI). Previous studies have suggested that obesity, assessed by body mass index (BMI), is more strongly associated with future risk of VTE than MI. Moreover, different obesity measures have displayed different impact on risk estimates for VTE and MI in independent studies. It is not known whether the relationship between obesity and future outcome is mediated by the same mechanism for VTE and MI. Investigation of obesity measures for VTE and MI within the same population ensures that the degree of known and unknown confounding is similar, and allows for comparison between the two outcomes.

Aims: To compare risk estimates for VTE and MI by various obesity measures in a prospective cohort recruited from a general population, and to explore whether adjustment for traditional atherosclerotic risk factors affected the risk estimates.

Methods: BMI, waist circumference (WC) and waist-to-hip ratio (WHR) were registered in 6708 persons without prior cardiovascular diseases, aged 25–84 years, who were enrolled in the Tromsø study in 1994–95. Obesity was classified according to predefined cut-off values for BMI, WC and WHR normally used for Caucasian populations. Incident events of VTE and MI during follow-up were registered through 2010 (study end). Cox-regression models were used to estimate age- and multivariable-adjusted hazard ratios (HR) with 95% confidence intervals (CI95). Analyses were stratified for sex. Multivariable analyses included age, smoking, systolic blood-pressure, total cholesterol, HDL cholesterol, diabetes mellitus and estrogen supplementation (women only) as covariates. The study was approved by the regional committee for research ethics, and participants gave informed written consent to participate.

Results: There were 288 validated incident VTE-events and 925 MIs during a median of 13.3 years of follow-up. BMI and WC exhibited higher risk estimates for VTE than for MI in both women and men, whereas WHR displayed similar risk estimates for VTE and MI. In women, BMI ≥ 30 kg/m^2 yielded a HR of 2.05 (CI95: 1.34–3.11) for VTE and 1.27 (0.97–1.66) for MI compared to BMI < 25 kg/m^2. For WC > 88 cm the HR was 2.44 (1.59–3.76) for VTE and 1.17 (0.91–1.51) for MI compared to WC < 80 cm. In men, BMI ≥ 30 kg/m^2 yielded a HR of 1.60 (0.91–2.82) for VTE and 1.61 (1.23–2.09) for MI. WC > 102 cm vs. < 94 cm yielded a HR of 1.96 (1.28–2.98) for VTE and 1.54 (1.25–1.90) for MI. Multivariable adjustments for traditional atherosclerotic risk factors substantially weakened all risk estimates for MI, whereas the risk estimates for VTE remained essentially unchanged.

Conclusions: Our study indicates that obesity, assessed by BMI and WC, displays higher risk estimates for VTE than for MI. The substantial weakening of risk estimates for MI, but not for VTE, by traditional atherosclerotic risk factors implies that these factors are key intermediates for the relationship between obesity and MI, and that other mechanisms are likely to mediate the risk of VTE by obesity.

PB 1.69-2

Postoperative complication is the main risk factor for venous thromboembolism after bariatric surgery

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Background: Deep vein thrombosis and pulmonary embolism are important causes of morbidity and mortality after bariatric surgery. The overall incidence of venous thromboembolism (VTE) after bariatric surgery and using thrombosis prophylaxis has been reported to range from 0.2% to 3.4%. Knowledge of factors associated with a higher risk of VTE after bariatric surgery may be essential to select patients who may benefit from either prolonged or intensified thrombosis prophylaxis.
Aims: The objective of this study is to determine the risk factors for VTE after bariatric surgery.
Methods: A retrospective multicenter case-control study was performed in patients who had bariatric surgery (gastric banding, gastric bypass, sleeve gastrectomy, duodenal switch, revisional procedures) between January 2008 and September 2011. All medical records were manually reviewed for the presence of all forms of VTE until 6 months after surgery, and all patients were called to ascertain the results. For every case of VTE after surgery, six control patients were selected who were matched for gender, age, participating center and type of surgery. Risk factors for VTE before and after surgery and duration of thromboprophylaxis were registered. Also postoperative complications (infection, major bleeding, leakage of the anastomosis, myocardial infarction), length of stay and intensive care admission were evaluated.
Results: A total of 2050 patients were included. In 12 patients a VTE occurred within 6 months after bariatric surgery (incidence 0.59%, 95% CI = 0.25–0.93). The majority of VTEs occurred within 1 month (67%). There was a very strong impact of complications after surgery (cases 83.3%, controls 9.7%, OR 46.4; 95%CI = 8.4–255.9) or intensive care admission (cases 58.3%, controls 6.9%, OR = 18.8; 95% CI = 4.3–81.1). The majority of postoperative complications were leakage or infection (90%), and these patients did not have a contraindication for therapeutic dose low molecular weight heparin. There was no clear association between classical thrombosis risk factors and postoperative VTE, only immobility was more present among the cases (16.7%) compared to the controls (2.8%, OR 7.0; 95% CI = 0.9–55.4).
Conclusions: The incidence of VTE is low after bariatric surgery using thrombosis prophylaxis. However, there is a very strong association between postoperative complications and VTE. Patients with a complication after surgery may benefit from more intensive thrombosis prophylaxis.

PB 1.69-3

In-hospital prevention of venous thromboembolism: how should we do it? Comparison of a surveillance project with the use of a validated risk score for thrombotic risk assessment

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Background: Venous thromboembolism (VTE) is a leading cause of mortality and morbidity in hospitalized patients. Despite evidence on the efficacy of prophylaxis in preventing VTE in the hospital, it is largely underused worldwide. The absence of widely accepted procedures to identify patients at risk contributes to this shortcoming. Aims. To analyze the validity of a program for in-hospital antithrombotic prophylaxis and to compare it with the use of the Padua Prediction Score (PPS).
Methods: A risk chart within the hospital prophylaxis project (STOP) was developed and used by physicians throughout the hospital to evaluate the thrombotic risk of all patients at admission (n = 5195 patients), from May 2010 to August 2011. It contained risk factors chosen among those reported in the literature that were the most frequent and/or had the highest associated risk. After filling in the risk chart, doctors administered prophylaxis based on their own judgment. Prophylaxis hospital guidelines were prepared together with the ward doctors. In a random sample of patients (n = 433), PPS results and the attending physician judgment, made through the STOP risk chart, were compared to the risk assessment of two experts who retrospectively evaluated risk factors and indication to prophylaxis from clinical records. The concordance between the two specialists was assessed using Cohen K statistic. Sensitivity and specificity in evidencing patients at risk for VTE were estimated for both the PPS and the attending physician, in the whole sample as well as stratifying for medical vs. surgical departments.
Results: Percentage of risk charts within the STOP project filled in by the attending physician over the study period and taken as a measure of adherence to VTE risk evaluation, decreased over time. In 2010, five wards were above 70%, considered the goal threshold, and 10 above the 50% threshold, while in 2011 there were three wards above 70% and five above 50%. No significant differences in adherence were observed between surgical and medical wards. Prophylaxis was provided in 46% of patients overall (32% medical, 52% surgical, 84% critical care). Concordance between the two hematologists in the whole sample was high (k = 0.78). Overall, the PPS had a high specificity (99%, 96%) and a lower sensitivity (66%, 76%) compared to the evaluation of each expert, while the opposite was true for the attending physician (specificity = 68%, 62%, sensitivity = 72%, 73%). The highest number of discrepancies between the evaluation of the PPS, the attending physician and the experts was observed for the risk factor 'acute infection'.
Summary/Conclusion: In-hospital VTE surveillance is time and resource consuming and most surveillance models fail over time. One of the problems that leads to failure is personnel turnover, which requires large investments in permanent education. We showed that the thrombotic risk assessment performed by the attending physician, even when educated within a hospital-wide surveillance project, performs worse than a validated risk score, that automatically provides an indication for prophylaxis. The PPS showed high specificity compared to expert evaluation, and can be used to select patients at thrombotic risk both in medical and surgical departments.

PB 1.69-4

Impact of chronic obstructive pulmonary disease on future risk of venous thromboembolism in a general population – the Tromsø study

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Background: Hospitalization for exacerbations of chronic obstructive pulmonary disease (COPD) is associated with a substantial risk of venous thromboembolism (VTE). VTE in patients hospitalized for COPD exacerbations is associated with prolonged hospital stay and increased 1-yr mortality rate. It is not known, however, whether the risk of VTE in patients hospitalized for exacerbations of COPD is caused by the respiratory failure or by other associated factors such as immobilization and bronchial superinfections.
Aims: We wanted to investigate the association between COPD and future risk of VTE in a prospective cohort recruited from the general population.
Methods: Spirometry were measured in 8645 unique men and women, aged 25–84 years, who participated in one or two surveys of the Tromsø study (Tromsø 5; 2001–2, and Tromsø 6;2007–8). COPD was defined according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) criteria. Incident VTE-events were registered from the date of inclusion through the end of follow-up, December 31, 2010. Cox-regression models were used to calculate crude and multivariable-adjusted hazard ratios (HR) with 95% confidence interval (CI). The analyses were adjusted for age, sex, body mass index, smoking, and cardiovascular diseases. Moreover, to minimize regression dilution effects, Cox-regression was performed in subjects with two recordings of spirometry during follow-up, allowing for time-dependency of the exposure variable and important co-variates. The study was approved by the regional committee for research ethics, and all participants gave their informed written consent to participate
Results: There were 1727 (20%) subjects with COPD (FEV1% < 70) at baseline, and in total 187 subjects in the population developed VTE (3.56 per 1000 person-years) of which 53 subjects with COPD (28.3% of VTE events) during a median follow-up of 6.72 years. Subjects with COPD did not have higher risk of total VTE (Multivariable HR 1.19, 95% CI 0.85–1.66) and provoked VTE (Multivariable HR 1.48, 95% CI 0.98–2.22) compared to subjects without COPD. Moreover, the risk of VTE did not increase across advancing stages of COPD (*P* for trend 0.5). Only minor changes in risk estimates were observed in time-dependent analyses.
Conclusions: There was no association between the presence of COPD and risk of total VTE in the general population, but subjects with COPD had higher risk estimates for provoked VTE. Our findings may suggest that the risk of VTE in patients hospitalized with exacerbations of COPD is precipitated by additional predisposing factors such as immobilization and respiratory infections.

PB 1.69-5

The number of venous valves in the legs does not explain the association between body height and risk of venous thrombosis

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Background: Several studies have shown that increasing body height is associated with risk of venous thrombosis, yet the mechanism remains unclear. It has been suggested that leg length could explain the association between body height and thrombotic risk. Thrombus formation often starts around venous valves, in local widenings of the veins called sinuses, where blood flow velocity is low and hypoxia adds to the thrombotic potential. When tall people would have more venous valves, the likelihood of thrombus formation increases, which could explain the risk. Alternatively, when relatively fewer valves are present, higher hydrostatic pressure and venous stasis may lead to more thrombus formation.
Aims: In this study we aimed to relate the number of valves in the upper leg deep veins to leg length, to increase our understanding of the mechanism underlying the association between tall stature and venous thrombosis risk.
Methods: This study was performed as a separate analysis in the Thrombo Genics study, a double blind, randomized clinical trial on a novel factor VIII inhibitor (TB-402). We assessed the number of valves in 54 patients who underwent contrast enhanced venography 3 weeks after elective hip surgery. Consent and ethical approval was obtained. We counted the number of venous valves in the deep veins from the popliteal fossa up to the level of the inguinal ligament. As in the calf superficial and deep veins are numerous, and their diameters are small, assessment of the number of venous valves is not possible here. Besides, we assessed whether there were double popliteal or superficial femoral vein segments. Body height and weight were documented, and leg length was measured. We analyzed the data using Poisson regression and assessed the effect of body height in tertiles.
Results: Patients came from six different countries and from 11 different medical centers. Mean age of the patients was 61 years (range 33–82). Twenty three (43%) patients were male. Body height was on average 1.68 m (range 1.52–1.85), whereas leg length was on average 88 cm (range 75–101). None of the patients had a history of PE or DVT. A positive history of varicose veins was present in nine of 54 (17%) patients. Patients had a median of three valves in the upper leg veins, with a range of 0–7 valves. Eighteen of 54 patients (33%) had a double venous segment, mostly the superficial femoral vein. We found no association between body height and the number of valves (RR 0.98; 95% CI 0.97–1.00). Stratified data for sex showed no association either.
Summary/Conclusions: We conclude that increased body height does not increase the probability of having more valves. The relatively lower number of valves present over an increased venous distance in people of tall stature suggests that higher hydrostatic pressure and venous stasis explain the increased thrombotic risk.

PB 1.69-6

Incidence of symptomatic venous thromboembolism among hospitalized patients with lung cancer in China

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Background: Cancer patients are at high risk of venous thromboembolism (VTE); however, the incidence, risk factors and prognosis of symptomatic hospitalized VTE in patients with lung cancer in China have not been well determined.
Aims: To determine the incidence of hospitalized symptomatic VTE of lung cancer in China and identify risk factors and prognostic impact of VTE.
Methods: A retrospective analysis was conducted to assess the incidence of VTE and clinical characteristics and laboratory test results associated with VTE in patients with new diagnosis of lung cancer in 2009. Univariate and multivariate logistic regression analysis was used to identify risk factors predictive of VTE. Kaplan-Meier analyses were performed to estimate and to compare death risk among patients with or without VTE. Cox proportional hazard models were used to identify the prognostic effect of VTE on survival.
Results: A total of 3741 patients with lung cancer were included in analyses. In all, 84 patients developed symptomatic VTE within two-

year follow-up, including 28 had pulmonary embolism (PE), 40 had deep venous thrombosis (DVT), and 16 had concurrent PE and DVT. The incidence of VTE was 2.2%. Multivariate analysis identified tumor stage IV, adenocarcinoma histology, and a baseline D-dimer level $\geq$ 500 μg/L as independent risk factors for VTE and VTE was a statistically significant factor affecting survival ($P < 0.001$).

Conclusions: The incidence of symptomatic VTE was about 2.2% in Chinese hospitalized patients with lung cancer. Advanced cancer stage, adenocarcinoma and high level of D-dimer were associated with increased risk of VTE and it is a poor prognostic factor for survival.

PB 1.70-1

Thyroid function, assessed by thyroid stimulating hormone, and future risk of venous thromboembolism -the Tromsø study

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Background: Thyroid hormones are known to affect the coagulation system by influencing plasma levels of factor VIII and von Willebrand factor, and low levels of these factors in hypothyroidism are normalized by hormone substitution. Thyroid stimulating hormone (TSH) is generally considered the most sensitive measure of thyroid function. However, a recent population-based nested case-cohort study reported that high levels of free thyroxine (FT4) were associated with increased risk of venous thromboembolism (VTE), whereas TSH levels were inversely and less pronouncedly associated with VTE. No prospective cohort study has so far investigated the association between TSH levels and future risk of VTE.

Aims: The purpose of the present study was to examine the association between TSH and future risk of VTE in a prospective cohort recruited from a general population.

Methods: TSH was measured in 11,965 subjects, aged 25–89 years, who participated in one or more surveys of the Tromsø study, starting in 1994–95 (Tromsø 4; 1994–95, Tromsø 5; 2001–2, and Tromsø 6; 2007–8). Incident events of VTE were recorded to the end of follow-up, December 31, 2010. Cox's proportional hazard regression models were used to estimate crude and multivariable-adjusted hazard ratios with 95% confidence intervals. The analyses were adjusted for age, sex, body mass index and smoking. TSH was analyzed in predefined categories (< 0.2 mU/L; low (11% of the population), 0.2–4.0 mU/L; normal, and > 4.0 mU/L; high (38% of the population) and as a continuous variable. Moreover, to minimize regression dilution effects, Cox-regression was performed in subjects with at least two recordings of TSH during follow-up, allowing for time-dependency of the exposure variable and important covariates. The study was approved by the regional committee for research ethics, and all participants gave their informed written consent to participate.

Results: There were 289 validated first VTE events (2.94 per 1000 person years), of which 114 were unprovoked, during a mean of 8.20 years of follow-up. TSH was not significantly associated with future risk of VTE in analyses treating TSH either as a continuous variable or in predefined categories. The risk of VTE decreased by 1% per 1 SD (2.04 mU/L) increase in TSH (Multivariable-adjusted HR 0.99; 95% CI 0.89–1.10). In subjects with low and high TSH levels according to predefined criteria, the risk estimates for VTE were somewhat increased (HR 1.13; 95% CI 0.47–2.76 and HR 1.15; 95% CI 0.71–1.86, respectively). The risk estimates for deep vein thrombosis (DVT) for low and high TSH levels vs. normal levels were even higher (HRs 1.73; 95%CI 0.64–4.72 and 1.59; 95% CI 0.90–2.82, respectively). All risk estimates were essentially similar in time-dependent analyses.

Conclusion: We found no significant associations between TSH levels and future risk of VTE in a prospective population-based cohort. However, low (< 0.2 mU/L) and high (> 4.0 mU/L) TSH levels were associated with higher risk estimates for sole DVT. The latter observation may imply that factors other than thyroid hormones alone contribute to increased risk of VTE in thyroid dysfunction.

PB 1.70-2

Cardiovascular profile of individuals with deep vein thrombosis: results of the Gutenberg Health Study (GHS)

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Background: Recent studies have shown that venous thromboembolism and arterial diseases may be related.

Aim: Our objective was to assess the cardiovascular (CV) profile of individuals with deep vein thrombosis (DVT) in a large-scale population sample.

Methods: GHS is an observational population-based cohort study (N = 15,010) comprising individuals aged 35–74 years selected representatively from local registry offices. Sociodemography, classical cardiovascular risk factors (CVRF), CV and other diseases were documented by computer-assisted personal interview, clinical assessment and laboratory examinations. DVT was defined as self-reported history of DVT, diagnosed by a physician in life time. Data underwent detailed quality control.

Results: The population-weighted prevalence for DVT was 3.6% (N = 534). The majority of individuals with DVT were women (62.1%; n = 362) and older than individuals without DVT (non-DVT; 60.2 ± 10.2 vs. 54.8 ± 11.1 years). CVRF like hypertension, diabetes, dyslipidemia, obesity and family history of myocardial infarction (MI), as well as cardiovascular comorbidities (coronary artery disease, MI, stroke, PAD, atrial fibrillation, heart failure) and cancer were more frequent in individuals with DVT. As expected, PE events were less frequently reported by non-DVT individuals (0.0% vs. 3.3%, $P < 0.0001$). In multivariable logistic regression analysis adjusting for all CVRFs, only sex (OR 1.86[1.55–2.22], $P < 0.0001$), age (OR 1.86 [1.55–2.22]; $P < 0.0001$) and obesity (OR 1.86[1.55–2.22]; $P < 0.0001$) were independently associated with DVT. Frequency of peripheral arterial disease (PAD) was remarkably higher in DVT compared to non-DVT individuals (19.1% vs. 2.7%, $P < 0.0001$). After adjusting for CVRF, PAD (OR 6.67[5.16–8.61]; $P < 0.0001$) was the strongest independently associated CVD with DVT (adjusted for all CVRFs), followed by heart failure (OR 2.54[1.51–4.26]; $P = 0.0004$) and atrial fibrillation (OR 1.86[1.29–2.64]; $P = 0.0095$). Stroke and cancer showed a weak association only. Overall, individuals with DVT presented a higher 10-year risk for incident cardiovascular disease (calculated by Framingham CVD Risk Score at time of data assessment) compared to non-DVT (9.0% vs. 7.2%; $P < 0.0001$).

Summary/Conclusion: DVT is related with a higher prevalence of CVRF and cardiovascular diseases. These findings may have implications for risk assessment and secondary prevention.

PB 1.70-3

Incidence of venous thromboembolism in patients undergoing laparoscopic surgery for colorectal cancer

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Background: The incidence of venous thromboembolism (VTE) after laparoscopic surgery for colo-rectal cancer is unknown.
Aims: We performed a prospective multicenter study aimed at assessing the incidence of and risk factors for VTE in patients undergoing elective laparoscopic surgery for colorectal cancer.
Methods: Complete compression ultrasonography of the lower limbs was performed at day 8 ± 2 after surgery. The primary outcome of the study was the incidence of any VTE at day 8 ± 2 after surgery. No restriction was given regarding antithrombotic prophylaxis.
Results: Overall, 298 consecutive patients were evaluated for inclusion in the study. Twenty-three patients were excluded (six conversion to laparotomic surgery; seven consent refusal; 10 indication for anticoagulant therapy). Thus, 275 patients were included in the study, 55% males, mean age 66 ± 11 years. Obesity (BMI ≥ 30) was found in 7%, bed rest in 1% and previous VTE in 2%. Mean surgery duration was 175 ± 74 min and mean post-surgery immobilization 33 ± 21 h. All patients received prophylaxis with low molecular weight heparin starting from 12 h after surgery to ultrasound assessment. After 6.2 ± 2.5 days, 46 VTEs were observed (16.7%), all deep vein thrombosis of the lower limbs. Thrombosis was symptomatic in two patients (0.7%) and proximal in two other patients (0.7%) for an overall incidence of major VTE of 1.4%. Bilateral DVT was found in 17 patients (6.2%). After surgery, one patients had a major bleeding and one patient had a clinically relevant non-major bleeding. Obesity (HR 3.4, 95% CI 1.5–7.6) and age ≥ 70 years (HR 3.7, 95% CI 1.9–7.1) were independent predictors for postoperative VTE. No correlation was observed between VTE and gender, comorbidities, surgical site, surgery duration or stage of malignancy.
Conclusion: The risk of developing VTE after laparoscopic surgery for colorectal cancer is substantial despite antithrombotic prophylaxis. Obesity and advanced age were risk factors for VTE.

PB 1.70-4

Outpatient treatment for PE and risk factors for mortality, recurrent VTE and pulmonary hypertension in a 6-month follow-up – results of a retrospective registry

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Background: Over the last 15 years, outpatient treatment for deep vein thrombosis (DVT) has become standard but most patients with pulmonary embolism (PE) are still hospitalized due to more severe comorbidities and a higher mortality. Recently, PE risk scores identified low-risk PE patients, for which outpatient treatment could be feasible. However, little is known about the distribution of PE risk categories, the management and outcome of unselected PE patients in daily care.
Aims: To evaluate distribution of PE risk categories, outcome and risk factors for mortality, recurrent VTE and pulmonary hypertension (PAH) in patients 6 month after PE.
Patients and Methods: Retrospective analysis of all outpatient PE patients presenting at our academic hospital between January 2000 and December 2010. Patients were identified in the PE-database of our Vascular Center and different hospital databases using ICD 10-code 'I26.x'. Patients with non-acute or asymptomatic PE were excluded. Data for 6 month FU were extracted from the PE-database of our Vascular Center, where all VTE patients are routinely seen after 6 month (echocardiography performed only in patients with suspected pulmonary hypertension).
Results: Between 2000 and 2010, a total of 439 patients presented as outpatients with symptomatic PE (mean age of 64.4 ± 16.7 years; 55.4% male; 82.7% with concomitant DVT). Of these, 49 patients (11.2%) were treated as outpatients (OP; in hospital < 24 h), 63 patients (14.4%) were discharged within 72 h (ED) and 327 patients (74.5%) were hospitalized for longer than 72 h (HO).
At 6 month, 17 patients (3.9%) had recurrent VTE, 48 (10.9%) had bleeding complications, 23 (5.2%) were found to have pulmonary hypertension and 47 (10.7%) were dead (0% HO; 3.2% ED and 13.8% HO, respectively).
In multivariate analysis, risk of recurrent VTE was significantly increased by low ESC-category (OR 2.4), initial RVESP > 40 mmHg (OR 3.2), symptomatic DVT (OR 2.4) and proximal DVT (OR 2.6). Increasing age was found to reduce the risk for recurrent VTE (OR 0.97; $P = 0.005$).
In all patients undergoing echocardiography at 6 month for suspected PAH, risk of PAH was significantly increased in patients with documented initial RVESP > 40 mmHg (OR 3.0), right heart strain in ECG at baseline (OR 6.6) and known thrombophilia (OR 8.4).
Mortality risk was significantly increased by age (OR 1.1/year; $P < 0.001$), concomitant diabetes mellitus (OR 4.2), underlying malignant disease (OR 4.4), right heart strain (OR 4.3), high ESC risk category (OR 8.7), but most strongly by INR values > 2 at diagnosis of PE (OR 59.0). In contrast, mortality was not increased by outpatient or early discharge treatment.
None of the tested parameters at initial VTE presentation were found to be predictive for bleeding complications.
Conclusion: We conclude that in cohorts of unselected PE patients, low risk PE can exactly be identified. Consequently, in daily care settings about 25% of patients can safely be treated as outpatients or early discharged. Our analysis identified several significant risk factors for unfavourable outcomes, some which are not described in the literature and might further improve decision making in PE treatment.

PB 1.70-5

Increased incidence of VTE prior to surgery in patients with renal cell carcinoma and tumor thrombus

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Background: Patients with kidney cancers are at increased risk of venous thromboembolism (VTE) with rates varying between 1% and 3% within 6 months of cancer diagnosis. Tumor thrombosis occurs frequently among patients with renal cell carcinoma (RCC) and has been reported to occur in 5–10% of all RCC patients. Venous tumor thrombosis disrupts vascular integrity and disturbs venous blood flow which could potentially increase the risk of VTE. The clinical impact of tumor thrombus and its potential association with increased risk of VTE in patients with RCC is unknown.

Aims: To determine if the presence of tumor thrombus in patients with RCC increases the incidence of VTE prior to radical nephrectomy and thrombectomy.
Methods: A retrospective cohort study of all late-stage (stage 3–4) RCC patients that underwent a radical nephrectomy at our institution between January 1, 2005 and July 1, 2012 was conducted. Radiological imaging data was reviewed for all patients to identify patients with tumor thombus seen on imaging. Tumor thrombus was defined as the presence of an intra-luminal filling defect in the renal, hepatic, portal or IVC directly extending from a renal mass detected on computed tomography or magnetic resonance imaging. Patients with VTE prior to or at cancer diagnosis were excluded. The primary outcome was the incidence of VTE between cancer detection and surgery. Venous thromboembolism was defined as proximal lower limb (popliteal vein or more proximal) deep vein thrombosis or pulmonary embolism. A two-sided Fisher exact test was used to compare the incidence of VTE between patients with and without tumor thrombus.
Results: A total of 179 RCC patients were included in the study. Fifty-six (31.3%) RCC patients had tumor thrombus diagnosed on imaging. The total population had a mean age of 62.5 years, men represented 68.2%. The number of patients at stage 3 was 51.4%. None of these patients received anticoagulation prior to surgery.
Three patients with tumor thrombus (5.4%; 95% CI: 1.1–14.6%) developed a VTE while awaiting surgery whereas none (0%; 95% CI: 0–2.9%) of the patients without tumor thrombus had an event ($P = 0.030$). Median time to VTE from tumor thrombus diagnosis was 15 days (range 5–21 days) and median time to surgery was 24 days (range 1 to 213 days) in the tumor thrombus group.
Conclusions: Tumor thrombus on imaging is a frequent finding among late-stage RCC patients awaiting radical nephrectomy. The presence of tumor thrombus in these patients increases the incidence of pre-operative VTE. Larger prospective studies are required to confirm these findings and assess the role of thromboprophylaxis in RCC patients with tumor thrombus.

PB 1.70-6
Incidence of Congestive Heart Failure-associated Venous Thromboembolism in Korean Population: from Health Insurance Review and Assessment Service database

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Background: Congestive heart failure (CHF) has been known to be a major risk factor for venous thromboembolism (VTE) in Western countries. The reported incidence of VTE in CHF has ranged 1.65-59% in Caucasian population. However, its contribution to VTE risk has not been established in an Asian population so far. To address this issue, a population-based study was carried out to know the incidence and risk factors of VTE in Korean patients with CHF.
Methods: This population-based study used the Korean Health Insurance Review and Assessment Service database. VTE patients with CHF from 2004 to 2008 were retrospectively identified by both diagnostic codes and medication codes for drugs used in initial treatment of VTE.
Results: During the 5 years of the study, 20,737 VTE cases were identified among 2,424,206 CHF patients and the incidence of VTE with CHF was 0.855% (95% confidence interval [CI], 0.844–0.867). The incidence increased steadily with advancing age, from 0.422% (95% CI, 0.405–0.439) at 40–59 years of age, 0.991% (95% CI, 0.974–1.008) at 60–79 years of age, up to 1.061% (95% CI, 1.029–1.094) in patients over 80 years of age. The annual VTE incidence in patients with CHF was 0.573% (95% CI, 0.552–0.594) in 2007, 0.713% (95% CI, 0.690–0.737) in 2008, 0.862 (95% CI, 0.836–0.889) in 2009, 0.971% (95% CI, 0.944–0.998) in 2010, and 1.188% (95% CI, 1.156–1.221) in 2011 which suggested a yearly increasing incidence of VTE in patients with CHF in the Korean population.
Conclusions: The incidence of VTE in patients with CHF was lower in the Korean population than in Caucasians, However, it also demonstrates a yearly increasing incidence of VTE in patients with CHF in the Korean population.

PB1.71 – Paediatric Thrombosis – I

PB 1.71-1
Vitamin K antagonists (VKAs) for venous thromboembolism (VTE) in a pediatric cohort with persistent antiphospholipid antibodies (aPL)

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Persistent aPL is associated with an increased risk of VTE in children, and high rates of recurrent thrombosis were described. The intensity of anticoagulation in childhood has been extrapolated from adults. However, the optimal duration of this therapy is currently uncertain, and should be evaluated for the risk-benefit of extended therapy.
Objective: To determine the rate of recurrent thrombosis and major bleeding in a prospective pediatric cohort with VTE and persistent APLs, receiving acenocoumarol until the clinical risk factor is resolved and until aPL disappears.
From May 1992 to July 2012, 371 children, < 18 years old with objectively confirmed VTE were evaluated. Newborns were excluded. All the patients (pts) were given acenocoumarol at a target INR range of 2.0–3.0. Age-adjusted initial doses were given according to published guidelines. Lupus anticoagulant (LA), anticardiolipin antibodies (aCL) IgG/IgM and anti-β2-glyprotein I (anti-β2GPI) IgG/IgM were tested, according to recommendations, every 3–6 months until LA became undetectable and low titers of aCL and anti-β2GPI were achieved. Inherited thrombophilia was also assessed. Age-related local cut-off values were used.
During a 20-year period, 24pts (6.5%) with VTE, 15females, median age 5.1 years (range 0.2–16.9) were identified with persistent aPL and were evaluated for a total of 64.1 patient-years. The patients are described according to their clinical conditions (unprovoked VTE, SLE, *Streptococcus* or *Staphylococcus* infections, uremic hemolytic syndrome (UHS) and catheters along with other risk factors) and aPL profiles. One pt with an unprovoked VTE and 3/7 pts with SLE had triple positivity (LA, IgGaCL, IgGanti-β2GPI). Another 3/7 pts with SLE had positive LA and IgGaCL, and the remaining pt. with SLE had LA alone. All these 8 pts. still showed persistent aPL at their last control or discharge to an adult center, median time positivity aPL of 22.3 months (range 7.8–91.2). Five pts. with infections showed extensive VTE and LA for a median time of 20.6 months (range 8.7–29.7); in one of them, an inherited combined prothrombotic disorder was also found. One pt. with UHS had IgGanti-β2GPI for 9.6 months. Ten pts with catheters showed aPL positivity for a median time of 9.7 months, (range 3.0–13.9), eight of them showed single aPL positivity. The INR values at the ranges of 2.0–3.0 or 1.5–1.9 were observed for 47.6% and 43.7% of the follow-up time respectively. One to three monthly controls were required to maintain INR in the target range. Two adolescents with SLE who discontinued acenocoumarol presented recurrent thrombosis and died afterwards of arterial ischemic stroke and sudden death, respectively. No pt. had major bleeding.
Conclusion: This is the first prospective pediatric cohort with VTE and persistent aPL who received VKAs until the clinical risk was resolved and LA became undetectable or low titers of aCL and anti-β2GPI were achieved. Neither recurrent thrombosis nor major bleeding was

observed in patients with different clinical settings, maintaining INRs values within moderate and low-intensities acenocoumarol most of the long follow-up time. Further prospective multicenter studies are needed to investigate the optimal intensity and duration of the antithrombotic therapy taking into account the clinical entities and aPL profiles.

PB 1.71-2

Evaluating the impact low molecular weight heparin on quality of life for children and their families

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Background: Low molecular weight heparin (LMWH) is a common treatment available for long term anticoagulation in infants and children. LMWH appears to be safe and effective in preventing thrombosis, requires two injections per day which are painful and cause bruising. How LMWH impacts families has not been evaluated and requires assessment considering the launch of new anticoagulants believed to provide similar efficacy. Given the expense of these new agents it is essential to demonstrate superiority through patient preference and patient quality of life The GRADE system for evaluating quality of evidence calls for appraisal of patient outcomes such as health-related quality of life (HRQOL). HRQOL describes the patients' perception of their physical, social, and mental wellbeing. HRQOL is influenced by type of treatment. The objective measurement of HRQOL in children receiving LMWH will provide important information to guide clinicans, funding agencies and the family in treatment choices. The KIDCLOT PAC QL© (Pediatric AntiCoagulation Quality of Life inventory) for pediatric patients on vitamin K antagonists has undegone both validity and realibility testing and is currently being employed internationally to assess the impact of oral anticoagulation on the HRQOL of children and their families. This study is the first to develop and validate an inventory that objectively measures HRQOL in children receiving LMWH. This information will provide guidance to improve anticoagulant treatment options for children.

Aims: To modify an existing pediatric HRQOL inventory that assesses the impact of oral anticoagulation (KIDCLOT PAC QL©) for LMWH use. The modified inventory will describe the variables which represent the impact of LMWH on the lives and well-being (HRQOL) of children and their families.

Methods: Modification of the inventory requires the removal of irrelevant questions and addition of therapy specific questions. The new questions will be validated using cogntive debriefing interviews with both children and parents. The cognitive debriefing interviews will ensure clarity of wording, sentence structure, and inclusion of all relevant information. If two or more participants have difficulty understanding a question the question will be rewritten and additional interviews will be conducted to confirm clarity. Any suggested areas of missing data will be added to the inventory and tested to ensure clarity. Informed consent and ethics approval was obtained.

Results: A final inventory was developed and validated to assess the impact of LMWH on HRQOL of children and their families. Parent cognitive debriefing interviews were held and child/teen interviews were conducted. The LMWH PAC QL inventory is complete and the inventory will be available to be used internationally to measure HRQOL in children receiving LMWH.

Summary: The HRQOL measure for children receiving LMWH provides a novel, important strategy for assessing treatment choices especially with the introduction of novel anticoagulant therapies. It is validated and available for use. Modifications for international use such as cultural adapation must be considered prior to implementation.

PB 1.71-3

Chylothorax in neonates and children: diagnosis and antithrombotic therapy of a complication associated with upper venous system thrombosis (UVST)

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Introduction: Chylothorax is a relatively rare complication with high morbidity and mortality, most frequently described in neonates and children after cardiothoracic surgery. Diet modification, octreotide and pleural drainage remain the standard initial therapy. Severe antithrombin loss in the chylous pleural effusion was reported. Only few cases of chylothorax related to UVST and its specific treatment have been published.

Objectives: To describe the demographic data, underlying diseases, clinical signs, imaging findings, treatment and outcomes of a series of neonates and children with chylothorax related to UVST evaluated in a single center.

From July 2006 to June 2012, 14 consecutive patients (pts.), seven females (50%), median age 51 days (range 10 days–8.8 years) with chylothorax and UVST were evaluated. The underlying diseases were: cardiac surgery, 7pts; infection, 2pts; preterm neonates with hyaline membrane disease, 2pts; hematopoietic stem cell transplant, 1pt., liver transplant, 1pt., congenital diaphragmatic hernia surgery, 1pt. All the patients had central venous access devices in the upper venous system. Twelve pts (85.7%) weighed < 10 kg. Clinical signs of UVST when the pleural effusion appeared were: swelling of the ipsilateral related limb, 2pts. and superior vena cava syndrome, 2pts. The median time between chylothorax onset and thrombosis diagnosis was 5 days (range 1–66), and it was longer than 7 days in 42.8% of the cases. The thrombosis was confirmed by ultrasonography, 11pts.; venography, 2pts. and angiotomography, 1pt. The involved veins were: subclavian, 13pts.; jugular, 6pts.; innominate, 1pt.; and extracardiac conduit following Fontan surgery, 1pt. The chylothorax was: right-sided, 3pts.; left-sided, 4pts. and bilateral, 7pts. All the pts. received standard initial management. Two children died due to their cardiovascular diseases before antithrombotic therapy could be started. Treatment in the remaining 12pts. was: Tissue plasminogen activator (tPA), median dose 0.2 mg/kg/h (range 0.1–0.5) for 3–6 h, 8pts.; heparin 12pts.; and vitamin K antagonists, 1pt. AT plasma levels were tested in 13pts. and its median minimum value was 0.33 U/mL (range 0.07–1.08). Levels below 0.45 U/mL were found in 11pts. AT concentrate or fresh frozen plasma were indicated in 11pts. to maintain AT levels above 50%. Chylothorax resolution was observed in the 12pts. in whom antithrombotic therapy was administered. The maximum drainage volume was, median 54 mL/kg/day (range 10–400). The median (range) duration of chylothorax in patients treated with tPA was 8 days (5–37) while in pts. without thrombolytic therapy, it was 17 days (11–24). Two pts. with Fontan surgery required pleurodesis.

Conclusion: Chylothorax related to UVST is underdiagnosed, especially in neonates and infants. Since extensive UVST was observed in most of the patients (71.4%) without clinical signs of thrombosis other than chylothorax, high clinical suspicion is necessary for an early diagnosis. UVST should be investigated even in patients with a recent cardiothoracic surgery. Acquired AT deficiency was found in most of the patients (84.6%), possibly increasing their thrombotic risk and heparin resistance. The use of antithrombotic therapy including thrombolytic agents and AT supplementation could reduce morbidity and mortality.

PB 1.71-4

Evaluating the impact of thrombophilia testing on health-related quality of life in children: development of an inventory

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Background: Thrombophilia testing is one of the most common types of genetic testing performed in children. The impact of this testing on health related quality of life (HRQOL) has not been evaluated. The concept of HRQOL focuses on dimensions of QOL related to health and therapeutic management strategies. HRQOL must be measured to develop effectual therapeutic choices, create research strategies, and change policies for improvement of health care adherence and outcomes. HRQOL is now considered the 'gold-standard' measurement for patient relevant outcomes. A HRQOL inventory for children with thrombophilia would assess general constructs that are salient for this population. Identification and evaluation of these constructs is critical to recognizing influences on patients HRQOL. This then facilitates improvements in care and helps to determine potential benefits and harms in testing for thrombophilia in families.
Aims: To develop a pediatric HRQOL inventory that describes variables which represent the impact of thrombophilia on the lives and well-being (QOL) of children and their families.
Methods: Focus groups identified items and themes significant to children and parents with thrombophilia. After items and themes were identified participants rated variables as to the level of frequency and severity using a Likert scale. Items were grouped into themes and a conceptual framework generated. The inventory was pre-tested to ensure that patients understood the questions and there is a full range of responses. Data was analysed using SPSS 20© for item level variability (frequency distributions, means, standard deviations and bar graph), and reliability (precision) for item reduction. Finally, cognitive debriefing interviews were held to ensure that patients understood the questions and there is a full range of responses for validity. Collected demographics include type of thrombophilia, age of patient, and gender. Informed consent and ethics approval was obtained.
Results: Focus groups with parents revealed concrete (medical, insurance, and activity related concerns), emotional (worry, stress, guilt), reproductive (concern regarding oral contraceptive use, future pregnancies and labour), and generational concerns (fear and guilt regarding passing the thrombophilia to child). While focus groups with adolescents had clearly positive (increased activity level and knowledge) and negative (restriction of activities, changes in lifestyle and emotional distress) themes in response to living with thrombophilia. Two inventories have been created a child/teen and a parent inventory. A total of 25 parents and 15 teens have completed the inventories. The final inventories had 35 and 43 questions on the child/teen inventory and the parent proxy respectively. Cronbach's alpha for the parent inventory is 0.899 and the child/teen is 0.934.
Summary/Conclusions: The newly generated HRQOL inventory for children with suspected or confirmed thrombophilia is validated and useful to determine confounders to diagnosis and facilitate partnership in care. Once confounders are identified the 'best' management (improved QOL associated with the safety and efficacy) for this patient population can be established.

PB 1.71-5

Warfarin knowledge retention in families participating in a home INR monitoring program

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Background: Treating children with warfarin is highly complex. The Royal Children's Hospital's home International Normalised Ratio (INR) program enables families to perform INR's at home, as required. This assists maintenance of the INRs within a strict target therapeutic range, with minimal impact on family life. Every family participates in an education program focusing on the mechanism of action, interactions and adverse effects of warfarin. Prior to commencing home INR monitoring, families must demonstrate a requisite level of knowledge and skill relating to warfarin management. Previous evaluations demonstrate this training program facilitates a high level of knowledge retention 6 months post training, however longer-term knowledge retention has not previously been measured.
Aim: To determine the long term warfarin knowledge retention of parents and patients participating in the Home INR Program.
Method: All parents and patients who successfully completed Home INR training between 2003 and 2011 were invited to complete a questionnaire evaluating the same knowledge content assessed at the time of completing their training. The results were compared to their original results using descriptive statistics, to assess knowledge retention.
Results: One hundred and one questionnaires were sent out to 88 families as in some families, both parents and older children had previously completed the education and questionnaire. Fifty-seven questionnaires (40 parents, 17 children) were returned (56%). The average length of participation in the Home INR program was 26.7 months (Range: 6.0–97.9). The mean questionnaire result at the time of original training was 29.1 (Range: 14–37) out of 40. The mean result from the repeat questionnaires mailed-out was 22.0 (Range: 14–30.5) out of 40, representing a statistically significant ($P < 0.001$) difference, with a mean decrease in knowledge of 7.1 marks. Of the 57 returned surveys, 24.6% ($n = 14$) said that they did not see a doctor in the clinical haematology outpatients department every 12 months. Furthermore, 38.6% ($n = 22$) of respondents said that they did not receive timely ongoing education about their child's warfarin therapy. The group who had an annual clinic review had an average questionnaire score decrease of −6.8. In comparison those who did not have an annual clinic review had an average decrease of −8.0. Whilst there was less of a knowledge reduction in those who had an annual clinic review compared to those that did not, this reduction was not statistically significant, most likely due to the small study numbers.
Conclusion: There was significant loss of warfarin knowledge between the two time points. Strategies need to be implemented to support families' ongoing knowledge retention and acquisition of new knowledge regarding warfarin management in children. This education could be delivered during annual clinic reviews, and we suggest these families attend a Haematology outpatient appointment at least every 12 months.

PB 1.71-6

A successful implementation of a pediatric thrombosis program to promote assistance to children, adolescent and young adults with cancer

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Background: Venous Thromboembolism (VTE) is a serious complication in patients with cancer and this problem is increasingly being recognized in pediatric practice. Recently, literature has demonstrated a dramatic increase in the prevalence of VTE at children's hospitals worldwide. It is also suggested by the American College of Chest Physicians Evidence-Based Clinical Practice Guidelines that pediatric hematologists with experience in VTE manage such patients. In order to fulfill this recommendation, the Instituto de Oncologia Pediátrica/Grupo de Apoio ao Adolescente e à Criança com Câncer (IOP/GRAACC), São Paulo, Brazil, organized a dedicated thrombosis team to promote the care of children with VTE within our institution.
Aims: To evaluate the VTE epidemiology and demographics findings by the thrombosis team since the program started at our institution.

Methods: Children, adolescences and young adults with cancer who developed VTE between February 2011 and December 2012 were studied retrospectively. Demographics and clinical characteristics related to thrombosis and cancer were evaluated. Chi-square test was performed to evaluate the relationship between low molecular weight heparin (LMWH) interval and recurrent thrombosis.
Results: The incidence of VTE was 5%. Of 34 patients diagnosed with symptomatic VTE, the median age at diagnosis was 12.3 years (IQR = 9), and 62% were female. The most common types of conditions associated with thrombosis were extra cranial solid tumor (56%), leukemia (26%), brain tumor (15%), and lymphoma (3%). Cancer and VTE were diagnosed at their initial presentation in 7 (21%) patients [extra cranial solid tumor (5)]. VTE were located in thorax (n = 17), lower venous system (n = 8), upper venous system (n = 7), neck vessels (n = 7), intra-abdominal vessels (n = 7), and CNS vessels (n = 3). Of note, pulmonary embolism (PE) and intra-cardiac thrombosis was documented in 44% (n = 15) of patients. An identified thrombosis risk factor was present in 88% of patients (central venous lines 26/34 [74%]). Anticoagulation therapy comprised LMWH [q24 h (n = 22), q12 h (n = 8)], oral anticoagulant therapy (n = 2) and others (n = 2). The duration of anticoagulation treatment was < 3 months in three patients, between 3 and 6 months in eight and more than 6 months in 23. VTE recurrence occurred in seven of 28 patients who survived. Six died due tumor progression. Heparin interval administration (q24 or q12) was not significantly associated with recurrent thrombosis (P = 0.71).
Summary/Conclusion: The creation of a new pediatric thrombosis program within a tertiary pediatric oncology academic center allowed a better characterization of VTE within our population. While some of our findings were consistent with the previously described findings in the literature, a much higher frequency of PE and intra-cardiac thrombus in children with extra-cranial tumors was noted. In terms of LMWH interval and VTE recurrence, an interesting information will merit further exploration.

PB1.72 – Thrombophilia – I

PB 1.72-1

The F2G1787T prothrombin gene mutation is not present in a representative cohort of Spanish patients with venous thromboembolism or controls

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Background: Known genetic abnormalities explain < 40% of patients with deep vein thrombosis. That is why there is an increasing interest in finding new mutations and new pathophysiologic explanations for this common disease. Recently, a new prothrombin gene mutation (p.Arg596Leu *F2* variant) has been identified in a Japanese family with hereditary thrombophilia. This mutant prothrombin was shown to have reduced activity in clotting assays and the produced thrombin was markedly resistant to inhibition by antithrombin (Miyawaki, NEJM 2012; 366: 2390).
Aims: We have investigated the presence of this mutation in a cohort of consecutive Spanish patients with venous thromboembolism previously reported (Tirado, Thromb Haemost 2005; 93:468).
Methods: We analyzed the F2G1787T prothrombin gene mutation by PCR. The fragment of 467 pb of exon 14 of F2 gene was amplified using these primers: forward F214-A-5′- AGGGCCTGGTGAA CACATCTTC-3′ and reverse F214-B-5′- CCAGGTGGTGGATTCT-TAAGTCTTC-3′. The 467-bp PCR product was digested with the restriction endonuclease *Msp*I (Biolabs) and electrophoresed in a Qiaxcel machine (Qiagen). The G allele leads to a cutting site for Msp I that digests the 369-pb fragments into 280 and 187 pb fragments. The pattern was confirmed by direct sequencing. Other classical thrombophilic parameters such as factor V Leiden mutation, PTG20210A mutation, antithrombin, protein C, protein S deficiencies, and ABO blood genotype were analyzed and reported previously (Tirado, Thromb Haemost 2005; 93:468).
Subjects: A Spanish cohort of Caucasian unselected patients with venous thromboembolism (n = 249; 111 males and 138 females; 47.1 ± 14.0 year) and unrelated matched controls (n = 248; 109 males and 139 females; 49.0 ± 14.9 year) was analyzed.
Results: The frequency of the classical parameters of thrombophilia in patients and controls was found to be consistent with a representative population of Spanish patients and controls. None of the patients or controls had the F2G1787T prothrombin mutation.
Conclusion: The F2C1787T prothrombin mutation seems to be very uncommon in the Spanish population and screening is not recommended in patients with venous thromboembolism.

PB 1.72-2

Thrombophilia in young patients with isquemic stroke. A prospective national study

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Background: A Thrombophilia (TF) panel is usually performed as part of the work up in young patients with stroke, especially when there is no apparent cause for the event. Even though a clear relationship between TF and stroke has not yet been established and prospective and adequate data is scarce, almost 25% of the TF studies are done in the arterial setting. The Argentinean Initiative of Stroke in the Young and Fabry disease (AISYF) is a unique opportunity for centralized and adequate testing of TF in this population.
Objective: To evaluate a TF panel in a prospective young population with confirmed ischemic stroke.
Methods: This is a Multicenter National Study for the detection of Fabry disease in young patients (18–55 years old) with Stroke. Twenty selected Centers from all over the country enrolled patients within 180 days of the event. All cryptogenic strokes were included in this analysis. Thrombophilia studies were centrally performed (Antithrombin, Protein C and free Protein S, Anti Cardiolipin Antibodies Ig G and M, Lupus Anticoagulant, Factor V Leiden (FVL), the Prothrombin G20210 A gene mutation (P20210) and the PAI 4G/5G mutation).
Results: One hundred and eighteen patients with stroke were evaluated in the last 2 years for Fabry disease in the AISYF study. Thirty patients were excluded from this analysis (six with a cardioembolic stroke, six with cerebral vein thrombosis, eight with a hemorrhagic stroke and 10 with arterial dissection). A thrombophilia panel was performed in the remaining 88 patients with a Cryptogenic ischemic stroke. Mean age was 41 years old, 39 women. Three patients (3.4%) had an Antiphospholipid Syndrome and warfarin was indicated, four (4.5%) had FVL (one Homozygous and also anticoagulated), two (2.2%) were heterozygous for the P20210 mutation, one patient who was not taking warfarin had a PC level of 24% and another patient Antithrombin of 35%. The PAI mutation was present in 50% of the idiopathic strokes (17% Homozygous 4G/4G and 33% heterozygous) just like in the normal Argentinean population.
Conclusions: (i) A positive TF test was found in 12.5% of the Cryptogenic strokes but only four patients (4.5%) were anticoagulated because of this. (ii) The frequency of the PAI mutation was similar to the frequency found in our normal population and should not be routinarly performed. (iii) We don‘t know yet the importance of testing TF as part of the screening work up in ischemic stroke, even in patients with an idiopathic event, since only a few patients required anticoagulation and without a clearly defined benefit in this setting.

PB 1.72-3

Prothrombin G20210A and oral contraceptives are risk factors for cerebral venous thrombosis

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Background: Prothrombin variant G20210A (PT G20210A) and the use of oral contraceptives (OC) are major risk factors for cerebral venous thrombosis (CVT). A synergism between these factors has also been reported. However, the role of factor V Leiden (FV Leiden) and PT G20210A as independent risk factors for CVT according to gender and the intensity of the synergism with OC remain controversial

Aims: This study aimed to evaluate the association between FV Leiden and PT G20210A with CVT in each gender and also the interaction with oral contraceptives. The results were also compared with those observed in patients with venous thromboembolism of the lower limbs (VTE).

Methods: Ninety-one patients (73 women and 18 men) diagnosed with CVT were studied. A control group of 284 individuals with no history of thrombosis and a group of 343 patients (218 women and 125 men) with VTE were also studied. Cases and controls were tested for the presence of FV Leiden and PT G20210A.

Results: The presence of PT G20210A in women using OC were associated with a high risk of CVT (OR, 45, CI 95%, 5–346) and also with VTE (OR, 14, CI 95%, 2–115).

Conclusions: Our results confirm that PT G20210A is an independent risk factor for CVT, with a much higher risk than that observed in patients with VTE. The risk was similar in men and women. The association of PT G20210A with OC use had a strong synergistic effect. In contrast with the observed for VTE, FV Leiden was not a risk factor for CVT.

PB 1.72-4

Thrombophilia screening in malaysian patients with arterial & venous thrombosis – a cause for concern?

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Background: The pathogenesis of arterial and venous thrombosis is quite complex involving universally recognized risk factors that are multifactorial. However, thrombosis may even occur under trivial and unusual or paradoxical conditions. The different pathogenesis for arterial and venous thrombosis warrants different clinical managements; the use of antiplatelet drugs in the former and anticoagulants for the latter. The complexity shrouding this clinical condition compounded with the lack of competent health care professionals in this specialty has impeded the development of thrombophilia-care in the country.

Aims: To investigate the relevance of thrombophilia screening in Malaysian patients with arterial and venous thrombosis and justify its incorporation as an important tool in the diagnosis and management of a clinical disorder that is considered a leading cause of morbidity and mortality in the world today.

Methods: Three hundred and seventy patients (236 arterial, 134 venous) with confirmed thrombosis were investigated for thrombophilia using recognized standard procedures. FVL and PTG20210A were confirmed by real-time PCR; protein C (PC), protein S (PS), antithrombin (AT), lupus anticoagulant (LA) and anti-phospholipid antibodies (APA) were identified using clotting, chromogenic and ELISA tests.

***Results*:** Eighty-three patients (42 males and 41 females) had abnormal thrombophilia profile. Thrombophilia was identified in 12.3% (29 of 236) of the patients with arterial thrombosis, of which 86.2% (25 of 29) were *inherited* and 13.8% (four of 29) *acquired*. Of the 134 patients with venous thrombosis, 40.3% (54 of 134) had thrombophilia with 87.0% (47 of 54) *inherited* and 13.0% (seven of 54) *acquired*. PC, PS and AT deficiencies were identified in 29, 18 and four patients respectively. LA and APA were identified in six patients. There were no patients with *acquired* and *inherited* thrombophilia occurring simultaneously, however, there were four patients who had both PC and PS deficiencies. None of the patients had FVL or PTG20210A mutations.

Conclusions: Abnormal thrombophilia profile was identified in two-fifths (40.3%) of the patients with venous thrombosis and in more than one-tenth (12.3%) with arterial thrombosis. The prevalence of *inherited* thrombophilia far exceeded the *acquired* form by a factor of at least 6.6. The significant prevalence of thrombophilia in the local thrombosis-patients is indeed a 'wake-up' call for health care professionals in the country, to warrant the inclusion of 'Thrombophilia profile' as an integral part of laboratory investigations for thrombosis. The results of which would contribute towards competent clinical management in terms of identifying high-risk patients and providing diagnostic, prophylactic and therapeutic services. Another finding of interest is the complete absence of FVL and PTG20210A mutations in all the 370 patients. This is contradictory to the numerous papers published in the West citing FVL mutation as the most prevalent *inherited* thrombophilia marker in the Caucasians. Is FVL and PTG20210A mutations confined to the Caucasian population only? Are Asians 'immune' to these two thrombophilia markers and are therefore less susceptible to thrombotic events? These are some of the pertinent questions that need to be verified with in-depth studies involving the local patient-population.

PB 1.72-5

Thrombophilia and cerebral venous thrombosis: a systematic review

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Background: Cerebral venous thrombosis (CVT) is a rare manifestation of venous thrombosis with considerable morbidity and mortality. Several factors contribute to the pathogenesis of this disease, among which thrombophilia.

Aims: To estimate the strength of the association between thrombophilia and incidence of CVT. Also, the strength of the association between thrombophilia and recurrence of (cerebral) venous thrombosis, as well as between thrombophilia and an adverse prognosis of CVT were explored.

Methods: We systematically searched The Cochrane Central Register of Controlled Trials (CENTRAL, *The Cochrane Library*, Issue 1 2013), MEDLINE (January 1966 to January 2013 (week 2); accessed via Pubmed) and EMBASE (January 1980–2013 (week 2); accessed via OVID). We also checked references of included studies. Studies were eligible if they met all of the following criteria: (i) diagnosis of CVT was objectively confirmed; (ii) patients were compared with a control group of non-genetically related subjects, or (iii) patients were analyzed in a moderate to large-size retrospective or prospective cohort study of consecutive patients ($n \geq 40$); (iv) prevalence of at least one of the following thrombophilic factors was assessed: factor V Leiden mutation; prothrombin G20210A mutation; antithrombin, protein C or protein S deficiency; antiphospholipid syndrome (presence of anticardiolipin antibodies, anti-β2 glycoprotein antibodies, and/or lupus anticoagulant); JAK2 mutations; hyperhomocysteinemia or MTHFR mutations; high factor VIII levels; or PAI-I polymorphisms; and (v) inherited or acquired thrombophilic factors were measured in an objectively and commonly accepted manner. Both studies that assessed prevalence of CVT or thrombophilia, as well as therapeutic interventions for CVT, such as anticoagulation treatment, were eligible. Two authors independently assessed eligible articles for inclusion in this review and systematically extracted data from selected articles. Conference abstracts were only considered if sufficient data on study design, participants, events and outcome measures was available for data

extraction. Duplicate reports of the same study were excluded to avoid publication bias and the most recent full-text paper was used. If necessary, study authors were contacted for more information. Discrepancies were resolved through discussion or with the opinion of a third author. Risk of bias, quality of evidence, potential heterogeneity and reporting biases were explored, a sensitivity analysis applied if applicable.
Results: Of 1355 identified citations 207 articles were selected for full-text evaluation. Cross-referencing of articles yielded another 10 articles. Finally, 66 papers enrolling 5898 patients with CVT and 8270 controls fulfilled our inclusion criteria and were included in this review.
Conclusions: Final results of this review will be published at the meeting.

PB 1.72-6

Thrombin generation in Cushing's and metabolic syndrome

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Background: The glucocorticoid excess found in patients with Cushing 's syndrome (CS) is thought to have both direct and indirect effects on the vasculature and the haemostatic system. CS is associated with an increased mortality, where hypercoagulability seems to play a crucial role in the development of both arterial and venous thrombosis.
In several recent studies in CS various abnormalities of coagulation have been described. These studies have concentrated on the use of single haemostatic and/or fibrinolytic marker. A better approach could be the use of a global test like the thrombin generation test (TGT).
Aims: To evaluate TGT in Cushing 's syndrome compared to patients with metabolic syndrome (MetS) and healthy individuals.
Methods: TGT (using the CAT method – Thrombinoscope, The Netherlands) as well as classical clotting markers (PT, aPTT, FVIII, antithrombin (AT), protein C and S) were evaluated in 33 CS patients and compared with both a group of 28 patients matched for the features of MetS and 31 healthy individuals. As well as the above haemostasis assays a full battery of endocrine and metabolic assays were measured using the routine hospital procedures – plasma, salivary and urinary cortisol, plasma ACTH, cholesterol, LDL, HDL and TG. All samples were only collected after obtaining informed consents from the patients.
Results: For the classical coagulation tests, PT was increased ($P < 0.0001$) in both CS and MetS patients, while PTT was shorter ($P < 0.0001$) in CS compared to both MetS and healthy group ($P < 0.0001$). FVIII, AT, protein C and S were increased only in CS patients ($P < 0.0001$). Both CS and MetS patients had a shorter TGT lag time ($P < 0.0001$), higher peak and ETP ($P < 0.0001$) than the healthy controls. The lag time was less reduced in CS ($P < 0.0001$) compared with the MetS group. BMI correlated negatively with lag time ($r = -0.40$; $P = 0.0001$) and positively to peak and ETP ($r = +0.34$; $P = 0.001$, $r = +0.28$; $P = 0.008$, respectively). Obese and diabetic patients had shorter lag time ($P = 0.0005$; $P = 0.0002$, respectively), higher ETP ($P = 0.0006$; $P = 0.007$, respectively) and peak ($P = 0.0003$; $P = 0.0005$, respectively) as well as a more prolonged PT ($P = 0.04$; $P = 0.009$, respectively). Hypertensive individuals had higher ETP ($P = 0.004$), Peak ($P = 0.0008$) and FVIII ($P = 0.001$).
Conclusions: Our findings confirm that a prothrombotic state could be seen in both CS and MetS patients using TGT. The lag time is shorter in the MetS than the CS patients indicating that the MetS patients may be at higher thrombotic risk then the CS patients. The high levels of endogenous physiological anticoagulants seen only in the CS patients could possibly represent a protective mechanism against the hypercoagulability in this patient group and account for the longer lag time in CS than MetS.

PB1.73 – Atherosclerosis: Miscellaneous – I

PB 1.73-1

Metabolic changes in rabbit atherosclerotic arteries: increased glucose uptake and metabolite levels of glycolysis, pentose phosphate pathway, tricarboxylic acid cycle and nucleotides

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Background: Glucose utilization in atherosclerotic arteries is largely affected by inflammation and possibly hypoxia, which could alter many metabolic systems. However, metabolic changes in atherosclerotic plaques remain unknown.
Aims: The present study aims to demonstrate changes in metabolic systems in atherosclerotic arteries of rabbits relative to glucose uptake and hypoxic condition.
Methods: Macrophage-rich or smooth muscle cell (SMC)-rich neointima was created by balloon injury with or without a 0.5% cholesterol diet in rabbit femoral arteries. We evaluated comprehensive arterial metabolism in the non-injured femoral artery and in femoral arteries with SMC- or macrophage-rich neointima by performing metabolomics analyses of central carbon metabolites, nucleotides, amino acids, and others (56 metabolites) using capillary electrophoresis-time of flight mass spectrometry. We also evaluated glucose uptake and its relationship to vascular hypoxia using ^{18}F-fluorodeoxyglucose (^{18}F-FDG) and pimonidazole, a marker of hypoxia.
Results: The levels of many metabolites increased in the femoral arteries with macrophage-rich neointima, compared with femoral arteries that were not injured and those with SMC-rich neointima (glycolysis, four of nine; pentose phosphate pathway, four of six; tricarboxylic acid cycle, four of six; glyconeogenesis/glycogenolysis, one of one; nucleotides, 10 of 20; amino acids five of six and nicotinamide, two of two). Only gluconic acid among metabolites differed between femoral arteries with or without SMC-rich neointima. Pimonidazole immunoreaction and nuclear translocation of hypoxia inducible factor-1a were closely localized with hexokinase II expression in macrophage-rich neointima, and were undetectable in femoral arteries that were not injured or had SMC-rich neointima. The uptake of ^{18}F-FDG in arterial walls measured by autoradiography increased in femoral arteries with macrophage- but not SMC-rich neointima (6.1 ± 3.6 [$n = 69$], vs. 1.8 ± 0.8%ID $\times$ kg/m^2 [$n = 72$]; $P < 0.001$), and positively correlated with macrophage- and pimonidazole-immunopositive areas ($r = 0.76$, and $r = 0.59$ respectively; $n = 69$ for both; $P < 0.0001$). Although more ^{18}F-FDG was uptaken in areas with, than without macrophages (3.9 ± 1.4 [$n = 24$] vs. 2.2 ± 0.8%ID $\times$ kg/m^2 [$n = 12$]; $P < 0.001$), ^{18}F-FDG uptake in macrophage areas did not change irrespective of hypoxia (non-hypoxic vs. hypoxic areas: 3.7 ± 1.3 vs. 4.1 ± 1.6% ID $\times$ kg/m^2; both $n = 12$).
Conclusions: Infiltrative macrophages and hypoxia in atherosclerotic arteries affect metabolic systems and glucose uptake in arteries.

PB 1.73-2

The transmembrane chemokine CXCL16 mediates platelet adhesion to von Willebrand factor, HUVECs and human arteries under physiologic flow conditions

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Background: CXCL16 is a member of the transmembrane chemokines and expressed at sites predisposed to atherosclerotic lesion formation and mediates monocyte adhesion to the endothelium.

Aim: In the present study platelet adhesion to CXCL16 is investigated.
Methods: Flow chamber experiments with CXCL16 immobilized in μ-slides and perfused with platelets in reconstituted were performed. Calcium Imaging was used to measure intra-platelet CXCL16 induced calcium flux. CXCR6 expression was demonstrated by western blotting and immunofluorescence techniques.
Results: Platelets in flowing blood adhered to CXCL16 transiently up to shear rates of 1800/s: platelets rolled on CXCL16 for 37 ± 3 μm before detachment (control was 13 ± 1 μm, $P < 0.001$) and rolling velocity was 0.43 ± 0.03 μm/s (control was 0.73 ± 0.12 μm/s, $P = 0.001$). However, for stable adhesion at these elevated shear rates the presence of additional adhesion ligands, such as von Willebrand factor (vWf) was mandatory (138.4 ± 6.2% of control, $P < 0.001$). CXCR6 is highly expressed in platelets and the cognate receptor for CXCL16 induced activation and intra-platelet calcium flux. CXCL16 is expressed on activated HUVECs and induced platelet adhesion in a CXCR6-dependent manner. Furthermore, CXCL16 is elevated expressed in TNF- and IFN-stimulated human internal mammaria arteries.
Conclusion: Therefore, CXCL16 is responsible for CXCR6 dependent platelet activation and more important, for platelet adhesion to HUVECs and vWf at shear rates up to 1800/s. Further flow experiments will be performed with human artery segments to evaluate CXCL16-CXCR6 dependent platelet adhesion to the endothelium of human vessels. These results demonstrate new mechanisms by which platelets might induce and accelerate atherosclerosis.

PB 1.73-3

Integrative bioinformatics analysis of genomic and proteomic approaches to understand the transcriptional regulatory program in coronary artery disease pathways

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Background: Patients with coronary artery disease (CAD) show a panel of differentially regulated serum biomarkers indicative of modulation of several pathways from disease onset to progression. Some of these biomarkers have been proposed for multimarker risk prediction methods. The underlying mechanism of the expression changes and modulation of the pathways in risk assessment needs to be addressed.
Aim: Our present work focuses on understanding the regulatory mechanisms at transcriptional level by identifying the core and specific transcription factors that regulate the CAD associated pathways.
Methods: Using the principles of systems biology we integrated the genomics (gene expression data of 10 CAD affected and 10 unaffected) and proteomics (400 affected and 400 unaffected) data with computational tools. The two sets of samples for gene expression and proteome analysis were selected from ongoing Indian Atherosclerosis Research Study (IARS) (Indian Heart J, 2010;6:286–95) which was designed according to the guidelines of World Medical Association Declaration of Helsinki and Indian Council of Medical Research and approved by Institutional Ethics Committee. Patients living in India for at least two generations were enrolled after signed informed consent was obtained (male ≤ 60 years and female ≤ 63 years at onset of disease, diagnosed as CAD) and all the blood samples were collected after overnight fasting of 12–14 h and stored at −80 °C. We selected biomarkers from seven different pathways based on their association with the disease and assayed 30 biomarkers by ELISA and built network modules using STRING database. The biomarkers selected were inflammation: Interleukin-6, Interleukin-8, Interleukin-10, Interleukin-12A, Interleukin-12B, Interleukin-18, Monocyte chemoattractant protein-1, High sensitive C reactive protein, Interferon gamma, Matrix metalloprotease-9 and secretary Phospholipase A2 and Gamma-glutamyltransferase-5, coagulation: Factor VII, Fibrinogen, Prothrombin, Plasminogen activator inhibitor-1, Plasminogen, Tissue factor, von Willebrand Factor, Platelet derived growth factor, cell adhesion: Clusterin and P-selectin, obesity: Adiponectin and Leptin, oxidative stress: Myeloperoxidase, stress: HSP27, HSP60, HSP70 and renal function: Cystatin C. The promoter sequence analysis for transcription factor binding sites was performed using Genomatrix software and microarray data was analyzed using R package v2.14.2.
Results: The Genomatrix software predicted 443 transcription factors binding profile in 30 different biomarkers, however, in global mRNA expression only 34 transcription factors were found to be differentially expressed in CAD affected and unaffected subjects. Out of these five transcription factors binding sites were highly represented in the promoter sequences of all 30 biomarkers thus forming the core regulatory transcription machinery which might influence the differential levels of biomarkers in serum of CAD affected subjects in comparison to unaffected. The five core transcription factors were PPARG, EGR1, ETV1, KLF7 and ESRRA and their combinatorial regulation may influence all the seven important pathways associated with CAD as outlined above.
Conclusions: Our analysis suggests that a profile of five core transcription factors binding to the promoters may play a major role in differential levels of biomarkers associated with CAD and thus use of this profile of transcription factors may be useful to identify new biomarkers which may play a role in risk assessment in CAD.

PB 1.73-4

CLL V-1, a synthetic phenanthrene compound, inhibits TNF-a-induced adhesion molecule expression through affecting reactive oxygen species production and NF-?B signaling in human endothelial cells

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Background: Cardiovascular diseases (CVD) are a major leading cause of death in the world and the dominant risk factor responsible for CVDs is atherosclerosis. Inflammatory mediators such as TNF-α and interleukins, which can upregulate expression of leukocyte adhesion molecules on endothelium and enhance circulating monocyes adhesion to endothelium, leading to a chronic inflammatory environment during atherogenesis.
Aim: This study was to investigate the anti-inflammatory effects of several synthetic and natural compounds on human umbilical vein endothelial cells (HUVECs) and to elucidate their underlying action mechanism.
Methods: Western blotting and RT-PCR were used to determine adhesion molecule protein and mRNA expression, respectively. Fluorescence microscopy and measurement was used for determining monocyte-endothelial cell interaction and intracellular ROS production. Western blotting and Electrophoretic mobility shift assay (EMSA) was used to examine NF-κB signaling and NF-κB-DNA interaction, respectively.
Results: Of the screened compounds, CLL V-1, a synthetic phenanthrene compound, was found to be the most potent on TNF-α-induced adhesion molecule expression. CLL V-1 inhibited TNF-α-induced ICAM-1 and VCAM-1 expression in a concentration-dependent manner, 1 μM was sufficient to exert its inhibitory effect. CLL V-1 also functionally reduced monocyte adhesion to endothelial monolayer. These effects did not result from its cytotoxicity. A further analysis revealed that CLL V-1 affected ICAM-1 and VCAM-1 mRNA expression in HUVECs. CLL V-1 attenuated TNF-α-induced IκB phosphorylation and degradation and NF-κB p65 and p50 translocation from cytosol to nucleus. In addition, CLL V-1 compromised TNF-α-induced NF-κB-DNA interaction and attenuated H_2O_2-

induced intracellular reactive oxygen species level and IκB phosphorylation.
Conclusion: We have demonstrated that CLL V-1 affected TNF-α-induced endothelial adhesion molecule expression through inhibition of ROS production and NF-κB signaling pathway.

PB 1.73-5

Early postoperative thrombosis following bypass surgery and remote endarterectomy in patients with peripheral arterial disease: a comparative analysis

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Background: Over the last decades there has been a tremendous increase in the number of with peripheral arterial disease (PAD). Results of surgical treatment of this common pathology are shadowed by high rate of early and late postoperative complications. Thrombosis of the reconstructed arterial segment following successful surgery remains one of the cornerstones in the management of patients with PAD. Controversies exist regarding optimal surgical treatment in patients with PAD suffering from isolated superficial artery occlusion.
Aims: To compare the rate of early postoperative thrombosis in patients with PAD following remote endarterectomy (RE) and femoro-popliteal bypass grafting (FPBG) for isolated superficial femoral artery (SFA) occlusion.
Materials and Methods: Fifty-seven consecutive PAD patients (57 lower limbs to be treated) from two vascular surgery units underwent surgical intervention for isolated SFA occlusion. FPBG was performed on 23 lower extremities while the alternative technique (RE) was used to revascularize remaining 34 limbs. RE as well as FPBG was done through a uniform surgical approach involving two vertical skin incisions: one in the groin and another one in the lower third of the thigh along its medial aspect. RE involved two longitudinal arteriotomies followed by the remote endartectomy of SFA using metallic Vollmar ring dissectors and autologous vein angioplasty at the site of arteriotomy incisions. FPBG was done using 8–9 mm synthetic (Dacron) grafts.
Results: Age median for RE and FPBG groups were comparable: 63.3 ± 6.5 and 6.7 ± 6.8 respectively. Early postoperative thrombosis defined as thrombotic occlusion of SFA or bypass graft within the first 6 months after surgery was diagnosed in four patients following RE (11.8%) and one patient (4%) after FPBG. Additional clinical parameters was brought to the analysis including duration of reconvalescence period, number of local complications, rate of repeated surgical interventions and dynamics in the degree of chronic arterial insufficiency during 6 months following successful revascularization. Two patients (5.9%) with early thrombosis following RE required repeated thrombectomies of the operated arterial segment, but developed irreversible ischemia of the extremity and further demanded major amputation. Early postoperative thrombosis in the group of FPBG was found in one patient (4%) and required a repeated femoral-popliteal below-the-knee bypass with complete resolution of symptoms.
Conclusions: FPBG is superior to RE in the management of PAD patients with isolated SFA occlusion since it is associated with lower risk of early postoperative complications including thrombosis of the operated arterial segment and better resolution of symptoms of chronic lower limb ischemia.

PB1.74 – Acquired Coagulation Disorders – I

PB 1.74-1

Acquired haemophilia: clinical manifestations and management. A 15 years experience from a single centre

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Background: Acquired haemophilia (AH) is a rare, often life-threatening haemorrhagic disorder, in individuals with no prior history of clinical bleeding. It is caused by autoantibodies commonly against coagulation factor VIII, rarely to other coagulation factors. Management of this disorder consists in rapid accurate diagnosis, control of bleeding and eradication of the inhibitor by immunosuppression.
Aim: We report a retrospective cohort of 28 patients with acquired haemophilia A and 1pt with acquired FXIII deficiency in a Greek haemophilia center.
Methods: Data derived from patient files. Inhibitor presence was suspected by the prolongation of aPTT, in patients with acquired bleeding tendency and confirmed by detection of FVIII inhibitor measured by NIijmegen modification of the Bethesda assay (cut off < 0.5 BU). An inhibitor titer > 5 BU was considered as high inhibitor. FXIII inhibitor was measured with a mixing test. Complete response (CR) to therapy was defined as negative inhibitor titer at the end of immunosuppressive regimen.
Results: The twenty-nine patients, median age 61 (range, 25–85) years, were diagnosed between 1997 and 2012. The most common symptoms in admission were spontaneous haemorrhages into the skin (80%), muscle or soft tissue haematomas (85%), mucosal bleeding (17%) and post trauma and surgical bleeding (3%). Haemarthroses were uncommon (2 pts). In 12 (40%) patients no underlying disease could be identified. 17/29 pts in whom a cause associated with inhibitor appearance was diagnosed, four had malignancies, six autoimmune disorder, one MGUS, four were postpartum and two had diabetes mellitus. Factor VIII:C was < 5% in 20 (74%) patients; An inhibitor titer > 5 Bethesda units (high responder) was measured in 23 (60%) patients. At the time of diagnosis a drop of Hb levels requiring RBC transfusions was observed in 20 (75%) patients. Bleeding in these patients was controlled with recombinant factor VIIa (NovoSeven), or activated prothrombin complex concentrate (FEIBA). No difference in efficacy was observed between the two agents. Inhibitor eradication strategy included: Prednisolone (1 mg/kg/day), with a 4–6 weeks tapering based on clinical status and FVIII inhibitor titre, (eight pts) or in combination with oral cyclophosphamide (1.5–2.0 mg/kg/day) for a maximum of 6 weeks (21 pts). Bleeding diathesis stopped by the time of inhibitor disappearance, independently of FVIII:C increase. Rituximab has been used to treat two of our patients (1pt with post-partum and 1pt with MGUS) with very high inhibitor and non response to common strategy. The post-partum inhibitor had a CR, while MGUS patient failed to response. Overall median time to CR was 4 weeks (range 2–6). Relapse has been reported in 20% of our patients most of them diagnosed with cancer and was associated with uncontrolled underlying disease. Mortality was associated with underlying disease. No patient died from bleeding complications.
Conclusions: Prompt recognition and early initiation of immunosuppressive treatment together with control of severe bleeding using by passing agents, are essential for the outcome of AH. Inhibitor eradication strategy can be adapted to clinical severity and patient's comorbidities.

PB 1.74-2

An immunological mechanism underlies the development of acquired von Willebrand syndrome (AvWS): clinical usefulness of an ELISA system detecting anti-VWF antibodies

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Background: Acquired von Willebrand Syndrome (AvWS) causes bleeding due to acquired reduction of von Willebrand factor (vWF). AvWS develops and is associated with underlining lymphoproliferative or myeloproliferative disorders, other neoplasia, immunological and cardiovascular disorders. Cause of AvWS is widely variable but it might depend either on immunological or non-immunological mechanisms. Under immunological mechanisms, anti-VWF immunoglobulin binds VWF and higher molecular weight VWF multimers might be extensively cleared from circulation.

Aims: We experienced a case of AvWS and examined the spectrum of anti-VWF antibody by specific ELIZA method and found auto-VWF IgG antibody caused severe AvWS.

Description of a Case: A 36 years old woman who had extensive purpula and mucosal bleeding during past 6 months and was referred to our hospital. She had been prescribed valproic acid due to depression but there was no previous history of typical underlining disorders of AvWS. She had no family history of hemorrhagic diseases, nor suffered from any hemorrhagic manifestations. At initial presentation, her activated partial thromboplastin time (APTT) was 48.3 s (35.2 s in normal), factor VIII procoagulant activity (FVIII:C) was 20%, plasma ristocetin cofactor activity (VWF:RCo) was below 6%, and immunoreactive VWF antigens (VWF:Ag) was 23%, respectively. APTT cross-mixing test showed factor deficiency pattern. Microtiter plates were coated with purified human plasma VWF obtained from commercial plasma VWF concentrates. After blocking, serially diluted plasma samples were overlaid for 1 h at 22 °C. Washed plates were added by HRP-conjugated either rabbit anti-human IgG serum (Dako) or goat anti-human IgM serum (Sigma) and incubated for 1 h 22 °C, followed by quantification of color development at 490 nm absorbance. Similarly, each IgG subclass was determined using anti-human IgG 1, 2, 3, 4 mouse serum. As a result, IgG1 type of anti-VWF antibody was detected and autoantibody against VWF was a cause of her AvWS. Soon after diagnosis, she needed immediate hospitalization because of left ovary hemorrhagic rupture. The bleeding stopped by administration of 2000 units (40 IU/kg) of plasma VWF concentrates twice a day for 3 days. One hour after infusion, VWF:RCo, VWF:Ag, and FVIII:C elevated to 27%, 107% and 52% of normal, respectively, suggesting that higher molecular weight VWF multimers might be selectively inhibited. Recovery of VWF and factor VIII, however, returned to baseline 15 h after administration, indicating shortened VWF half life. Seven days after the first bleeding, right ovary bleeding happened and we tried DDAVP and her abdominal pain was relieved. Two hours after infusion of DDAVP, VWF:RCo, VWF:Ag, and FVIII:C elevated to 46%, 10% and 46%, respectively. VWF:RCo returned to baseline level 4 h after administration, whereas VWF:Ag and FVIII:C returned to baseline level 12 h after administration. Although several reports have indicated that children prescribed with valproic acid had lower VWF activities, this is the first report of severe von Willebrand disease with autologous anti-VWF antibody that appeared to be associated with valproic acid usage.

Conclusion: The AvWS diagnosis were rapidly capable of by using specific ELISA method and may be useful and sensitive for screening of suspected von Willebrand syndrome with bleeding diathesis.

PB 1.74-3

Managing acquired haemophilia A: relating the European acquired haemophilia registry (EACH2) data to experience in a South London centre, St George's Hospital

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Background: Acquired haemophilia A (AHA) remains a rare and potentially fatal bleeding disorder characterised by the development of autoantibodies directed against coagulation factor VIII. Recently, the EACH2 Registry has provided the largest observational dataset from 117 European centres on the management of AHA. As a large tertiary referral unit in South London, AHA is a part of our regular practice.

Aims: To audit our AHA experience and to compare it to the data presented in the EACH2 registry.

Method: A single centre retrospective analysis of all AHA patients registered at our centre between 2006 and 2012. Data collected through paper and electronic records included demographics, aetiology, bleeding characteristics, immunosuppression and haemostatic agents used to control the first bleeding episode.

Results: We identified 11 female and five male patients with AHA. The median age at diagnosis was 78 years (40–99). At presentation, the median haemoglobin concentration, FVIII:C and inhibitor titre were 94 g/L (46–138) 2 IU/dL (0–20) and 14 BU/mL (< 1 to > 6000).

2/11 patients required surgical intervention for life-threatening bleeding. Severe bleeding, as defined by the EACH2 registry, was observed in 62% of patients. This group had higher peak inhibitor titres compared to those without severe bleeding (32 vs. 9 BU/mL). The overall survival was 75% with one acute fatality.

Only one patient did not receive any haemostatic agents. Of the remainder, 8/15 were treated with aPCC only, 3/15 received rFVIIa only and 5/15 required sequential therapy using both agents, primarily for invasive procedures. No patients were treated with desmopressin or factor VIII concentrate. To control the first bleeding episode, the median dose of aPCC was 47000iu. Of the few patients (3/15) who received rFVIIa, the median dose was 20 mg.

11/16 patients received dual immunosuppressive therapy with cyclophosphamide and prednisolone. Rituximab was reserved for poor response and administered to 3/16 patients.

Those treated with Rituximab took longer to achieve remission (negative inhibitor 183 days, normal FVIII 120 days).

An inhibitor titre < 16 BU/mL (8/16) was associated with quicker inhibitor eradication (10 days vs. 90 days) and quicker normalisation of FVIII:C (23 vs. 48 days)

Relapse was observed in five patients with the majority receiving subsequent rituximab. This group had a higher historical peak inhibitor titre than those who had sustained remission.

Summary: Our findings are not dissimilar to the data in the EACH2 registry. Despite the older age of our patients, all other baseline patient demographics and bleeding characteristics were similar. Bypassing agents were used to treat all patients with clinical bleeding, unlike only 70% in the EACH2 registry. Our patients received a higher total dose of haemostatic agent, which may reflect the frailty of our older patient population. Unlike the EACH2 registry, severe bleeding was more common in those with a higher peak inhibitor titre. However, our study did reinforce the finding of the EACH2 registry that an inhibitor titre < 16BU/mL was associated with reduced time to remission. Given the limitations of patient number, this single centre audit supports the importance of collecting registry data in rare diseases.

PB 1.74-4

Acquired hemophilia A: retrospective analysis of 49 cases from a single Chinese hemophilia center

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Background: Acquired hemophilia A (AHA) is a rare bleeding disorder caused by the autoantibody directed against factor VIII in patients without previous history of a bleeding disorder. Its clinical features range from harmless bruises to life-threatening bleedings with a high mortality rate.
Aims and Methods: We retrospectively analyzed the characteristics and outcomes of 49 patients with AHA diagnosed in our center from February 1994 to December 2012. This study was approved by the medical ethic committee of Hospital of Blood Diseases, Peking Union Medical College. All patients signed an informed consent.
Results: The median age was 49 years (range 14–78 years). Associated diseases were observed in 9 (18%) patients. Nine patients had comorbidities. The most common symptoms were ecchymosis and hematoma. Twenty-four patients with acute bleeding episodes were treated with prothrombin complex concentrate (PCC) at a relative low dose of 30–50 U/kg/day and achieved good outcomes without adverse reaction. Corticosteroids alone or in combination with cyclophosphamide were used as first-line therapy to eradicate the inhibitors. In 39 evaluable patients, 35 (89.7%) achieved complete remission (CR). Four patients died of hemorrhage. The response time was not related to the titers of FVIII:C and inhibitor.
Conclusions: This study demonstrates that when bypassing agents such as recombinant activated factor VII and activated prothrombin complex concentrate are not affordable or available, low dose PCC is effective and safe to control acute bleeding in AHA patients. Corticosteroids alone or in combination with cyclophosphamide used as first-line therapy achieved good outcomes with CR rate of 89.7%. The comorbidities and side-effect of immunosuppressive agents in these patients probably have adverse impact on the prognosis.

PB 1.74-5

Telemedicine: physician supported rural hemophilia treatment center (HTC) making a difference for patients

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Background: Telemedicine is defined as 'the use of medical information exchanged from one site to another via electronic communications to improve patients' health status.'[1] Persons with bleeding disorders (PWBD) live in wide geographic areas and often have to travel great distances to see specialists who understand and are able to treat their disorders.
The Northern Regional Bleeding Disorders Center (BDC) is located in Northern Michigan and provides services to approximately 200 patients covering a geographic area of > 11,000 sq miles/28,490 sq km. It is the only HTC in the USA that does not have a physician on site but collaborates with physicians from other HTCs to provide care for patients closer to their home. Traveling to distant HTCs would significantly add hours, if not days, to their schedules.
The HTC began telemedicine in 2010 with both pediatric and adult patients. Presented are metrics of the program including: demographics of patient population, description of miles/km traveled and miles/km saved by the patient, number of labs ordered, and patient/provider satisfaction scores.
Aim: Describe metrics from telemedicine visits at a BDC over a period of 2 years (2011–2012).
Methods: Pediatric and adult patients seen via telemedicine over a period of 2 years (2011–2012) were offered a patient satisfaction survey after their visit. Data was collected from their visit including: labs ordered, mileage, estimated time and travel costs saved by participating in telemedicine visit. Participating providers were queried on their satisfaction with telemedicine.
Results: Between 2011 and 2012, 48 patients (pediatric-11, adults-37) were seen via telemedicine. A total of 191 labs were ordered (pediatric-44, adults-147) for diagnostic, continued monitoring, and surgical planning. These 48 patients traveled 2419 actual miles/3893 actual km, saving a cumulative total of 17,980 miles/28,936 km by utilizing telemedicine. Eight 40 h work weeks were saved over 2 years by patients not traveling to a distant site to see the bleeding disorder specialist (17,980 m/55 mph = 326.9/40 h work week = 8.2 work weeks over 2 years). This equates to one working month per year saved by not traveling to a distant site. No adverse outcomes were experienced by any patients utilizing telemedicine.
Patients either agreed or strongly agreed in all areas of the patient satisfaction survey: they could clearly see and hear the distant provider, they knew what to expect and felt comfortable discussing their issues during the interview, they felt their questions were answered, their privacy was respected, the provider answered their questions thoroughly, by using telemedicine they were able to see the specialist sooner than if they had traveled to the distant site, they would recommend telemedicine to others, and the quality of their medical care met their expectations. Providers expressed satisfaction with telemedicine.
Conclusion: Telemedicine is an effective tool to utilize for evaluation and follow up in PWBD given the distance many patients live from an HTC. Patients, families and providers are very satisfied. Significant time and travel was saved. High quality care, including surgical and birth planning was provided with no adverse outcomes.

PB 1.74-6

Diagnosis and management of acquired haemophilia A (AHA) patients: experience of a single center

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Background: AHA is a rare bleeding disorder with an incidence of 1.5/million/year. Mortality rate is high (9–22%), if diagnosis is delayed and treatment is not promptly established.
Aims: Retrospective evaluation of AHA affected patients diagnosed and followed at our Institution. Patients characteristics, bleeding symptoms and treatment, eradication therapy and response, occurrence of possibly related diseases have been considered.
Patients. Between 1984 and 2012, 34 patients (M, 14; F, 20), were diagnosed with AHA. Diagnosis median age: 70.2 years (25–89); median time from the first bleeding symptoms and diagnosis: 48.5 days (4–264); median follow-up (FU): 19.7 months (2.6–215.4).
Results: At diagnosis, bleeding symptoms were present in 33/34 patients (97%) (28 muscle haematomas, three haematuria, two postpartum haemorrhages, 1GI bleeding, one haemoperitoneum, one haemarthrosis, two post-surgery bleedings); median inhibitor title was 9.6 BU/mL (2.5–138), median FVIII:C level, 2% (0.01–21); in 16 patients FVIII:C level was < 1%. Clinical conditions triggering the inhibitor appearance were present in 17 cases (50%): previous delivery in 9, autoimmune diseases in 4, cancer in 4.
First-line eradication therapy was prescribed in all patients: prednisone (PDN) (median dose 1 mg/kg/day, range 0.5–2) for 4 weeks, in 23 (68%), dexamethasone (DXM) (median dose 24 mg/day, range 24–40) for 4-day courses, in 7 (20%), azathioprine (AZA) 100 mg/day for 3 months, in 2 (6%), cyclophosphamide (CTX) (0.5 and 1.5 mg/kg/day) plus PDN (standard doses) for 2 months, in 2 (6%). Thirty-one patients are evaluable for response, 27 treated with PDN/DXM, four

with other immune suppressive drugs. Inhibitor eradication (undetectable inhibitor, normal FVIII:C levels, absence of bleedings), was obtained in 24/31 patients (77%) (22/27 [81%] on PDN/DXM, 2/4 [50%] on other immune suppressants).
Second-line therapy was administered in 4/7 no responder patients: CTX + PDN in 2, DXM in 1, PDN in 1. Inhibitor eradication was obtained in 1. Third-line therapy was performed in 2/3 second-line no responder: 1 CTX + PDN, 1 AZA + PDN. No one responded. One of these two patients was treated with Rituximab, obtaining finally persistent inhibitor eradication. Three patients relapsed after first-line treatment (3/24, 12.5%).
At last control, 23 patients maintained persistent inhibitor eradication (median FU: 25.9 months [2.6–150.9]).
Bypassing agents (rFVIIa or FEIBA) were used in 21 patients with a high efficacy to control bleeding symptoms: FEIBA in 11, rFVIIa in 7, either FEIBA or rFVIIa in 3. A severe bleeder no responder, is on FEIBA prophylaxis three times a week with a good control of hemorrhagic events.
During FU, occurrence of diseases possibly related to the inhibitor presence (cancer) was recorded in 2/17 idiopathic cases (11.7%); 5/34 deaths, not bleeding related, were recorded.
No relapses were observed in two post-partum inhibitor affected patients who underwent three deliveries after a period of 2, 3.6, 7.3, years respectively after inhibitor eradication.
Conclusions: We confirm literature data as regard as idiopathic AHA cases percentage (50%). We observed a high response rate after steroid administration (81%). Bypassing agents were efficacious in all treated patients. Relapse rate was relatively low (12.5%). A good management of AHA reduce the mortality risk bleeding related.

PB2.21 – Antiplatelet Agents: ADP Receptors – III

PB 2.21-1

Absence of off-target prolongation of the bleeding time in prasugrel-treated rats and mice: additional evidence for selective P2Y12 inhibition by prasugrel

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Background: Prasugrel is the 3rd generation thienopyridine prodrug exhibiting more optimal pharmacokinetics and pharmacodynamics compared with clopidogrel. This profile results in both more effective platelet inhibition and greater clinical benefit in acute coronary syndrome (ACS) patients undergoing percutaneous coronary intervention. Ticagrelor is a cyclopentyl-triazolo-pyrimidine and non-competitive $P2Y_{12}$ antagonist recently approved for reducing the rate of thrombotic cardiovascular events in ACS patients.
Aims: Recent data suggest that thienopyridines result in off-target effects at the vessel wall that contribute to bleeding in mice (J. Pharmacol. Exp. Ther. 338, 22–30, 2011). However, our pilot non-clinical studies showed that high-dose prasugrel resulted in a similar prolongation of the bleeding time (BT) as a non-thienopyridine $P2Y_{12}$ antagonist, ticagrelor, suggesting no off-target effect of prasugrel. In the present study, we further examined the individual and combined effect of prasugrel and ticagrelor on BT in rats, wild-type mice and in $P2Y_{12}$ knock-out mice.
Methods: Four hours after the administration of single or combined agents to mice or rats, a needle (23 or 21G, respectively) was advanced 0.5 or 1 cm into each tail vein at a 4.5 or 3 cm point from the tail end and immediately withdrawn. Blood was blotted every 5 s with a filter paper and when blood no longer appeared the bleeding time was recorded with control (vehicle) values of 92 ± 5 s ($n = 10$) and 104 ± 6 s ($n = 10$) in mice and rats, respectively. In mice, additional experiments included measurement of platelet aggregation in platelet-rich plasma stimulated with 20 μM ADP 30 min after BT determination.
Results: A single dose of prasugrel alone (10 mg/kg, *p.o.*) or ticagrelor alone (30 mg/kg, *p.o.*) resulted in significant prolongation of the bleeding time in rats to 228 ± 23 s ($P < 0.001$ vs. vehicle, $n = 10$) and 231 ± 23 s ($P < 0.001$ vs. vehicle, $n = 10$), respectively. Combined administration of prasugrel (10 mg/kg) with ticagrelor (30 mg/kg) resulted in similar extent of prolongation of bleeding time (255 ± 21 s, $P < 0.001$ vs. vehicle, $n = 10$) compared to each individual dosing group. In wild-type mice, both prasugrel (3 and 10 mg/kg, *p.o.*) and ticagrelor (10 and 30 mg/kg, *p.o.*) prolonged BT in a dose-related manner with high-dose values for prasugrel and ticagrelor being 148 ± 7 s ($P < 0.001$, $n = 10$) and 149 ± 10 s ($P < 0.001$, $n = 10$), respectively. In $P2Y_{12}$ knock-out mice, similar prolongation of the bleeding time was observed in the vehicle group (146 ± 7 s, $n = 10$) and neither prasugrel nor ticagrelor had any significant prolongation of the bleeding time compared to the vehicle group. Platelet aggregation studies showed that both prasugrel and ticagrelor substantially inhibited platelet aggregation in wild-type mice, and these values were similar to those in all groups of $P2Y_{12}$ knock-out mice including the vehicle group.
Summary/Conclusion: These results support the contention that prasugrel is a selective $P2Y_{12}$ inhibitor with an apparent absence of off-target effects that influence the bleeding time or inhibition of platelet aggregation.

PB 2.21-2

Impact of cigarette smoking on clopidogrel antiplatelet effect in diabetic patients

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Background: High on-clopidogrel platelet reactivity (HPR) is associated with an increased risk of adverse events after coronary stenting and acute coronary syndromes. Pharmacodynamic studies have shown that residual HRPP is more common in patients with diabetes. Recent data suggest that cigarette smoking might enhance clopidogrel mediated platelet inhibition. Indeed, cigarette smoking is an inducer of cytochrome P450 1A2, a hepatic enzyme involved in clopidogrel metabolism.
Objective: The aim of our study was to investigate the influence of cigarette smoking on clopidogrel mediated platelet inhibition in diabetic patients presenting with an acute coronary syndrome.
Methods: This is a prospective study including 30 diabetic patients (mean age: 58.9 ± 9.7 years, 17 men) admitted with ACS without ST elevation who received 600 mg clopidogrel loading dose followed by a maintenance dose of 150 mg. 60.8% of patients were active smokers. Evaluation of platelet function was performed by the VerifyNow P2Y12 Test, 24 h after the loading dose and at the third day. Residual HPR under clopidogrel was defined as a PRU value > 230 at H24.
Results: The mean H24 PRU was significantly lower in diabetic smokers compared to non smokers (172.1 ± 61.7 vs. 255.6 ± 62.4, $P = 0.001$). HPR rate on clopidogrel in diabetic patients smoking was significantly lower on day 1 (17.6% vs. 69.2%, $P = 0.008$). Only 5.9% of diabetic smokers had HPR at the third day. In the multivariate analysis, current smoking has emerged as a protective factor of HPR on clopidogrel (OR = 0.08, CI 95% [0.007–0.9]; $P = 0.04$).
Conclusion: This pilot study confirms the enhancement of the antiplatelet effect of cigarette smoking even in coronary diabetic population admitted for ACS. This confirms that the main mechanism of HPR in this population is associated with a reduced production of active metabolite.

PB 2.21-3

The experience of the antiplatelet therapy by clopidogrel in the West-Siberian region of Russia: the effects of CYP2C19 and ABCB1 allelic variants

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Background: Clopidogrel is known to be one of the principal drugs for modern antiplatelet therapy. However, the interindividual variability of the response to clopidogrel has been also shown. This variability depends both of the presence of certain genetic polymorphisms, in particular, allelic variants of CYP2C19 and ABCB1 genes, and of clinical factors. Clinical trials revealed the regional features of abundance of genetic polymorphisms influencing clopidogrel metabolism.

Aims: To examine frequencies of occurrence of allelic variants CYP2C19 *2, *17 and ABCB1 C3435T among the patients of the West-Siberian region of Russia receiving clopidogrel and to determine contribution of these markers to the clopidogrel laboratorial efficacy.

Methods: One hundred and fifty-eight patients with cardiologic pathology receiving clopidogrel were enrolled. The allelic variants of ABCB1 and CYP2C19 were determined by means of real-time PCR. The relative change of platelet aggregation with ADP before and 48 h after taking of loading dose of clopidogrel (600 mg) referred to as resistance index (RI) was assessed. RI was assumed to be a measure of the clopidogrel laboratorial efficacy. The relation of allelic variants of ABCB1 C3435T and CYP2C19 *2, *17 to RI was estimated. The study protocol was approved by the local ethic committee of the ICBFM SB RAS.

Results: Depending on RI the following subgroups of patients were assigned: sensitive (RI > 30%, 58.9%), semi-responders (RI = 10–30%, 20.9%), non-responders (RI = 0–10%, 7.6%), and 12.7% of patients demonstrated paradoxical laboratorial response: platelet aggregation increased after clopidogrel taking.

According to genetic analysis data, the CYP2C19 gene was presented by allelic variants *1/*2 (24.7%), *2/*2 (0.6%); *1/*17 (17.0%) and *17/*17 (1.0%), and the ABCB1 gene by 3435 CC (20.3%), CT (41.8%) and TT (37.9%) variants. The correlation analysis revealed the connection between CYP2C19*2 and RI. Both CYP2C19*17 and ABCB1 C3435T allelic variants have not been shown to make an effect in changes of platelet aggregation after clopidogrel taking. None statistically significant connection between considered genetic variants and paradoxical laboratorial response on clopidogrel was found.

Depending on the type of drug metabolism patients were divided into five subgroups: fast (*17/*17, *1/*17), extensive (*1/*1), intermediate (*1/*2, *1/*3), slow (*2/*2), 'uncertain' (*2/*17). Significant differences ($P < 0.05$) were found between average RI in groups of fast and extensive metabolizers and in groups of fast and «uncertain» metabolizers. As a result, the influence of allelic variant CYP2C19*2 is more significant than the influence of the variant CYP2C19*17 in the group of patients under study.

Summary/Conclusions: The frequencies of occurrence of CYP2C19 and ABCB1 allelic variants in a group of patients from West-Siberian region of Russia receiving clopidogrel therapy were calculated and the effect of these genetic variants on clopidogrel laboratorial efficacy was examined. 12.7% of patients in the group under study were found to demonstrate paradoxical laboratorial response on clopidogrel. Such unusual response has not been shown to be associated with genetic variants considered. CYP2C19*2 has been shown to make an effect on the laboratorial response on the clopidogrel. RI in the subgroup of fast metabolizers is higher than in subgroups of extensive and «uncertain» metabolism.

PB 2.21-4

The VerifyNow PRU is associated with optimal duration of clopidogrel interruption prior to CABG surgery: sub-Analysis of TARGET CABG study

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Background: The utility of thrombelastography (TEG) to determine the timing of CABG in pts treated with clopidogrel was evaluated in the first prospective study, TARGET CABG which showed that pts non-responsive by TEG had no greater chest tube output when operated within 24 h of last clopidogrel dose compared to clopidogrel naïve pts. The utility of VN to optimally time CABG is unknown.

Methods: In TARGET CABG, we also analyzed platelet function recovery (PFR) using VerifyNow P2Y12 assay in 81 patients on aspirin and clopidogrel therapy undergoing elective first time isolated on-pump CABG. CABG was done within 1 day (nonresponse), 3–5 days (moderate response), and > 5 days (high response) based on ADP induced platelet-fibrin clot strength (MA-ADP) as measured by thrombelastography. Proportion of patients achieving pre-surgical PRU > 208 was used indicate PFR.

Results: Baselines PRUs were in agreement with MA-ADP within each response group and there was significant difference in mean baseline PRUs between response groups ($P < 0.05$) (Figure 1). Ninety-seven percent of patients demonstrated PFR prior to surgery. The total amount of red blood cells transfused did not differ between response groups.

Conclusions: PFR by VN-P2Y12 assay agrees with TEG, suggesting that PFR specific to the P2Y12 receptor significantly influenced the primary results of TARGET-CABG. The use of platelet function testing may allow for improved pre-surgical management of $P2Y_{12}$ inhibitor therapy prior to major surgery, and further investigation is warranted.

PB 2.21-5

Comparison of the antiplatelet effect of crushed clopidogrel vs. whole tablet in diabetic patients presenting with an acute coronary syndrome

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Background: Pharmacodynamic studies have shown that persistently high residual platelet reactivity (HRPP) is common in patients with diabetes in spite of clopidogrel treatment. Even, a 600-mg clopidogrel loading dose is not sufficient to overcome this effect, especially in diabetic patients with ACS. This can be explained by altered resorption, altered liver metabolism, or altered platelet response. Recently, it was demonstrated that clopidogrel given crushed provided faster absorption and greater rate of clopidogrel inactive metabolite in healthy subjects.

Objective: The hypothesis of the study was that 600 mg clopidogrel loading dose (LD) administered crushed may provide more effective platelet inhibition than an equal clopidogrel dose taken orally as whole tablets in patients with type 2 diabetes mellitus presenting with ACS.

Methods: In a prospective, single-center, single-blind study, 30 (58.9 ± 8.4 years; 17 males) ACS diabetic patients were randomized to either 600 mg crushed clopidogrel ($n = 15$) or whole tablets ($n = 15$) follow by a maintenance dose of 150 mg, respectively. Pharmacodynamic assessment was performed by VerifyNow P2Y12 system accumetrics at 24 h and 3 days post LD. High Residual platelet reactivity (HRPR) was defined as platelet reactivity units (PRU) > 235.

Results: The baseline characteristics were no different in the two groups. We noted no difference between the antiplatelet effect produced by 600 mg crushed clopidogrel compared with whole tablets at both 24 h (PRU = 199.73 ± 79.3 vs. 216.9 ± 70.1; $P = 0.53$) and at 3 days (PRU = 164.3 ± 92.9 for crushed clopidogrel vs. 160.5 ± 73.5 for whole tablet; $P = 0.9$). HRPR rate also was similar in the two groups, 40% ($P = 1$) at 24 h and diminished significantly in both groups at 3 days to 20% of patients ($P < 0.001$).
Conclusions: Despite the small sample size, the present study demonstrates that clopidogrel given crushed in diabetic ACS patients is as effective as whole tablets.

PB 2.21-6

Rebound of ADP mediated platelet aggregation after abrupt cessation of long-term clopidogrel therapy positively correlate to thrombin and arachidonic-acid dependent platelet aggregation

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Background: The interplay between various platelet agonist-receptor interactions during and after stopping antiplatelet therapy is insufficiently known and possible rebound phenomena may have important adverse consequences.
Aim: To investigate the influence of abrupt cessation of long-term clopidogrel therapy on platelet aggregation to adenosine diphosphate (ADP), arachidonic acid (AA) and thrombin receptor-acivating peptide (TRAP) in patients with coronary heart disease on aspirin.
Patients and methods. Two-hundred patients who underwent to elective PCI with stent who were on 1 year dual antiplatelet therapy (aspirin + clopidogrel) and had no adverse events during that time were enrolled in the study. Platelet function was measured by multiple electrode aggregometry using three agonists [ADP with PGE1 (ADPHS-test), AA (ASPI-test), and TRAP (TRAP-test)] at the last day of clopidogrel therapy and 90 days after stopping the drug.
Results: Platelet aggregation on ADP [320.5 AU × min (212.7–461.7) vs. 838.5 AU × min (728.0–968.7), $P < 0.001$], AA [155.0 AU × min (115.0–211.0) vs. 228.0 AU × min (154.0–375.7), $P < 0.001$] and TRAP (1134.7 ± 176.6 vs. 1158.1 ± 175.3 AU × min, $P = 0.041$) significantly increase after 90 days. Levels of TRAP and ASPI at 90 day significantly increase according to quartiles of ΔADP (difference between the ADP test at 90 day and baseline) $P < 0.001$ and $P = 0.021$, respectively.
Conclusion: Patients with higher increase of ADP dependent platelet aggregation after stopping clopidogrel had higher platelet aggregation on TRAP and AA.

PB2.22 – Platelet Function Tests: Clinical

PB 2.22-1

Platelet-reactivity tests identify patients at risk of secondary cardiovascular events: a review and meta-analysis

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Background: Antiplatelet therapy is standard treatment for the prevention of cardiovascular events (CVE). High-on-treatment platelet reactivity (HPR) is a risk factor for secondary CVE in patients prescribed aspirin and/or clopidogrel. Numerous platelet-function tests are available that assess HPR for aspirin (HAPR) or clopidogrel (HCPR).
Aims: The present review and meta-analysis aimed to assess the ability of individual platelet-function tests to reliably identify patients at risk of secondary CVE while being treated with aspirin and/or clopidogrel.
Methods: *Data Sources:* A systematic literature search using the terms 'aspirin resistance' and 'clopidogrel resistance' was conducted to identify studies on platelet-reactivity measurements and CVE. The years of publication ranged from January 1966 to September 2012; the publications were in English.
Study Selection: Main inclusion criteria were: (i) study design was prospective; (ii) study medication was aspirin and/or clopidogrel; (iii) a platelet-function test was performed at baseline; and (iv) data on baseline platelet-function-test outcome and CVE during follow-up available. The latter criterion was necessary to extract dichotomous frequency data. Of 2676 identified studies, 89 (3.3%; reporting on 29,018 patients) were included in the meta-analysis.
Data Extraction: Data abstraction and data-quality and -validity assessment were executed independently by two observers.
Results: With regard to HAPR, 21 different tests were discussed in 49 studies (12,687 patients). Pooled analysis showed that HAPR was diagnosed in 29.0% of patients, and associated with an increased risk of developing CVE (RR: 2.08; 95%CI: 1.74–2.48). The negative predictive value (NPV) was 89.8%, the positive predictive value (PPV) 19.0%. When the individual HAPR tests were compared, 11 showed an increased risk of CVE in patients with HAPR. As regards HCPR, 50 studies (21,037 patients) discussed 14 different tests and reported HCPR being present in 38.5% of patients and associated with an increased CVE risk (RR: 2.90; 95%CI: 2.44–3.44). The NPV of the HCPR tests was 95.2%, the PPV10.6%.
Conclusion: HPR is seen in a large number of patients on antiplatelet therapy. Patients with HPR are inadequately protected against future cardiovascular complications. Some HAPR and HCPR tests can identify patients at higher risk of secondary CVE.

PB 2.22-2

The real world relationship between VerifyNow PRU and device-reported percent inhibition: analysis from the GRAVITAS trial

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Background: The VerifyNow P2Y12 Test is a rapid, point-of-care platelet function test that has been extensively validated as a tool for measuring the antiplatelet effect of P2Y12 receptor inhibitors. The VN P2Y12 Test reports results as P2Y12 Reaction Units (PRU) and device-reported percent inhibition of platelet reactivity (%I), based on using thrombin receptor-induced platelet aggregation as a substitute for a baseline, P2Y12 inhibitor naïve PRU result. The PRU result is highly specific for P2Y12 receptor blockade due to the effect of a P2Y12 inhibitor and is an absolute measure of the drug effect. The%I result is a relative measure of the drug effect because the absolute effect is normalized using baseline, thrombin-receptor induce platelet aggregation. PRU results have been clinically validated to identify patients at increased risk for thrombosis and bleeding due to the presence of a P2Y12 inhibitor antiplatelet effect.%I results have been clinically validated to correlate to a return to baseline platelet function following P2Y12 inhibitor cessation. The relationship between these two results has not been extensively described.
Aims: The objectives for this study were to (i) compare the actual relationship of PRU and%I results to a model based on true baseline platelet reactivity and (ii) determine the agreement of PRU and &I results at published clinical decision points.
Methods: Matched PRU and%I results were evaluated using measurements obtained from the GRAVITAS trial. A total of 10,375 Verify-

Now P2Y12 Test measurements from 5429 subjects were used for the analysis. The manufacturer-reported 95% confidence interval PRU reference range of baseline platelet reactivity (194–418) represents the range of PRU results when the 'true' %I is 0%. A PRU result of 0 is the expected result when 'true' %I is 100%. Taken together, a model for the relationship of PRU and%I was constructed using an inferred range of PRU results at each level of 'true' %I. Because the reference range is the 95% confidence interval of baseline PRU results, the model is therefore a prediction of the relationship between PRU and% I for at least 95% of the observations. The percent of actual measurements that were in agreement with the model was determined, and the agreement of results using PRU < 208 and % I > 20% cutoffs also was determined.

Results: 96.8% of the matched PRU and%I results were within the theoretical model, which was within the expected > 95% of results. The PRU > 208 cutoff reported to define high on-treatment platelet reactivity was 95% specific for the %I < 20% reported to define a baseline platelet function.

Summary/Conclusion: The results of this investigation confirm there the PRU and device-reported%I results from the VerifyNow P2Y12 Test are highly correlated and are consistent with a prediction model of their relationship. These observations suggest that device-reported %I is an acceptable surrogate for true percent inhibition calculated from a pre-drug and on-treatment PRU result. In addition, the results indicate that there is consistency between the clinically validated PRU and device-reported%I decision points.

PB 2.22-3

High platelet reactivity is associated with increased D-dimer levels in acute coronary sindrome patients

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Background: High platelet reactivity (HPR) on dual antiplatelet treatment is an independent predictor of adverse ischemic events in acute coronary syndrome (ACS) patients undergoing percutaneous coronary intervention (PCI). Few data are available on the possible association of this phenotype with coagulation and inflammation.

Aims: To investigate the relationship between HPR – by both arachidonic acid and ADP – and markers of clotting and fibrinolysis activation and inflammatory response.

Methods: We recruited 646 ACS patients undergoing PCI on dual antiplatelet therapy. Platelet reactivity was assessed by light transmission aggregometry by 10 μM ADP and by 1 arachidonic acid (AA). D-dimer levels were assessed by immunoturbidimetric method (IL Laboratory, Milan). HPR by ADP was diagnosed in the presence of LTA by ADP ≥ 55% according to our previous published data; HPR by AA in the presence of LTA ≥ 20%.

Results: D-dimer, fibrinogen and ESR levels were respectively: (median and interquartile range) 229.5 (119.7–413.2) ng/mL, 534.0 (451.5–612.2) mg/dL, 15.0 (11.0–21.0) mm/h.

HPR by ADP was detected in 357/646 (55.2%) and HPR by AA in 189/646 (29.2%).

LTA by AA values significantly correlated with D-dimer, fibrinogen and ESR values (D-dimer: $r = 0.15$, $P < 0.001$; fibrinogen: $r = 0.16$, $P = 0.02$, ESR: $r = 0.25$, $P < 0.001$).

D-dimer, fibrinogen and ESR levels were significantly higher in patients with HPR by AA than in patients without HPR by AA [D-dimer: 293 (156.5–537) ng/mL vs. 194 (107.8–376.3) ng/mL, $P < 0.001$; Fibrinogen: 549 (467–627.5) mg/dL vs. 525 (437.5–598.2) mg/dL, $P = 0.04$; ESR 25 (15–45) mm/h vs. 17.5 (8–27.7) mm/h, $P = 0.004$].

According to HPR by ADP we found significantly higher D-dimer and ESR levels in patients with HPR than in patients without HPR by ADP [D-dimer: 238 (124–456.5) ng/mL vs. 204 (112.7–363.2) ng/mL, $P = 0.03$; ESR 22 (12–36.5) mm/h vs. 16 (8–30) mm/h, $P = 0.03$].

Conclusions: HPR is associated with a prothrombotic and proinflammatory state suggesting a possible role of the antithrombotic therapy in modulating this phenotype associated with a higher incidence of ischemic events.

PB 2.22-4

Aspirin resistance and C-reactive protein predict long term mortality in STEMI patients

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Background: Data on long term prognostic value of high platelet reactivity (HPR) on aspirin in patients with acute coronary syndrome (ACS) undergoing PCI are limited. On the other hand, C reactive protein levels has been suggested to be associated with post-PCI atherothrombotic events.

Methods and Results: We evaluated 494 STEMI patients (366 M/128 F; age: 65.8 ± 12.4 years) undergoing PCI with stent implantation.

Platelet reactivity was assessed by light transmission aggregometry by 1 arachidonic acid (AA). HPR by AA was diagnosed in the presence of LTA ≥ 20%. At a median follow-up of 27.8 months (13.1–48.8), 58 patients died (11.7%).

Median values of LTA by AA were significantly higher in patients died with respect with the alive: 24.18 ± 21.1% vs. 16.2 ± 11.4%, $P > 0.001$. By the ROC curve analysis, 18% was found to be the value of LTA by AA associated with the higher specificity and sensitivity for death.

CRP values were significantly higher in patiebts died with respect with the others: 55.86 ± 62.46 mg/L vs. 24.68 ± 37.12 mg/L, $P < 0.001$. By the ROC curve analysis, 12 mg/L was found to be the value of CRP associated with the higher specificity and sensitivity for death.

Patients with HPR by AA were not at significantly higher risk of death [HR = 1.57 (0.5–4.9); $P = 0.440$]; patients with elevated levels of CRP were at higher risk of death [HR = 2.72 (1.1–6.76); $P = 0.031$]; the presence of both HPR by AA and elevated CRP levels was associated with the highest risk of death [HR = 4.19 (1.67–10.55); $P = 0.002$].

Conclusion: These results document that both HPR by AA and elevated CRP levels identify a subgroup of STEMI patients at significantly higher risk of death, for whom it might be crucial to optimize antithrombotic therapy.

PB 2.22-5

Pharmacogenomic study of clopidogrel poor response

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Background: The use of antiplatelet therapy with aspirin and clopidogrel, reduces significantly recurrent cardiovascular events in patients with acute coronary syndrome. Clopidogrel is a pro-drug, its absorption in the intestine is limitedby the P-Glycoprotein, encoded in the ABCB1 gene. It ismetabolized in the liver by theenzymes of the cytochrome P450 superfamily (CYP) to produce the activemetabolite that antagonizes the P2Y12 platelet receptor. There is a wide interindividual variability in response to clopidogrel. The poor response to clopidogrel has been associated with polymorphisms in ABCB1, CYP and P2RY12 genes. In Mexican population the frequency of these polymorphisms and their relation with clopidogrel poor response is unknown.

Aims: Determine the frequency of clopidogrel poor response and its relation with polymorphisms of the ABCB1, CYP2C19, CYP3A5 and

P2RY12 genes in patients underwent percutaneous coronary intervention.

Methods: A total of 276 patientswho underwent percutaneous coronary intervention and received a clopidogrel loading dose of 300 or 600 mg were included. Platelet aggregation was performed by a turbidimetric method on a Lumiaggregometermodel 810 AC AGGRO software/LINK Chrono-log, Havertown, PA, USA. Different concentrations of ADP (0.58–10 μM) were used as agonist to determinate clopidogrel's poor response. Single nucleotide polymorphisms (ABCB1, CYP2C19*2, CYP2C19*3, CYP3A5*3 and two polymorphic sites of the P2RY12 gene G52T and C34T) in genomic DNA were performed using 5′exonuclease TaqMan assays by a 7900HT Fast Real-Time PCR System (Applied Biosystem, Foster City, CA, USA). All patients signed informed consent, this protocol is according to the Helsinki declaration and it was approved for the ethics committee of the National Institute of Cardiology.

Results: Sixty one (22.1%) of the 276 patients included in the study, were clopidogrel poor responders. ABCB1 TT allelehas a frequency of 18.1% in our studied population OR2.4P = 0.015 (CI 1.23–4.66). A logistic regression model showed that omeprazole increase the risk to 7.82 ($P = 0.023$) of being clopidogrel poor responder.

The studied CYP and P2RY12 polymorphisms were not related with clopidogrel poor response, and had frequencies below 9% in our population.

Conclusions: Carriersof ABCB1 TT allele had a higher risk of being clopidogrel poor responders, compared with CC and CT carriers. Polymorphisms of the CYP and P2RY12 genes were not related to clopidogrel poor response and their frequencies were lower than reported for Caucasian populations.

PB 2.22-6

Prevalence of CYP2C19 variant alleles and pharmacodynamic variability of aspirin and clopidogrel in American Indians

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Background: Extensive variability to clopidogrel has been identified, and genetic variation in the metabolic enzyme cytochrome P450 (CYP) 2C19 is one important factor. The prevalence of important genetic polymorphisms of the CYP2C19 gene has been determined in most groups, but not in American Indians. Furthermore, the overall effectiveness of clopidogrel and aspirin has not been studied in the American Indian population, even though this group has some of the highest mortality rates in the U.S.A. for cardiovascular disease and diabetes.

Aims:

1 Identify the prevalence of CYP2C19 variant alleles in American Indians of the Oglala Sioux Tribe of South Dakota, U.S.A.
2 Assess the antiplatelet pharmacodynamic variability and the influence of candidate clinical and genetic predictors in American Indians.

Methods: Our protocol was approved by the appropriate medical ethics committees. We recruited and obtained consent from 50 American Indians of the Oglala Sioux Tribe with coronary artery disease (CAD) taking aspirin and clopidogrel. Whole blood was collected for analysis by the VerifyNow P2Y12 and aspirin tests. Saliva samples from the CAD patients and 50 additional healthy volunteers ($n = 100$ total) were genotyped for known CYP2C19 variants (*2, *3, and *17). Genotyping was conducted by real-time polymerase chain reaction (TaqMan assay) at a regional Tribal college. We analyzed single predictors of platelet reactivity with the Kruskal-Wallis and chi-square tests.

Results: The observed genotype distributions were in Hardy-Weinberg equilibrium. Genotype frequencies were: CYP2C19 *1/*1, $n = 62$; *1/*2, $n = 20$; *2/*17, $n = 2$; *1/*17, $n = 13$; *17/*17, $n = 1$. Twenty-two percent of American Indians were heterozygous for the *2 loss-of-function allele (allele frequency = 11%; 95% CI = 7–16%), and 16% of American Indians carried at least one CYP2C19*17 allele (allele frequency = 9%; 95% CI = 5–13%). The pharmacodynamic effectiveness of clopidogrel in American Indians (median = 194 P2Y12 reaction units (PRU), range = 29–400) was not statistically different from a historical control of primarily Caucasian patients (median = 186 PRU, range = 85–331; $P = 0.70$). There was no significant effect of genotype on platelet aggregation as measured by the VerifyNow P2Y12 test ($P = 0.72$; median and range for CYP2C19*1/*1 and *2/*17 = 218, 29–400 PRU; *1/*2 carriers = 194, 108–242 PRU; *17 carriers = 171, 74–354 PRU). The median aspirin reaction units (ARU) for American Indians was 437 (range = 350–659), and 73% had ARU values < 550. Higher PRU values were associated with never smoking ($P = 0.018$), a history of coronary artery bypass grafting ($P = 0.043$), no previous percutaneous coronary intervention ($P = 0.042$), and low aspirin dose (81 mg; $P = 0.013$). Never smokers ($P = 0.044$), low aspirin dose ($P = 0.037$) and age ≤ 65 years ($P = 0.005$) were significant predictors of high ARU values.

Conclusions: The variability to aspirin and clopidogrel in American Indians of the Oglala Sioux Tribe is consistent with reported values for other groups as measured by the VerifyNow assay. However, the tested CYP2C19 genotypes (*2, *3, and *17) did not explain the variability of clopidogrel response. Future work will further characterize genetic variation in a larger sample of this population with significant health disparities.

PB2.23 – Platelet Activation: Receptor Changes – I

PB 2.23-1

Modulation of α-thrombin function by platelet glycoprotein Iba

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Background: The protease α-thrombin (FIIa) interacts with several substrates to orchestrate biochemical reactions and cellular functions in response to vascular injury. Platelets – key participants in such events, including hemostasis and thrombosis – express glycoprotein (GP) Ibα on their membrane; this is the most abundant FIIa binding site, but its function relative to that of protease activated receptors (PARs) signaling after proteolytic cleavage remains unclear. We previously demonstrated that FIIa binding to GPIbα concurrently involves two independent sites – site 1 in the proximity of exosite I and site 2 involving exosite II. Thus, one FIIa interacts with two distinct GPIbα molecules and each GPIbα interacts with two distinct FIIa molecules.

Aims: The long term goal of our studies is to characterize in detail the functional consequences, *in vivo* and ex vivo, of FIIa binding to GPIbα.

Methods: To achieve our goal, we have generated mice expressing human (hu) GPIbα with wild type (WT) sequence or mutated at residues interacting selectively with FIIa site 2 (D277N) or both site 1 and 2 (Y276/278/279F).

Results: Mouse platelets expressed ≈9000 WT or mutant huGPIbα molecules; ≈10,000 FIIa molecules bound to huGPIbα-WT (with an apparent 1:1 stoichiometry and K_D of ≈3 nM), while binding to mutant huGPIbα was essentially abolished. Mice with defective FIIa binding to platelet GPIbα exhibited a pronounced prothrombotic phenotype, with a significantly ($P < 0.01$) shorter time to carotid artery occlusion following ferric chloride injury (median 550.5 s; $n = 18$) than mice with normal FIIa binding to platelets (median 1980 s; $n = 19$). Accordingly, the platelet-rich plasma (PRP) of mice with defective FIIa binding exhibited a significantly shorter clotting time in the presence of 4 nM FIIa and significantly enhanced intracytoplasmic Ca^{2+} transients and platelet aggregation following stimulation by 0.5 nM

FIIa. Mouse and human platelets differ significantly with respect to expressed PAR receptors, as the former express PAR3 and PAR4, while human platelets express PAR1 and PAR4. Human platelets bind FIIa like mouse platelets, with a 1:1 stoichiometry relative to expressed GPIbα and K_D of ≈3 nM. FIIa binding to human platelets is abolished by the previously characterized monoclonal antibody LJ-Ib10. In agreement with the results obtained with mutant mouse PRP lacking FIIa binding to platelets, addition of LJ-Ib10 significantly shortened the clotting time. However, in marked contrast, this antibody greatly reduced intracytoplasmic Ca^{2+} transients and platelet aggregation following stimulation by 1 nM FIIa.

Summary/Conclusions: Our findings identify GPIbα as a relevant FIIa activity modulator with respect to platelet activation and clotting. However, the consequences on platelet aggregation vary depending on the specific PAR type expressed. Human platelets depend on FIIa binding to GPIbα for efficient PAR1 cleavage and subsequent signaling. In contrast, binding to GPIbα competes for FIIa cleavage of PAR4 on mouse platelets and subsequent activation. Common in the two species is the competing effect of GPIbα binding of FIIa on fibrinogen clotting. Thus, through distinct mechanisms influenced by the expression of specific PAR subtypes, GPIbα can modulate FIIa function in hemostasis and thrombosis both with prothrombotic or antithrombotic consequences.

PB 2.23-2

Thrombin is a selective inducer of heparanase release from platelets via protease-activated receptor-1

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Background: Heparanase, known to be involved in angiogenesis and metastasis, was shown to form a complex with tissue factor (TF) and to enhance the generation of factor Xa (Nadir *et al*, Haematologica, 2010). Platelets contain abundant amount of heparanase and were the primary source for the protein purification.

Aims: To identify the inducer and pathway of heparanase release from platelets.

Methods: Pooled platelet rich plasma from platelet units obtained from the blood bank or healthy donors was incubated with ADP, epinephrine, collagen, ristocetin, arachidonic acid and thrombin. Level of heparanase released from platelets was studied by ELISA and western blot analysis. The effect of selective protease-activated receptor-1 (PAR-1) inhibitor (FR171113) and thrombin receptor activator peptide (TRAP) were assessed using platelet aggregometry and heparanase procoagulant activity assays. In-house synthesized inhibitory peptides to TF/heparanase complex were used to evaluate platelet heparanase involvement in activation of the coagulation system.

Results: Heparanase was released from platelets only by the induction of thrombin while ADP, epinephrine, collagen, ristocetin and arachidonic acid exerted no effect on heparanase release. Level of heparanase in the plasma after thrombin induction was 250 folds higher compared to heparanase baseline plasma level. Activation of PAR-1 by TRAP dramatically increased heparanase procoagulant activity and was significantly decreased by the addition of PAR-1 inhibitor or TF/heparanase complex inhibitory peptides.

Conclusions: Heparanase is selectively released from platelets by thrombin *via* PAR-1 receptor. Platelet heparanase is involved in activation of the extrinsic coagulation pathway. The present study widens our understanding regarding potential anticoagulant effect, in addition to anti-platelet effect, of the new clinically studied PAR-1 inhibitors.

PB 2.23-3

Can platelet surface markers synchronize differential platelet secretion?

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Background: Platelet a-granules contain a number of pro-angiogenic (e.g., vascular endothelial growth factor, VEGF, and stromal cell-derived factor-1a, SDF-1a) and anti-angiogenic regulators (thrombospondin and endostatin). Recent evidence indicates that different angiogenic regulators may be stored in different a-granules and differentially released upon different stimuli. Platelet surface expression of granule membrane embedded P-selectin is widely used as an indicator of platelet secretion/degranulation. Platelets also express specific receptors to some platelet angiogenic regulators (such as glycoprotein IV/CD36 for thrombospondin), which allows surface binding of those angiogenic regulators upon release. Our hypothesis is that distinct release of angiogenic regulators can lead to their distinct expression/binding on platelet surface.

Aims: To explore the possibility of using a multicolor flow cytometric assay of platelet surface markers to monitor differential release of platelet angiogenic regulators and the underlying signaling mechanisms.

Methods: Hirudinized or citrated whole blood samples from 15 healthy volunteers were stimulated at 37 °C for 10 min with a panel of platelet agonists: the thrombin receptor PAR1-activating peptide (PAR1-AP; 10 μM), PAR4-AP (100 μM), collagen-related peptide (CRP; 10 μg/mL), the thromboxane A2 analogue U46619 (1 μM), and ADP (100 μM). The samples were then labeled with various panels of different fluochrome-conjugated antibodies detecting P-selectin (a general marker of platelet secretion), fibrinogen (a platelet aggregability marker), SDF-1a (a proangiogenic regulator), and thrombospondin (an antiangiogenic regulator).

Results: Platelet activation by PAR1-AP led to marked elevations of platelet P-selectin expression (e.g., from 1.9 ± 1.0% of rest to 95.0 ± 3.1%), but milder elevation in fibrinogen binding (4.0 ± 2.7% to 77.5 ± 15.0%) and thrombospondin expression (1.9 ± 0.4%–50.7 ± 11.8%), and a minimal increase in SDF-1a expression (2.5 ± 1.0%–4.2 ± 2.4%). PAR4-AP stimulation elevated P-selectin, fibrinogen, and thrombospondin expression similarly (98.2 ± 0.4%, 99.0 ± 0.5%, and 89.6 ± 5.5%, respectively), but did not increase SDF-1a expression (2.5 ± 1.0% vs. 3.3 ± 1.6%). CRP stimulation induced similar responses as by PAR4-AP, while U46619 and ADP arose milder responses (e.g., 57.9 ± 33.8% and 68.8 ± 1.8% P-selectin expression, respectively). Moreover, multicolor analyses showed that there were not distinct platelet subpopulations expressing thrombospondin but not SDF-1a or vice versa. Follow-up work was hence focused on possible different impacts of intracellular signaling interventions on platelet P-selectin and thrombospondin expression using a panel of inhibitors: Src (PP2, 50 μM), PI3K (LY294002, 50 μM), Akt (Akt1/2 kinase inhibitor; 50 μM), and p38 (SB203580; 25 μM). None of these inhibitors influence PAR1-AP or PAR4-AP-induced P-selectin expression, whilst p38 inhibition mildly reduced PAR1-AP-induced thrombospondin expression. In CRP, U46619, and ADP-stimulated platelets, Src and PI3K inhibition reduced, while p38 inhibition most markedly decreased platelet P-selectin and thrombospondin expression in a similar manner. Hence, above signaling interventions did not elicit any distinct effects on the two platelet secretion markers.

Conclusion: Multicolor flow cytometry using whole blood samples seems to be less than an optimal approach to demonstrate differential releases of platelet angiogenic regulators, albeit it has the advantage displaying multiple surface markers on individual platelets.

PB 2.23-4

The effect of omega-3 supplementation on platelet GPVI levels

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Background: The primary platelet receptor for collagen, glycoprotein (GP) VI plays a critical role in arterial thrombosis. GPVI is extremely stable on the surface of circulating platelets but is rapidly shed by a disintegrin and metalloproteinase (ADAM) sheddases, primarily ADAM10, upon GPVI ligand engagement or exposure of platelets to elevated shear. Statin therapeutics which work to reduce membrane cholesterol levels, have been shown to elevate ADAM10 activity in cell culture. Common therapeutic strategies to prevent cardiovascular disease such as cholesterol reduction and omega-3 supplementation may affect membrane GPVI and plasma soluble GPVI (sGPVI) levels.
Aims: To assess the effect of modulation of platelet cholesterol and membrane biochemistry on surface and soluble GPVI levels in platelet-rich plasma (PRP) under resting and shear-activated conditions.
Methods: In experiments conducted *in vitro*, PRP from healthy donors was treated with 5–15 mM methyl-β-cyclodextrin (MβCD) to remove cholesterol then resuspended in autologous plasma. Samples of citrated PRP were isolated from healthy donors before and after 1 month daily ingestion of 120 mg eicosapentaenoic acid (EPA) and 520 mg docosahexaenoic acid (DHA). All PRP samples were either untreated or exposed to 7500–10,000/s uniform shear stress using a cone-plate viscometer for up to 10 min. Shear-activated metalloproteolytic shedding of GPVI was monitored by flow cytometry and sandwich ELISA, and platelet-associated ADAM10 activity was measured using a quenched fluorescent peptide with unique sequence matching the ADAM10 cleavage site within GPVI (GPVI-FRET).
Results: Platelet suspensions that were treated with 5–15 mM MβCD had up to 8-fold increases in sGPVI in the absence of shear activation. Exposure to 10,000/s shear stress resulted in 16-fold increases in shedding of GPVI that was enhanced a further 20% by cholesterol depletion. Increased GPVI-FRET cleavage was observed in PRP samples that had been treated with 5- 15 mM MβCD compared to untreated PRP indicating that platelet ADAMs activity was enhanced by cholesterol depletion. Depletion of platelet membrane cholesterol may render GPVI more susceptible to proteolysis under both resting and high shear conditions. Levels of platelet GPVI on resting platelets were reduced by 11% in samples from eight healthy donors after 1 month ingestion of omega-3 fatty acids. The plasma lipid composition, as ascertained by mass spectrometry, was altered by omega-3 fatty acid ingestion.
Summary/Conclusion: Common therapeutic approaches to reduce cholesterol levels also modulate GPVI receptor density on resting platelets and accentuate shear-induced GPVI shedding, potentially reducing the risk of arterial thrombosis in patients with coronary artery disease. Future experiments will assess the consequences of accelerated loss of GPVI on platelet function, however our data imply an additional pleiotropic effect of omega-3 fatty acids *in vivo* that is cardioprotective.

PB 2.23-5

Lipid rafts in platelets: their role for TXA2-induced platelet activation

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Background: Aspirin is the mainstay antiplatelet treatment in patients with cardiovascular disease; its best-characterized effect is the irreversible inhibition of cyclooxygenase 1 and the subsequent formation of thromboxane A_2 (TXA_2), a potent vasoconstrictor and platelet agonist acting on its specific receptor. Lipid rafts are cholesterol-rich microdomains present in the plasma membrane that are believed to serve as platforms for signal transduction processes by localizing or excluding receptors and signaling molecules. Lipid rafts play an important role in signal transmission in different cell types, including platelets. Currently is unknown if they regulate TXA_2-induced platelet responses.
Aim: To analyze the participation of lipid rafts in platelet responses to TXA_2.
Methods: Platelets from normal subjects were washed and aggregation was assessed by optical aggregometry. The release of adenine nucleotides was quantified by HPLC, the cytosolic concentration of calcium by fluorimetry in FURA 2AM-loaded platelets, and platelet activation (P-selectin and PAC-1) by flow cytometry. Lipid rafts isolation was performed by sucrose gradient ultracentrifugation of platelet lysates. The different fractions were analyzed for specific proteins by immunoblotting. The fractions corresponding to lipid rafts were identified by the presence of CD36, a protein commonly used as a marker of lipid rafts. The functional role of lipid rafts was studied by disrupting them with methyl-β-cyclodextrin (MβCD) prior to platelet activation.
Results: Disruption of lipid rafts with MβCD decreased platelet aggregation induced by the TXA_2 analogs U46619 or IBOP (> 85%), dense granule release (> 70%) and P-selectin exposure (> 80%). This demonstrates the importance of TXA_2 receptor association with lipid rafts for TXA_2-induced platelet function. However, platelet aggregation induced by U46619 could be at least in part mediated by the platelet-released ADP through the ADP receptor P2Y12, which has been previously demonstrated to be associated with the lipid rafts (1). Therefore, disruption of lipid rafts could also affect P2Y12 mediated effects. To elucidate this question, we explored two platelet responses known to be directly dependent on TXA_2 receptor: the cytosolic calcium increase and the activation of the integrin IIbIIIa (PAC-1+) (1). We found that disruption of lipid rafts also decreased platelet responses directly linked to the TXA_2 receptor, supporting the concept that lipid rafts are involved in signal transmission mediated by the TXA_2 receptor. In addition, the isolation of lipid rafts allowed the detection in the fraction corresponding to the rafts (CD36+) of a significant percentage (38%) of total TXA_2 receptor as determined by densitometry. MβCD-treatment strongly reduced the presence of both CD36 and the TXA_2 receptor in the lipid rafts. Using cholera toxin-Alexa488 as a marker of rafts simultaneously with the TXA_2 receptor antibody, we confirmed by confocal mycroscopy the co-localization of TXA_2 receptor associated with platelet lipid rafts.
Conclusion: These results demonstrate for the first time the importance of lipid rafts for the TXA_2-induced platelet responses, a process which could be related to the presence of the TXA_2 receptor associated with lipid rafts. Grants: PI07/0463; Retics06/0026. (1) Dorsam RT, JCI 2004;113:240

PB 2.23-6

Soluble CLEC-2 was released upon platelet activation and detected in human plasma

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Background: CLEC-2 is a recently-identified platelet activation receptor, which binds to snake venom rhodocytin or a membrane protein podoplanin. Soluble membrane proteins were released from activated platelets. However, whether soluble human CLEC-2 is released upon platelet activation has not been fully investigated.
Aims: The aim of this study is to investigate whether soluble CLEC-2 is released upon platelet activation and whether soluble CLEC-2 is detected in human plasma.
Methods: Human washed platelets were stimulated with various agonists for 2 h at 37 °C. Reactions were terminated by addition of EDTA.

The stimulated platelets were centrifuged at 13,000 G for 1 min. Resultant supernatants were further ultra-centrifuged at 100,000 G for 3 h. Supernatants after centrifugation (Sup), pellets after centrifugation (Pel), supernatants after ultra-centrifugation (ultra-Sup), and pellets after ultracentrifugation (ultra-Pel) were separated by SDS-PAGE and Western blotted with anti-CLEC-2 or anti-GPVI antibody. ELISA system to measure soluble CLEC-2 was established using mouse monoclonal anti-CLEC-2 antibodies. Blood samples from 40 healthy volunteers were taken into plastic tubes with EDTA, sodium citrate, or inhibitor cocktails for measurement of platelet factor-4 (PF4) and beta thromboglobulin (betaTG) to obtain plasma. Then, soluble CLEC-2, PF4, and betaTG in three kinds of plasma were measured by ELISA. Written informed consent was provided according to the Declaration of Helsinki.

Results: In the Sup fraction of rhodocytin-stimulated platelets, anti-CLEC-2 antibody detected 32/40 kD and 25 kD bands. However, only 32/40 kD bands were observed in the ultra-Sup fraction, and only 25 kD bands were observed in the ultra-Pel fraction, suggesting that soluble CLEC-2 is released both as a shed form and a membrane-associated form on the surface of microparticles. In the case of GPVI, however, membrane-associated form was only faintly observed. CLEC-2 shedding was almost equally observed upon platelet activation induced by various agonists including rhodocytin, thrombin, collagen-related peptide, whereas GPVI shedding was significantly increased in the presence of the GPVI agonist. CLEC-2 shedding, but not GPVI shedding, was significantly inhibited by recombinant tissue Inhibitors of metallo-proteinase 2, an intrinsic MMP-2 inhibitor. Concentrations of PF4 and betaTG were markedly increased in EDTA serum and sodium citrate serum compared with that in inhibitor cocktail plasma. On the other hand, concentration of soluble CLEC-2 is similar among three kinds of plasma. Concentration of soluble CLEC-2 in EDTA plasma of healthy volunteer was 63 $\pm$ 80 pg/mL.

Conclusions: Soluble CLEC-2 and soluble GPVI are released upon platelet activation by a different mechanism. Soluble CLEC-2 measurement is more convenient than measurement of PF4 and betaTG, because soluble CLEC-2 can be measured using frequently used EDTA or sodium citrate plasma. CLEC-2 can be used as a useful biomarker for platelet activation *in vivo*.

PB2.24 – Platelet mRNA/Protein Expression

PB 2.24-1

Differences in platelet microRNA profiles after aspirin use are associated with differences in whole blood aggregation and might identify aspirin resistance

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Background: MicroRNAs have been established as markers for cardiovascular disease in many studies. However, most of the patients included in these studies were treated with aspirin for secondary prevention reasons. It is known, that there are individual differences in the response of platelets to aspirin. Whether the response of platelets to aspirin shows differences in miRNA expression is not know. We therefore, studied the influence of aspirin on microRNA expression levels, in isolated platelets of healthy volunteers. Additionally we analyzed, whether the *in vitro* response of platelets to indometacine, a surrogate for aspirin, in ADP-mediated whole blood aggregation was related to differences in miRNA profiles.

Methods and Results: We measured relative expression levels of platelet microRNAs using microarrays before and after 2 weeks of aspirin use (100 mg, once daily) in 15 healthy individuals (age 45–65 years). Based on aspirin-induced changes in microRNA profile, two subgroups could be distinguished: a group of individuals (n = 9) with similar and a group with different (n = 6) miRNA profiles before and after aspirin use. Additionally, we performed *in vitro* whole blood aggregation studies to determine whether the observed difference in microRNA profile before and after aspirin use, could be linked to platelet function. Whole blood from the same individuals, in the absence of aspirin, was tested using Multiplate® aggregometry and was incubated with indometacine to mimic aspirin use. The subjects with a similar microRNA profile had significantly reduced ADP-mediated aggregation as compared to the group in which the microRNA profile was different before and after medication.

Conclusion: Differences in platelet microRNA profiles after aspirin use are related to differences in ADP-mediated indometacine-treated whole blood aggregation. This could imply that differences in miRNA profiles might identify individuals with aspirin resistance.

PB 2.24-2

Next generation sequencing analysis of human platelet polyA+ mRNAs

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Background: Platelets are small anucleate cells circulating in the blood vessels where they play *a key role in hemostasis and thrombosis*. Platelet gene expression has been investigated in the past mainly by mRNA quantification using microarray and qPCR techniques, and the results published thus far contain considerable disagreements in both the magnitude and the nature of the platelet transcriptome. Here we used next generation sequencing (RNA-seq) to characterize the profile of the polyadenylated (polyA+) mRNA in purified human blood platelets.

Results: We utilized the Illumina HiSeq 2000 platform to sequence cDNA converted from platelet polyA+ RNA. We detected 58,155,680 high-quality reads which were mapped against the set of chromosomes of the Human Feb. 2009 (GRCh37/hg19) assembly. We found 35,322,009 (60.7%) reads of ~100 bp long that could be uniquely mapped to the human genome. Analysis of this data generated a list of highly abundant platelet transcripts (> 10,000 reads) encoding annotated as well as novel genes. We identified high transcript levels for genes related to the cytoskeleton function, chemokine signalling, cell adhesion, aggregation, as well as receptor interaction between cells. Consistent with previous findings our data also suggests that nearly two-thirds of the platelet transcriptome comprise mitochondrial-expressed genes.

Conclusion: We report on the global architecture of platelet transcriptome and identify genes that may hold significant functions in platelets in addition to several novel transcripts, at single-base resolution. We conclude that platelet-expressed transcripts are well-defined but limited in number, presumably reflecting the lack of ongoing transcription in the anucleate platelets combined with selective degradation of those transcripts that are unnecessary for platelet function.

PB 2.24-3

Mass spectrometry analysis showed comparable releasates and phosphorylation profiles after PAR-1 or PAR-4 stimulation of platelets

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Background: Platelets play a crucial role in haemostasis and the regulation of inflammation, angiogenesis and atherogenesis. It has

been proposed that differential release profiles of pro- and anti-angiogenic factors in response to stimulation of the protease activated receptors (PAR)-1 and -4 is important for the regulation of angiogenesis. Such analyses have been performed only for a small number of granule proteins. Such phenomena could be better studied in the context of the entire releasate. In addition, if differential release indeed occurs, clues as to how differential release is regulated should be obtained.

Aims: To comprehensively analyze differences in the releasates and intra-cellular phosphorylation profiles of unstimulated, and PAR-1 and PAR-4 stimulated platelets.

Methods: Isolated platelets from three different donors were maximally stimulated with PAR-1 agonist, PAR-4 agonist, or left unstimulated. These were lysed and subjected to differential stable isotope labelling. For determining platelet releasate, isotope-labeled samples were subjected to 2-dimensional chromatography and LC-MS/MS analysis. PF-4, β-TG, PDGF-AB, RANTES and thrombospondin levels were measured in the platelet releasate from four different donors with ELISA. Global analysis of serine/threonine phosphorylation was performed with Western blots. A more detailed analysis of the platelet phosphoproteome occurring upon PAR-1 and PAR-4 stimulation was performed using differential stable isotope labeling of the PAR-1, PAR-4, and control samples followed by strong cation exchange chromatography based enrichment, and LC-MS/MS analysis.

Results: We measured the quantitative release of 93 proteins from platelets stimulated with PAR-1 and PAR-4. A strong correlation between the proteins released after either stimulus was observed (Spearman's r: 0.93, r^2: 0.86, $P < 0.0001$). Quantification of the released proteins after stimulation with PAR-1 or PAR-4 with ELISAs showed a comparable release for PF-4 (PAR-1: 103.7 ± SEM 2.7 ng/mL, PAR-4: 119.2 ± SEM 5.1 ng/mL; not significant), β-TG (PAR-1: 141.5 ± SEM 15.7 ng/mL, PAR-4: 107.0 ± SEM 0.8 ng/mL; not significant), PDGF-AB (PAR-1: 4.0 ± SEM 0.1 ng/mL, PAR-4: 4.9 ± 0.3 ng/mL; not significant), and RANTES (PAR-1: 32.8 ± SEM 3.7 ng/mL, PAR-4: 45 ± SEM 6.7 ng/mL; not significant). Release of thrombospondin was slightly reduced for PAR-1 induced release (7.2 ± SEM 0.3 μg/mL), compared with PAR-4 (9.8 ± SEM 6.7 μg/mL; $P < 0.05$). Downstream signal transduction measurements after stimulation with PAR-1 and PAR-4 agonist revealed similar phosphorylation patterns on Western blot. With quantitative mass spectrometry 244 phosphorylation events on 183 proteins were quantified upon PAR-1 and/or PAR-4 stimulation. A significant correlation between PAR-1 and PAR-4 phosphorylation quantity was observed (Spearman's r: 0.49, r^2: 0.24; $P < 0.0001$).

Conclusions: After proteomics we did not observe different release patterns for any of the 93 proteins analyzed. PAR-4 stimulation showed stronger release of granules that PAR-1 stimulation. The difference in thrombospondin is probably due to overall stronger activation of platelets by PAR-4. Furthermore, we did not detect differences in signal transduction downstream of PAR-1 and PAR-4

PB 2.24-4

Proteins' synthesis in platelets: the key role of platelet's receptors

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Background: Although platelets lack a nucleus or genomic DNA, they are able to translate mRNA into proteins *de novo*. Megakaryocytes and their progeny, circulating anucleate platelets, retain a subset of pre-mRNAs and it is spliced into mature mRNA in response to cellular activation.

The Aim of Our Study: To analyze the platelet's receptors GP IIb-IIIa, GP VI, GP Ia-IIa, GP Iba, P2Y1, P2Y12, P2X1 including numbers on platelet's membrane and pre-mRNA and mRNA of their genes in resting and activated platelets.

Methods: cDNA prepared from total RNA isolated from platelets of 50 healthy donors, the level of mRNA was analyzed by RT-PCR using the TaqMan assay, the presence of pre-mRNA and mature mRNA was detected by PCR with primers specified to exon-exon junction or intron. The GP IIb, GP IIIa, GP Iba, GP VI, GP Ia and P2Y12 genes' polymorphism were determined by PCR-RFLP. The numbers of GP IIb-IIIa, GP Iba, GP VI, GP Ia-IIa and P2 platelet's receptors and IL-1beta were analyzed by flow cytometry with FITC labeled antibodies as mean fluorescence intensity. The platelet's activation was analyzed both by photometric method (ADP-induced aggregation) and by flow cytometry (P-selectin expression per platelet).

Results: We found Spearman correlation between the levels of pre-mRNA GP IIIa, GP IIb, GP Iba (R from 0.67 to 0.87, P from 0.02 to 0.0004). And the level of mature mRNA was higher in donors with high level of platelet aggregation (R from 0.31 to 0.52, P from 0.08 to 0.003). Genetic variants Leu33Pro GP IIIa, Ile843Ser GP IIb, G36T P2Y12 and Thr145Met GPIba didn't influence gene expression and level of mRNA. At the same time, in resting platelets from carriers of mutant allele C18T P2Y12 the level of mature mRNA was higher compared with non-carriers, who had pre-mRNA mainly ($R = 0.59$, $P = 0.04$). The numbers of GPIIb-IIIa, GPIba and P2Y12 on platelet's membrane didn't depend on the level of respective mRNA. Only the number of GP IIb-IIIa receptors on platelet correlated weakly with level of mRNA of GP IIb gene – $R = 0.4$ ($P < 0.1$). However, we found strong positive correlation between the level of mRNA GP IIIa and numbers of ADP-receptors P2Y1 ($R = 0.71$; $P = 0.008$) and P2Y12 ($R = 0.71$; $P = 0.009$). On the other hands, the level of mRNA P2Y12 correlated with numbers of GP IIb-IIIa on platelet's membrane – $R = 0.34$; $P = 0.09$. The correlation between numbers of receptor GP IIb-IIIa and receptor P2Y12 on platelet's membrane was detected too ($R = 0.55$, $P = 0.00002$). According to above mentioned we may conclude that synthesis of GP IIb-IIIa and P2Y12 proteins is interdependent on levels of transcription and translation. The numbers of GP IIb-IIIa, GP Iba, P2Y1, P2Y12, P2X1 and IL-1beta were changed in activated platelets (by 10 mkM ADP). Moreover, in activated platelets we observed transformation of GP IIIa, GP IIb, GP Iba pre-mRNAs into mature mRNAs. This process was inhibited by selective blocker of GP IIb-IIIa receptors.

Conclusion: the GP IIb-IIIa receptor may play a key role in regulation of proteins' synthesis in platelets *de novo*.

PB 2.24-5

Quantitative PCR of platelet specific mRNA reveals significant upregulation of TLR-2 in patients with non-ST elevation myocardial infarction

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Background and Aim: Recent evidence highlights the importance of platelets as part of the innate immune system in the pathogenesis of atherosclerosis and its clinical sequelae the acute coronary syndrome. Platelets do express several functionally active immune receptors of the toll-like receptor (TLR) family including TLR-2, which has been linked to the development of atherosclerosis. Platelets are capable of de novo protein synthesis via platelet specific mRNA. We hypothesized that platelet TLR-2 is involved in the pathogenesis of atherosclerosis and its clinical sequelae myocardial infarction.

Methods: Highly purified platelets from patients with NSTEMI and patients without coronary artery disease (CAD) sampled after coronary angiography were used for extraction of platelet specific mRNA.

Analysis of human TLR-2 mRNA expression was done by plasmid-based quantitative real-time PCR with beta-actin as house-keeping gene. Soluble TLR-2, which may act as a decoy receptor, was assessed by ELISA in serum of patients with NSTEMI and non-CAD controls.
Results: Mean age of patients was 59.4 ± 2.9 (SEM) years. Mean platelet count was 236.1 ± 21.25 K/μL for NSTEMI patients (n = 13) and 227.4 ± 12.06 K/μL for non-CAD patients (n = 11, P = ns for patients vs. controls). NSTEMI patients included patients with 3-vessel (31%), 2-vessel (46%) and single vessel (23%) disease. Mean CK and CK-MB levels for patients with NSTEMI were 321.8 ± 108.9 and 76.33 ± 28.02 U/L respectively. Platelet TLR-2 mRNA was significantly elevated in NSTEMI patients (12.10 e-005 ± 2.391e-005 copies in relation to platelet beta-actin) vs. non-CAD controls (4.738e-005 ± 7.128e-006 copies in relation to platelet beta-actin, P = 0.012). Plasma soluble TLR-2 was not significantly different in patients with NSTEMI (0.38 ± 0.17 ng/μL) compared to non-CAD (0.52 ± 0.21 ng/μL, P = 0.679) patients.
Conclusion: We identified significantly elevated platelet TLR-2 mRNA expression in Patients with NSTEMI compared to non-CAD patients, which was not accompanied by increased serum soluble TLR-2. Up-regulation of TLR-2 mRNA in platelets indicates a crucial role of platelet mediated innate immunity in the pathogenesis of ACS. Further exploration of platelet TLR-2 function in atherosclerosis is warranted as platelet targeted anti-inflammatory therapies may represent a novel therapeutic approach in the preventive treatment of ACS.

PB 2.24-6
Regulatory role of proteasome in determination of platelet life span

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Limit of platelet life span (8–10 days) is determined by the activity of a putative 'internal clock' composed of Bcl-2 family proteins, while role of other molecular players in this process remains obscure. Here, we sought to establish a central role of proteasome in platelet life span regulation.
Administration of mice with inhibitors of proteasome peptidase activity induced significant thrombocytopenia. This was associated with enhanced clearance of biotin-labeled platelets from circulation and reduction in average platelet half life from 66 to 37 h. Cells pretreated *in vitro* with proteasome inhibitors exhibited augmented annexin V binding and drop in mitochondrial transmembrane potential indicative of apoptotic cell death and decreased platelet life span. These cells were preferentially phagocytosed by monocyte-derived macrophages, thus linking proteasome activity with platelet survival. Decisive role of proteasome in this process was underscored from enhanced expression of conformationally active Bax in platelets with attenuated proteasome activity, which was consistent with pro-apoptotic phenotype of these cells. The present study establishes a critical role of proteasome in delimiting platelet life span ostensibly through constitutive elimination of the conformationally active Bax. These findings bear potential implications in clinical settings where proteasome peptidase activities are therapeutically targeted.

PB2.25 – Platelet Disorders: Screening

PB 2.25-1
Evaluation of the diagnostic potential of a whole blood remote assay in assessment of platelet function in bleeding disorders

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Background: Platelet function tests are widely used to diagnose inherited platelet function disorders. Light transmission aggregometry (LTA) is still regarded as the gold standard, however it is time consuming, requires a considerable volume of blood and samples must be processed within a finite time period after venepuncture. Among patients with excessive bleeding and suspected platelet dysfunction only around 60% are found to have an abnormality in platelet responses with lumi-aggregometry, implying that the remaining 40% may have defects in other parts of the haemostatic pathway (Dawood et al, Blood 2012; 120(25):5041–5049). We have developed an assay for platelet function where fresh whole blood samples are manipulated in a simple way that does not require specialized staff or equipment. The samples are then fixed, which stabilizes them for up to 9 days, and can be shipped for analysis to a central laboratory.
Aim: To assess the diagnostic potential of remote platelet function testing (RPFT) in the diagnosis of platelet function defects in patients with excessive bleeding.
Methods: Participants were recruited to the Genotyping and Phenotyping of Platelets study (GAPP, ISRCTN 77951167) from April 2012 to January 2013. This study was approved by the National Research Ethics Service Committee and all participants signed informed consent. Platelet function was assessed in 55 patients who had a history of excessive bleeding, and in 44 healthy volunteers using RPFT and lumi-aggregometry used as the reference method. For RPFT, blood was stimulated with the combinations of ADP (10 μM)/U46619 (1 μM) and arachidonic acid (0.5 mM)/epinephrine (100 μM), with TRAP (20 μM) alone and vehicle. Following stimulation blood samples were stabilized with a patented fixative solution (Platelet Solutions, Nottingham) and the expression of P-selectin and CD63 was measured by flow cytometry.
Results: Reference ranges for all measured parameters were set as mean ± 2SD obtained from healthy volunteers. Overall agreement between lumi-aggregometry and RPFT was good, with the diagnosis of a platelet function defect matching in 80% of cases (κ = 0.602, P < 0.0001). In 26 patients, platelet function appeared normal using lumi-aggregometry and RPFT. Twenty-eight patients were identified as having a platelet defect with lumi-aggregometry and 18 of these were also detected by RPFT. The majority of patients who had an abnormal pattern of response on lumi-aggregometry but not on RPFT had a mild platelet function defect based on dynamic kinetic information from lumi-aggregometry. In one further patient with a normal pattern of response in lumi-aggregometry, an isolated defect in alpha granule secretion (P-selectin) was detected using RPFT, whereas dense granule secretion was normal by both lumi-aggregometry and RPFT (CD63).
Conclusion: Our observations suggest that remote platelet function testing may be useful as an easy to use pre-test to select which patients with bleeding disorders would benefit from further extensive platelet phenotyping. This assay can be performed remotely and requires a small volume of blood, both of which offer significant advantages over lumi-aggregometry. Expression of glycoprotein receptors and immunologic platelet count can also be assessed in the fixed samples for RPFT and further evaluation of the test in patients with thrombocytopenia is warranted.

PB 2.25-2

Investigation of the utility of the ISTH bleeding assessment tool (BAT) in predicting platelet defects in participants with clinically diagnosed bleeding disorders

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Background: The ISTH/SSC Bleeding Assessment Tool (BAT) was developed as a tool to accurately record bleeding symptoms and to aid diagnosis in patients referred with a possible bleeding disorder. It is also a valuable research tool in studies that examine patients with excessive bleeding, allowing documentation of the severity of their bleeding. However, it has not been validated in patients with suspected inherited platelet function disorders.
Aims: To investigate the utility of the ISTH BAT in predicting platelet defects in participants with clinically diagnosed bleeding disorders.
Methods: Participants with clinically diagnosed excessive bleeding and healthy volunteers were recruited to the Genotyping and Phenotyping of Platelets study (GAPP, ISRCTN 77951167) from October 2011 to December 2012 from UK Comprehensive Care Haemophilia Centres. The study aimed to determine whether these patients had underlying platelet function defects and what the prevalence of these defects were in this patient group. The ISTH BAT was carried out in all participants by experienced personnel. Platelet function testing was carried out by light transmission aggregometry alongside measurement of ATP secretion. The bleeding assessment scores were compiled at entry to the study and therefore blinded to the platelet function testing results. This study was approved by the National Research Ethics Service Committee and all participants gave written informed consent.
Results: One hundred participants were included ($n = 79$ with suspected platelet function defects and $n = 21$ healthy volunteers). A platelet defect was found on platelet function testing in 56% of participants with bleeding symptoms (44% had no demonstrable platelet defect, 20% had a Gi-type defect, 18% had a dense granule secretion defect, 8% had a defect in the thromboxane pathway and 10% had a complex phenotype). The score obtained through the ISTH BAT in participants with suspected platelet function defects (12; IQR 8–16) was significantly higher than in healthy volunteers (0; IQR 0–0) ($P < 0.001$), thus confirming the clinical diagnosis of a bleeding disorder. There was no significant difference between participants in whom a platelet defect was detected by platelet function testing (11; IQR 8–16) and in whom platelet function was deemed normal (12; IQR 9–14) ($P > 0.05$). There was no association between the type of platelet defect detected and the ISTH BAT score. A ROC curve analysis was carried out to evaluate whether the ISTH BAT could discriminate between patients with and without a demonstrable platelet defect on platelet function testing. The area under the curve was 0.513 (95% CI 0.383–0.642, $P = 0.85$) thus demonstrating a lack of discriminative ability. Moreover, the ISTH BAT score was not associated with a demonstrable platelet defect on platelet function testing in a simple logistic regression model (OR 0.997 [95% CI 0.92–1.08], $P = 0.95$).
Summary/Conclusions: The ISTH Bleeding Assessment Tool is a useful tool in assessing and recording recurrent mild bleeding symptoms and appropriately documents the lifelong bleeding history. However, the score obtained is not predictive of the presence of a platelet defect on platelet function testing.

PB 2.25-3

Five year report of a multicenter project for characterization of inherited platelet disorders (IPD) in the Iberian Peninsula. Diagnosis of 23 severe IPD and identification of 14 new genetic variants

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Background: Inherited platelet disorders (IPDs) comprise a heterogeneous group of uncommon diseases characterized by abnormalities of platelet function/production giving rise to lifelong bleeding diathesis of variable intensity. Notwithstanding a century of research on IPDs, early identification and characterization of affected patients remain a challenge due to: heterogeneity of clinical and laboratory presentation; lack of specificity and complexity of platelet function assays; large number of causative genes; and absence of genotype-phenotype correlations. Multicentre projects on IPDs, consensus guides for diagnosis, and reference centers networks, could help to overcome this challenge.
Objective: To assist and to encourage the characterization of IPD patients in the Iberian Peninsula, we set up a project, under the scientific sponsorship of the Spanish Society of Thrombosis and Haemostasis.
Methods: In 5 years, around seventy unrelated patients with suspected IPD were referred from hospitals of twenty cities of Spain and Portugal. Clinical features were assessed by their hematological and bleeding manifestations graded by a unique simple scale. We received blood samples and performed a platelet phenotype characterization including: full blood count and film examination; PFA-100 tests; light transmission aggregation (LTA) with distinct agonists (mostly: arachidonic acid, ADP, epinephrine, TRAP, ristocetin, and collagen); flow cytometry (FC) assessment of surface receptors (Ib/IX/V, IIb/IIIa, Ia/IIa), activation status and granule secretion (PAC-1, CD62, CD63); and in some cases vWF binding, clot retraction assay, ^{14}C-serotonin uptake/release, and electron microscopy. A patient's parallel control and a healthy volunteer freshly extracted were always studied in parallel. DNA and RNA were extracted from peripheral blood. If laboratory results guided diagnosis of a particular IPD, candidate causative genes were chosen for PCR amplification and sequencing.
Results: Variable degree of platelet dysfunction, not attributable to acquired factors, was confirmed in most patients. In about one third of them, laboratory findings suggested defective platelet secretion or signalling of unknown aetiology. Platelet function testing (PFT) and molecular studies confirmed diagnosis of severe IPD as follows: Glanzmann Thrombastenia (GT) in 12 out of 18 suspected cases; Bernard-Soulier Syndrome (BSS) in eight out of 12; Hermansky-Pudlak syndrome (HPS) in one out of three; and Chediak-Higashi Syndrome (CHS) in the two suspected cases. Among these, we found eight novel molecular variants of GT: three in *ITGB3* [c.773–774delTG, c.647A/G (p.Tyr190Cys), c.431T/G (p.Met118Arg)]; and five in *ITGA2B* [c.1644G/T (p.Glu507STOP), c.2637delC, c.2964delG, g.9860–9862delAT, c.2473_2481del insTCACCTGGTC]. We identified three new mutations causing BSS: c.259T/C in *GP9* [p.Trp71Arg], 268 C/T [p.Pro90Ser] in *GPIBB*, and a rare heterozygous 358–366del9pb [p.Gln90_Leu92del] in *GPIBA*. Lastly, we recognised two new mutations in *LYST*, c.11362 G>A [p.Gly3725Arg] and c.961T>C, [p.Cys258Arg], in patients presenting with 'severe accelerated phase' and 'mild' forms of CHS, and a novel HPS type-1 caused by c.844G>T [p.Glu204Stop] change in *HPS1*. One suspicion of platelet-type-vWD and two of MYH9 were not confirmed.
Summary: This work illustrates the feasibility and usefulness of multicenter projects at international level to assist biological and molecular characterization of IPDs. Clinical suspicion of severe IPD was confirmed by both PFT and genetic studies in 23 (60%) out of 38 patients, among which 14 represent novel molecular variants.

PB 2.25-4

Storage pool disorder: a continuous whole blood flow cytometric method for the measurement of platelet calcium flux using an Accuri C6 cytometer

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Background: Storage Pool Disorder (SPD) results from abnormalities in the secretory mechanism or from deficiencies of platelet specific granules. Platelet dense granules are intracellular storage sites for calcium (Ca^{2+}), ADP, ATP, pyrophosphate and serotonin. The laboratory diagnosis of SPD is normally based upon abnormal platelet aggregation and demonstration of platelet nucleotide deficiencies. Other specialised investigations include the analysis of dense granule contents (serotonin, Ca^{2+}), flow-cytometry of mepacrine-treated platelets and electron microscopy of ultrastructure. A problem with measuring Ca^{2+} flux by flow-cytometry is that most analysers use a closed sampling system making analysis non-continuous.

Aim: The Accuri C6 Cytometer operates using an open fluid system. This allows the continuous recording of dynamic Ca^{2+} measurement after the addition of agonists. To validate the system and investigate its utility in the diagnosis of SPD, platelets in whole blood were exposed to different agonists and calcium modifying agents: ADP, Bovine Thrombin, Thrombin receptor-activating peptide (TRAP), U46619 (thromboxane receptor agonist), Calcium Ionophore (A23187) and EGTA a Ca^{2+} chelating agent.

Method: Citrated whole blood was diluted 1:10 using Ca^{2+}free Tyrode's buffer and loaded with an equal volume of 5 mM Fluo-4 (a Fluo calcium indicator) at 37 °C for 30 min. Cells (100 μL) were then stained with $CD41^{FITC}$ and $CD62^{PE}$ to identify the platelet population and to detect activated samples. Cells were resuspended in 4 mL Ca^{2+}free Tyrode's buffer. Ca^{2+} fluorescence was detected through the 585/40 band pass filter and collected in FL1-A. Fluorescence levels were recorded for 2 min at baseline and then a further 2 min following the addition of 5 or 10 μM ADP or other agonists. The difference between baseline and post agonist peak fluorescence was Ca^{2+} indicative of flux.

Results: Platelet Ca^{2+} flux was investigated in two groups: (i) Normal aggregation trace and normal nucleotide ADP (0.22–0.59 pmoles/10^9 platelets), $n = 12$ (ii) Abnormal aggregation trace and low nucleotide ADP and clinical features of a bleeding disorder, $n = 5$. The results are expressed as a ratio ± 2SD (Peak fluorescence induced by agonist/Baseline fluorescence) and compared using the t-test. Normal platelets responded in a dose-dependent manner to ADP (2 – 100 μM). The difference between the two groups was significant ($P < 0.05$ and $P < 0.005$) using 5 and 10 μM ADP, 1.86 ± 1.15 verses 1.10 ± 0.04 and 2.48 ± 1.41 verses 1.13 ± 0.05, respectively. Suggesting that 10 μM ADP is the concentration that discriminates SPD best. In both groups 10 mM EGTA totally inhibited Ca^{2+} release, 0.99 ± 0.08 and 1.00 ± 0.05, respectively. TRAP (500 μM) produced an impaired Ca^{2+} flux response in the SPD group, 1.23 ± 0.14 verses 2.66 ± 1.46, respectively ($P < 0.005$). In normal platelets Ca^{2+} flux could be induced by bovine thrombin (1 IU/mL), U46619 (10 μM) and A23187 (10 μM).

Conclusion: The Accuri C6 allows the continuous monitoring of Ca^{2+}flux in platelets and has utility in the diagnosis of dense granule SPD. Because of the heterogeneity of SPD further evaluation of this method is ongoing.

PB 2.25-5

Prevalence of disease and relationships between laboratory phenotype and bleeding severity in platelet primary secretion defects

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Background: The prevalence of platelet primary secretion defects (PSD) among patients with bleeding diathesis is unknown. Moreover, there is paucity of data on the determinants of bleeding severity in PSD patients.

Aims: To determine the prevalence of PSD in patients with clinical bleeding and to study the relationships between the type of platelet defect and bleeding severity.

Methods: Data on patients referred for bleeding to the Angelo Bianchi Bonomi Hemophilia and Thrombosis Center, Milan (Italy) in the years between 2008 and 2012 were retrieved to study the prevalence of PSD. Demographic, clinical and laboratory information on 32 patients with a diagnosis of PSD was used to compare patients with or without associated medical conditions and to investigate whether or not the type and extension of platelet defects were associated with the bleeding severity score (BSS; crude and age-normalized) or with the age at first bleeding requiring medical attention.

Results: The estimated prevalence of PSD among 207 patients with bleeding diathesis and bleeding severity score above 4 was 18.8% (95% confidence interval [CI]: 14.1–24.7%). Patients without associated medical conditions had earlier age of first bleeding (18 vs. 45 years; difference: −27 years; 95% CI: −46 to −9 years) and different platelet functional defect patterns (Fisher's exact test of the distribution of patterns, $P = 0.007$) than patients with accompanying medical conditions. The number of agonists eliciting reduced secretion was not associated with BSS, age-normalized BSS or age at first bleeding requiring medical attention at linear regression analysis (BSS, beta: −0.4; 95%CI: −2.0 to 1.3; $P = 0.675$; age-normalized BSS, beta: −0.05; 95%CI: −0.19 to 0.09; $P = 0.442$; age at first bleeding requiring medical attention, beta: 5.8; 95%CI: −2.1 to 13.8; $P = 0.144$). The median values of the different proxies of bleeding severity were not different across patterns of platelet defect (pattern type vs. BSS, $P = 0.635$; pattern vs. age-normalized BSS, $P = 0.301$; pattern vs. age at first bleeding requiring medical attention, $P = 0.277$; all P-values calculated by Kruskal–Wallis test).

Summary/Conclusions: PSD is found in approximately one fifth of patients with clinical bleeding. PSD patients display mild to moderate bleeding tendency, the severity of which is largely unrelated to the type and extension of laboratory defect.

PB 2.25-6

Lumi-aggregation is useful for better defining platelet function defects in response to weak agonists

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Background: Platelet function studies are important for the diagnosis of patients with disorders of primary hemostasis. The diagnostic interpretation of platelet aggregation in response to weak agonists is often problematic, and it is uncertain if an evaluation of platelet dense granule secretion is useful in this regard.

Aims: To evaluate whether measurement of platelet dense granule ATP secretion by lumi-aggregation (Chrono-Log Corp., Havertown, PA) would clarify platelet aggregation defects in response to weak agonists (4 μM ADP, 100 μM epinephrine, 1.6 mM arachidonic acid), especially when the response to only one of these agonists is abnormal.

Methods: Platelet function studies (n = 146) in non thrombocytopenic patients with bleeding histories, who were referred for hemostasis consultation, were reviewed retrospectively. All patients were thought to have mild bleeding disorders by their consulting hematologist. Results from platelet aggregation and secretion studies in response to weak agonists were compared in 119 adults (29 male, 90 female, ages 21–85 years) and 27 children (13 male, 14 female, ages 7 months–18 years). Repeat testing was performed on 19 patients within 8 days to 11 months of the initial platelet function study, including seven studies ordered for confirmation of an isolated platelet function defect in response to a single agonist.
Results: Concordant aggregation and secretion findings were confirmed in 125/146 cases, including 25 (17%) that were classified as normal. Isolated abnormal aggregation in response to a single agonist was identified in 35/146 (24%) cases: 27 (77%) abnormal responses to ADP alone, 7 (20%) abnormal responses only to epinephrine, and 1 (3%) abnormal response to arachidonic acid alone. Both abnormal platelet aggregation and secretion in response to the same single agonist were demonstrated in 14/35 (40%) cases. In the remaining 21/35 (60%) cases, which comprised all of the platelet function studies with discordant platelet aggregation and secretion findings, abnormal platelet aggregation with normal dense granule secretion was observed in 5/21 (24%) cases, and secretion defects in response to multiple agonists were documented in 16/21 (76%) cases. Repeat analysis confirmed the original platelet function abnormality in six of 7 (86%) of the original 14/35 cases with isolated abnormal platelet aggregation in response to a single agonist, when concomitant abnormal secretion was present. Clinicians did not repeat platelet function testing on patients with isolated abnormal platelet aggregation in response to a single agonist when secretion studies were normal. Similar findings were obtained in a subanalysis of results from pediatric patients.
Conclusion: Platelet dense granule secretion measured by lumi-aggregation is useful for better defining platelet function defects in response weak agonists, ADP, epinephrine, and arachidonic acid, especially when an isolated aggregation defect in response to a single agonist is noted. In our case series, nearly one quarter of aggregation studies performed resulted in inconclusive findings (abnormal aggregation in response to a single weak agonist). In 60% of these cases, lumi-aggregation allowed discrimination between normal and abnormal platelet function.

PB2.26 – Platelet Procoagulant Activity

PB 2.26-1

Targeting platelet GPIbβ decreases GPIb-dependent signaling, platelet procoagulant activity and arterial thrombosis

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Background: The GPIb-V-IX complex regulates the adhesion, activation and procoagulant activity of platelets. We previously reported that RAM.1, a rat monoclonal antibody directed against the extracellular domain of mouse GPIbβ, diminished adhesion of platelets and CHO cells transfected with the human GPIb-IX complex to von Willebrand factor under flow conditions.
Aims: The aim of the study was to evaluate the functional importance of GPIbβ by studying the impact of RAM.1 on GPIb-mediated platelet responses and *in vitro* and *in vivo* thrombus formation.
Methods: GPIb-dependent signaling was monitored on the ability to extend filopodia and by measuring intraplatelet Ca^{2+} variations after adhesion of platelets or GPIb-IX transfected CHO cells to immobilized von Willebrand factor under static conditions. Procoagulant activity was measured with the Calibrated Automated Thrombin Generation method. PS exposure was determined by Annexin-V binding using flow cytometry. *In vitro* thrombus formation was assessed following perfusion of hirudinated blood over a collagen matrix. Thrombus formation was also evaluated in two *in vivo* models, after a laser-injury of mesenteric arteries and a forceps-injury of the aorta. Hemostasis was evaluated using a tail bleeding assay.
Results: RAM.1 dramatically reduced GPIb-mediated filopodia extension of murine platelets and CHO GPIb-IX cells and inhibited GPIb-mediated Ca^{2+} signaling of murine platelets after adhesion to von Willebrand factor. RAM.1 inhibited thrombin generation triggered by tissue factor or collagen in platelet-rich plasma, but interestingly did not impair phosphatidylserine exposure in response to strong agonists such as thrombin, CRP and A23187. In addition, RAM.1 reduced thrombus formation after perfusion of mouse whole blood over collagen in a shear-dependent manner. This effect was also observed *in vivo*, since injection of RAM.1 F(ab)'2 diminished thrombus formation induced by laser beam injury of mesenteric arterioles and forceps injury of the abdominal aorta. In contrast, RAM.1 F(ab)'2 did not increase the tail bleeding time and the volume of blood lost.
Conclusions: These findings provide new evidence that targeting a subunit other than GPIbα can lead to an antithrombotic effect via the GPIb-V-IX complex. This could represent an alternative way to reduce thrombus formation without impairing hemostasis.

PB 2.26-2

Aggregation of PS exposing platelets and activated (non-PS exposing) platelets

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Background: A common feature of platelets with high levels of PS exposure, whether initiated by agonist or ABT-737 mediated pathways, is a loss of mitochondrial membrane potential. Recently a novel form of PS exposing platelets with intact mitochondrial membrane potential and active integrin $\alpha_{IIb}\beta_3$ was described by Topalov et al (ATVB, 2012). Formation of this novel procoagulant platelet ($PS^{\Delta\psi m+}$) was promoted by an increased platelet concentration or shaking and mediated by integrin $\alpha_{IIb}\beta_3$.
Aims: To investigate the molecular mechanisms responsible for formation of the novel PS exposing platelet subpopulation in human and murine models.
Methods: Platelet activation and mitochondrial membrane potential were evaluated by flow cytometry. To assess platelet aggregation, separated platelets (1×10^8 cells/mL) were labeled with either Cell tracker green or violet. The labeled platelets were washed twice, and then incubated with TMRM for evaluating loss of $\Delta\psi_m$. The separate populations were mixed, stimulated for 15 min with thrombin, labeled with APC-annexin V and/or PE-JON/A, and evaluated either by standard flow cytometry or ImagestreamX[TRADEMARK]. ImagestreamX [TRADEMARK] images were analyzed with IDEAS[TRADEMARK] software.
Results: As reported by Topalov et al stimulation of either human or murine platelets with 100 nM thrombin resulted in a concentration-dependent increase of a PS exposing platelet subpopulation with intact mitochondrial membrane potential, low levels of intracellular calcium, and integrin $a_{IIb}b_3$ activation ($PS^{\Delta\psi m+}$). Absence of Bax/Bak did not affect $PS^{\Delta\psi m+}$ platelet formation. Somewhat surprisingly, absence of cyclophilin D (CypD) markedly decreased $PS^{\Delta\psi m+}$ platelet formation, despite CypD's known role in mediating mitochondrial permeability transition pore formation and $\Delta\psi_m$ loss.
A feature of the $PS^{\Delta\psi m+}$ platelet subpopulation was its characteristic high side scatter and forward scatter within the activated platelet subpopulation. This phenomenon suggested the possibility that $PS^{\Delta\psi m+}$ platelets might represent an aggregate. Cell tracker green or violet (CTG or CTV)-labeled platelets were examined after thrombin stimu-

lation. Evaluation of the PS $^{\Delta\psi m+}$ platelet subpopulation revealed its close correspondence with a CTG^+/CTV^+ platelet subpopulation. ImageStreamX[TRADEMARK] was used to directly visualize the $PS^{\Delta\psi m+}$ platelet subpopulation. This visual evaluation by high resolution microscopy revealed that almost the entirety of the $PS^{\Delta\psi m+}$ platelet subpopulation consisted of a platelet aggregate of an annexin V^+, JON/A^-, $\Delta\psi m^-$ platelet and an annexin V^-, JON/A^+, $\Delta\psi m^+$ platelet, thus lending the $PS^{\Delta\psi m+}$ subpopulation its fluorescence characteristics.

Conclusions: Formation of the $PS^{\Delta\psi m+}$ platelet subpopulation is dependent on the mitochondrial protein CypD. However, ImageStreamX[TRADEMARK] and population labeling experiments suggest that the $PS^{\Delta\psi m+}$ platelet subpopulation may represent a misleading result of heterogenously aggregated platelets, thus explaining this platelet subpopulation's fluorescence characteristics, concentration and integrin $\alpha_{IIb}\beta_3$ dependence, and regulation by CypD.

PB 2.26-3
Signalling via CLEC-2 generates a procoagulant response in human platelets

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Background: The C-type lectin receptor CLEC-2 is a recently described platelet receptor that plays important roles in platelet cross talk and in development. It has also been shown to play a crucial role in thrombus stability. A natural ligand for CLEC-2 is podoplanin which is expressed on lymphatic endothelial cells, where it plays a role in vascular development, and on inflammatory macrophages, where it can activate platelets via CLEC-2. CLEC-2 can also be activated by the snake venom, rhodocytin. CLEC-2 signalling results in Src and Syk-dependent tyrosine kinase signalling and activation of PLCγ2 via a single YxxL hemITAM sequence. The downstream signalling pathway through CLEC-2 resembles signalling through another tyrosine kinase, ITAM-signalling receptor, the collagen GPVI/FcγR receptor complex, which is the major receptor that, on its own, is capable of generating a procoagulant response in platelets.

Aim: We sought to determine whether signalling through CLEC-2 is also capable of generating a procoagulant response in human platelets.

Methods: Platelets from healthy consenting donors were stimulated with either rhodocytin or podoplanin, and analyzed by flow cytometry for markers of a procoagulant response (Annexin V binding) and degranulation (P-selectin). Procoagulant phospholipid (PPL) activity was measured by the Calibrated Automated Thrombogram (CAT) assay using 1 pM TF reagent to trigger thrombin generation, and using peak thrombin (PT) as a measure for PPL activity.

Results: Rhodocytin induced Annexin V binding in a subset of 24 $\pm$ 4.9% of platelets (n = 5) accompanied by the release of Annexin V^{+ve} microparticles, with a maximum effect at 30 nM. At the same dose P-selectin was expressed on the majority (81 $\pm$ 4.9%) of the platelets (n = 5). These levels were similar to those induced by the GPVI-specific collagen-mimetic peptide CRP-XL. Addition of ADP, thrombin, U-46619 and CRP-XL showed synergism with rhodocytin resulting in a 1.47, 3.10, 1.81 and 2.52, fold increase in the percentage of Annexin V positive platelets respectively (P = 0.0003). The platelet procoagulant response to rhodocytin was inhibited by 37% with aspirin (P = 0.0337; n = 5) and was enhanced 1.61 $\pm$ 0.07 fold by platelet-platelet aggregation by (P = 0.0019; n = 7). Mouse podoplanin (20 μg/mL) also generated platelet Annexin V binding in 26 $\pm$ 3.1% of platelets (n = 6). In addition, 41 $\pm$ 12.4% of platelets became Annexin V positive (n = 3) when incubated with human podoplanin-expressing CHO cells. Both mouse podoplanin and human podoplanin-expressing CHO cells induced platelet microparticle generation. Rhodocytin-activated platelets showed significant PPL activity in the CAT assay with PT of 356 $\pm$ 2 compared to control samples 172 $\pm$ 6 (P = 0.0019; n = 3). Additionally, platelet-derived microparticles generated either by rhodocytin or human podoplanin-expressing CHO cells showed significant increase in PT with 88 $\pm$ 1 and 121 $\pm$ 4 compared to 17 $\pm$ 1 and 33 $\pm$ 5 respectively in control samples (P = 0.0003 and 0.0024 respectively; n = 3). Immunofluorescence studies showed that platelets bound to podoplanin-expressing CHO cells and expressed a procoagulant surface and P-selectin.

Conclusion: Signalling in platelets via CLEC-2 induces a procoagulant response in platelets and the generation of procoagulant microparticles. The response of platelets to CLEC-2 resembles signalling through GPVI. These data suggest novel findings of a procoagulant role for CLEC-2 in human platelets.

PB 2.26-4
Evidence for necrotic platelets in collagen dependent thrombus formation *in vivo*

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Background and Aims: Platelets display heterogeneity in response to agonist stimulation with regards morphology and surface characteristics. Activation by strong agonists ex-vivo results in a phosphatidylserine expressing, highly procoagulant platelet subpopulation that has recently been proposed to be necrotic. It has been difficult to track the contribution of the 'necrotic' population to thrombus formation due to an overlap between markers of activation and necrosis, thus the functional relevance of these platelets is uncertain. Fluorescent tagged small trivalent arsenical (4-(N-(S-glutathionylacetyl) amino) phenylarsonous acid) named 'tagged-GSAO' is known to label late apoptotic and necrotic nucleated cells via covalent bonding with closely spaced di-thiol proteins in the cytoplasm. Here we aim to investigate tagged-GSAO as a potential marker of activated, 'necrotic' platelets after agonist stimulation and to determine if we could use this marker to explore the contribution of necrotic platelets to thrombus formation *in vivo*.

Methods and Results: Flow cytometry analysis of washed human platelets stimulated with dual agonists, collagen 5 μg/mL and thrombin 1 U/mL generated a subpopulation of P-selectin positive platelets that co-labelled with tagged-GSAO compared to its control, tagged-GSCA. This subpopulation is characterised by high annexin-V staining and a spectrum of calcein retention suggesting it encompasses platelets in the process of losing viability. Pre-incubation with control vs. pancaspase inhibitor did not affect this GSAO+ve population (74.1% vs. 74.5%) suggesting a caspase independent pathway. The GSAO+ve population was collagen dependent. Thrombin alone at doses up to 10 U/mL generated a minor GSAO+ve population compared with control (2.5 $\pm$ 1.2% vs. 0.2 $\pm$ 0.3%, P = 0.03), however collagen 5 μg/mL resulted in a significant increase in GSAO+ve platelets (0.2 $\pm$ 0.3% vs. 33 $\pm$ 5.7%, P = 0.0015). Dual stimulation increased this subpopulation of platelets further (33 $\pm$ 5.7% vs. 49.03 $\pm$ 16%, P = 0.0002). Generation of GSAO+ve platelets was dependent on exogenous calcium (20.42 $\pm$ 0.25% vs. 49.52 $\pm$ 0.34%, P = 0.0002). Treatment with calcium ionophore resulted in > 90% GSAO+ve platelets with high annexin- V binding. This data supports the concept that these platelets are necrotic.

To determine if tagged-GSAO compound could be used to track this platelet sub-population during thrombus formation *in-vivo*, we demonstrated that tagged-GSAO did not affect *in-vitro* platelet aggregation or coagulation studies. To assess the contribution of necrotic platelets *in-vivo*, we used confocal intra-vital microscopy to compare two mouse models of thrombosis: laser injury (thrombin dependent) and ferric chloride (collagen and thrombin dependent). We used platelet marker anti-CD42b-dylight 649 and tagged GSAO. Rhodamine-2 was used to monitor platelet calcium response. The laser injury model of thrombosis showed no co-localisation of tagged-GSAO and platelets in the first

3 min of thrombus formation, consistent with our thrombin alone *in-vitro* data. This is in contrast to the ferric chloride model, where platelet aggregates co-labelled with tagged-GSAO indicating a potentially necrotic platelet clot. GSAO+ve platelet aggregates were also Rhodamine-2 positive indicating prolonged high calcium state. The non-aggregatory platelets did not label with GSAO and were Rhodamine-2 negative.

Conclusion: Tagged-GSAO labels a platelet subpopulation likely undergoing necrosis. These platelets are seen to play a role in collagen dependent thrombosis, but not in early thrombin mediated aggregation *in-vivo*.

PB 2.26-5

Quantification of the platelet channelome in ultra-purified human platelet samples highlights the high expression level of TMEM16F

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Ion channels play crucial roles in many aspects of cellular function. Approximately twenty ion channels have already been reported in platelets and/or megakaryocytes, although for many their relative expression and importance remains unclear. The human genome encodes more than 400 proteins that form or regulate functional ion channels, therefore platelets are likely to express additional ion channel proteins with as yet unidentified roles. We recently demonstrated that quantitative-PCR was a key tool in identifying Kv1.3 (KCNA3) as the sole K^+ channel responsible for one of the largest ionic conductances of platelets and megakaryocytes. Nevertheless, quantification of the platelet channelome using quantitative-PCR raises two main challenges; ion channels need only be present at small levels to regulate function, and platelets contain lower levels of mRNA than other blood cells.

We aimed to further develop our methodology to isolate ultrapure platelet mRNA, for use in assessing the platelet channelome using quantitative-PCR.

Platelet rich plasma (PRP) from healthy consenting donors was prepared from Acid-Citrate-Dextrose-anti-coagulated whole blood. Platelet activation was further inhibited with 2 mM EDTA, 0.1 μM PGE_1 and 0.3 μM acetylsalicylic acid and only the upper portion of PRP isolated. A series of immunomagnetic depletion steps removed leukocytes and platelet-leukocyte complexes using CD45, and erythrocytes using CD235a. Platelets were positively selected using CD42b-tagged magnetic beads, then washed and lysed for isolation of mRNA. Following reverse transcription, platelet cDNA was analysed by quantitative-PCR using 400 in-house-validated QuantiTect primer assays (Qiagen) for ion channel or channel regulatory genes (the 'channelome'). Expression levels were normalised to GAPDH.

All platelet cDNA samples were screened for expression of platelet, erythrocyte and leukocyte markers. No sample was positive for CD235a, and any that showed trace CD45 levels were discarded. The top 33 most abundantly expressed channelome transcripts included 10 channels previously reported in platelets. Of note, by far the most abundantly expressed was TMEM16F (2.685 ± 0.847 relative to GAPDH). Originally described in platelets as an anion channel (Suzuki et al, 2010, Nature, 468, 834), a recent study suggests this is a Ca^{2+}-permeable cation channel (Yang et al, 2012, Cell 151, 111). The order of expression of other known channelome genes was P2RX1 (0.529 ± 0.269) > KCNA3 (0.332 ± 0.083) > ORAI1 (0.165 ± 0.038) > ITPR2 (0.120 ± 0.055) > STIM1 (0.114 ± 0.030) > TPCN1 (0.076 ± 0.052) > TRPC6 (0.068 ± 0.042) > CLIC1 (0.041 ± 0.007) > ITPR1 (0.020 ± 0.002) relative to GAPDH. These results correlate closely with estimations of relative channel density reported in patch clamp recordings and/or relative importance of the channels in functional studies. In addition, we identified several transcripts that encode for ion channels or their regulators, which have not previously been reported in platelets.

We have developed an approach to ultra-purify platelet mRNA for the purpose of detecting transcripts for proteins that may play a role even at low levels of expression, such as ion channels. A channelome screen confirmed the expression of 10 channel-related proteins previously reported in the platelet or megakaryocyte and highlights the high expression of TMEM16F. Further ion channel-related proteins were also detected which require additional work to validate the presence of functional protein and potential roles in platelet/megakaryocyte function.

Funded by the British Heart Foundation (PG/11/56).

PB 2.26-6

Perturbations in local clot hemodynamics triggers intraluminal thrombus contraction

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Background: Hemodynamic conditions vary throughout the vasculature, creating diverse environments in which platelets must respond. To prevent blood loss, a growing platelet deposit must be assembled in the presence of fluid wall shear stress (t_w) and a transthrombus pressure gradient (ΔP) that drives bleeding.

Aims: Our aim was to investigate clot development and contraction in response to controlled perturbations in transthrombus pressure gradient and wall shear stress.

Methods: We designed a microfluidic device capable of side view visualization of a developing thrombus forming on collagen ± tissue factor (TF), while independently controlling ΔP and t_w. Unlike traditional *in vitro* clot contraction studies, this device incorporates the critical mechanical and biological properties that can only be obtained under flow conditions. Platelet deposits were formed at an arterial shear rate (1130/s) for 10 min while maintaining a physiologic transthrombus pressure gradient (ΔP = 0–23.4 mm Hg). Side view visualization of thrombosis with transthrombus permeation allowed for quantification of clot structure, height, and composition at various ΔP.

Results: When ΔP was increased from 20.8 to 23.4 mm Hg at constant arterial shear stress, clot height was reduced 20% on collagen/TF and 28% on collagen alone. Using a fluorescent reporter of thrombin production, we observed a 62% reduction in thrombin's ability to penetrate into the luminal side of the clot as ΔP was increased from 0 to 23.4 mm Hg. This was consistent with convective removal of thrombogenic solutes due to pressure-driven permeation. Following thrombus development, the clot was perturbed by either reducing or increasing flow. Unexpectedly, the 20-μm thick platelet layer underwent massive platelet retraction upon flow reduction. Contraction rates 1–2 min after flow reduction/cessation significantly increased by 6.5-fold (upstream region) and 4.6-fold (downstream region) ($P < 0.05$), compared to the rate before perturbation. In our rigid wall microfluidic device we added 1 μM SQ-29,548 (TXA_2 receptor antagonist) or 10 μM MRS-2179 and 50 μM 2-MeSAMP ($P2Y_1$ and $P2Y_{12}$ platelet receptors, respectively) to investigate the role of soluble autocrine mediators ADP and TXA_2. Both antagonists significantly reduced the total clot contraction as compared to buffer. ADP antagonists had the largest effect, reducing the total contraction nearly 75% over 7 min, whereas TXA_2 antagonist reduced the contraction by 44%. Interestingly, when flow was increased to pathophysiological shear rates (15,000/s), vWF mediated platelet aggregation resulted in clot structures exceeding 100 μm in height. These protruding deposits diverted flow from the downstream portion of the clot to allow rapid trailing edge retraction.

Summary/Conclusions: We have recently described a novel microfluidic platform that allows for side view visualization of thrombus growth in the presence of a transthrombus pressure gradient. Using this technique we report that small reductions in transthrombus pressure drop

(11%) result in significant increases in average clot height (20–28%). We observed that reducing flow causes a rapid clot contraction mediated by ADP and TXA_2. Flow dilution of these platelet autocrine mediators during intraluminal clotting balances the platelet contractile apparatus with prevailing hemodynamics, a newly defined flow sensing mechanism to regulate clot function.

PB2.27 – Platelets in Cardiovascular Disease

PB 2.27-1

Search for a predictive signature of coronary artery disease in platelet transcriptome

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Background: Platelets retain a small but significant amount of functionally active megakaryocyte-derived mRNA as well as protein and molecular machinery necessary for translation. The platelet transcriptome may change in coronary artery disease (CAD). We previously showed that differentially expressed transcripts do exist in platelets from patients with non-ST elevation acute coronary syndrome (NSTE-ACS) compared to those with stable angina (SA), suggesting that ACS platelets are potentially preconditioned at the transcriptional level to a higher degree of reactivity, which eventually lead to the thrombotic event.
Aims: (i) To assess in the platelet transcriptome a predictive signature of the CAD type. (ii) To identify transcriptional variations related to the CAD type as compared to healthy subjects.
Methods: Total RNA was isolated from leukocyte-depleted platelets from 41 NSTE-ACS and 37 SA patients, age- and sex-matched and with no comorbidities (diabetes and kidney disease). Gene expression profiling was performed using the HumanHT v.12 BeadChips (Illumina). Data variance stabilizing transformation and robust spline normalization were conducted with the lumi R package. Genes were filtered out if < 10% of expression data have at least a 1.35-fold change in either direction from gene's median value, and statistical differences and prediction classifiers were assessed using the BRB-ArrayTools v.4.3.0-beta2 package. Functional annotation clustering was done using the DAVID v.6.7 Bioinformatics Resources. Platelet activation state was assessed by flow cytometry, using anti-CD41, CD62, PAC1, and Tissue factor antibodies.
Results: Microarray analysis identified 4000 ± 670 transcripts expressed in patients' platelets. Interestingly, the number of transcripts that passed the quality control criteria was lower in NSTE-ACS than in SA patients (3770 ± 598 and 4141 ± 733, respectively, $P < 0.02$). Differential analysis was performed on the 2687 transcripts expressed in all samples: 138 were found significant with a permutation P-value ≤ 0.001 and a FDR < 2% (121 decreased and 17 overexpressed in NSTE-ACS vs. SA platelet). Notably, 58 of these transcripts are unannotated genes. Functional annotation analysis of the remaining 73 unique genes revealed that the Gene Ontology classes 'Translation and Elongation' and the KEGG pathway 'Ribosome' were significantly enriched in NSTE-ACS downregulated transcripts (17 and 10 genes, respectively, adjusted P-value 2.36E-10 and 1.14E-08). Prediction analysis of microarray (PAM) identified a classifier composed of 17 unique genes: cross-validation, used to assess the performance of the PAM classifier, showed that it had a rate of correct classification of 70%.
Comparison with 10 age- and sex-matched healthy subjects and functional analysis showed that several biological processes and cell compartments are altered in CAD platelets (adjusted P-value < 0.05), e.g. 'Wound healing', 'Glycolysis', 'Regulation of metabolic processes', 'Membrane-bound vesicle', and 'Platelet alpha-granule' genes. Of interest, CD41 mRNA is overexpressed in NSTE-ACS compared to both SA and healthy platelets, and this is paralleled by the higher antigen level assessed by flow cytometry.
Conclusion: The observed decrease of translational elongation genes in NSTE-ACS might point out an exhausted capability of *de novo* protein synthesis, which has been already exploited to sustain the platelet hyperreactive state found in unstable patients. The partial predictive capacity of the PAM classifier suggests that other variables may act as confounders.

PB 2.27-2

Plasma-derived microparticles as a source of biomarkers in acute coronary syndromes: a proteomic study comparing ST-elevation myocardial infarction patients and stable coronary artery disease controls

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1 Microparticles (MP) are cell-derived submicron size membrane vesicles known to be increased in the blood of patients with acute coronary syndromes [Mallat Z et al. (2000) Circulation 101:841–843]. They derive from various cells, most notably platelets, but also leucocytes, lymphocytes, erythrocytes and endothelial cells. MP are present in a small amount in the circulation of healthy individuals, but their levels increase in a high range of pathological conditions.
2 Our objective was to compare the proteome of plasma-derived microparticles from ST-elevation myocardial infarction (STEMI) patients and stable coronary artery disease (SCAD) controls. Our goal was to detect MP proteins differentially regulated that could serve as biomarkers for the acute event.
3 Plasma MP were isolated from 10 STEMI patients and 10 SCAD matched controls. We followed a standardized procedure to guarantee the purity of the microparticle population [Ramacciotti E et al. Thromb Res. 2010;125:e269-e274]. MPs were characterized by FACS with FITC-conjugated mouse anti-human CD41 monoclonal antibody, and by electron microscopy. Proteome analysis was based on two-dimensional differential in-gel electrophoresis (2D-DIGE) and mass spectrometry (MALDI-MS/MS and LC-MS/MS). For the DIGE analysis, CyDye-labeled proteins were separated by 24 cm 4–7 IPG strips for the first dimension, and 11% SDS-PAGE for the second. Image analysis was performed with Progenesis SameSpots software (NonLinear Dynamics). Validations were by 1D- and 2D-western blotting in an independent cohort of patients.
4 Due to the low amount of protein obtained from the patients, two pools were done, one for each condition (STEMI and SCAD). The DIGE analysis included four gels (technical replicates). Following image analysis, 1228 spots were detected per gel. The number of differentially regulated spots was of 115 (fold change ≥ 2 and $P < 0.05$). Form those, 71 were up-regulated in MP from STEMI patients, whereas 44 were up-regulated in MP from SCAD controls. So far we successfully analyzed 76 differentially regulated spots by mass spectrometry. Proteins identified include complement C4-A, fibrinogen, haptoglobin, apolipoprotein A-1, etc. Interestingly, alpha-2-macroglobulin and cardiotrophin-like cytokine factor 1 (CLCF1) were found to be up-regulated in STEMI samples. The former plays an important role in thrombogenesis and has been recently found to be up-regulated in plasma MP from deep venous thrombosis patients. The latter is a cytokine with B-cell stimulating capability that we recently found to be present in platelet-derived MP.

5 We performed the first high-resolution proteome analysis of MP from STEMI and SCAD patients. We provide a repertoire of proteins up-regulated in STEMI MP that could serve as biomarkers for the acute event. We believe our data open a new line of investigation that will allow a better understanding of the pathophysiological mechanisms underlying acute myocardial infarction. Our results demonstrate plasma-derived MP are a promising source of biomarkers for the acute event.

PB 2.27-3

P-selectin compared to other tests as a simple-to-use approach for assessing platelet function in cardiac patients

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Background: There are several tests of platelet function that are used to monitor the effects of antiplatelet agents, particularly aspirin and $P2Y_{12}$ antagonists, in patients with acute coronary syndromes. We have developed tests based on measurement of platelet surface-bound P-selectin following platelet stimulation. The tests differ from other approaches to measuring platelet function in that they are simple-to-use and measurements can be performed remotely, e.g. in GP's surgeries or in people's homes. Treated blood samples are stable for up to 9 days and are posted to a central laboratory for analysis. All tests, including the P-selectin tests, demonstrate that high residual platelet function in patients taking the $P2Y_{12}$ antagonist clopidogrel have a significantly increased risk of further thrombotic events.
Aim: The P-selectin test is being developed for commercial use by Platelet Solutions Ltd. The aim of this study was to compare the results obtained with other forms of platelet function testing in the same patients.
Methods: For measurement of P-selectin platelets in citrate anti-coagulated whole blood are activated and fixed after 5 min using a unique stabilising agent (patent applied for). We have compared the P-selectin aspirin and $P2Y_{12}$ tests with three aspirin and four $P2Y_{12}$ commercial tests including PRP aggregometry (PRP), Multiplate (MP), VerifyNow (VN) and Biocytex VASP (VASP). Blood samples were obtained from patients following treatment for 1 month with either aspirin plus clopidogrel ($n = 78$) or aspirin plus the more potent $P2Y_{12}$ antagonist, prasugrel ($n = 28$).
Results: All of the $P2Y_{12}$ tests demonstrated variability in the effectiveness of clopidogrel to inhibit platelet function while prasugrel treatment resulted in greater inhibition. Spearman correlations of P-selectin $P2Y_{12}$ test results compared with the other $P2Y_{12}$ tests were all good with r values of 0.5958 (PRP), 0.6268 (VN), 0.6227 (MP) and 0.4986 (VASP) all $P < 0.0001$. All of the aspirin tests consistently identified four patients as either non-responders or non compliant while all other patients demonstrated inhibition by aspirin using all tests.
Conclusions: The P-selectin tests, with their potential for remote testing, were at least as effective in determining the inhibitory effects of antiplatelet therapies as the other tests used in this study.

PB 2.27-4

Comparison of the optimul assay with light transmission aggregometry for the detection of aspirin resistance in patients with stable coronary artery disease

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Background: Clinical studies into aspirin resistance (ASA-R) predominantly utilise point-of-care tests based on surrogate measures of platelet function, as light transmission aggregometry (LTA) which is widely considered the gold standard, is not suitable for clinical application. These surrogate tests demonstrate poor correlation with LTA and so the clinical need for an improved standardised test remains. The 96-well plate assay (Optimul) is based on LTA but requires less technical expertise and enables rapid testing at a range of agonist concentrations. In healthy subjects, it has demonstrated good ability to determine response (both *in vitro* and *in vivo*) to antiplatelet agents and good correlation with LTA in both healthy subjects and in patients with mild bleeding defects. ASA-R by LTA is generally defined as > 20% aggregation in response to 1.6 mM arachidonic acid (AA). No such criteria have been defined for the Optimul assay.
Aims:
1 To assess the correlation between LTA and Optimul following *in vivo* aspirin therapy in subjects with cardiovascular disease
2 To define criteria for ASA-R by Optimul
Methods: Whole blood was drawn into citrate (final concentration 0.32%) from patients with coronary artery disease receiving 75 mg aspirin daily ($n = 15$). Aggregation of platelet-rich plasma was performed by LTA in a PAP-8E aggregometer (Bio/Data) in response to 0.6, 1.0 and 1.6 mM AA (Sigma-Aldrich). Aggregation using the 96-well technique was performed using a plate reader (Tecan Sunrise) at absorbance 595 nm in response to 0.03, 0.1, 0.3, 0.6, 1.0 and 1.6 mM AA. Hundred percent aggregation was defined as the optical density of platelet-poor plasma.
Correlation of Optimul and LTA was performed using a Pearson correlation and paired t-test. Receiver operating characteristic (ROC) analysis curves were performed to assess the ability of 96-well aggregometry to identify ASA-R. Statistical analyses were performed using Prism 4 (GraphPad). Statistical significance was taken as $P < 0.05$. The study was approved by local ethics committee and all subjects provided informed consent.
Results: Optimul was found to be positively correlated to LTA for all AA concentrations tested (0.6 mM, r^2 0.59, $P < 0.001$; 1.0 mM, r^2 0.82, $P < 0.001$; 1.6 mM, r^2 0.81, $P < 0.001$). No significant difference was found between the two assays ($P = 0.1868$). ROC analysis using above the criteria to define ASA-R, yielded non-significance at all concentrations ($P > 0.05$). Modifying ASA-R criteria to > 30% aggregation by LTA improved significance, in particular at 1.6 mM AA (0.6 and 1.0 mM, AUC 0.91, 95%CI 0.75–1.01, $P = 0.063$; 1.6 mM, AUC 1.00, 95%CI 1.00–1.00, $P = 0.024$). For the 1.6 mM ROC at 30% LTA aggregation, a cut-off of < 27.73% yielded 100% sensitivity and specificity.
Summary/Conclusions: Optimul and LTA correlate well within patients with stable coronary artery disease, with no significant difference seen in results. ROC analysis trended to significance for all concentrations of agonist, but with significance only achieved following acceptance of a more stringent LTA definition of ASA-R. A degree of this discrepancy is likely to reflect measurement error from an imperfect gold standard. These findings suggest that the Optimul assay may be a suitable clinical tool for the detection of ASA-R.

PB 2.27-5

Role of platelets in driving the thrombotic risk and protective processes associated with exposure to diesel exhaust particles

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Background: There is an established link between exposure to air born pollution and the incidence of respiratory and cardiovascular diseases. Inhalation of ambient particulate matter enhances the risk of platelet-driven cardiovascular diseases such as myocardial infarction in at-risk individuals. The nanoparticulate fraction of ambient air (i.e. particulate matter of diameter < 100 nm) is thought to be the most damaging

to health. Nanoparticles induce inflammation and cytotoxicity and have been shown to translocate the pulmonary epithelium to blood and internal organs. Urban ambient air is rich in nanoparticulate diesel exhaust particles (DEP) produced from industrial, public and private vehicular emissions.

Aims: We aimed to investigate the ability of DEP to interact physically and functionally with platelets as well as the ability of endothelial mediators to offer protection against DEP-induced platelet activation.

Methods: The interaction of DEP and carbon black (CB, which consists of the carbon core of DEP without the transmission metals and organic compounds associated with the surface of DEP) with platelets was examined by transmission electron microscopy, while the functional consequences of exposure were assessed by measuring *in vitro* and *in vivo* platelet aggregation using established methods.

Results: Electron microscopy revealed DEP and CB to be present in a mixture of individual, chained and agglomerated nanoparticles. Following exposure, both DEP and CB were internalised and seen in proximity with the open canalicular system in platelets. DEP induced concentration-dependent human platelet aggregation *in vitro* whereas CB had no effect. DEP induced Ca^{2+} release, dense granule secretion and surface P-selectin expression, but not toxicologic membrane disruption. Low concentrations of DEP potentiated agonist-induced platelet aggregation *in vitro* and *in vivo*. A nitric oxide donor was found to exert a similar inhibitory effect upon DEP-induced platelet aggregation to that occurring with a conventional platelet agonist.

Summary/Conclusions: DEP associate physically with platelets in parallel with a Ca^{2+}-mediated aggregation response displaying the conventional features of agonist-induced aggregation. The ability of DEP to enhance the aggregation response to platelet stimuli would be expected to increase the incidence of platelet-driven cardiovascular events should they be inhaled and translocate into the blood and provides a potential mechanism for the increased thrombotic risk associated with exposure to ambient particulate air pollution. Our study also suggests that the healthy vascular endothelium would be expected to provide protection against the platelet-mediated pro-thrombotic effects of DEP. A lack of endothelial-mediated inhibition of nanoparticle-induced platelet aggregation during endothelial dysfunction may provide a mechanism for the enhanced risk observed in individuals with underlying cardiovascular conditions upon exposure to particulate ambient pollution.

PB 2.27-6

Platelet and leukocyte ROS production and lipoperoxidation are associated with high platelet reactivity in acute coronary syndrome patients on dual antiplatelet treatment

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High platelet reactivity (HPR) on dual antiplatelet therapy is a risk factor for adverse vascular events in acute coronary syndrome (ACS) patients.

Several studies have shown that reactive oxygen species (ROS) may be involved in modulating platelet function.

In ACS patients we investigated: (i) the relationship between the amount of ROS production/lipoperoxidation of cellular elements (by FACS analysis) and platelet activation through different pathways; (ii) the association of cellular ROS production with the presence of high platelet reactivity to ADP and arachidonic acid (AA) stimulation in ACS patients on dual antiplatelet therapy.

Significantly higher levels of platelet and leukocyte-derived ROS were detected in 49 dual HPR (with platelet aggregation by 1 mM AA $\geq$ 20% and by 10 μM ADP $\geq$ 70%) with respect to non-HPR patients (n = 49). Similarly, dual HPR patients had significantly higher platelet and leukocyte lipoperoxidation than non-HPR patients.

After adjustment for several potential confounders, platelet-, leukocyte-derived ROS and platelet and leukocyte lipoperoxidation remained significantly associated to dual HPR [platelet-ROS:2.53 (2.28–5.01), $P < 0.001$; leukocyte-ROS:1.13 (1.06–1.21), $P < 0.001$; platelet-lipoperoxidation:1.92 (1.10–1.35), $P < 0.001$; leukocyte lipoperoxidation: 1.59 (1.28–1.98), $P < 0.001$].

The significant predictors of ADP, AA, and collagen platelet aggregation at multiple linear regression analysis, after adjusting for age, cardiovascular risk factors, procedural parameter, medications, leukocyte number and MPV, were platelet-, leukocyte-derived ROS and platelet and leukocyte lipoperoxidation.

Our results demonstrate that in patients with ACS on dual antiplatelet therapy ROS production by and lipoperoxidation of platelets are strictly correlated to the different pathways of platelet aggregation and that ROS production and lipoperoxidation of platelets and leukocytes are predictors of nonresponsiveness to dual antiplatelet treatment.

PB2.28 – Platelet Activation: Miscellaneous – I

PB 2.28-1

Streptococcus pneumoniae triggers platelet activation and platelet-leucocyte complex formation in a strain dependent and toll-like receptor 2 independent manner

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Background: *Streptococcus pneumoniae* strains vary considerably in their ability to cause invasive disease, and this is associated with the capsular serotype. The *S. pneumoniae* capsule inhibits complement- and phagocyte-mediated immunity. Septic patients often develop thrombocytopenia, which is associated with higher mortality. Besides the role of platelets in hemostasis, platelets exert a role in the immune continuum through release of regulatory proteins, platelet-neutrophil complex formation, and secretion of antimicrobial peptides.

Aims: To determine how *S. pneumoniae* activates human platelets.

Methods: *Streptococcus pneumoniae* serotypes used: D39, TIGR4 and 6303 and mutant unencapsulated D39. Human platelet aggregation was measured in citrate-anticoagulated platelet rich plasma by light transmission aggregometry. Platelet degranulation and platelet-leukocyte complexes were measured by flow cytometry. Platelet and neutrophil killing capacity was determined in incubation experiments with *S. pneumoniae*.

Results: Unencapsulated *S. pneumoniae* induced platelet aggregation in a capsule-dependent manner; aggregation was not induced by encapsulated strains, or by direct toll-like receptor (TLR) ligands. Aggregation was inhibited by IgG receptor blocking antibody anti-FcγRII, but not by anti-TLR2. Whole blood incubation with all *S. pneumoniae* serotypes resulted platelet degranulation and platelet-granulocyte and platelet-monocyte complex formation. These reactions were inhibited by PGE1 but not by anti-FcγRII, anti-TLR2 and 4. Platelets had no antimicrobial effect on *S. pneumoniae*, nor did platelet-neutrophil complex formation enhance neutrophil killing capacity.

Conclusions: *Streptococcus pneumoniae* causes FcγRII mediated platelet aggregation through a mechanism that is inhibited by its capsule. All *S. pneumoniae* serotypes are potent inducers of platelet degranulation and platelet-leukocyte complex formation in a TLR2 independent manner.

PB 2.28-2

Multiple sites on *Streptococcus gordonii* surface protein PadA mediate outside-in signaling in platelets

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Background: Infective endocarditis (IE) is a life threatening disease caused by a bacterial infection of the endocardial surfaces of the heart. The oral pathogen, *Streptococcus gordonii* is amongst the most common pathogens isolated from IE patients. Previously we identified a novel cell wall protein expressed on *S. gordonii* called platelet adherence protein A (PadA). This protein interacts with platelet GPIIb/IIIa resulting in platelet adhesion, dense granule secretion and platelet spreading. Proteomic analysis of the PadA protein identified two short amino acid motifs (RGD and AGD) which have been previously shown to be important for fibrinogen binding to GPIIb/IIIa and contributing to the generation of outside-in signalling.

Aim: The aim of this project was to determine if either or both of these motifs found on S. gordonii PadA have any role in mediating outside in signalling in human platelets.

Methods: Bacteria were grown in Brain Heart Infusion Broth over night under static conditions. The regions encoding potential platelet interactive motifs AGD and RGT were subjected to alanine-substitution mutagenesis. Dense granule secretion was measured by luminometry using a luciferin/luciferase assay. Platelet interactions with immobilized bacteria under shear were assessed using a parallel flow chamber. Platelet spreading on native and mutated PadA was visualised by confocal microscopy with differential interference contrast or using an argon laser at 488 nm.

Results: Site directed mutagenesis on the PadA protein in which the $_{454}$AGD and $_{383}$RGT was substituted to AAA resulted in the RGT motif having no role in supporting platelet adhesion (P = NS) however plays a role in dense granule secretion ($P < 0.005$) and platelet spreading ($P < 0.005$). The AGD motif on the PadA protein however has no role to play in supporting firm platelet adhesion or dense granule secretion (P = NS) however plays a role in platelet spreading ($P < 0.01$).

Conclusions: The work presented here extends previous knowledge in *S. gordonii* platelet interactions by identifying two motifs on PadA that are similar to the known binding motifs in fibrinogen that recognise GPIIb/IIIa. Of particular interest is the role these motifs play in platelet function. It appears that the RGT motif has no role in supporting platelet adhesion however plays a role in dense granule secretion and platelet spreading. In contrast to this, AGD has no role in supporting platelet adhesion or dense granule secretion, however plays a role in platelet spreading. These results suggest for the first time that multiple sites on *S. gordonii* PadA generate outside in signals through GPIIb/IIIa to mediate platelet responses that likely contribute to the thrombotic complications of IE.

PB 2.28-4

Effects of high-amount high-intensity exercise on *in vivo* platelet activation: modulation by lipid peroxidation and AGE/RAGE axis

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Background: Physical activity is associated with cardiovascular risk reduction, but the effects of exercise on platelet activation remain controversial.

Aims: We investigated the effects of regular high-amount, high intensity aerobic exercise on *in vivo* thromboxane (TX)-dependent platelet activation and plasma levels of platelet-derived proteins, CD40L and P-selectin, and whether platelet variables changes may be related to changes in HDL and in the extent of oxidative stress and oxidative stress-related inflammation, as reflected by urinary isoprostane excretion and endogenous soluble receptor for advanced glycation end-products (esRAGE), respectively.

Methods: Urinary excretion of 11-dehydro-TXB_2 and 8-iso-prostaglandin (PG) F_{2a} and plasma levels of P-selectin, CD40L and esRAGE were measured before and after a 8-week standardized aerobic high-amount-high-intensity training program in 24 low-to-intermediate-risk sedentary subjects with low HDL cholesterol.

Results: Exercise training had a clear beneficial effect on HDL cholesterol (+11%, $P = 0.014$) and triglyceride (−27%, $P = 0.009$) concentration. In addition, a significant ($P < 0.0001$) decrease in urinary 11-dehydro-TXB_2 (27%), 8-iso-PGF_{2a} (22.4%), plasma P-selectin (29%), CD40L (35%) and a 61% increase in esRAGE were observed. Multiple regression analysis revealed that urinary 8-iso-$PGF_{2\alpha}$ [beta = 0.393, SEM = 73.4, $P = 0.004$] and esRAGE ($\beta = -0.30$, SEM = 48.2, $P = 0.024$) were the only significant predictors of urinary 11-dehydro-TXB_2 excretion rate over the training period.

Conclusions: Regular high-amount-high-intensity exercise training, with minimal weight change, in sedentary, low- or intermediate-risk people, together with increases in fitness (as measured by peak oxygen consumption), has broad beneficial effects on platelet activation markers, paralleled and possibly associated with changes in the lipoprotein profile and in markers of lipid peroxidation and AGE/RAGE axis. Our findings may help explaining why a similar amount of exercise exerts significant benefits in preventing cardiovascular events, and may guide the optimization of the exercise protocol in patients with different degrees of cardiovascular burden, including those with chronic ischemic heart disease, based on the effect on lipoprotein profile as well as on platelet activation and oxidative stress markers.

PB 2.28-5

Effect of hydrogen sulphide (H2S) on human platelet adhesion and clot retraction

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Background: Hydrogen sulphide (H_2S) is increasingly recognized as a member of a growing family of 'gasotransmitters', together with its two counterparts, NO and CO. Actually, H_2S is emerging as an important endogenous modulator which exhibits beneficial effects on the cardiovascular system, without producing toxic metabolites.

Objective: The present work was designed *in vitro* to study the effect of H_2S on human blood platelets adhesion and clot retraction.

Methods: Tests were performed using platelet rich plasma (PRP) or washed platelets (WP) (300×10^9 plat/L). Sodium hydrogen sulfide (NaHS) was used as H_2S donor. A platelet suspension was treated with 10 mM NaHS or buffer.

Adhesion: Microplate wells were coated with 100 μL of fibrinogen solution (20 μg/mL) and blocked with 200 μL of BSA solution. WP (100 μL) preincubated with buffer or 10 mM NaHS were added to wells coated with fibrinogen and incubated at room temperature for 1 h. The wells were rapidly filled with 100 μL of 0.1 M citrate buffer, pH 5.4, containing 5 mM p-nitrophenyl phosphate and 1% Triton X-100 (v/v). After 60 min incubation at room temperature, the reaction was stopped by the addition of 70 μL of 2 M NaOH. The p-nitrophenol produced was measured with a microplate reader at 405 nm against a platelet-free blank.

The amount of platelets adherent to protein-coated wells was estimated by measurement of the platelet acid phosphatase activity using a calibration curve.

Clot retraction: PRP pre incubated with buffer or 10 mM NaHS. Plasma was induced to coagulate with 0.4 UI/mL thrombin. The clots were allowed to retract at 37 °C and were photographed at various times (15, 25, 35 and 45 min). Two-dimensional sizes of retracted clots on photographs were quantified using NIH ImageJ software, and retraction was expressed as percentage retraction [1 − (final clot size/initial clot size)].

Statistical significance was determined using Mann-Whitney test, control vs. H_2S ($n = 5$).

Results: H_2S significantly inhibited platelet adhesion to fibrinogen coated plates ($P < 0.01$). In clot retraction, no difference was observed at the end point (45 min). However PRP incubated with NaHS had a delay with statistical significance at 25 and 35 min ($P = 0.001$ and $P = 0.04$ respectively), changing from hyperbola to sigmoid the kinetic curve of clot retraction.

Conclusion: It has been demonstrated that ligand binding to integrin aIIbb3 induces outside-in signaling-mediated Rap1b. It is crucial for the activation of a second pool of Rac1, which is important for clot retraction and platelet spreading. Our results suggest that a pathway, such as CalDAG-GEFI/Rap1-Rac1, could be affected by H_2S. In conclusion, several evidences are accumulating to demonstrate that H_2S donor compounds exert significant effects on antiadhesive activity in prevention of thrombotic states.

PB 2.28-6

A novel real-time whole blood flow cytometric assay identifies a sub-population of platelets that rapidly bind Annexin-V upon stimulation

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Background: The rate of platelet activation may be critical in determining the dynamics of thrombus formation, yet the regulation of platelet kinetics has received less investigation than the maximum extent of platelet response.

Aims: Here we report the development a novel flow cytometric assay designed to measure the kinetics of platelet activation, with the aim of measuring makers of platelet activation that occur rapidly upon activation.

Methods: Whole citrated blood was diluted in Hepes buffered saline containing (i) Fluo4-NW (Invitrogen), or (ii) FITC-anti-fibrinogen antibodies (Dako), PE-anti-P-selectin antibodies (BD), and Cy5-Annexin-V (BD) in combination. All reagents were dialyzed prior to use. Samples, maintained at 37 °C during data acquisition, were stimulated with CRP-XL (1 μg/mL) together with $CaCl_2$ (2 mM) and gly-pro-arg-pro peptide (0.5 mg/mL) (Sigma). Data were acquired for 5 min at approximately one platelet per 1 ms. All data were analysed using R (www.r-project.org) by fitting LOESS curves to the raw data from which the maximum response, the rate and acceleration (rate at which the rate changes) of platelet response were derived.

Results: Coefficient of variation (CV) was below 10% for all assays except acceleration in P-selectin exposure (CV = 16%) which showed increased variability due to the slow rate of P-selectin exposure. No correlation existed between the maximum level and the rate or acceleration of calcium flux, fibrinogen binding, P-selectin exposure or Annexin-V binding ($n = 10$). This assay revealed a small (< 2%) population of platelets that bind Annexin-V rapidly following stimulation. This population is reduced at lower concentrations of CRP-XL, absent with $CaCl_2$ alone, they are small, have high levels of P-selectin exposure, and curiously show differential levels of fibrinogen binding.

Summary/Conclusions: We have developed a robust, reproducible and sensitive assay for the measurement of the kinetics of platelet activation, and identified a sub-population of platelets that bind Annexin-V rapidly after activation. It is intriguing to postulate how this negatively charged population of platelets appearing early in thrombus formation might alter thrombus growth and development.

PB2.29 – Vascular Progenitor and Stem Cells

PB 2.29-1

Evaluation of endothelial dysfunction and regeneration in an elderly population: the Mugello Study

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Background: The precise mechanisms underlying the relationship between increasing age and increased risk for vascular diseases are not completely elucidated.

Circulating progenitor cells (CPC), endothelial progenitor cells (EPCs), and circulating endothelial cells (CEC) may play a role in the development and progression of atherosclerosis.

Clinical studies have demonstrated in young and middle-aged population that EPCs inversely correlated with the presence of traditional cardiovascular risk factors and with circulating pro-inflammatory molecules, but scarce data in the elderly are available.

Aims: We used data from the Mugello Study, a prospective study which enrolled ultranonagenarian subjects, who lived in the Mugello Area to evaluate the relationship between EPC, CPC, CEC and advanced age.

Methods: Circulating CEC, CPCs and EPCs were assessed by flow cytometric analysis and were defined as CD146+/CD31+/CD45−/CD61− (CEC), CD34+, CD133+, CD34+/CD133+ (CPC) and CD34+/KDR+, CD133+/KDR+, CD34+/CD133+/KDR+ (EPC) respectively. We also evaluate circulating levels of C-reactive Protein (CRP) and white blood cell count.

Results: We enrolled 270 ultranonangenarians (198F/72M) with a median age of 92 (90–103) years.

Ultranonagenarians showed a significant higher number of CEC[3 (0–187) vs. 1 (0–23) cells/10^6 events, $P < 0.001$] and a lower number of EPC[CD34+/KDR+:2 (0–28) vs. 10 (3–43) cells/10^6 events; CD133+/KDR+:3 (0–23) vs. 10 (4–45) cells/10^6 events;CD34+/CD133+/KDR+:2 (0–23) vs. 9 (3–43) cells/10^6 events, $P < 0.001$] with respect to younger (median age 65 years, 45–78 years) control population. In elderly and younger subjects CPC number was similar [CD34+:340 (30–1030) cells/10^6 events vs. 336 (259–414) cells/10^6 events; CD133+:314 (30–1030) vs. 321 (246–396) cells/10^6 events; CD34+/CD133+:307 (30–1010) vs. 294 (224–364) cells/10^6 events].

In the Mugello population CEC, CPC and EPC number was not affected by traditional cardiovascular risk factors. Furthermore, we found a significant relationship between CPC and EPC and leukocyte number [CPC: CD34+:$r = -0.162$, CD133+: $r = -0.165$, $P = 0.005$; CD34+/CD133+:$r = -0.190$, $P < 0.01$; CD34+/KDR+:$r = -0.142$; CD133+/KDR+:$r = -0.148$; CD34+/CD133+/KDR+:$r = -0.144$, $P < 0.05$ respectively] and between CEC, EPC number and CRP serum levels [CEC: $r = 0.142$, $P = 0.050$; CD34+/KDR+: $r = -0.317$, $P < 0.001$; CD133+/KDR+: $r = -0.306$, $P < 0.001$; CD34+/CD133+/KDR+: $r = -0.312$, $P < 0.001$ respectively].

Conclusions: Our results demonstrate the presence of an endothelial dysfunction, as documented by high CEC and low EPC number in ultranonagenarians. Moreover the positive relationship between CEC and CRP and the negative relationship between EPC and CRP suggest that an inflammatory state, which is commonly present in the elderly, significantly affects the endothelial function by promoting vascular damage and by reducing the endothelial regenerative capacity.

PB 2.29-2

Adherence to lifestyles' modifications after a cardiac rehabilitation (CR) program and endothelial progenitor cells (EPCs): a 6-months follow-up study

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Background: A significant increase of EPCs number among acute coronary syndrome (ACS) patients participating in a CR program has recently been reported, but no data on the impact of adherence to lifestyle recommendations provided during a CR program on EPCs number are available.

Aim: To investigate the effects of adherence to lifestyles' recommendations on EPCs, inflammatory and functional parameters after 6 months from a CR program in ACS patients.

Methods: One hundred-ten out of 112 ACS patients (90M/20 F; mean age 57.9 ± 9.4 years) completed the study. EPCs, high sensitivity C-reactive protein (hsCRP), NT-ProBNP levels, and cardiopulmonary testings were determined at the end of the program (T1) and at a 6 month follow-up (T2). At T2, a questionnaire assessing dietary habits and physical activity in the previous 6 months was obtained. EPCs were defined as CD34+KDR+, CD133+KDR+ and CD34+CD133+KDR+. hsCRP and NT-ProBNP were measured by nephelometric and immunometric method, respectively.

Results: At T2, we observed a significant decrease of all the three types of EPCs [CD34+KDR+ 10 (0–37) vs. 7 (0–43) $P = 0.01$; CD133+KDR+ 10 (0–33) vs. 7 (0–43) $P = 0.03$; CD34+CD133+KDR+ 10 (0–33) vs. 7 (0–43) $P = 0.03$], of CRP ($P = 0.009$) as well as of NT-ProBNP ($P < 0.0001$). On the basis of dietary habits and physical activity we divided patient population into three categories (no/low adherence to lifestyles' recommendations, moderate adherence; high adherence). An increased adherence to lifestyle recommendations associated to a significant increase in EPCs and Watt max variations between T1 and T2 was observed (Δ CD34+KDR+ p for trend = 0.04; Δ CD133+KDR+ p for trend = 0.02; Δ CD34+CD133+KDR+ P for trend = 0.02; Δ Watt max P for trend = 0.003).

Moreover, the group of patients who reported at the end of the CR program an increase of EPCs and baseline levels of CRP in the lowest tertile (< 2.2 mg/dL) showed, at a 6-month of follow-up, significant higher levels of EPCs ($P = 0.03$), Watt max ($P = 0.003$) and VO2max ($P = 0.002$) and significant lower CRP levels ($P = 0.01$).

Conclusions: Adherence to lifestyles recommendations provided during a CR program is able to influence variations of EPCs' and Watt max, at 6-month follow-up.

PB 2.29-3

Extracellular acidosis inhibits the proangiogenic responses and the tissue regeneration capacity of endothelial progenitor cells

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Background: We have previously demonstrated that extracellular acidosis, a characteristic feature of inflammation, induces apoptosis of hematopoietic progenitors (CD34+ cells). Endothelial progenitor cells (EPC) are a subpopulation within the CD34+ cells which are mobilized from the bone marrow after vascular damage to the site of injury and contribute to tissue revascularization.

Aims: To study the impact of acidosis on the proangiogenic responses and the tissue regeneration capacity of EPC.

Methods: EPC were seeded in EBM medium supplemented with fetal bovine serum, VEGF1, SDF1 and bFGF. The pH of the culture medium (normal value: 7.4) was adjusted at pH values of 7.0 and 6.6 by the addition of an isotonic solution of HCl ($n = 4$–5. *$P < 0.05$ vs. pH 7.4, ANOVA).

Results: We found that while the survival of EPC was not affected by pH, the exposure to acidic medium for 24 h inhibited the proliferation (18 ± 1 and 26 ± 1*% of inhibition at pH 7 and 6.6 respectively, pNPP assay), migration (55 ± 3* and 61 ± 6*%, Boyden chamber), wound repair (9 ± 1 and 69 ± 4*%, mechanical damage of the monolayer) and tubule formation (58 ± 2* and 77 ± 5*%, Matrigel). We also evaluated how acidosis influences the EPC contribution to tissue regeneration in a murine model of hind limb ischemia induced by ligation of the femoral artery. To this end, EPC were previously incubated for 24 h at pH 7.4 or 6.5 and then transplanted intravenously (5 h post-surgery). After 7 days, the percentage of blood flow recovery within the ischemic area was analyzed (Doppler scanning). While the percentage of flow recovery in non-transplanted mice was 49 ± 6%, in those transplanted with EPC grown at pH 7.4 the flow recovery was 78 ± 10*%. However, when the EPC were exposed at pH 6.6, the flow recovery was similar to the non-transplanted animals (48 ± 8%, $n = 4$ nude mice per group).

Summary/Conclusion: Our results suggest that extracellular acidosis inhibits EPC proangiogenic responses *in vitro* and the tissue regeneration capacity of EPC in a murine model of hind limb ischemia.

PB 2.29-4

Down-regulation of TIPE2 in peripheral blood mononuclear cells is associated with low levels of circulating endothelial progenitor cells in patients with type 2 DM

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Backgroud: Activation of the innate immunity system and systemic inflammation are closely involved in the pathogenesis of diabetes and its vascular complications. Circulating endothelial progenitor cells (EPCs) play an important role in the neovasculogenesis and maintenance of vascular homeostasis. Tumor necrosis factor alpha induced protein-8 like-2 (TIPE2) is a very recently identified negative regulator of inflammation that maintains immune homeostasis.

Aims: This study was performed to investigate the relationship between peripheral blood mononuclear cells (PBMCs) TIPE2 and circulating EPCs number in Type 2 Diabetes Mellitus (T2DM).

Methods: Peripheral blood mononuclear cells (PBMCs) were collected from 36 normal subjects and 31 patients with T2DM. Mononuclear cell TIPE2 was measured by real-time PCR. EPCs were defined by CD133(+)/CD34(+)/VEGFR2(+) and quantified by flow cytometry.

Results: The TIPE2 mRNA and the EPCs number in the T2DM group were significantly lower than those in normal controls ($P < 0.01$). The changes of TIPE2 showed significant correlation with the changes of EPC number ($r = 0.341$, $P = 0.003$). Furthermore, the urine albumin/creatinine ratio (UACR) negatively correlate with the TIPE2 mRNA levels ($r = -0.5221$, $P = 0.0013$) and the number of circulating EPCs ($r = 0.8441$, $P = 0.0003$) in all the T2DM patients.

Conclusion: TIPE2 and circulating EPCs number are closely related and could be considered as potential markers of inflammation and renal impairment in T2DM.

PB 2.29-5

Mechanistic study of the proangiogenic effect of osteoprotegerin

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Background: Osteoprotegerin (OPG), a soluble tumour necrosis factor receptor family member, inhibits RANKL-mediated osteoclastogenesis. We have previously reported that OPG enhances the proangiogenic properties of endothelial colony-forming cells (ECFC) *in vitro*, and promotes vasculogenesis *in vivo*. Here we investigated how OPG promotes neovascularisation.

Methods: Umbilical cord blood samples were collected from consenting mothers. Endothelial cells were isolated from human umbilical cords and ECFC from human umbilical cord blood. One day before experiments, cells were growth-arrested for 18 h in EBM2, 3% FCS and released from growth arrest by adding EBM2, 5% FCS, with or without various concentrations (0–0.4 nM, 0–25 ng/mL) of OPG at 37 °C. Proteomic analysis was used to reveal and identify proteins that were differentially expressed in OPG-stimulated and control ECFC. Some of the proteins involved in angiogenesis were further validated and characterized by RT-qPCR, western blot and apoptosis experiments. *In vitro* angiogenesis assay and *in vivo* Matrigel plug assay were performed to validate OPG mechanism of action.

Results: Proteomic experiments showed that OPG pretreatment affected ECFC protein expression in two ways, 23 spots being downregulated and six upregulated. These spots corresponded to proteins involved in cell motility, adhesion, signal transduction and apoptosis. In keeping with these proteomic results, we found that OPG induced ECFC adhesion to activated endothelium in shear stress conditions, promoting intermediate but not focal adhesion to fibronectin and collagen. Treatment with OPG induced a reorganization of the ECFC cytoskeleton, with the emergence of cell protrusions characteristic of a migratory phenotype. These effects correlated with decreased FAK phosphorylation and enhanced integrin $\alpha_V\beta_3$ expression. OPG drastically reduced caspase-3/7 activities and maintained ECFC viability after 48 h of treatment. All these effects were significantly attenuated by ECFC incubation with the CXCR4 antagonist AMD-3100, and by prior heparan sulphate proteoglycan disruption. The proangiogenic properties of OPG appeared to be mediated by the proteoglycan syndecan-1, although OPG 1-194 lacking its heparin-binding domain still had pro-vasculogenic effects *in vitro* and *in vivo*.

Conclusion: These results suggest that OPG may interact with ECFC by binding to HSPGs/syndecan-1, thereby induce an anti-adhesive effect and promoting ECFC migration through a SDF-1/CXCR4 dependent pathway.

PB 2.29-6

Protease-activated receptor 1 and 2 stimulate endothelial colony forming cell vasculogenesis in a vascular endothelial growth factor- and extracellular signal-regulated kinase-dependent manner

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Background: Endothelial cells line the inner side of blood vessels and perform critical physiological functions. Besides guaranteeing normal blood flow through the inhibition of platelets, endothelial cells also play a critical role in vascular repair and angiogenesis. Endothelial dysfunction is associated with atherosclerotic and ischemic syndromes of the cardiovascular system and is a critical cause of cardiovascular diseases. Endothelial progenitor cells (EPCs) are circulating stem-like cells able to differentiate into mature endothelial cells and replenish the endothelial lining at the sites of vascular damage. Their utilisation for cell therapies aiming to restore healthy endothelial lining of blood vessels and stimulate neovascularisation of ischemic tissues has been the object of intense investigation. Different types of EPCs exist depending on the isolation and culture procedure. In this study, we utilise the most recent and promising type of EPCs denominated endothelial colony-forming cells (ECFCs), which is characterised by the ability to proliferate efficiently *in vitro*, to differentiate into fully functional mature endothelial cells, and to form a vascular capillary network both *in vitro* and *in vivo*.

Aims: Following the identification of both protease-activated receptor (PAR) 1 and 2 in ECFCs, we investigated the role of these receptors in the regulation of proliferation, differentiation and tubulogenesis within this cell type.

Methods: The stimulation of PAR1 or PAR2 was obtained with selective activating peptides (H-Thr-Phe-Leu-Leu-Arg-NH2 and 2-Furoyl-Leu-Ile-Gly-Arg-Leu-Orn-NH2, respectively). The effect of PAR stimulation on ECFC proliferation was assessed using a metabolic tracer, while the effect on ECFC differentiation was assessed by immunoblotting for mature endothelial markers, such as platelet endothelial cell adhesion molecule (PECAM-1), vascular endothelial growth factor receptor 2 (VEGFR2), VE-cadherin and von Willebrand Factor (VWF). The effect on the vasculogenic activity of ECFCs was tested in a Matrigel[TRADEMARK]-based tube formation assay. Finally, the molecular mechanism underlying ECFC regulation by PAR1 and PAR2 was dissected using phospho-specific immunoblotting for active extracellular signal-regulated kinase (ERK) and by inhibiting either ERK (e.g. PD98059) or VEGF (e.g. VEGF inhibiting antibody) in different functional assays.

Result: ECFC proliferation was not affected by PAR1 or PAR2 stimulation. On the other hand, both PAR1 and PAR2 stimulation resulted in a significant increase of mature endothelial marker expression. In particular, VEGFR2 expression was between 2 and 4 times higher in ECFCs treated with PAR1- or PAR2-activating peptides compared to controls. This upregulation of VEGFR2 was abolished by the ERK inhibitor PD98059. The stimulation of either PARs also resulted in a significant increase in the activation of ERK and in a potentiation of the vasculogenic response of ECFCs cultured on Matrigel[TRADEMARK]. Interestingly, the inhibition of ERKs with pharmacological tools (e.g. PD98059) or the antibody-based inhibition of VEGF resulted in the ablation of the PAR-stimulated tube formation.

Summary/Conclusions: Taken together, PAR1/2 activation promotes the maturation of ECFCs and stimulates their vasculogenic activity in an ERK- and VEGFR2-dependent manner. Therefore, activating PAR1 and PAR2 either *in vivo* or *ex vivo* may represent a valid approach for the stimulation of ECFC-dependent vascular repair or ischemic tissue revascularisation.

PB2.30 – Microparticles and Disease – II

PB 2.30-1

Characterization of circulating microparticles in pancreatic and colorectal cancers and chronic inflammatory diseases

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Background: Microparticles have intensively been described as potential biomarkers and biovectors in coagulation, inflammation and

cancer. Pancreatic and colorectal cancers (CRC) are known to have elevated annexin V-positive microparticles in the plasma. Procoagulant activity of microparticles seems also to be elevated in these two types of cancer and in an inflammatory context. However, to date, no study has compared the quantity and the repartition of circulating microparticles in cancer and chronic inflammatory diseases.

Aims: The aim of the present study was to compare the quantity and the origin of circulating microparticles between cancers associated with thrombosis and chronic inflammatory diseases.

Methods: A prospective cohort included patients with pancreatic adenocarcinoma ($n = 29$), CRC ($n = 83$), chronic pancreatitis ($n = 14$) and Crohn's disease ($n = 14$). Healthy controls ($n = 19$) were also included. Microparticles were isolated from blood samples. High-sensitivity flow cytometry using fluorescents beads with appropriate sizes was performed to identify and quantify annexin-V-, platelet-, endothelial-, erythrocyte- and leukocyte-derived microparticles.

Results: Patients with a pancreatic cancer had significantly increased levels of annexin-V positive microparticles compared to patients suffering from a CRC ($P < 0.001$). Surprisingly, we didn't observe significant difference in the level of annexin-V positive microparticles, between each cancer (pancreas and CRC) and its equivalent inflammatory state (chronic pancreatitis and Crohn's disease, respectively). Although the repartition of microparticles according to their origin was similar in pancreatic and CRC, there was a significant difference compared to chronic pancreatitis or Crohn's disease. Platelets-derived microparticles were significantly increased and endothelial cell-derived microparticles were significantly decreased in chronic inflammatory diseases in comparison with cancers ($P < 0.03$). Theses significant differences were amplified with healthy controls.

Conclusions: The repartition of cells-derived microparticles significantly differs between cancer, chronic inflammatory disease and healthy controls. This difference allows allocating a hallmark for the different pathologies.

PB 2.30-2

Increased microparticles activities as a marker of platelet activation after percutaneous coronary intervention

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Background: Percutaneous coronary intervention (PCI) is a procedure to open the occlusive coronary artery. It was reported that restenosis of coronary artery may occurred after PCI. Balloons pumping during PCI can cause de-endothelialisation that result in exposure of subendothelial tissue which may activates platelets. There are several biomarkers of platelet activation, among others is microparticle which is mainly consist of phospholipid with procoagulant activities.

Aims: To compare microparticles activities in coronary artery before and after percutaneous coronary intervention as an evidence of platelet activation due to de-endothelialisation.

Methods: Twenty three MCI patients consisted of seven female and 16 male who underwent PCI were enrolled in this study. Three mL of blood were withdrawn from coronary artery just before ballooning and immediately after stenting, and then mixed with sodium citrate as anticoagulant. Determination of microparticle or phospholipid (PPL) activities were done based on clotting time of a mixture of plasma sample and phospholipid deficient plasma with the addition of activated factor X and calsium ion. The result of PPL activities were expressed as PPL ratio, i.e. clotting time of patient plasma divided by average of clotting time in healthy subjects. The proposal of this study has been approved by the ethical committee of Harapan Kita Cardiac Center and all of the subjects has given informed consent.

Results: The median (range) of PPL ratio before PCI (ballooning) was 1.3 (0.7–3.1) while after PCI it was 1.1 (0.5–1.5). The difference was statistically significant. The clotting time after PCI was shorter compared with that before PCI indicated higher phospholipid activities in the plasma sample after PCI.

Conclusions: The microparticle activities after PCI was higher than that before PCI indicated platelet activation occurred during PCI.

PB 2.30-3

Microparticle detection in human plasma using nanoparticle tracking analysis and scanning ion occlusion sensing

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Background: In recent years cell-derived microparticles (MPs) have attracted growing attention as possible contributors to a wide range of physiological and pathological processes in the human body, including coagulation and thrombogenesis. Although showing promising potential as biomarkers and therapeutic targets the diversity of the size and composition of MPs as well as their susceptibility to being affected by pre-analytical handling entail a major challenge when it comes to standardizing their measurement and interpreting the results.

Aims: We evaluate the ability of two recently developed methods applicable for detection of MPs at the lower end of the MP size range to perform precise particle size and concentration measurements as well as the influence of pre-analytical parameters and instrument settings on the results. In continuance we will establish preliminary reference intervals for MP concentrations for the two methods using defined instrument settings.

Methods: Within- and between-day variation, effect of freezing at −80 °C, storing and diluting (with DPBS buffer) plasma samples as well as the impact of collecting postprandial vs. fasting samples is assessed using nanoparticle tracking analysis (NTA; Nanosight®) and scanning ion occlusion sensing (SIOS; qNano®). Preliminary reference intervals for MP concentration in fasting as well as non-fasting individuals will be calculated from measurements performed on plasma samples from 20 healthy individuals.

Results: Whereas NTA and SIOS do not agree on the absolute MP concentrations and size distributions they separately exhibit a within- and between-day variation that suggests they will be applicable for plasma MP level measurements in studies of patients. For SIOS we find direct proportionality between the calculated and measured MP concentration within the examined range of dilution factors. Freezing significantly changes and generally increases MP levels but variably. Preliminary results show that postprandial samples have a significantly higher concentration of MPs compared to fasting samples, and with the rise of MP concentration as a result of food intake being of substantially varying magnitude the reference intervals for non-fasting individuals is expected to be considerably broader than for fasting individuals.

Conclusions: NTA and SIOS display promising abilities as methods for MP level detection in plasma samples and while they cannot be expected to provide exact absolute concentrations and size distributions over the whole range of MPs present in plasma they complement each other and existing methods for MP measurement such as flow cytometry in order to identify differences in MP levels between healthy persons and relevant patient populations. Based on our preliminary results we recommend MP measurements to be performed on fresh plasma samples from fasting individuals as results from non-fasting as well frozen samples should be interpreted with certain reservations.

PB 2.30-4

Microparticles do not modify the antithrombotic properties of brain microvascular cells

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Background: High levels of membrane-shed submicron microparticles (MP) circulate in the peripheral blood of patients with thrombotic diseases including ischemic stroke. While circulating MP are useful biomarkers of cell activation/apoptosis, their role in the development of thrombosis remains uncertain.

Aims: To study the potential effects of MP from ischemic stroke patients on the antithrombotic properties of brain microvascular compared to macrovascular endothelial cells focusing on the interactions between platelets and endothelial cells under static and flow conditions and on thrombin generation.

Methods: MP were purified from lipopolysaccharide-stimulated blood or from blood of patients with ischemic stroke ($n = 16$) or stroke mimics ($n = 8$). MP were then quantified and characterized by flow cytometry. After 24 h co-incubation of MP (3000 annexin V+/μL) with human brain microvascular endothelial cells (hCMEC/D3) or human umbilical vein endothelial cells (HUVEC), adhesion of platelets to endothelial cells under static conditions was quantified by fluorescent microscopy using a CD42b labelling. Platelet adhesion was also assessed under high shear stress (1800/s) in a parallel flow chamber and strings of von Willebrand factor covered by calcein-loaded platelets were quantified by fluorescence microscopy. Modulation of the antithrombotic properties of endothelial cells by MP was assessed by measuring thrombin generation at the cell surface by means of the Calibrated Automated Thrombography method in platelet-rich plasma in the absence of tissue factor.

Results: In resting conditions, platelet adhesion was less intense on hCMEC/D3 than on HUVEC under both static and flow conditions (covered surface: 0.3 ± 0.1 vs. 1.6 ± 0.5%, $P = 0.05$ and 0.9 ± 0.03 vs. 5.8 ± 1.9 arbitrary units, respectively). Platelet adhesion increased after agonist stimulation of HUVEC only (4.7 ± 1.4% vs. 1.6 ± 0.5%, $P = 0.09$ after stimulation with tumor necrosis factor/interferon-gamma for static experiments and 28.9 ± 11.6 vs. 5.8 ± 1.9 arbitrary units after stimulation with IBMX/forskolin for flow conditions). These differences could be related to the less intense expression of von Willebrand factor by hCMEC/D3 than by HUVEC. When preincubated with endothelial cells, MP from stimulated whole blood did not modify platelet adhesion on either hCMEC/D3 or HUVEC. To evaluate the modification of antithrombotic properties of the endothelium by MP, we measured thrombin generation at the endothelial cell surface. Under resting conditions, thrombin generation was higher at the surface of hCMECs/D3 compared to HUVEC (peak 47.9 ± 14.7 vs. 9.5 ± 4.3 nM, $P = 0.02$) but lower than in absence of endothelial cells (peak 60.4 ± 6.3). Incubation with whole blood-derived MP for 4 h prior to thrombin generation led to a slight but not significant increase in peak (18.8 ± 3.7 vs. 9.5 ± 4.3 nM, $P = 0.15$) and decrease of lagtime (17.3 ± 2.3 vs. 25.4 ± 6.2 min, $P = 0.2$) only on HUVEC. MP isolated from stroke patients or stroke mimics did not modify thrombin generation on both cell lines.

Conclusions: In this study, we found that MP isolated from stimulated whole blood or from stroke patients did not promote either platelet adhesion or thrombin generation on endothelial cells from brain microvascular (hCMEC/D3) or from macrovascular (HUVEC) origin. However, we provide evidence that these endothelial cells differ regarding their relation to haemostasis that could be useful in understanding the physiopathology of stroke.

PB 2.30-5

Circulating microparticles in patients with colorectal cancer

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Background: Microparticles (MPs) are subcellular fragments shed from cell membranes of different cell types through a process of vesiculation and are believed to play a role in thrombus formation *in vivo*. Numerous studies have found increased levels of microparticles in patients with solid and haematological malignancies. Few of these studies, however, have focused on patients with colorectal carcinoma (CRC).

Aim/Objective: In this prospective study we aimed to investigate whether levels of endothelial (EMPs; CD62+), platelets (PMPs; CD41+) and Tissue Factor-bearing MPs (TF + MPs) are elevated in patients with stage 1–3 CRC compared to healthy subjects and to determine whether these levels decrease significantly after surgical resection of the tumor.

Materials and Methods: MPs plasma levels were measured in 36 patients with colorectal cancer and 16 healthy controls, using a multiparameter flow cytometric assay. Blood samples were taken pre-operatively and during 5 months after surgery.

Results: When compared to controls, patients showed significantly higher median levels of PMPs (2268 and 280079 mp/L; $P < 0.0001$) and EMPs (2268 and 2807 mp/L; $P < 0.0001$). This was also the case for TF + MPs (1613 and 15028 mp/L; $P < 0.0001$). Patients with more advanced stages of cancer seemed to have higher levels of MPs, but no significant difference was found.

There was no significant decrease in any of the MPs levels during the 5 months after surgery. Subanalysis of groups with early and more advances stages of CRC did not change these results.

Conclusions: Our results show that patients with CRC have significantly higher levels of circulating MPs when compared to controls. No significant decrease in MPs levels were found In the 4 months after surgery.

PB 2.30-6

Biological markers in the progression of asymptomatic carotid stenosis

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Background: Asymptomatic carotid stenosis presents a high risk of suffering an ischemic stroke, and it is important to stratify said risk in order to select the cases that could benefit from preventive interventional treatment.

Aim: To determine biomarkers that relate to the progression of carotid stenosis.

Patients and Methods: We studied 40 patients with asymptomatic > 50% carotid stenosis and 40 healthy subjects. Circulating markers of endothelial damage (circulating endotelial cells, CECs; circulating microparticles, MPs; von Willebrand factor, vWF), inflammation (Interleukin-6, IL6; C-reactive protein, CRP; Fibrinogen, Fg) and tissue factor (TF) were determined.

Results are expressed as mean values ± standard deviations (SD). Data were analyzed using SPSS version 15.0., P-values < 0.05 were considered statistically significant. Statistical comparisons were performed using the Student's t test, and the analysis of variance (ANOVA) with Bonferroni *post hoc* analysis. Spearman's correlation test (IC 95%) was used.

Results: The levels of all markers studied were statistically higher in patients compared to controls: (i) endothelial damage markers, CECs (90 ± 61 vs. 10 ± 6 cell/mL, $P < 0.0001$), MPs (13344 ± 7944 vs.

8916 ± 5820 event/120s, $P < 0.05$) and vWF (220 ± 60 vs. 102 ± 29%, $P < 0.0001$); (ii) inflammatory markers: CRP (3.7 ± 5.3 vs. 1.1 ± 0.87 mg/dL, $P < 0.01$); Fg (316 ± 45 vs. 248 ± 37 mg/dL, $P < 0.0001$), and IL6 (2.5 ± 2.9 vs. 0.8 ± 0.7 pg/mL, $P < 0.02$).
MP levels showed a positive, significant correlation with TF ($r = 0.43$) and CRP ($r = 0.42$) ($P < 0.05$). Furthermore, levels of IL6 correlated with those of TF ($r = 0.61$; $P < 0.001$) and CRP ($r = 0.39$; $P < 0.5$), and Fg levels with CRP ($r = 0.42$; $P < 0.05$).
Conclusions: Asymptomatic carotid stenosis patients show elevated circulating markers of endothelial damage and inflammation. Our results indicate that markers of endothelial damage are those with the greatest increase, supporting, therefore, its role in carotid stenosis. We have presented initial data from a study whose purpose is to ascertain which set of biological markers and parenchymal or functional MRI findings can serve as predictive factors in asymptomatic carotid stenosis.
Supported by grant FIS PI10/00473.

PB2.31 – Endothelium and Disease

PB 2.31-1
Circulating endothelial cells in venous thromboembolism

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Background: The sequence of events leading to venous thrombosis is not well known. Recently it was suggested that activation of the coagulation system mediated by tissue factor precedes platelet activation in venous thrombosis. The source of tissue factor is not clear. Activated endothelial cells have been shown to express tissue factor *in vitro* and probably they may promote expression of tissue factor in intact venous endothelium.
Aims: To quantify CEC (circulating endothelial cells) and CEP (circulating endothelial progenitors) and evaluate soluble markers of endothelial activation levels in patients with venous thromboembolism (VTE). To characterize the CEC for the expression of activation (CD54, CD62E) and procoagulant (CD142) markers and investigate whether the expression of these markers correlates with other clinical and laboratorial data.
Methods: We recruited 16 patients with previous VTE (at least 1 month after the thrombosis) and 20 healthy individuals. The CEC were characterized and quantified by flow cytometry (FACS Calibur®, Becton Dickinson) using a single-platform method. The antibodies used were anti-CD146, anti-CD45, anti-CD133, anti-CD54, anti-CD62E and anti-CD142. CEC were identified as CD146 + CD45-CD133-. The soluble markers of endothelial activation evaluated by ELISA essays were: sP-selectin, sE-selectin, sICAM-1, sVCAM-1 (eBioscience); IMUNOBIND Tissue Factor (American Diagnostica Inc); vWF Ag (Siemens). We also collected the number of thrombosis episodes and the levels of fibrinogen, homocysteine, D-dimmers, FVIII, protein S, protein C and anti-thrombin.
Results: In VTE patients the number of CEC was increased ($P < 0.001$) and the number of CEP was decreased ($P = 0.029$). There was a statistically significant increase in the number of activation (CD54, CD62E) and procoagulant (CD142) markers, although the soluble markers levels (sICAM-1, sE-Selectin and sTF) weren't increased. There was a statistically significant increase in the levels of sVCAM-1 and vWF:Ag. The number of CD142+ CEC and the level of sTF were correlated with the number of thrombosis.
Conclusion: In our study, the number of CEC in patients with previous VTE is increased and they express activation and procoagulant markers. These results suggest that CEC are involved in the pathophysiology of venous thrombosis.

PB 2.31-2
Endotelial nitric-oxide synthase gene polymorphisms in early and late-onset severe preeclampsia

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Backgound: Preeclampsia (PE) is a systemic syndrome that occurs in 3–5% of pregnant women and classically manifests as new-onset hypertension and/or proteinuria after 20 weeks of gestation. PE is a leading cause of maternal and neonatal morbidity and mortality. PE progresses with vasoconstriction, hipercoagulability and endothelial dysfunction, but the mechanisms are not fully understood. The L-arginine-NO pathway appears to play a central role in both normal pregnancy and PE. Several polymorphisms affecting endothelial nitric oxide synthase (eNOS) production levels or function were described, including G894T (Glu298Asp; exon 7), VNTR b/a (variable-number 27-bp tandem repeat; intron 4) and T-786C (promoter) polymorphisms in eNOS gene.
Aim: To investigate the frequency of these polymorphisms in early and late-onset severe preeclamptic and normotensive women.
Methods: This case-control study enrolled 53 women with early-onset severe PE, 45 women with late-onset severe PE and 103 nomotensive pregnant. DNA was extracted from whole blood using a mini Spin Kit. G894T (*Mbo*I) and T-786C (*Msp*I) genotyping was performed by PCR-RFLP analysis. The VNTR b/a polymorphism was detected by PCR allele specific. Statistical analysis was performed by qui square test (SPSS 13.0).
Results: The frequency of TT genotype (G894T) in the late-onset group (20%) was higher than control group (1.9%) ($P = 0.002$). The frequency of aa genotype (VNTR b/a) in the early-onset group (13.2%) was higher than late-onset group (2.2%) and control group (1.0%) ($P = 0.002$). No difference was observed for T-786C polymorphism among the groups.
Conclusion: Considering these genotypes frequencies, these results suggest that NO synthesis can be involved in vasoconstriction, hipercoagulability and endothelial dysfunction in PE cases, mainly in the late-onset form.
Keywords: Preeclampsia, nitric oxide, polymorphisms, endothelium.

PB 2.31-3
Evidence of endothelial dysfunction in patients with major depression: recovery after treatment with a selective serotonin reuptake inhibitor (SSRI)

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Background: New emerging concepts suggest an association between major depression, inflammation and cardiovascular risk. Alterations of serotonergic mechanisms and its modulation could be involved not only in the enhanced cardiovascular risk in major depression, by promoting the activation of platelets and coagulation mechanisms, but also inducing endothelial dysfunction at the vascular level.
Aims: To investigate the possible association between major depression and endothelial dysfunction and the potential corrective effect of an antidepressive treatment with a selective serotonin reuptake inhibitor (SSRI).
Methods: To compare the degree of endothelial damage in samples from healthy subjects (CON) and major depressive patients at the time of diagnosis (P0), and after 8 (P8) and 24 weeks (P24) of treatment with a SSRI (S-citalopram), different markers were analyzed: (i) circu-

lating endothelial cells (CEC) and endothelial progenitor cells (EPC), by flow cytometry, (ii) soluble levels of von Willebrand factor (vWF) and VCAM-1 in plasma samples, by ELISA techniques; and (iii) changes in the expression of the adhesion receptor ICAM-1 on cultured endothelial cells (HUVEC) exposed to sera from both groups by immunofluorescence.

Results: Levels of CEC in P0 samples were increased with respect to healthy controls (99.9 ± 11.5 vs. 48.5 ± 7.2 CEC/mL, $P < 0.01$). Along the SSRI treatment, levels of CEC decreased progressively reaching values similar to controls (P8: 81.2 ± 15.6 and P24: 49.7 ± 7.5 CEC/mL, respectively). The EPC values were lower in P0 samples compared to healthy subjects, without reaching statistical significance. Plasma levels of VWF and VCAM-1 in P0 samples were increased vs. healthy controls (vWF: 126.5 ± 11.7% vs. 87.1 ± 8.1%, $P < 0.01$; and sVCAM-1: 768.7 ± 110.4 vs. 532.8 ± 31.2 ng/mL, $P < 0.05$ respectively), but no significant differences were observed during the SSRI treatment. The exposure of HUVEC to P0 sera samples resulted in an increment of the expression of ICAM-1 on the cell surface with respect to CON cells. Expression of ICAM-1 was normalized when HUVEC were exposed to samples from patients under SSRI treatment for 24 weeks (P24).

Conclusions: Our results confirm the presence of endothelial dysfunction associated with major depression and indicate that treatment with a selective serotonin reuptake inhibitor have a potential protective role in the modulation of the pro-inflammatory state developed in these patients.

PB 2.31-4

Endothelial activation in patients with superficial vein thrombosis (SVT) of the lower limbs

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Background: The association between venous thromboembolism (VTE) and arterial cardiovascular events has been confirmed by several epidemiologic and experimental studies in the last few years. We have previously shown that patients with spontaneous VTE present an endothelial dysfunction, suggesting that endothelium is the link between venous and arterial thrombosis. Superficial vein thrombosis (SVT) of the lower limbs has long been considered a benign disorder, but more recently several studies have demonstrated that SVT shares the same risk factors of VTE and carries a relevant risk of thromboembolic complications. As a result, it has been advocated that SVT should be considered the superficial venous manifestation of VTE. No data are available on SVT and endothelial function.

Aims: Aim of the present study was to establish whether patients with symptomatic SVT of the lower limbs have altered circulating markers of endothelial activation.

Methods: One hundred and twenty-four patients with SVT of the internal or external saphenous veins or their collaterals of at least 4 cm in length underwent blood sampling for measuring soluble P-selectin and von Willebrand factor antigen (vWFAg), markers associated with damage of the endothelium, on the day of diagnosis, before starting treatment. The severity of symptoms was also determined using the Visual Analogue Score (VAS) for pain.

One hundred and seventeen healthy controls, matched for sex and age (M/F:53/64, mean age 65), were simultaneously studied. A subgroup of SVT patients ($n = 22$) was reassessed at a subsequent time, distant from the event (2 months later).

Results: Soluble P-selectin was signficantly higher in SVT patients (M/F:56/68, mean age 66), than in controls (mean ± SD: 38 ± 13.4 ng/mL vs. 34 ± 25 ng/mL; $P = 0.029$). vWFAg was also higher in patients than in controls (mean ± SD: 209 ± 82% vs. 154 ± 75%; $P < 0.0001$). At follow up, P-selectin and vWFAg remained significantly higher in SVT patients than in controls (42.2 ± 3.2 ng/mL and 189.1 ± 8.2% respectively), without significant differences with the acute phase.

No correlation was observed between the levels of vWFAg or P-sel and the extension of SVT or the rate of subsequent recurrent SVT events.

sPsel levels, but not VWF:Ag levels, were significantly higher in patients with a Visual Analogue Score for pain > 5 (27.4 ± 2.5 ng/mL vs. 22 ± 2.55 ng/mL; $P = 0.047$).

Conclusions: Patients with symptomatic SVT of the lower limbs have a systemic perturbation of endothelial function, as revealed by significantly higher plasma levels of markers of endothelial activation. Endothelial dysfunction is not due to the inflammatory reaction accompanying acute SVT. Increased endothelial-activation markers do not seem to predict recurrent superficial events

PB 2.31-5

Toll-like receptor 4 and the inflammasome NALP3 mediate the activation and damage of endothelial cells in advance chronic kidney disease: protective effect of defibrotide

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Background/Aim: Endothelial dysfunction (ED) is a common pathologic condition for the development of atherothrombotic complications in many diseases. Our group has been able to reproduce *in vitro* and characterize the ED associated with chronic kidney disease (CKD). However, the relative contribution of the uremic environment, the impact of the substitutive therapies, and the precise molecular mechanisms involved are still under investigation. Involvement of the innate immunity receptor Toll like receptor (TLR) 4 and the inflammasome NALP3 in the inflammatory response of endothelial cells was explored.

Methods: Cultured endothelial cells were exposed to sera from three groups of CKD patients, under: conservative treatment (predialysis) (PreD), undergoing hemodialysis (HD) and peritoneal dialysis (PD). Activation of the inflammation related proteins p38MAPK, Akt, and the transcription factor NFkappaB, changes in the expression of TLR4 and ICAM1 and activation of the NALP3 components were evaluated in these cells. The effect of the novel pharmacological agent with pleiotropic actions on hemostasis, defibrotide, was also investigated in this *in-vitro* model.

Results: TLR4 was found to be expressed in EC both in the cytoplasm and at the surface. After exposure to the sera under study, there was an increase in the expression of TLR4 and a redistribution to their luminal surface in the three situations (PreD, HD and PD). This effect was paralleled by activation of the stress protein Akt and the transcription factor NFκB, together with a higher expression of ICAM1 at the EC surface, which was partially prevented by an antibody to TLR4. Exposure of EC to the sera from PreD, HD and PD groups led to the assembly of NALP3 and TXNIP proteins, with the activation of caspase-1 and release of IL-1β. This inflammatory response was observed in association with the three uremic groups, being more intense in response to the sera from dialyzed patients. Defibrotide was able to prevent these changes.

Conclusion: TLR4 and the multiprotein oligomer NALP3 inflammasome, two crucial elements of the innate immune response, were found to be associated with the development of endothelial activation and damage in response to chronic inflammation and oxidative stress. Further research is needed to explore their usefulness as potential targets to prevent the development of endothelial damage. In addition, defibrotide showed a preventive effect on the inflammatory reaction observed.

PB 2.31-6

Biomarkers of endothelial dysfunction in cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL)

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Background: Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL), is an inherited disease due to cerebral microangiopathy presenting with variable pictures, including stroke, progressive cognitive impairment and disability. Among pathogenic processes, endothelial dysfunction has been hypothesized.

Aim: To evaluate the role of biomarkers of endothelial dysfunction (EPCs and CPCs, von Willebrand factor [vWF], and thrombomodulin [TM]) in a large CADASIL series.

Methods: Forty-five CADASIL patients and 33 sex and age-matched controls were enrolled. EPCs were measured by using flow cytometry and defined as positive for CD34/KDR, CD133/KDR and CD34/CD133/KDR; CPCs as positive for CD34, CD133 and CD34/CD133. vWF and TM were measured using commercially available ELISA kits.

Results: In comparison with controls, CADASIL patients presented significantly lower EPCs levels [CD34/KDR:0.07 vs. 0.1 cells/μL, $P = 0.009$; CD133/KDR:0.07 vs. 0.1 cells/μL, $P = 0.008$; CD34/CD133/KDR:0.05 vs. 0.1 cells/μL, $P = 0.002$], significantly higher vWF activity [130.2 vs. 91.7%, $P = 0.014$] and similar levels of TM [29.2 vs. 27.4 ng/mL, $P = 0.955$].

CPCs were not significantly lower in CADASIL, but patients with stroke or dementia had significantly reduced CPCs levels than patients without [CD34:1.85 vs. 2.83 cells/μL, $P = 0.014$; CD133:1.77 vs. 2.78 cells/μL, $P = 0.009$; CD34/CD133:1.77 vs. 2.73 cells/μL, $P = 0.009$]. Both vWF and TM were not significantly higher in the same subgroup of CADASIL patients with a more severe phenotype.

Conclusions: This is the largest series of CADASIL patients in which biomarkers of endothelial dysfunction have been studied. We confirmed the previously reported association between EPCs and CPCs and the disease, and we found an association with vWF, supporting the hypothesis of the presence of endothelial dysfunction in this disease and its potential role in modulating phenotype.

PB2.32 – Immune Thrombocytopenia Purpura – II

PB 2.32-1

Restrospective analysis of the incidence of refractory ITP and response to second line treatments. Experience in a single centre.

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Background: Primary Immune Thrombocytopenia (ITP) is an acquired autoimmune disease. It is characterized by an accelerated destruction and an inadequate production of platelets. Corticosteroids are commonly used as first line treatment but the incidence of recurrent ITP and response to second line treatments is not well established.

Aims: The aim of this review is to analyze the incidence of recurrent ITP and its response to second-line treatments.

Methods: We conducted a retrospective, observational study based on the follow-up of patients diagnosed with ITP at our centre during the last 28 years. Patients were classified by first and second line treatment administrated and by the obtained response and its duration. We defined a complete response as platelet count > 100×10^9/L for more than 6 months without treatment and with no bleeding symptoms. Maintained response was defined as platelet count > 50×10^9/L, for more than 6 months without treatment and with no bleeding symptoms.

Results: Seventy-one patients were diagnosed with ITP, receiving treatment 90.1% (64 patients). Sixty-two of them (96.8%) were treated with corticosteroids as first line treatment. Global response was 74.2%, but only 11.3% could be classified as complete responses and 19.3% as maintained responses. The rest of the patients did not achieved response according to the criteria defined above. Average time of response were 48.5 months (8–156) and 58.1 months (16–156) for complete and maintained response respectively.

Most frequent second line treatments were romiplostim, rituximab and splenectomy. Twenty four patients (37.5%) received romiplostim, 70.1% of them as second line treatment. 8.3% obtained a complete response with an average duration of response of 18 months (10–26). Twenty-four percent of them (10 patients) obtained response but are still on treatment. A synergistic effect seems to exist in associating low dose corticosteroids and romiplostim, but this fact is not analyzed in this review due to short time follow-up.

Twelve patients (18.7%) received rituximab. Three of them (25%) achieved complete response, that lasted an average time of 62 months (25–80 months). One of them achieved a maintained response that lasted for 24 months.

A total of twenty one patients (32.8%) underwent splenectomy. 28.6% of them (six patients) reached complete response. Average time of response was 53 months (10–108). One patient reached a maintained response that lasted 14 months.

Conclusions: First-line treatment for ITP was corticosteroids in the majority of patients. However, the incidence of recurrent ITP is higher than expected, reaching 76%. Almost 20% of the patients reached a maintained response.

Second line treatments rescued 31.4%, all of them reaching long term complete responses. Rituximab and esplenectomy achieved the best durable responses.

It is too soon to analyze the incidence of long term complete responses related to romiplostim, although an 8.3% of the patients reached a complete response. It is an effective salvage therapy, although it is hard to discontinue treatment.

16.2% of the patients receiving second line treatments achieved a maintained response, concluding that 47% of refractory/recurrent patients were rescued with second line treatments

PB 2.32-2

Differential effects of All-Trans Retinoid on expansion of CD4+CD25+Foxp3 Treg subpopulations in patients with chronic immune thrombocytopenia

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Objective Immune thrombocytopenia (ITP) is an immune-mediated disorder. In addition, T-cell is also involved in platelet destruction or marrow suppression. During immune responses CD4+ T cells can differentiate into a range of cell types, including T helper type 17 (Th17) and regulatory T cells (Treg). The goal of the present study is to investigate the therapeutic effects of all-trans-retinoic acid (ATRA) plus prednisone treatment on adult refractory idiopathic thrombocytopenic purpura (RITP) and to further explore the underlying mechanisms.

Methods: The concentrations of the peripheral blood $CD4^+$ T cells (TH17,nTREG and mTREG) were analyzed by flow cytometry,the levels of IL-17, IL-10, TGF-β cytokines were confirmed by enzyme-linked immunoassay (ELISA), and the expression of RORγ, Foxp3 were detected by semiquantitative reverse-transcription polymerase chain reaction (RT-PCR) in 45 RITP patients before and after treatments, with 20 normal individuals as respective controls.

Results: The overall response rate of ATRA plus prednisone treatment in RITP patients was 48.9%, 22.2% ($n = 10$) of patients with a complete response and 26.7% ($n = 12$) of patients with a partial response. The levels of regulory T cells were significantly increased in patients after ATRA plus prednisone treatment,especially $CD31^{-}CD4^{+}CD25^{+}CD127^{+}CD45RO^{+}$ mTreg ($P < 0.05$); The concentrations of IL-10, TGF-β was increased ($P < 0.05$),whereas IL-17 factor showed no obvious change in the effective groups after treatment ($P > 0.05$). The expression levels of Foxp3 enhanced dramatically after treatment in real-time RT-PCR analysis ($P < 0.05$). However, the concentrations of ROR-γ showed no obvious change after the therapy in real-time RT-PCR analysis ($P > 0.05$).
Conclusions: Sixty percent of ARITP patients recovered after ATRA plus prednisone treatments. The therapeutic effects of ATRA plus prednisone may be involved in the increased levels of mTreg cells in peripheral blood.

PB 2.32-3
Nonclassical monocytes are expanded and involved in the pathogenesis of immune thrombocytopenia

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Background: Human monocytes are heterogeneous and play an important role in the inflammatory and autoimmune response. CD16+ monocytes have been described to increase in active immune thrombocytopenia (ITP) patients. Recently, based on function and phenotype, monocytes are classified into three subsets, with the CD16+ subset further subdivided into the intermediate (CD14++CD16+) and the nonclassical (CD14+CD16++) group.
Aim: To investigate the distribution of monocyte subsets in ITP patients and their possible role in the pathogenesis of this disease.
Methods: Seventy-three patients diagnosed with ITP (47 females and 26 males, median age: 40 years; median platelet counts: 23×10^9/L) were enrolled in this study after informed consent obtained. This study was approved by the medical ethic committee of our hospital. All patients had not been treated for at least 1 month before sampling. The frequencies of monocyte subsets were determined by flow cytometry. Cytokine production from monocyte subsets was measured by ELISA. The sorted monocyte subsets were co-cultured with CD4+T cells and autologus platelets ($n = 12$) respectively. The proliferation of auto-reactive CD4+ T cells was determined by BRDU ELISA. To detect the relationship between monocyte subsets and T cell differention, the intracellular IL-17A, IFNG and IL-4 levels of T cells co-cultured with different monocyte subsets were analyzed by flow cytometry. Nonparametric statistic tests were used: Mann-Whitney U test or Wilcoxon test was performed for independent or related two-group analysis, respectively. $P < 0.05$ was considered significant and results were described as median [inter-quartile range].
Results: The frequency of nonclassical monocytes was significantly increased in active patients ($n = 73$) compared with controls ($n = 49$), (untreated ITP: 5.44% [7.52%] vs. Ctrl: 1.83% [2.34%] $P < 0.001$). In 23 patients, the monocyte subsets were reevaluated after treatment with glucocorticoids for 7 days, nonclassical monocytes were decreased markedly (untreated: 4.95% [4.7%] vs. treated: 0.96% [4.35%] $P < 0.001$). The frequency of intermediate monocytes was increased in active patients (untreated ITP: 10.54% [3.58%] vs. Ctrl: 7.6% [6.2%] $P < 0.001$) and remained stable after glucocorticoids therapy (untreated: 10% [4.2%] vs. treated: 9.79% [9.18%] $P = 0.798$). The frequency of classical monocytes was decreased in active patients (untreated ITP: 82.6% [7.21%] vs. Ctrl: 90.18% [7.31%] $P = 0.001$) but recovered after glucocorticoids therapy (untreated: 83.15% [9.32%] vs. treated: 88.62% [7.73%] $P = 0.019$). Compared to the controls, serum TNF-α ($P = 0.004$) and IL-1β ($P < 0.05$) levels in ITP patients were elevated. After stimulation with LPS for 20 h, nonclassical monocytes produced more TNF-α ($P < 0.05$) and IL-1β ($P = 0.057$) than the intermediate. However, the three subsets showed no difference in the proliferation of platelet reactive T cells. Further studies demonstrated that nonclassical monocytes skewed Th1/Th2 response (Th1/Th2: nonclassical 15.36 ± 4.25 vs. Ctrl 8.92 ± 3.67, $P < 0.05$) and promote the expansion of Th17 cells (nonclassical/T cells 1.26%[1.02%] vs. Ctrl 0.73% [0.67%], $P < 0.05$).
Conclusion: This study demonstrated that nonclassical monocytes may hallmark the activity of ITP and be involved in the immune disfunction of this disease.

PB 2.32-4
CXCL-10/IP-10 chemokine expression in patients with immune-mediated thrombocytopenia

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Background: Immune thrombocytopenic purpura (ITP) is a heterogeneous clinical syndrome characterized by immune-mediated increase in platelet clearance through the production of autoantibodies against platelet glycoproteins, resulting in enhanced Fc-mediated destruction of platelets by reticuloendothelial system macrophages. CXC chemokine ligand-10/interferon-γ-inducible protein-10 (CXCL-10/IP-10), the prototype of the CXC family, is a T-helper (Th) 1 cytokine that exhibits a chemotactic activity for activated Th1 cells and is suggested to be involved in various Th1-dominant autoimmune diseases including ITP.
Aims: This study aimed to evaluate the plasma level of CXCL-10/IP-10 in patients with ITP as well as investigate its role in the pathogenesis and diagnosis of the disease.
Methods: Plasma CXCL-10/IP-10 was evaluated using enzyme-linked immunosorbent assay (ELISA) in 90 patients with ITP; 60 with de-novo active untreated ITP and 30 with remitted ITP, compared with 20 healthy volunteers.
Results: Significantly higher levels of CXCL-10/IP-10 were detected in both active ($P < 0.001$) and remitted ($P = 0.01$) ITP compared with controls, and in active compared with remitted patients ($P < 0.001$). The best cut-off value of 120 and 70 pg/mL were able to sharply discriminate between active ITP and controls, and remitted ITP and controls, respectively. No significant correlation was found between CXCL-10/IP-10 and platelet counts of either active or remitted patient subgroups.
Conclusions: CXCL-10/IP-10 is significantly elevated in active ITP, consistent with the Th1 characterization of the disease, suggesting CXCL-10/IP-10 as a pathogenetic and diagnostic marker of ITP.

PB 2.32-5
Detection of autoantibodies against platelet glycoproteins in patients with idiopathic thrombocytopenic purpura by a flow cytometric bead array

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Background: Idiopathic Thrombocytopenic Purpura (ITP) is an autoimmune disorder characterized by premature platelet destruction induced by autoantibodies directed against platelet glycoproteins. Despite being a clinically important disorder, ITP lacks a feasible diagnostic assay for routine clinical use.
Aims: The aim of this study is to establish a flow cytometric bead array (CBA) to detect autoantibodies against platelet glycoproteins (GPs) in patients with ITP.
Methods: Polystyrene beads of different fluorescent intensities were coated with monoclonal antibodies against human platelet GPs Ib/IX (SZ1), Ib (SZ2), IIIa (SZ21), GPIIb (SZ22), and P-selectin (SZ51), and

incubated with platelet lysates from patients with ITP or control individuals. The captured platelet antigen-autoantibody complexes were detected by an FITC-labeled goat anti-human IgG antibody by flow cytometry. The mean fluorescence intensity (MFI) in samples from 50 ITP patients, 37 non-ITP patients and 49 healthy controls was determined. The results were compared with that from the traditional monoclonal antibody immobilization of platelet antigen (MAIPA) technique.

Results: The newly established CBA was designed to detect five platelet-specific autoantibodies against GPIb/IX, GPIb, GPIIIa, GPIIb, and P-selectin simultaneously. Coefficients of variation (CVs) of intra-assay were 9.25%, 5.25%, 3.32%, 2.33% and 2.51%, respectively, and those of inter-assay were 10.89%, 6.58%, 8.65%, 7.12% and 7.98%, respectively. The MFI values of SZ1, SZ2, SZ21, SZ22 and SZ51 in ITP group were all higher than those in non-ITP or healthy groups (SZ1: 5.03 ± 3.90 vs. 1.91 ± 0.64 or 1.88 ± 0.84, $P < 0.01$; SZ2: 4.67 ± 3.66 vs. 2.36 ± 1.16 or 2.16 ± 0.93, $P < 0.01$; SZ21: 2.70 ± 1.31 vs. 1.08 ± 0.32 or 1.14 ± 0.41, $P < 0.01$; SZ22: 2.52 ± 1.19 vs. 1.10 ± 0.41 or 1.28 ± 0.57, $P < 0.01$; and SZ51: 2.33 ± 1.19 vs. 0.96 ± 0.40 or 1.06 ± 0.67, $P < 0.01$). In ROC analysis, with a cut-off value of 1.35, 0.60, 0.49, 0.48 and 0.63, respectively, the area under curve of the CBA was 0.84 (SZ1), 0.81 (SZ2), 0.89 (SZ21), 0.90 (SZ22) and 0.92 (SZ51). When the data of five antibodies were combined, the sensitivity of the CBA was 72.0% (36/50), significantly higher than that of MAIPA (48.0% (24/50), $c2 = 6.00$, $P < 0.05$).

Conclusion: We developed a CBA with five monoclonal antibodies to detect autoantibodies against human platelet GPs. Our results show that this assay was more sensitive than traditional methods, indicating that the assay may be used to improve the diagnosis of ITP.

PB2.33 – ADAMTS13: Clinical – II

PB 2.33-1

Importance of measuring circulating ADAMTS13 immune complexes in addition to free antibodies in patients with acquired TTP

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Background: A functional deficiency of ADAMTS13 results in accumulation of uncleaved, highly adhesive Willebrand factor (VWF) multimers in plasma leading to increased platelet aggregation and thrombus formation in the microcirculation. Anti-ADAMTS13 autoantibodies are the main cause of severe ADAMTS13 deficiency in acquired thrombotic thrombocytopenic purpura (aTTP). Circulating ADAMTS13-specific immune complexes (IC) as a potential factor in disease pathogenesis have been described in the plasma of aTTP patients.

Aim: We analyzed plasma samples from a large cohort of patients with aTTP for the presence of circulating IC and correlated these data with free anti-ADAMTS13 antibodies, ADAMTS13 activity (ADAMTS13:Ac), ADAMTS13 antigen (ADAMTS13:Ag) and inhibitor.

Material and Methods: We developed an ELISA assay to specifically determine IC levels in which the antigen portion is captured by a polyclonal anti-ADAMTS13 IgG and the immunoglobulin component detected by a class-specific antibody. Co-immunoprecipitation of ADAMTS13 with total IgG was employed as an orthogonal method. All other ADAMTS13 parameters were determined by standard protocols.

Results: Eighty-eight patients were analyzed in the acute phase. Free and ADAMTS13-bound IgG anti-ADAMTS13 antibodies were detected in 94% and 96% of patients, respectively. In both instances, IgG4 was the most prevalent IgG subclass (free: 94%, complexed: 93%). ADAMTS13:Ac and ADAMTS13:Ag were undetectable in most patients or very low. In remission (25 patients, median length of remission 12 months), 80% of patients still had detectable free IgG antibodies, mainly IgG4 (70%) followed by IgG1 (50%). ADAMTS13-specific ICs were found in 96% of patients, with IgG4 being again the most prevalent subclass (92%). Importantly, in 20% of patients, ADAMTS13-IgG IC were detected in plasma although free IgG antibodies were absent.

A comparison of ADAMTS13:Ac and ADAMTS13:Ag levels between acute and remission phase samples showed a statistically significant increase in both parameters at remission, whereas ADAMTS13 inhibitor and IgG anti-ADAMTS13 (total and subclasses) antibody titers were decreased. A trend towards reduced or undetectable titers at remission was also observed for ADAMTS13-specific IC of the IgG1, IgG2 and IgG3 subclasses, but not for the IgG4 subclass.

Conclusions: Our analysis provided for the first time a complete picture of the autoantibody response in aTTP. ADAMTS13-specific IC were mainly of the IgG4 subclass and persisted for a long time, as they could be detected in plasma of aTTP patients not only in the acute phase but also in remission. Our study suggests that in addition to characterization of autoantibodies against ADAMTS13, ADAMTS13-specific IC also need to be determined at the time of diagnosis and during remission to obtain a full picture of disease status.

PB 2.33-2

Mutations and polymorphisms in adult onset pregnancy related TTP

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Background: Thrombotic thrombocytopenia purpura (TTP) is a rare but potentially fatal microangiopathic haemolytic anaemia (MAHA) which afflicts approximately six individuals per million per year. It is characterised by a congenital, autoimmune or idiopathic reduction in ADAMTS13 (a disintegrin and metalloprotease with thrombospondin type 1 motif). Adult onset TTP is often precipitated by physiological stress and pregnancy related TTP is a subgroup which may represent up to one third of annual TTP cases. It can be difficult to diagnose and is characterised by recurrent foetal loss, maternal morbidity and mortality.

Aims: We examined the differences in the ADAMTS13 gene in our subjects compared to wild type, including intronic, silent and missense polymorphisms and mutations.

Method: DNAwas extracted from EDTA samples in 15 pregnancy related TTP subjects on the UK TTP Registry after informed consent. ADAMTS13 exons and exon- intron boundaries were amplified using polymerase chain reaction (PCR) and sequenced. Any variations between the ADAMTS13 genotype of patients from our reference sequence were recorded. The variations found were assessed for their frequency in the general population using the 1000 genome project and Hapmap.

Results: All 15 patients had an ADAMTS13 activity of < 5% (NR 66–120%) on admission. We found that our cohort had a number of ADAMTS13 variations in common which have a very low prevalence in the general population. For example the R7W polymorphism is present in 13% of the European population, but occurred in 11/15 (73%) of our patients. Each patient had at least two variations from wild type ADAMTS13. We focused on three variations which occur in exons 23 and 24: the A1033T SNP is often co-inherited with the R1060W mutation (which is associated with decreased ADAMTS13 activity due to decreased secretion); 12/15 (80%) of our cohort had both variations, where the general prevalence is 2% and < 1% respectively. None of our cohort had inherited one variation without the other. Upstream from this pairing, the V970V SNP was found in 9/15 (60%), but occurs at a frequency of 2% across all ethnic groups. Of the 12 patients who had inherited both A1033T and R1060W, 8/12 (67%) also had inherited V970V.

Conclusion: The potential impact of ADAMTS13 non synonymous polymorphisms has received little investigation and synonymous poly-

morphisms have been regarded as devoid of clinical significance. However both appear to occur far more often in the disease state than anticipated from general population frequencies. Some of these may potentially have a real effect on the secretion or activity of ADAMTS13 whereas others may be linked to the mutations responsible for the disease. Further investigation would be needed to determine if this is the case. It is of particular interest that patients with the R1060W mutation presented later on in life during pregnancy and that this mutation was found to occur frequently with the A1033T polymorphism and V970V synonymous polymorphism. This is something that should be considered for *in vitro* studies as there may be possible modulatory effects.

PB 2.33-3

Variability in measurement of ADAMTS13: a UK NEQAS multicentre exercise for ADAMTS13 assays

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Background: ADAMTS13 is a plasma metalloprotease which regulates the multimeric size of VWF by cleaving the protein at Tyr1605-Met1606. ADAMTS13 deficiency (acquired or congenital) is frequently observed in thrombotic thrombocytopenic purpura (TTP). This disease primarily requires a clinical diagnosis, but ADAMTS13 assays can provide confirmation, help characterise the phenotype of the deficiency, may be useful in monitoring treatment, and can be used to identify family members with ADAMTS13 deficiency.

Methods: We describe here a multicentre exercise for ADAMTS13 assays, in which 22UKand International centres performed assays on three lyophilised plasma samples. AD12:01 was a normal plasma sample; AD12:02 was from a 22 year old female diagnosed with TTP, who had received 8 days of plasma exchange and Rituximab; AD12:03 was from a 58 year old female with acquired TTP, who had 10 days of plasma exchange prior to sample collection.

Results: All centres performed activity assays, with six also performing antigen assays, and nine performing IgG anti-ADAMTS13 assays. Median (and range) ADAMTS13 activity in each sample was: AD12:01 78% (25.3–104.0), AD12:02 60.5% (33.0–71.5), and AD12:03 < 3% (0.0–37.1). All but one centre reported ADAMTS13 activity below 10% for sample AD12:03. However, two centres reported higher ADAMTS13 activity levels in sample AD12:02 than in sample AD12:01. One of these also reported a level of 37% activity in sample AD12:03. In total, three centres reported activity assay results on sample AD12:01 below the lower limit of their reference range. Seven centres reported results below their reference range for sample AD12:02. Results amongst centres using collagen binding assay (CBA)-based methods were lower than for centres using other methodologies by an average 46%. Nine centres used the Technozym ADAMTS-13 INH kit for anti-ADAMTS13 assays, assays (manufacturer's cut-off 15 μ****/mL), but agreement was suboptimal. Median (CV) anti-ADAMTS13 activity in each sample was: AD12:01 9 μ**/mL (37%), AD12:02 14.5 μ***/mL (29%), and AD12:03 26.7 μ***/mL (27%). Frequency of testing clinical samples in different centres varied markedly – two centres reported that they perform fewer than two assays per annum, with five centres reporting more than 100 assays each year.

Conclusions: Currently no international standards or any other external quality assessment programme are available for ADAMTS13 assays. Development of both will assist in standardisation and therefore improved clinical utility of these assays.

PB 2.33-4

Decreased ADAMTS13 activity and its clinical significance in patients with idiopathic portal hypertension

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Background: Idiopathic portal hypertension (IPH) is characterized by a noncirrhotic portal hypertension with hypersplenism, but is distinctly different from liver cirrhosis (LC) with regenerative nodules. The characteristic hepatic lesions in IPH are the occulusive changes of intrahepatic portal branches and portal fibrosis, but its pathogenesis has not been fully clarified. Deficiency of ADAMTS13 activity (ADAMTS13:AC) results in the accumulation of unusually large von Willebrand factor multimers (UL-VWFM) and platelet thrombi formation. We demonstrated that an imbalance between the decreased ADAMTS13:AC and the increased substrates may reflect the predisposing state for platelet thrombi formation in advanced LC patients (Thromb Haemost, 2008; 99:1019).

Aims: The aim of this study was to analyze ADAMTS13:AC and its related parameters, and to explore the clinical significance in patients with IPH in comparisons with LC.

Methods: Patients studied were chronic hepatitis (CH) in 33, LC in 109 (Child A-LC in 35, Child B-LC in 33 and Child-LC in 41), and IPH in 15. The diagnosis of IPH was based on the definition and diagnostic criteria described in the Japan IPH Research Committee. Liver biopsy under laparoscopic examination and angiography were performed in all patients with IPH. Plasma ADAMTS13:AC was determined by both a classic VWFM assay and a chromogenic ELISA. VWF:Ag was measured by ELISA, and VWFM patterns were analyzed by SDS-0.9% agarose gel electrophoresis.

Results: The ADAMTS13:AC measured by ELISA strongly correlated with those determined by the VWFM assay. ADAMTS13:AC was 57% (mean) in IPH compared to 100% in healthy controls, 87% in CH, 79% in Child A-LC, 63% in Child B-LC, and 31% in Child C-LC. No patients with IPH whose ADAMTS13:AC showed severe deficiency (< 3% of controls) were found, while five end-stage LC had severe deficiency. VWF:Ag was 254% in IPH compared to 100% in normals, 245% in CH, 320% in Child A-LC, 436% in Child B-LC, and 486% in Child C-LC. As a result, the ratio of VWF:Ag to ADAMTS13:AC was 5.4 in IPH, 1.0 in normals, 2.9 in CH, 4.3 in Child A-LC, 13.3 in Child B-LC, and 43.2 in Child C-LC. UL-VWFM was not found in IPH, whereas it was detected in LC with the lowest ADAMTS13:AC (23%) and the highest values of Child-Pugh score (12.4), serum creatinine (2.4 mg/dL) and blood ammonia (144 μg/dL), which corresponded to 16% of LC patients whose ADAMTS13:AC showed < 50%. In IPH, ADAMTS13:AC decreased in patients with hepatic encephalopathy and severe liver atrophy than those without (41% vs. 67%, $P < 0.05$). The ratio of VWF:Ag to ADAMTS13:AC inversely correlated with platelet count ($r = -0.534$, $P < 0.05$), hemoglobin ($r = -0.501$, $P < 0.05$), and serum albumin ($r = -0.579$, $P < 0.05$).

Conclusions: In IPH, ADAMTS13:AC decreased to 57% of normals, the value of which corresponded to Child B-LC patients. VWF:Ag increased 2.5 times higher than normals. The imbalance of enzyme to substrate indicates platelet hyperaggregability, and may contribute to anemia, reduced functional liver capacity, and the occurrence of hepatic encephalopathy together with the development of liver atrophy in patients with IPH.

PB 2.33-5

An unbalance between von Willebrand Factor and its cleaving protease ADAMTS13 in acute liver failure: implications for hemostasis and correlations with clinical outcome

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Background: Liver diseases are frequently associated with substantial changes in the hemostatic system. Traditionally these changes were believed to result in a hypocoagulable state. Recent clinical and laboratory studies have challenged this concept an it has now been well established that patients with chronic liver diseases are in 'hemostatic rebalance', which results from a commensurate decline in pro- and antihemostatic drivers, and from the presence of compensatory mechanisms sustaining an appropriate hemostasic function. In acute liver failure (ALF), the net effect of all hemostatic changes is not clear, partly because these alterations in the hemostatic system in these patients have been less well defined when compared to those in patients with chronic liver failure. In an effort to elucidate this issue, we are systematically studying consequences of hemostatic defects in patients with ALF.

Aims: In the present study, we assessed levels and functionality of the platelet-adhesive protein von Willebrand factor (VWF) and its cleaving protease ADAMTS13 in plasma of patients with acute liver injury and acute liver failure (ALI/ALF). Furthermore, we explored possible associations between VWF, ADAMTS13 and disease outcome, as plasma levels of these molecules have been associated with progressive liver failure.

Methods: We measured functional parameters of VWF and ADAMTS13 in plasma of 50 patients taken on the day of admission for ALI/ALF. In addition, we investigated the ability of patient plasma to support platelet adhesion and aggregation in a flow-based model. Finally, we explored possible relationships between VWF/ADAMTS13 and ALI/ALF specific complications, frequency of liver transplantation, spontaneous survival and death rates. Plasma of 40 healthy volunteers served as controls.

Results: VWF antigen levels were highly elevated in patients with ALI/ALF. In contrast, the collagen binding activity as well as the VWF ristocetin cofactor activity/VWF antigen ratio were significantly decreased when compared to healthy controls. Also the proportion of high molecular weight VWF multimers was reduced, despite severely decreased ADAMTS13 levels, suggesting proteolysis of VWF by enzymes other than ADAMTS13. In spite of these functional defects, platelet adhesion and aggregation were better supported by plasma of patients with ALI/ALF when compared to control plasma. Low ADAMTS13 activity, but not high VWF antigen, was associated with poor outcome in patients with ALI/ALF as evidenced by higher grades of encephalopathy, higher transplantation rates and lower survival.

Summary/Conclusion: Strongly elevated levels of VWF in plasma of patients with ALI/ALF support platelet adhesion, despite a relative loss of function of the molecule, suggesting a rebalanced primary hemostatic function in ALF. Furthermore, low ADAMTS13 activity is associated with progressive liver failure in the patient cohort. An unbalance between VWF and ADAMTS13 has been shown to lead platelet-induced thrombosis, and intrahepatic thrombosis has been shown to accelerate liver failure progression. We therefore speculate that a substantially unbalanced VWF/ADAMTS13 ratio, as observed in the present study, results in the formation of platelet-rich microthrombi in the diseased liver and in turn adversely affects disease progression in patients with ALI/ALF.

PB 2.33-6

Relationship of von Willebrand factor, D-Dimer and ADAMTS13 with hypercoagulability in type 1 diabetic patients with and without nephropathy

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Background: Diabetic nephropathy is an important risk factor for cardiovascular complications. Some studies have shown that increased plasma levels of Von Willebrand factor (VWF) and reduced of ADAMTS13 are associated with diabetic nephropathy and an increased risk of cardiovascular events in type 2 diabetic patients. However, these relationships have not been investigated in type 1 diabetic patients and a better understanding of the role that these parameters play in type 1 diabetic patients is still lacking.

Aims: Evaluate plasma levels of VWF, ADAMTS13 and D-Dimer (DDi), and VWF/ADAMTS13 ratio in type 1 diabetic patients.

Methods: Patients were classified according to glomerular filtration rate (GRF) and urinary albumin excretion in three groups: without renal dysfunction (GFR $\geq$ 90 mL/min/1.73 m^2 and normoalbuminuria, $n = 49$), with mild renal dysfunction (GFR $\geq$ 60 and $<$ 90 mL/min/1.73 m^2 and/or microalbuminuria, $n = 35$) and with severe renal impairment (GFR $<$ 60 mL/min/1.73 m^2 and/or proteinuria, $n = 41$). A group of 35 individuals without diabetes or renal dysfunction was also included. Individuals undergoing hemodialysis and those with liver disease, alcoholism, coagulation disorder, cancer, history of renal transplantation and pregnancy were excluded. VWF, ADAMTS13 and DDi were measured by ELISA method. Statistical comparisons were performed by Mann-Whitney test. Differences were considered significant when $P < 0.05$.

Results: Diabetic patients without renal dysfunction had higher levels of VWF, 1031.600 mU/mL (778.100–1142.200 mU/mL), than individuals without diabetes, 730.800 mU/mL (575.600–962.600 mU/mL), $P = 0.002$. VWF levels increased with renal function impairment, being increased in diabetic patients with mild renal dysfunction, 1233.200 mU/mL (904.900–1486.400 mU/mL), compared with those without renal dysfunction, $P = 0.013$, and even higher in patients with severe renal impairment, 1358.000 mU/mL (1164.000–1751.600 mU/mL), $P = 0.029$. Levels of ADAMTS13 were reduced in patients without renal dysfunction, 296.760 ng/mL (221.335–440.415 ng/mL), compared to healthy subjects, 444.960 ng/mL (362.180–594.345 ng/mL), $P < 0.001$. However, an increase in ADAMTS13 levels was also detected in patients with mild, 502.600 ng/mL (293.730–601.975 ng/mL), and severe renal impairment, 567.640 ng/mL (363.050–654.365 ng/mL), compared to those without renal dysfunction, $P < 0.001$. Furthermore, VWF/ADAMTS13 ratio was increased in patients without renal dysfunction, 3.161 (2.028–4.204), and with mild, 2.569 (1.878–3.122), and severe renal dysfunction, 2.427 (1.908–3.454), compared to non-diabetic subjects, 1.653 (1.368–1.881), $P < 0.001$, but no significant differences were observed between diabetic patients with and without renal dysfunction. DDi levels were higher only in patients with severe renal impairment, 363.607 ng/mL (223.470–508.604 ng/mL), compared to other groups, 177.055 ng/mL (130.350–264.494 ng/mL), 176.845 ng/mL (131.935–259.828 ng/mL) and 217,251 ng/mL (142.361–297.972 ng/mL), $P < 0.001$.

Conclusion: The gradual increase in VWF levels as nephropathy progress suggests that endothelial dysfunction may contribute to the development and progression of vascular complications in type 1 diabetic patients. However, increased levels of VWF are accompanied by a concomitant increase in ADAMTS13 levels, the enzyme responsible for cleavage and inactivation of VWF, either as a compensatory mechanism or due to a compromised renal function that may affect the

renal excretion of these proteins. This hypothesis is corroborated by increased levels of DDi observed in the severe renal impairment group, indicating a hypercoagulable state in these patients.

PB2.34 – Fibrinolytic System: Basic – II

PB 2.34-1

Regulation of fibrinolysis by polyphosphate is dependent on the plasminogen activator

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Background: The modes of action of the plasminogen activators (PA), tissue plasminogen activator (tPA) and urokinase (uPA) differ. Efficient tPA-mediated plasminogen activation requires binding to fibrin. In contrast, uPA is fibrin independent but is associated with its cellular receptor uPAR. We have previously shown that polyphosphate (polyP), a biomolecule released from platelet dense granules, alters fibrin structure and attenuates tPA and plasminogen binding to fibrin thereby down-regulating fibrinolysis.
Aims: The aim of this study is to determine if polyP-induced changes in fibrin structure influence uPA-mediated fibrinolysis
Methods: Fibrin clots were formed from fibrinogen (2.4 μM), glu-plasminogen (0.24 μM) and tPA (20 pM) or uPA (180 pM) ± $polyP_{65}$ (5 μM). Clotting was initiated by thrombin (0.25 U/mL) and $CaCl_2$ (5 mM) and fibrinolysis monitored at 340 nm. Fibrin degradation products (FDPs) were collected and analyzed by Western blotting using an anti-human fibrin(ogen) antibody. Plasmin generation in clots was quantified by incorporating the fluorogenic substrate D-VLK-AMC (excitation 360 nm emission 460 nm). Confocal microscopy was used to visualize fibrin structure and localization of proteins during fibrinolysis. Clots were prepared with fibrinogen (2.65 μM, 9% was DyLight 488-labelled), plasminogen (1.25 μM, 20% was DyLight 633-labelled), thrombin (0.25 U/mL), $CaCl_2$ (5 mM) and fibrinolysis initiated by exogenous PA (75 nM).
Results: As previously reported $polyP_{65}$ delayed tPA-mediated clot lysis (50% lysis times 102.6 ± 2.3 vs. 69.8 ± 1.9 min; $P < 0.001$). Plasmin generation during tPA-mediated fibrinolysis was delayed and reduced 1.3-fold in $polyP_{65}$ containing clots (1860 ± 82 vs. 2475 ± 155 average FU/min $P < 0.001$). Confocal microscopy also showed delayed tPA-mediated fibrinolysis in clots containing $polyP_{65}$. Plasminogen was visualized at the edge of the lysis front as a tight band. In marked contrast to tPA, uPA-mediated clot lysis was enhanced by $polyP_{65}$ (50% lysis times 64.5 ± 1.7 vs. 108.2 ± 3.8 min; $P < 0.001$). This was reflected as an increase in FDPs generated during uPA-mediated lysis of clots containing $polyP_{65}$ and a 1.6-fold increase in plasmin generation (3961 ± 73 vs. 2434 ± 123 average FU/min; $P < 0.0001$). Confocal microscopy also showed enhanced uPA-mediated lysis by $polyP_{65}$, with a broader zone of plasminogen visible compared to that observed with tPA. The signal of 633-plasminogen was unchanged upon conversion to plasmin, suggesting the observed differences result from accumulation of plasminogen on fibrin at the lysis front.
Varying the plasminogen concentration (0.125–1 μM) in clot lysis only marginally affected tPA-mediated fibrinolysis, conversely, uPA-mediated lysis was dramatically accelerated by increased plasminogen. The effect of $polyP_{65}$ on uPA-mediated fibrinolysis and plasmin generation was overcome by additional plasminogen, while the down-regulation of tPA-mediated lysis and plasmin generation was unaffected.
Summary: $PolyP_{65}$ has opposing effects on tPA- and uPA-mediated fibrinolysis. The cofactor function of fibrin in tPA-mediated plasminogen activation is attenuated by polyP. In contrast, as uPA does not bind fibrin, it is feasible that polyP may act as a template to facilitate the interaction between uPA and plasminogen thereby accelerating fibrinolysis.

PB 2.34-2

The expression of plasminogen activator inhibitor type-1 is post-transcriptionally regulated by sphingosine-1-phosphate and SERPINE1 mRNA binding protein

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Background: Plasminogen activator inhibitor type-1 (PAI-1), the major physiologic inhibitor of fibrinolysis and proteolysis produced in liver and vascular cells, is implicated in thrombosis and atherosclerosis. Sphingosine-1-phosphate (S1P) is a breakdown product of sphingosine released from platelets upon activation and regulates various gene expressions and alters PAI-1 expression in several cells. Nevertheless, posttranscriptional regulation of PAI-1 by S1P has not been elucidated.
Aims: We aimed to determine whether S1P is involved in posttranscriptional regulation of PAI-1 expression.
Methods: We analyzed the promoter activity, mRNA levels, 3′-untranslated region (UTR) activity and protein levels of PAI-1 using human liver derived HepG2 cells.
Results: S1P (500 nM) increased PAI-1 promoter activity by three fold (luciferase assay) and the expression of PAI-1 mRNA by two fold (RT-PCR) at 4 h. Transcriptional induction of PAI-1 by S1P was transient. S1P decreased expression of PAI-1 mRNA to 0.6 fold and inhibited the accumulation of PAI-1 protein into the conditioned media at 24 h (Western blot). Human PAI-1 mRNA exists in two subspecies (3.2 and 2.2 kb) and S1P decreased the baseline luciferase activity of 1 kb fragment of the 3′ terminus (+2177–3176 nt) of 3′-UTR of the 3.2 kb PAI-1 mRNA [3′-UTR (+2177–3176)]. Mutation of AU-rich element 3 (ARE3) inhibited the decreased activity of PAI-1 mRNA [3′-UTR (+2177–3176)] by S1P. S1P destabilized 3.2 kb PAI-1 mRNA (Northern blot), suggesting that S1P decreased the expression of PAI-1 protein by regulating PAI-1 expression at the posttranscriptional level and thereby affecting mRNA stability. S1P increased SERPINE1 mRNA binding protein (SERBP1) through $S1P_1$ receptor and facilitated binding of SERBP1 to PAI-1 mRNA (pull-down assay). Small interfering RNA against SERBP1 prevented the decrease of PAI-1 mRNA induced by S1P, suggesting that SERBP1 and ARE3 in the 3′-UTR were cooperatively involved in the posttranscriptional regulation by S1P.
Summary/Conclusion: Our data indicate that brief transcriptional induction of PAI-1 by S1P is followed by S1P-mediated destabilization of 3.2 kb PAI-1 mRNA mediated by specific effects on the 3′-UTR through SERBP1 and ARE3. These effects are associated with decreased expression of PAI-1 protein potentially leading to regulation of fibrinolysis.

PB 2.34-3

Investigation of possible exosite interactions between plasmin(ogen) and antiplasmin

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The inhibition of plasmin by antiplasmin (AP) is usually understood as a two step-process; the binding of the C-terminal region of AP to the kringle domains of plasmin(ogen) followed by the inhibitory interaction between the AP reactive centre loop and active site of plasmin. Our previous work has shown that the binding affinity between α2-antiplasmin lacking the C-terminal region to active site-blocked plasmin remains strong, K_d = 50 nM. This raised the possibility that additional exosite region(s) may exist beyond the C-terminus/kringle domain interaction. To further explore this possibility we performed binding studies of AP with various plasminogen conformations (Glu and Lys-

plasminogen (PLG), active plasmin, micro-plasmin, active site-blocked plasmin and micro-plasmin). Binding of these plasmin(ogen) variants with full-length and C-terminally truncated antiplasmin was assessed by surface plasmon resonance. Our results show that in the closed, Glu-PLG conformation, where the kringle domains and protease active site are unavailable the binding affinity for AP(wt) remains high (K_D = 30 nM) and that this interaction is not influenced by the removal of the C-terminus of AP (K_D = 50 nM). As expected the binding affinity is increased in the open and active conformations (K_D = 7.8 nM and 1.6 nM) with Lys-PLG and active site blocked plasmin respectively. However, C-terminal truncation of AP resulted in a 20–30 fold decrease in binding affinity for Lys-PLG and active site blocked plasmin. A dramatic loss in binding affinity was observed for micro-PLG (K_D = ~1.4 μM) with both AP(wt) and AP(c-trunc). These results suggest that the high affinity interaction observed between AP and Glu-PLG may involve exosites within the AP core domain and the kringle domains of plasmin(ogen). This is further supported by the observation that there is a 3-fold loss in binding affinity of plasmin-CMK with AP(C-trunc) compared to micro-plasmin-CMK with AP (C-trunc). Mutagenesis of residues in β-sheet C of human α2-antiplasmin was also carried to further explore regions in AP involved in exosite interactions with plasmin(ogen).

PB 2.34-4

Comparison of thrombolytic efficacy of plasmin and rt-PA in an *in vitro* flow system

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Background: Plasmin, a directly acting thrombolytic agent has been recently tested in phase I clinical studies. While plasmin demonstrated a very favorable safety profile upon intra-arterial delivery to the clot site, its thrombolytic efficacy remains to be further assessed.
Aim: To test in a model system if there are differences in thrombolysis between clots exposed to equimolar concentrations of plasmin and rt-PA after partial vessel recanalization.
Methods: Model blood clots were prepared in a glass chamber enabling direct observation by dynamic optical microscopy. The incubation of clots with plasmin (2.4 mg/mL) or rt-PA (2.63 mg/mL), allowing for the initial biochemical clot degradation, was followed by 'flushing' the clots with tangentially directed plasma flow devoid of a thrombolytic agent, mimicking blood flow after partial vessel recanalization. The acquired images were analyzed for non-dissolved blood clot area as a function of time.
Results: With both thrombolytic agents, the relative clot area decreased rapidly in the first 30 s after initiation of perfusion due to 'flushing' the degraded clot fragments (after plasmin by 0.26 ± 0.22 and after rt-PA by 0.34 ± 0.21, P = 0.60). In the following minutes the clot size showed a linear time dependence: after incubation with plasmin the clot size did not change substantially any more, while after incubation with rt-PA the clot size continually decreased. The slopes of the regression lines differed significantly ($r_{pl} = -8.9 \times 10^{-3}$/min vs. $r_{rtPA} = -44.1 \times 10^{-3}$/min, $P < 0.01$).
Conclusion: The thrombolytic action of plasmin was terminated rapidly by contact with flowing blood plasma, while the thrombolytic action of rt-PA was prolonged.

PB 2.34-5

Antibody-engineered bispecific inhibitor against TAFI and PAI-1 with improved expression and efficacy

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Background: Targeting Thrombin Activatable Fibrinolysis Inhibitor (TAFI) and Plasminogen Activator Inhibitor-1 (PAI-1) constitutes a potential strategy to increase thrombolytic efficiency of plasminogen activators without aggravating safety concerns. Additionally, the synergistic profibrinolytic effect caused by dual targeting of human TAFI and PAI-1 has been demonstrated *in vitro* to be even more pronounced when using a diabody (Db) than the combined addition of the parental monoclonal antibodies (MA) (Develter et al., JTH, 2008). Db's are bispecific antibody fragments with a size of 58 kDa allowing better clot penetration. To evaluate this dual targeting strategy *in vivo*, a novel Db was generated from two potent MA's against rat TAFI and PAI-1. However, this Db encountered stability problems hampering further evaluation.
Aims: Improving the plasma stability of a bispecific Db against rat TAFI and PAI-1 by adding a stabilizing flexible linker (single-chain diabody, scDb) and by CDR-grafting on a stable scaffold in order to evaluate this dual targeting strategy *in vivo*.
Methods: Db and scDb were constructed by cloning and assembling the variable domains of MA-33H1F7 (inhibiting rat PAI-1) and MA-RT36A3F5 (destabilizing rat TAFIa), followed by expression in bacteria (Db) and HEK293T cells (scDb), and subsequent purification. Since instability of (sc)Db is caused by the variable domains of MA-RT36A3F5, CDR-grafted variants (CDR-Db and CDR-scDb) were constructed on a stabilized scaffold (scFv-4D5 derived from Herceptin, Kelley et al., Biochemistry 1992). Functional stability was determined through an ELISA assessing simultaneous binding to rat TAFI and PAI-1. Inhibitory properties against rat TAFI and PAI-1 were measured via chromogenic assays. An *in vitro* clot lysis assay was used to evaluate the profibrinolytic effect of parental MA (0.7 μM) or (sc) Db (1.4 μM, corresponding to an equal amount of binding sites as 0.7 μM MA) in rat plasma.
Results: CDR-grafting improved expression of CDR-Db in bacteria (three-fold increase vs. Db) and of CDR-scDb in HEK293T cells (10-fold increase vs. scDb). Upon incubation of Db for 3 h at 37 °C in citrated plasma, 13% residual binding capacity was observed. In contrast, residual binding capacity under these conditions was 44 ± 9%, 62 ± 10% and 88 ± 13% for scDb, CDR-Db and CDR-scDb, respectively. Inhibitory properties of the parental antibodies were preserved in Db, CDR-Db and CDR-scDb, but could not be determined for scDb due to insufficient production. The profibrinolytic effect of Db, CDR-Db and CDR-scDb was 2%, 46% and 85%, respectively, compared to that of MA-RT36A3F5 against TAFI. The contribution of the effect of the PAI-1 inhibiting moiety could not be evaluated in the plasma-based assay system due to the low baseline plasma levels of PAI-1.
Conclusion: Our efforts to increase the plasma stability of an unstable bispecific antibody-based inhibitor against rat TAFI and PAI-1 resulted in a CDR-scDb, exhibiting a seven-fold increased stability and a 43-fold increased profibrinolytic effect. In order to explore the full potential of the dual TAFI/PAI-1 inhibitor, *in vivo* thrombolysis experiments in which both TAFI and PAI-1 contribute, are in progress.

PB 2.34-6

Plasminogen alterations mediated by N-homocysteinylation processes

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Background: Hyperhomocysteinemia is a risk factor for atherothrombosis. Plasma homocysteine exists in several chemical species such as protein bound, oxidized to form dimers, free reduced homocysteine and as its cyclic thioester homocysteine thiolactone (HTL). N-homocysteinilation reactions are mediated by HTL, which is N-linked to epsilon amino group of lysine residues resulting in modified proteins named HTL adducts.
Aim: To evaluate the effects of HTL on plaminogen (Plg) structure, activation kinetics and plasmin activity.
Methods: Human purified Plg (1.5 mg/mL) was incubated with HTL (molar ratio Plg:HTL = 1:150; 3 h 37 °C); saline solution was used as control. Samples were dialyzed to remove unreacted HTL. The homocysteinylated Plg (Plg-HTL) was analyzed by native and denaturing polyacrylamide gel electrophoresis (PAGE and SDS-PAGE), crossed immunoelectrophoresis (CIE) and capillary electrophoresis. Plg-HTL (0.3 μM) activation with tissue Plg activator (t-PA, 360 U/mL) was studied through kinetic amydolitic assays with chromogenic substrate S-2251 (0.5 mM), evaluating paranitroaniline release over time. Lysis activity of t-PA (11 U/mL) activated Plg-HTL over fibrin obtained in the presence of purified fibrinogen (2.6 mg/mL), thrombin (0.5 U/mL) and $CaCl_2$ (20 mM), was kinetically studied. All experiments were performed in quadruplicate.
Results: There were no significant differences in the electrophoretic profiles between Plg-HTL and control in PAGE, SDS-PAGE and capillary electrophoresis. However, CIE showed two fractions for Plg-HTL, one coinciding with the fraction of Plg control and another with a different displacement, related to the heterogeneity of the homocysteinylated protein. Amydolitic assays showed an increased activation time for Plg-HTL compared to control (41 ± 2 vs. 34 ± 1 min; $P = 0.01$) and a decreased enzyme activity (0.0053 ± 0.0002 vs. 0.0066 ± 0.0004/min; $P = 0.01$). In accordance, fibrin lysis times with Plg-HTL were significantly prolonged respect to control (51 ± 2 vs. 40 ± 1 min; $P < 0.05$).
Conclusions: Lysis of fibrin networks was diminished by N-homocysteinylated plasminogen. It can be suggested that Plg of patients with high homocysteine plasma levels would have less fibrinolytic activity and therefore, be associated to the prothrombotic state of hyperhomocysteinemia.

PB2.35 – Haemophilia A: Basic Research – II

PB 2.35-1

Prediction by cellular expression of impact of four novel F8 molecular variations identified in isolated female with low FVIII:C levels suspected of being haemophilia A carriers

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Background: Haemophilia A (HA) is an inherited X-linked disorder that typically affects males, while females are carriers. However, carrier status of HA may also be raised in females with low clotting factor VIII (FVIII) level and without HA family history once no alternative diagnosis can be retained.
Aim: The aim of the present study was to predict the carrier status in four female without family history of bleeding with decreased FVIII:C levels (< 50 IU/dL) in whom we identified unreported *F8* gene variation.
Methods: Prediction of the causal impact of these four novel molecular variations was studied by two strategies: bioinformatics approaches and site-directed mutagenesis followed by cellular expression. Molecular modelling was performed using Alamut®-Mutation Interpretation Software 2.1 and sequences alignments of the homologous FVIII (human, pig, canine, mouse and fish FV/FVIII). Mutant plasmids were generated through site-directed mutagenesis. Plasmid DNA of FVIII WT and FVIII mutants were transfected into COS-1 monkey cells. For each construct, three series of duplicate assays were performed. To evaluate specific activities, FVIII activity (FVIII:C) and FVIII antigen (FVIII:Ag) levels were measured in the conditioned media of COS-1 by using one-stage aPTT clotting assay and ELISA.
Results: We identified four novel nucleotide variations. Three were missense substitution: p.Pro521Leu, p.Ser577Tyr and p.Leu2032Pro and one was a duplication: p.Ala315dup. No other nucleotide change was found in the *F8* coding region or in exon-intron boundaries.
Analysis with molecular modelling tools showed that p.Pro521Leu and p.Ser577Tyr substitutions were probably damaging for the FVIII function. The p.Leu2032Pro substitution analysis led to discrepant results, but impact on splicing cannot be excluded. All novel missense changes were fully conserved among mammalian species. Specific activity of factor VIII mutants relative to WT showed that expression of FVIII mutant p.Leu2032Pro was similar to WT in COS-1 ($P = 0.69$) while FVIII mutants p.Pro521Leu, p.Ser577Tyr and p.Ala315dup led to a significant decrease ($P < 0.01$). FVIII activities were undetectable in conditioned media for FVIII mutant p.Ser557Tyr and p.Ala315dup while p.Pro521Leu variant displayed a mild impairment of WT FVIII activity (38% of WT FVIII:C).
Conclusion: In this study we identified four novel mutations detected in female with low FVIII level. The p.Pro521Leu substitutions could lead to a mild HA while p.Leu2032Pro could be considered as *F8* polymorphism. Both p.Ser577Tyr and p.Ala315dup lead to severe dysfunctions of FVIII. These two mutations could be responsive of severe HA. A prenatal diagnosis could be offered to carriers with these mutations.

PB 2.35-2

Cellular stress in synoviocytes, chondrocytes and osteoblasts and increased osteoclast activity induced by plasma derived factor VIII products *in vitro*

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Aim: By effective treatment of bleeding and thereby reducing blood induced joint damage factor concentrate replacement therapy has dramatically improved the quality of life of patients with haemophilia. However, plasmatic factor concentrates contain many co-purified proteins that might impact the influence of these blood products on joint cells after bleeding. As these products induce mild cellular stress in blood cells, we compared the effect of factor VIII products on joint cell viability and morphology.
Method: The effect of three different recombinant FVIII (rFVIII) products and four plasma derived FVIII (pdFVIII) products was investigated. Primary cells, straight from the human knee tissue (PromoCell, Heidelberg, Germany), were cultured and factor VIII concentrates were added to a final factor FVIII concentration of up to 1 IU/mL. Osteoclast formation was induced from human peripheral blood mononuclear cells. Cell viability was tested by cell viability assay (MTT) and cell counting. The influence of FVIII products on synoviocytes, chondrocytes, osteoblasts and osteoclasts was investigated in addition by microscopy.

Result: About 1 IU/mL of all different pdFVIII concentrates strongly increased human synoviocytes proliferation, while all different rFVIII concentrates (1 IU/mL) had no influence. The effect of the pdFVIII concentrates on chondrocytes and osteoblasts was dramatic. Twenty-four hours incubation with pdFVIII products induced cell membrane blebbing and cell shrinkage in many osteoblasts. The incubation of pdFVIII products induced detachment of many chondrocytes from the matrix. RFVIII products did not induce any of these effects. RFVIII treated osteoblasts and chondrocytes showed a typical picture of undisturbed human cells in culture with a 'smooth' membrane surface. Osteoclasts differentiated in the presence of plasma derived factor VIII concentrates showed after 7 days significantly more nuclei as osteoclasts that were differentiated in the presence of recombinant factor VIII concentrates.
Conclusion: Enhanced synoviocyte proliferation clearly indicates that the tested pd FVIII products, in contrast to the rFVIII products, might amplify the damaging effect of blood on synoviocytes. As chondrocytes produce all the components of cartilage and as bone destruction is induced by severely impaired osteoblast activity, chondrocyte and osteoblast destruction by the tested pdFVIII concentrates might lead to an enhanced destruction of the cartilage and bone, while the tested rFVIII concentrates do not. As a positive correlation between the number of nuclei per osteoclast and the volume of the pit (resorption bays) made has been described in the literature, this result again indicates a potential harmful effect of plasma derived factor VIII concentrate in comparison to recombinant factor VIII.

PB 2.35-3

An aberrant pattern for intron 1 inversion with concomitant large duplication and deletion within the F8 gene in a severe hemophilia A

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Background: Hemophilia A (HA) is the most common X-linked recessive bleeding disorder and caused by mutations in the F8 gene, resulting in a quantitative and qualitative abnormality of coagulation factor VIII. There is a wide spectrum of mutations in the F8 gene causing HA. Inversion of intron 1 is responsible for about 2% of HA cases and results from intrachromosomal homologous recombination between a 1041 bp region (int1h-1) of intron 1 and an extragenic copy (int1h-2) approximately 140 kb telemetric to the F8 gene.
Aims: To disclose the complex molecular mechanism for the aberrant pattern of intron 1 inversion associated with gross genomic rearrangements causing severe HA.
Methods: All 26 exons and flanking intron/exon boundaries of F8 gene were amplified and sequenced directly. Intron 1 inversion was detected by long-fragment PCR described by Bagnall et al. (2002). We used the AccuCopy technology, a copy number variation (CNV) genotyping method based on multiplex competitive amplification, for quantitative analysis of copy number of all 26 exons in the F8 gene. Affymetrix CytoScan HD array was also applied to detect CNVs of the whole genome. Breakpoints of gross genomic rearrangements were identified by primer walking strategy and genome walking technique.
Results: No point mutation was identified in all 26 exons, flanking intron/exon boundaries, promoter, 5′ and 3′ regions of the F8 gene. An abnormal pattern of intron 1 inversion was detected in the index patient: one corresponding to the normal int1h-2 region (1191 bp) and the other corresponding to the band with the inversion of the int1h-2/1 sequence (1776 bp). AccuCopy showed normal dosage of all 26 exons in the F8 gene. Affymetrix CytoScan HD array revealed a duplicated region between F8A2 and int1h-2, which included three genes: VBP1, RAB39B and CLIC2. In addition, a deleted region between int1h-1 and exon 2 of F8 gene was detected. We successfully characterized the breakpoints of the deleted region, revealing that a genomic rearrangement occurred between the deleted region and the additional copy of the fragment between F8A2 and Int1h-2 in the process of intron 1 inversion.
Conclusions: We presented the case of a patient with an aberrant pattern of intron 1 inversion in the F8 gene causing severe HA and illustrated the complex molecular mechanism of the inversion associated with gross genomic rearrangements.

PB 2.35-4

Hemarthrosis-induced heme oxygenase-1 reduces the immune response to FVIII in hemophilic mice

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Background: Major bleedings in patients with hemophilia A are treated by on-demand administration of therapeutic FVIII. The role of bleeding severity has been evoked as a possible risk factor for the development of anti-FVIII 'inhibitory' antibodies. It remains unclear however whether the increased risk for inhibitor development is a direct consequence of bleeding, that presumable creates a local inflammatory microenvironment, or of the elevated dose of infused FVIII.
Aim: We evaluated the impact of induced hemarthrosis on the development of an anti-FVIII immune response in FVIII-deficient mice.
Methods: Hemarthrosis was induced in the left knee of 6–7-week old FVIII-deficient mice by intra-articular space insertion of a needle. One week later, mice received 1 IU FVIII once a week for 3 weeks. The levels of inhibitory anti-FVIII IgG titers were evaluated by ELISA and Bethesda assay. Induction of arthropathy was validated 3 weeks after injury by measuring knee diameters. Expression of heme oxygenase-1 (HO-1) was assessed by Western-blot in spleen, liver and knee joint extracts 1 week after hemarthrosis induction.
Results: Induction of hemarthrosis lead to the development of arthropathy 3 weeks after injury with knee diameters of 3.48 ± 0.08 mm in the injured left knee and 3.07 ± 0.02 mm in the untouched right knee ($P < 0.0001$). Unexpectedly, levels inhibitory anti-FVIII IgG were significantly lower in injured mice as compared with sham-treated mice after 3 weeks of FVIII administration, as measured by ELISA (mean ± SEM: 132 ± 32 vs. 334 ± 46 μg/mL, respectively, $P < 0.05$) and Bethesda assay (29 ± 8 vs. 111 ± 22 BU/mL, respectively, $P < 0.005$). We then assessed the induction of the heme detoxifying enzyme HO-1. Induction of hemarthrosis was associated with the induction of HO-1 in the injured knee, but not in the untouched knee, spleen or liver.
Conclusions: The induction of hemarthrosis in hemophilic mice reduces the intensity of the anti-FVIII immune response against exogenously administered FVIII. This reduction is associated with a local induction of HO-1 on week after injury, at the time of the first FVIII administration. Our previous data had shown that the systemic induction of HO-1 upon heme injection was able to drastically reduce the onset of the anti-FVIII immune response in experimental hemophilia A. The present data indicate that the local induction of HO-1 in a non-immunological compartment is sufficient to modulate the immune response to FVIII. Our results showing that major bleeds in mice, leading to extravascular blood release and hemolysis, induce HO-1, thus reducing the immune response to FVIII, are in contrast with empirical observations in hemophilia A patients. Indeed, in patients, major bleeds and the ensuing administration of large doses of FVIII, have been proposed as predisposing factors for inhibitor development. Of note, while patients are generally treated as soon as bleeding occurs, the mice received FVIII 1 week after injury. Whether differences in timing of bleeding induction and FVIII administration, or in the intrinsic capacity to induce HO-1 are responsible for the observed discrepancy, remains to be elucidated.

PB 2.35-5

The development and binding mechanism of FVIII Trp1707Ser related inhibitors

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Background: Haemophilia A is due to the mutations of F8 gene. The replacement of factor VIII is the most effective treatment now. But FVIII antibodies develop in approximately 20–50% of patients with HA, which complicates the management of hemorrhage.
Aims: To investigate the binding mechanism of inhibitors in FVIIITrp1707Ser missense mutation.
Methods: LD-PCR and PCR were adopted for the screening of the INV22 and INV1 respectively. The F8 coding and boundary sequences were analyzed by sequencing. Inhibitors were reacted with different segments of FVIII (Heavy chain, light chain, A1, A2, A3, C1, C2), and corrected test was used to measure the remaining FVIII:C(%) by adding pooled plasmas of normal persons. Inhibitors in plasma were purifed by affinity chromatography. After labeling purified inhibitors with biotin, wesern blot was used to further confirm the binding reactions between inhibitors and segments which employed each segment as an antigen and biotin-labeled inhibitors as antibodies.
Results: F8 gene direct sequencing showed that the HA patient with inhibitor had the missence mutation c.97223G>C in exon 14 of F8 gene (p.Trp1707Ser). Corrected method detected that the remaining FVIII:C were increased when inhibitors reacted with FVIII heavy chain and light chain, and the reaction with heavy chain was increased more obvious. The remaining FVIII:C were also increased in the A2 and C2 domain reactions, and the A2 domain reaction was increased more obvious. No significant differences were seen in the A1, A3 and C1 domain reactions. The western blot results further confirmed the previous results as bands were seen when degenerated B-domain deleted recombinant FVIII, heavy chain, light chain, A2 and C2 were used as antigens. The bands were more darker when heavy chain and A2 were used as antigens. No band was detected in the reaction with A1, A3 and C1 domain.
Conclusions: The binding sites of the FVIII Trp1707Ser related inhibitors were in the A2 domain of FVIII heavy chain and C2 domain of the light chain. The binding reaction was more intense with the A2 domain of the heavy chain. Inhibitors binding to A2 domain may affect FVIII to activate FIX as a cofactor, and inhibitors binding to C2 domain may block the interations between FVIII and phospholipids or VWF.

PB 2.35-6

Functional FVIII assays and bleeding phenotype in 13 patients with mild haemophilia A due to the p.Tyr365Cys mutation

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Aims: The aim of this study was to define the laboratory characteristics and bleeding phenotype of a cohort of individuals with an identified p.Tyr365Cys mutation.
Methods: The Institute of Genetic Medicine at the International Centre for Life, Newcastle upon Tyne, identified all individuals with the p.Tyr365Cys mutation registered on our North of England database. Their bleeding history was evaluated and historical one stage and chromogenic FVIII assays were recorded. Factor XI assay, von Willebrands disease screen and platelet aggregation studies were performed to rule out a coincidental additional bleeding disorder.
Results: Thirteen individuals had haemophilia A with the p.Tyr365Cys mutation. Median age at diagnosis was 34 years: range 1–69 years. Six were identified incidentally when coagulation screening showed a prolonged APTT, three were identified by family screening and three presented with bleeding symptoms. The route to diagnosis for one patient was unclear.
Recurrent severe epistaxis was reported by one individual; further investigation confirmed the co-existence of Type 2N von Willebrands disease. A second patient had recurrent spontaneous muscle haematomas and was found to have an additional F8 mutation associated with a moderate bleeding phenotype.
Prior to diagnosis six individuals experienced bleeding (five dental, four trauma-induced, one post-surgical); in only one case was this bleeding sufficient to prompt investigation and diagnosis of a bleeding disorder. Two of these patients had experienced haemostatic challenges (surgical and dental) at other time-points with no significant haemorrhage. Two individuals had undergone dental extraction as their only haemostatic challenge without any bleeding.
Median One Stage FVIII level was 33% (range: 8–46%) and Chromogenic assay 62% (range: 30–113%) with inverse discrepancy seen in all patients. FXI level and platelet aggregation studies were normal. One individual was found to have von Willebrands disease (type 2N).
Nine individuals received haemostatic treatments (DDAVP or FVIII concentrate) following diagnosis. In nine of thirteen treatment episodes the treatment was prophylactic: dental extraction (3), spinal surgery or regional anaesthesia (3), amputation of limb (1), orthopaedic surgery (2). Two individuals were treated following significant trauma and one was managed with FVIII concentrate for spontaneous haematomas (this patient had a second F8 mutation and moderate haemophilia A). No haemorrhagic complications occurred.
Conclusions: Our data support previous publications in which patients with the p.Tyr365Cys mutation are reported to have minimal bleeding symptoms and a significantly higher chromogenic FVIII concentration. Prophylactic haemostatic treatment prior to intervention may not be required and may represent unnecessary exposure to FVIII concentrate. In addition we can confirm the value of carefully investigating patients with significant bleeding in this patient group, as coexistent bleeding disorders are likely to be identified and will require specific treatment.

PB2.36 – Haemophilia A: Clinical Research – VI

PB 2.36-1

Low titre FVIII inhibitor assay may substitute the recovery and half-life measurements in detection of tolerance after immune tolerance induction therapy

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Background: Immune tolerance induction is so far the only proven method to eradicate FVIII inhibitors in patients with severe haemophilia A. Tolerance is currently defined as normalization of FVIII pharmacokinetic parameters after administration of FVIII, especially FVIII half-life and 30-minutes FVIII recovery. FVIII recovery below 66% and half-life < 6 h were taken as indicative of the presence of FVIII inhibitors in the International Immune Tolerance Study (I-ITI Study). However, pharmacokinetic evaluations in patients are burdensome because of the need for multiple blood samples.

Methods: In order to investigate whether inhibitors assays can be used as significant parameters to detect the state of tolerance, the Nijmegen assay (NA), the Bethesda assay (BA) and the Low titre assay (LTA) were performed on plasma samples from 13 pharmacokinetic studies on those subjects in the I-ITI study who had successfully achieved immune tolerance. Both the sensitivity and specificity of each FVIII inhibitor assays were investigated relative to the pharmacokinetic parameters. In addition, the usefulness of measuring FVIII-recovery within 30 min was explored.

Results: The inhibitor data were all within the normal range (0.0–0.2 BU/mL) using the NA but varied from 0.2 to 0.9 BU/mL with the BA including two subjects with abnormal inhibitor values (0.8 resp.0.9 BU/mL). The LTA varied between 0.00 and 0.18 BU/mL. Neither BA levels ($P = 0.70$) nor NA levels ($P = 0.91$) correlated significantly with FVIII half-life. In contrast, the LTA correlated significantly with the corresponding half-life ($P = 0.02$).

FVIII-recovery was normal in all patients and no correlation was detected between the half-life and recovery 30 min post-infusion. Furthermore, FVIII half-life of 7.0 h was established as the minimum normal half-life and was used for further analysis of the results. The Receiver Operating Characteristic (ROC) curves of the BA, the NA, LTA and FVIII recovery were calculated using a half-life of 7.0 h as inhibitor status criterion. The highest specificity (75%) combined with a sensitivity of 100% was observed for the LTA representing a cut off value of 0.04 BU/mL.

Conclusion: The low titre assay is a reliable tool to exclude the presence of low titre of FVIII inhibitors, and therefore, may represent a new and important parameter in the determination of successful immune tolerance induction outcome.

PB 2.36-2

A retrospective observational multicenter cohort study on peri-operative factor VIII consumption in hemophilia A ('OPTI-CLOT' studies)

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Background: Hemophilia A is an X-linked bleeding disorder, caused by a deficiency of clotting factor VIII (FVIII). Treatment of hemophilia with coagulation factors is costly. Previous studies have demonstrated that FVIII consumption in prophylactic treatment can be significantly reduced by individualized dosing regimens. However, data on peri-operative FVIII consumption, clearance and modifying factors are scarce.

Aims: To evaluate peri-operative FVIII consumption and achieved FVIII levels in hemophilia A with attention to patient and surgical characteristics.

Patients and Methods: In this retrospective observational multicenter cohort study, we included patients with severe and moderate hemophilia A (FVIII levels < 0.05 IU/mL), without an inhibitor, undergoing elective, and low or medium risk surgery from January 2000 until July 2012, from three Dutch Hemophilia Treatment Centers. The study was not subject to the Medical Research Involving Human Subjects Act and was approved by the Medical Ethics Committee. FVIII concentrates were administered with the aim to achieve the following target levels advised by the Dutch Hemophilia Consensus: day 1: 0.8–1.0 IU/mL; day 2–5: 0.5–0.8 IU/mL; >day 6: 0.3–0.5 IU/mL. The achieved FVIII plasma levels were generally checked daily. Data were collected on clinical and surgical characteristics, peri-operative dose and mode (bolus infusion: BI or continuous infusion: CI) of FVIII concentrate administration, achieved FVIII levels and bleeding complications.

Results: A total of 176 surgical procedures was performed in 99 patients: 61 adults (123 surgeries; median age 43 years; median weight 80 kg) and 38 children (53 surgeries; median age 4 years; median weight 18 kg). In adults, mainly orthopedic surgeries were performed (46%), in children mainly intravenous catheters were inserted or removed (55%). Peri-operative bleeding complications were reported in 28 procedures (16%), not associated with low FVIII levels; in seven patients (4%) reoperation was required for persistent bleeding.

Median amount of infused FVIII concentrate during hospitalisation was 20,063 IU (443 IU/kg) per surgical procedure. Sixty-one percent of achieved peri-operative levels in steady state was above highest required levels (median +0.27 IU/mL) according to the Dutch Hemophilia Consensus. Only 25% was within target range and 14% of levels was below the target range (median −0.14 IU/mL).

CI was used more frequently (64%) than BI (36%), especially in children compared to adults (51% vs. 31%). CI and BI did not differ in bleeding complications or achieved FVIII levels. For adults the median amount of infused FVIII concentrate was significantly higher when treated by CI (445 IU/kg) compared to BI (161 IU/kg) ($P < 0.001$), after correction for duration of hospitalisation this difference did not persist; CI: 40 IU/day and BI: 38 IU/day ($P = 0.51$). Further analyses are ongoing to differentiate between indications for surgery.

Conclusion: Sixty-one percent of peri-operative FVIII levels was above the predefined target levels. These data support that there is room for improvement in the efficacy of peri-operative dosing. PK-guided dosing with iterative pharmacokinetic modelling in the peri-operative period is therefore promising.

PB 2.36-3

Bleeding frequency and consumption of FVIII concentrate during on-demand and prophylactic treatment with Human-cl rhFVIII in prospective clinical studies in adult patients with severe haemophilia A

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Background: There is ample evidence to support prophylactic treatment with factor VIII (FVIII) in children with severe haemophilia A (HA). Adults with severe HA, however, are often treated on-demand only and the potential benefit of regular prophylaxis is linked to a higher consumption of costly FVIII concentrates. During the clinical development of Human-cl rhFVIII, the first recombinant FVIII concentrate expressed in a human cell line, its efficacy and safety was evaluated in previously treated adult patients (PTPs) during on-demand treatment only (GENA-01) and prophylaxis (GENA-08). The two studies were similarly designed, e.g. regarding required study duration, and analyzed, e.g. regarding documenting, characterizing and treating bleeding episodes (BE).

Aims: To compare *post-hoc* the BE frequency and the use of FVIII concentrate in patients treated exclusively on-demand with those treated prophylactically.

Methods: Both prospective multi-centre studies were approved by the Ethics Committees of each participating institution and informed consent was obtained from the patient prior to any trial-related activity. In GENA-01, patients were to be treated on-demand for ≥ 6 months and ≥ 50 exposure days with protocol recommended doses ranging from 20 to 60 IU/kg, depending on the severity of the BE. In GENA-08, patients were to be treated prophylactically with Human-cl rhFVIII every other day with 30–40 IU/kg for ≥ 6 months. Human-cl rhFVIII was also to be used in case of breakthrough BEs using the same doses as in GENA-01.

Results: Twenty-two PTPs with severe HA were enrolled in GENA-01, and 32 in GENA-08. The study populations were reasonably well com-

parable to each other (GENA-01 vs. GENA-08), regarding age (39.6 ± 14.1 vs. 37.3 ± 13.6 years), BMI (23.9 ± 4.8 kg/m^2), haemophilia joint health score (38.4 ± 30.3 vs. 34.6 ± 32.2), race (> 80% White in both studies) and historical bleeding sites. In GENA-08, the majority of patients (65.6%) had been treated prophylactically prior to study entry. Their historical mean BE rate/month was 0.540, and their mean prophylactic dose/month was 293 IU/kg. The other patients who had been treated on-demand had a mean BE rate/month of 3.924. In GENA-01, all but two patients were treated on-demand prior to study entry. The historical mean BE rate/month of all GENA-01 patients was 4.07. During the studies, the GENA-08 patients experienced a mean of 0.19 ± 0.31 BEs per month, and the GENA-01 patients 4.77 ± 2.54 BEs per month. Their respective mean drug consumption was 475.5/kg/month (466.1 for prophylaxis, 9.4 IU/kg/month for treatment of breakthrough BEs) and 156.9 IU/kg/month, respectively.

Conclusion: The data suggest that regular prophylactic treatment with Human-cl rhFVIII in adult PTPs with severe HA results in an approximately 25-fold reduction of BE rate, and 4-fold increase of FVIII concentrate consumption.

PB 2.36-4

Atrial fibrillation in people with hemophilia: a cross-sectional evaluation in Europe

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Background: With increasing life expectancy of people with hemophilia (PWH) in developed countries, the number of PWH affected with age-related diseases is also increasing. Atrial fibrillation is a common health problem in the general population, but in PWH, evidence-based guidelines for the management of AF are lacking.

Aims: The aim of this cross-sectional pan-European study is to analyze the prevalence of AF and risk factors for stroke in our adult hemophilia population and to document current anticoagulation practice.

Methods: The ADVANCE Working Group consists of members from 14 European hemophilia centers. Each center retrieved data on the number of PWH with AF in their hemophilia population, as well as their total number of adult PWH. For each person with AF, a case report form was completed.

Results: In total, 29 PWH with AF were documented of whom 25 (86%) with hemophilia A and 4 (14%) with hemophilia B. The mean age was 68.2 years (IQR 62–75.5). Hemophilia was severe in 6 (20.6%), moderate in 6 (20.6%) and mild in 17 (58.6%) patients. The prevalence in the total studied hemophilia population was 0.94% (29/3094) and increased with age; in patients > 40 years it was 1.7% (29/1723) and in patients > 60 years 3.6% (23/635). The median CHA_2DS_2-Vasc score was 1.0 (IQR 0–2). Hypertension was reported in 12 patients (41.4%), diabetes in 3 (10.3%), previous stroke or TIA in 1 (3.4%), peripheral vascular disease in 4 (13.8%). In 11 patients (37.9%), anticoagulation was started of whom nine low dose aspirin and two vitamin K antagonists. Of these 11 patients, nine had mild hemophilia, one moderate and one severe with FVIII prophylaxis. During follow-up after diagnosis (mean follow-up 52.9 months), there were no thrombotic events reported, nor increases in bleeding severity.

Conclusion: In this largest cohort of PWH with AF so far, the prevalence of AF in hemophilia increases with age and is predominantly present in mild hemophilia. Based on the population based CHA_2DS_2-Vasc risk scores, PWH have a low stroke risk that might be even lower considering the hypocoagulable state. Hemophilia doctors prescribe anticoagulation therapy approximately in half of their mild hemophilia patients and very few in moderate and severe.

PB 2.36-5

Beriate® P in the treatment of patients with haemophilia A: update of a long-term pharmacovigilance

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Pharmacovigilance studies are effective tools to collect data on products in the post authorization period. This study assessed the long-term efficacy, tolerability and safety of a high purity plasma-derived FVIII concentrate, Beriate® P (CSL Behring GmbH, Marburg).

Previously untreated – and previously treated patients at any age with haemophilia A, who had received Beriate® P and who fulfilled the inclusion criteria were enrolled. Based on the proceeding at the centres, patients were routinely screened every 3–12 months. Parameters documented comprised efficacy-, safety-, pharmacoeconomic- and pharmacokinetic data.

Up to now, 86 patients were included into this study [median duration: 44 months (0–115)] and data from 809 visits were available for analysis. Seventy-one patients suffered from severe-, 10 patients from moderate- and four patients from mild haemophilia A. In one patient, the information on severity of haemophilia A was missing. There was no proven virus transmission for hepatitis A-, hepatitis B-, hepatitis C- or human immunodeficiency virus. Median patient age was 19.8 years (0.1–74.6).

Eighty-two percent of the patients (N = 68) received prophylaxis with at least one infusion per week. Median average number of bleeds per year was 3.23 (0–42.98) and a median of 2.0 infusion per bleeding was administered. Seventy-four percent of patients (N = 72) experienced no major bleeds and 11% experienced no bleed at all with regard to all sorts of bleeding. Efficacy of Beriate® P was assessed as good or excellent in 98.5% of patients with minor bleeds and 94% in patients with major bleeding.

Two cases of inhibitor development were reported; one inhibitor was transient and the second inhibitor was treated with immune tolerance therapy. The results included in this interim analysis confirm the very good efficacy, tolerability and safety of Beriate® P.

PB 2.36-6

Comorbidities in patients with haemophilia and other rare bleeding disorders: a cross sectional study

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Background: Broad availability of virus-inactivated clotting factor concentrates and newer treatment options for HIV and HCV infected individuals has led to a near normal life expectancy in people with haemophilia (PWH) and other rare bleeding disorders (RBD). Thus, this patient cohort is facing comorbidities that are common in the general population but may pose a challenge to healthcare professionals due to the underlying bleeding disorder.

Aims: The aim of this study was to quantify comorbidities among PWH and patients with other RBD aged over 40 years who are regularly treated at the Haemophilia Center Bonn. The present sub-analysis focused on cardiovascular risk factors and diseases.

Methods: We conducted an observational, descriptive, cross-sectional study in PWH and patients with other RBD who were treated at the Haemophilia Center Bonn in 2011. We considered inclusion of patients with haemophilia A and B, type 2 and 3 von Willebrand disease (VWD) as well as patients with deficiency of clotting factors I,

VII, XI or XIII. Patients with type 1 VWD and carriers of haemophilia were excluded. Eligible patients were identified in our electronic patient database and comorbidity information was extracted from medical records.

Results: In total, 348 (42.8%) out of 814 patients were aged over 40 years. From these 348 patients 165 (47.4%) had severe forms of haemophilia or other RBD, 100 (28.7%) were moderate and 83 (23.9%) had a mild form of disease. One hundred and twenty-one (34.8%) patients had active HCV, 52 of these patients had mild to moderate liver fibrosis (F1-F2) and 43 patients had high-grade liver fibrosis or cirrhosis (F3-F4) as documented by transient elastography. HIV-infection was present in 87 (25%) patients over 40 years of age. Body mass index (BMI) was calculated in 343 out of 348 patients: four patients (1.2%) were underweight (BMI < 18.5 kg/m^2), 143 (41.7%) were of ideal body weight (BMI 18.5–25 kg/m^2), 152 (44.3%) were overweight (BMI 25–30 kg/m^2) and 44 (12.8%) were obese (BMI > 30 kg/m^2). The following cardiovascular diseases were identified: arterial hypertension in 109 (31.3%) patients, coronary heart disease in 19 (5.5%) patients, myocardial infarction in 8 (2.3%) patients, atrial fibrillation in 7 (2%) patients and apoplexy in 3 (0.9%) patients. Mean age at diagnosis of coronary heart disease was 63.12 years (42–83 years, standard deviation 11.93) and for atrial fibrillation 65.57 years (50–79 years, standard deviation 11.01).

Summary/Conclusion: Comorbidities, especially cardiovascular risk factors and diseases are present in a substantial proportion of patients with haemophilia and other RBD aged over 40 years. Careful assessment of comorbidities and guidance of affected patients is of increasing importance in haemophilia care.

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PB 2.37-1

FXIII- a novel treatment in haemophilia

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Background: Clot stability and structure are normalised if factor XIII (FXIII) is added to Factor VIII (FVIII) deficient plasma. FXIII could be an effective, novel therapy in haemophilia.

Aims: Investigate the effect of supra-physiological levels of FXIII in whole blood from patients with severe haemophilia A and evaluate the mechanisms of FXIII in haemophilia.

Methods: Six patients with severe haemophilia A and four healthy controls were included. Blood was drawn into citrate and CTI 30 μg/mL and spiked with rFVIII (0, 0.025, 0.05, 0.1, 1 IU/mL), plus FXIII (0, 0.5, 1 or 2 IU/mL). Clotting was triggered with tissue factor (TF, Innovin, 1:50000) plus calcium and measured using thromboelastometry. To assess clot stability the above assay was performed with t-Pa 1.5 nM. Area under the elasticity curve (AUEC) was the endpoint.

Thrombin generation (TG) was recorded in FVIII deficient plasma spiked with FVIII and FXIII as above using TF (1PM) and the CAT method (Thrombinoscope BV).

Effects of increasing concentrations of FXIII on FXIII activation were investigated using FXIIIa fluorogenic sustrate. TF (1:50000), Phospholipid (4 nM) and FXIIIa fluorogenic substrate peptide (A101, Zedira, 50 μM) were combined in microtiter wells. FVIII deficient plasma spiked with FVIII plus FXIII as above was added and coagulation triggered with calcium. FXIII activation was recorded as changes in the fluorescent signal.

A bleeding model in FIX deficient mice was used to see if infusion of FXIII (up to 50–200 IU/kg) could improve average time to haemostasis following saphenous vein incision compared to controls.

Results: FXIII added to whole blood resulted in a significant dose-dependent increase in clot stability at all concentrations of FVIII ($P < 0.05$, ANOVA). Mean clot stability in haemophilia patient samples could be normalised at low concentrations of FVIII when FXIII was administered as an adjunct as shown by a normalised AUEC with 0.08 IU/mL of FVIII plus FXIII 0.5 or 0.05 IU/mL FVIII combined with 2 IU/mL FXIII. FXIII had no significant effect of parameters of clot formation. FXIII significantly enhanced thrombin generation, shortening ttPeak and lagtime ($P < 0.05$). ELISA excluded FXIII contamination by FVIII.

The dynamic FXIII activation assay showed that addition of FXIII stoichiometrically regulates efficiency of conversion to FXIIIa, even at low FVIII concentrations.

The data from the mouse-bleeding model indicated that FXIII did not significantly change average time to haemostasis. Infusion of FXIII did not result in animal death.

Conclusion: The study shows that exogenous FXIII is able to normalise clot stability in whole blood samples from haemophilia patients. The failure to enhance haemostasis in the mouse model may reflect high endogenous FXIII and an inability to induce supra-physiological levels. FXIII enhances TG and this may provide a novel explanation for the clot modifying effects previously reported. We demonstrated that supra-physiological FXIII stoichiometrically enhances FXIII activation, providing a rational for the clot stabilising effects of FXIII in the presence of the abnormal thrombin generation of haemophilia. These results support the use of FXIII as a novel treatment in haemophilia and merits initiation of clinical trials.

PB 2.37-2

Bleeding pattern and median time interval between bleeding episodes amongst patients receiving on-demand and prophylaxis therapy

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Background: Prophylaxis with factor VIII (FVIII) is considered the optimal treatment for the management of hemophilia A patients without inhibitors. The recently published ADVATE (rAHF-PFM) Prophylaxis Study[1] compared the efficacy of two prophylaxis regimens (pooled prophylaxis) and between on-demand and either standard or PK-tailored prophylaxis treatments. Previously on-demand-treated patients of median age 26 (range: 7–59 years; ($N = 53$ PP population) with FVIII levels ≤ 2% received 6 months of on-demand treatment and then were randomized to 12 months of either standard (20–40 IU/kg every other day) or pharmacokinetic (PK)-tailored (20–80 IU/kg every third day) prophylaxis, both intended to maintain FVIII trough levels ≥ 1%. Twenty-two (33.3%) patients on prophylaxis experienced no hemorrhages, whereas no patients treated on-demand were hemorrhage-free. The annual bleeding rate (ABR) for the two prophylaxis regimens were comparable, whereas differences between on-demand and either prophylaxis regimen were statistically significant ($P < 0.0001$).

Objectives: To evaluate the bleeding pattern and median bleed-free intervals (days) in patients who have taken part in the rAHF-PFM Prophylaxis Study[1].

Results: Herein, we report on an analysis of the joint bleeding and total hemorrhages among patients in the ADVATE (rAHF-PFM) Prophylaxis Study. Among all patients in the per protocol analysis set ($N = 53$), 1351 total hemorrhages occurred during the on-demand

phase, of which 1164 were hemarthroses. Seventy-seven hemorrhages (55 hemarthroses) occurred among patients randomized to the standard prophylaxis arm and 75 (72 hemarthroses) among patients in the PK prophylaxis arm ($P < 0.0001$ for both total and joint bleeding between either prophylaxis arm and on -demand).
The median differences in the interval (days) between bleeding events was significant comparing on-demand to either prophylaxis arm: with median time (days) between bleeding for on-demand of 6.3 days vs. 184.5 days in the standard prophylaxis arm and 183 days in the PK prophylaxis arm ($P < 0.0001$). When investigating the trough levels in the two prophylaxis arms the median trough levels differed: 2.5 IU/dL in the standard prophylaxis arm ($N = 24$) and 1.0 IU/dL in the PK prophylaxis arm ($N = 23$). It appears that trough level of 1.0 IU/dL in the PK prophylaxis arm was enough to achieve 1 ABR in this study.
No deaths, inhibitors or cases of hypersensitivity related to rAHF-PFM occurred during the conduct of this study.
Conclusions: These results provide additional evidence for the superiority of prophylaxis compared to on-demand treatment in severe hemophilia A and further indicate that a PK-tailored regimen may be effectively administered every third day.

PB 2.37-3

AURIGA: study of adherence to prophylaxis in patients with severe haemophilia A

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Background: An international study performed in 18 countries showed reduced adherence to prophylaxis in adolescent patients compared with patients aged < 12 years (Geraghty et al. *Haemophilia*. 2006;12:75–81).
Aim: To assess adherence to prophylaxis with factor VIII (FVIII) in patients with severe haemophilia A aged 6–21 years.
Methods: We assessed adherence to prophylaxis by comparing the amount of FVIII administered by the patient with the amount prescribed by their physician over a 2-year period. To evaluate adherence from the patient's perspective, we developed a questionnaire ad hoc to assess the benefits of prophylaxis, barriers to adherence, patient perception of self-efficacy (patient's belief that he is able to self-administer the treatment and take care of his own health), and self-care practices. The Haemo-QoL questionnaire was administered to assess patient quality of life. The study was approved by the medical ethics committees of participating hospitals. Informed consent was obtained of each patient.
Results: Seventy-six patients were recruited from 14 centres in Spain between November 2011-November 2012. Patient data were retrospectively collected over a 2-year period before the date of inclusion in the study. Patient adherence ranged from 35.6% to 100%, with a mean (SD) of 95.0% (11.5%). Children aged < 12 years had a slightly higher adherence rate (95.7%) than adolescents (94.9%) ($P = 0.64$). Adherence scores ranged from 25 to 119 (mean, 87.3; higher scores indicate better adherence). The mean score for patients aged ≤ 7 years was 88.9; for patients aged 8–12 years, 86.4; for patients aged 13–16 years, 85.6; and for patients aged ≥ 17 years, 80.9 ($P = 0.18$). Adherence was higher when parents were responsible for administration (95.7%) than when patients self-infused (93.8%) ($P = 0.50$). Patients with lower adherence had more joint problems than those with better adherence ($P > 0.01$). The average Haemo-QoL score for patients aged ≤ 7 years was 74.5; for patients aged 8–12 years, 77.9; for patients aged 13–16 years, 78.8; and for patients aged ≥ 17 years, 89.0 ($P < 0.05$). The quality of life reported by patients (77.6) was higher than that reported by their parents (73.8) ($P = 0.03$).
Conclusions: Minimal differences in adherence between age groups were observed in Spanish patients with haemophilia A, unlike studies published in other European countries, in which adherence dramatically decreased during adolescence. This may be because in Spain, the transfer of responsibility for treatment from the parent to the child does not occur until early adulthood. Regarding the influence of psychological variables, patient-reported scores on the adherence questionnaire indicate that, as patients become older, they perceive more barriers to continuing treatment (time, effort, and day-to-day interferences), fewer benefits of prophylaxis, and less self-efficacy (ie, they believe they are less able to take care of their own health). If this trend continues, it could lead to adherence issues in adulthood. As expected, patients with lower adherence had more joint problems and, therefore, worse general health. The improvement in quality of life seen with patient age may reflect that patients are better able to accept and adapt to haemophilia as they get older.

PB 2.37-4

Major surgery in severe haemophilia A with inhibitors using a recombinant factor VIIa/activated prothrombin complex concentrate hybrid regimen

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Background and Aim: Major surgery in haemophilia A and inhibitors is increasingly performed with recombinant FVIIa (rFVIIa) or activated prothrombin concentrate (APCC). Evidence of superiority of one agent over the other is lacking. Dosing recommendations are available but the optimal regimen remains unclear. We report a hybrid regimen of rFVIIa and APCC (FEIBA®) in six total knee replacements (TKR), one emergency orchidectomy and one emergency open appendectomy in four patients with haemophilia A and inhibitors performed at our centre.
Methods: All patients received rFVIIa 90 μg/kg 2 hourly for at least 48 h and switched to FEIBA between days 2 and 5. RFVIIa was reduced to 90 μg/kg 3 hourly on the days 2 and 3 and 90 μg/kg 4 hourly on days 4 and 5. All received tranexamic acid 1gram QDS and this was stopped 12 h before switching to FEIBA®. FEIBA® 200 U/kg/day in three divided doses was given when switched up to day 5 and 100–150 U/kg/day in two divided doses thereafter.
Results: The joint functionality was excellent after all TKR at 1 year. In three (patients 1 and 4) there were no haematological bleeding complications (one had a surgical bleed that stopped immediately after ligation of a spurting vessel). Patient 2 developed a possible muscle bleed on day 5 following a TKR on 150 U/kg/day FEIBA® and was switched back to rFVIIa, 90 μg/kg 2 hourly for 24 h after which FEIBA® was resumed without further complications. Patient 3 developed a quadriceps haematoma on day 6 after a TKR whilst on 100 U/kg/day FEIBA®. He was switched to rFVIIa in reducing frequency for the remainder of his treatment without further complications. Patient 4 had three other procedures. An emergency orchidectomy was complicated by a large hemiscrotum haematoma on day 10 after stopping treatment with FEIBA® on day 9. An emergency open appendectomy was complicated by a large intra-abdominal haematoma on day 1 on 2 hourly rFVIIa 90 μg/kg. This settled on increasing doses, 150 μg/kg 2 hourly for eight doses, after which he was switched to FEIBA® without further complications. Finally, wound site bleeding developed on day 1 after a second TKR on 2 hourly rFVIIa 90 μg/kg, unresponsive

to increased doses of rFVIIa, but settled after switching to FEIBA® 200 U/kg/day. He rebled on day 6, on the same dose of FEIBA®. Sequential treatment with rFVIIa (105 μg/kg 2 hourly and FEIBA® 62.5 U/kg 12 hourly) was given for 12 h. The bleeding settled and treatment with FEIBA® was continued.

Conclusion: We report a hybrid regimen of rFVIIa and APCC in major surgery. There was bleeding in two patients (five procedures, including one surgical bleed). There were no long term sequelae and joint functionality after TKR was excellent. To our knowledge there have been no reports of the systematic use of a hybrid regimen combining the flexibility of rapid dose changes of rFVIIa and less frequent dosing with APCC. A further advantage is a significant cost saving compared to the use of monotherapy with rFVIIa.

PB 2.37-5

Helixate® NexGen for the treatment of hemophilia A: update of a long-term pharmacovigilance project

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This multinational (Austria, France, Germany, Italy, Sweden) project is assessing the long-term efficacy, tolerability and safety of Helixate® NexGen (CSL Behring, Marburg), a recombinant FVIII concentrate, in the post authorization period.

PUPs and PTPs with hemophilia A at any age treated with Helixate® NexGen are eligible for enrollment. Patients are routinely screened every 3–12 months.The following parameters, as determined routinely for these patients (non-interventional design), were documented: overall clinical response, bleeding events, adverse drug reactions including the incidence of inhibitors, laboratory safety and virus safety parameters, relevant concomitant diseases, relevant concomitant medication. Pharmacokinetic data were also collected if available. Treatment modalities with Helixate® NexGen, including average factor consumption per month and exposure days were recorded.

Data from a total of 223 patients with a total of 2306 visits were available for this update. The majority of the patients (82%) had severe hemophilia A. The median age was 23.0 years (range: 15 days–68 years). Median time between visits to the respective center was 4 months (range: 0–48 months).

The majority of patients are under prophylactic treatment (83%). The most common prophylaxis regimen was 2–3 infusion (64.5%) per week. The median number of bleeds per year was 2.57 per patient. A median number of two infusions were administered per bleeding. Eighty-two percent of the patients had no major bleeding and 14% experienced no bleed at all. During the up to 11-year observation period only three cases of inhibitor development were reported.

Efficacy of Helixate® NexGen in the treatment of bleeding was assessed as good or excellent in 97% of all documented bleeds. The results included in this interim analysis confirm the excellent efficacy, tolerability and safety of Helixate® NexGen.

PB 2.37-6

Applicability of the ESC guidelines on management of acute coronary syndromes to people with haemophilia – an assessment by the ADVANCE Working Group

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Background: There are no evidence-based guidelines for antithrombotic management in people with haemophilia (PWH) presenting with acute coronary syndrome (ACS)

Aim: Review of current European Society of Cardiology guidelines, and to consider how best they should be adapted for PWH.

Methods: Structured communication techniques based on a Delphi-like methodology were used to achieve expert consensus on key aspects of clinical management.

Results: The main final statements are: a) ACS and myocardial revascularization should be managed promptly by a multidisciplinary team that includes a haemophilia expert, b) Each comprehensive care centre for adult PWH should have a link to a cardiology centre with an emergency unit and 24 h availability of PCI, c) PCI should be performed as soon as possible under adequate clotting factor protection, d) Bare metal stents are preferred to drug eluting stents, e) Anticoagulants should only be used in PWH after replacement therapy, f) Minimum trough levels should not fall below 5–15% in PWH on dual antiplatelet therapy, g) The duration of dual antiplatelet therapy after ACS and PCI should be limited to a minimum, h) PWH receiving antiplatelet therapy should be offered gastric protection, i) The use of GPIIb-IIIa inhibitors is not recommended in PWH other than in exceptional circumstances, j) The use of fibrinolysis may be justified in PWH when primary PCI (within 90 min) is not available ideally under adequate clotting factor management.

Conclusion: It is hoped that the results of this initiative will help to guide optimal management of ACS in PWH.

PB2.38 – Haemophilia A: Clinical Research – VIII

PB 2.38-1

Heterogeneity of bleeding classification among randomized studies in hemophilic patients

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Background: Bleeding in patients with hemophilia includes intraarticular bleeding, intramuscular bleeding, and mucocutaneous bleeding. Bleeding classifications, as defined by EMEA and ISTH, do not correlate well with the type of bleeding as experienced by patients with hemophilia. This makes it difficult, if not impossible, to make clear comparisons between studies evaluating bleeding events in this population.

Aim: This is a systematic review to determine the degree of homogeneity of bleeding classifications among randomized interventional studies for patients with hemophilia.

Methods: A literature search was conducted utilizing Pubmed database, retrieving the publications between 1981 and 2012 using 'hemophilia' 'bleeding' and 'randomized' as keywords. Only original randomized studies were included in the final review. Meta-analyses,

antiviral studies and studies that did not define bleeding as the end point of the study or did not include details regarding bleeding definition were excluded. Data regarding the definition of bleeding in hemophilic patients were extracted and classified into two major categories: those defining the location of bleeding and those defining the severity of bleeding. The I^2 statistic was used to assess the heterogeneity of bleeding classifications. An I^2 index of > 50% was considered as large heterogeneity.

Results: Two hundred and twenty-nine publications were found in the primary search and 28 eligible trials were identified. There were three studies on perioperative managements and 25 studies on non-operative hemophilic patients. Thirteen studies used nominal data, nine used ordinal data and one used combined data to analyze bleeding. The I^2 statistic index for perioperative studies and non-operative studies was 50% and 77.5% respectively. Even among the definitions regarding joint bleeding, the I^2 index was as high as 79%.

Summary/Conclusion: Among the randomized studies on hemophilic patients, highly variable characteristics were used to report bleeding. Although bleeding into the joints is the most common hemorrhagic complication in these patients, the studies did not have universal and objective criteria when describing its symptoms or signs, disallowing commensurable analysis. Various scoring systems have been developed to evaluate chronic joint deformity, but acute hemorrhage into the joint is usually dependent on subjective, self-reporting mechanisms. The data in this systematic review serves as a call for collaboration in our community to reach a consensus so that a reproducible evaluating system for haemorrhages in patients with hemophilia can be established.

PB 2.38-2

Combined T- and B-cell immunomodulatory therapy for immune tolerance induction in four hemophilia patients with poor risk inhibitors

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Background: Development of inhibitory antibodies in patients with hemophilia remains the most significant complication of treatment with coagulation factor concentrates. Immune tolerance induction (ITI) using high-dose factor VIII (FVIII) or factor IX (FIX) is now considered the standard of care for children with inhibitors. However, ITI is associated with a failure rate of ~30% in hemophilia A and up to 70% in hemophilia B. Individuals with factor IX inhibitors (who have a risk of anaphylaxis and nephrotic syndrome with standard ITI) and individuals with factor VIII inhibitors who have failed tolerance attempts with high dose clotting factor and anti-CD20+ are unlikely to gain tolerance without immunomodulation (altering the T cell interaction with B cells).

Aim: To describe four consecutive patients with high-titer inhibitors treated with ITI and combined immunomodulatory therapy.

Methods: Patients were treated with high-dose daily FVIII/IX plus the combined immunomodulatory (IP) regimen described by Beutal (*Haemostaseologie*, 2009): rituximab 375 mg/m^2/dose × 4 doses, mycophenolate mofetil (MMF) 300 mg/m^2/dose b.i.d × 50 days, dexamethasone 12 mg/m^2/day divided b.i.d for 4 days in weeks 2, 4, and 7, and intravenous immunoglobulin (IVIg) 1 g/kg/day × 2 doses with each dexamethasone pulse. Hemophilia B patients with inhibitors were started on IP simultaneously with ITI, whereas only those hemophilia A patients who were refractory to standard ITI plus rituximab received ITI + IP.

Results: *Patient 1* is a 33-month old boy with severe hemophilia B due to whole gene deletion. ITI + IP resulted in complete remission of inhibitor that persists 10 months later, after full recovery of CD20+ cells. He currently receives FIX 100 unit/kg q.o.d. *Patient 2* is a 5 year old boy with severe hemophilia B due to a frameshift mutation (F9 c.97delT (p.Leu-24HisfsX10)). ITI + IP resulted in complete remission of inhibitor that persists 4 months later, with partial recovery of CD20+ cells. He receives daily FIX 60 units/kg. *Patient 3* is a 5 year old boy with severe hemophilia A due to intron 22 inversion who failed ITI plus rituximab. ITI + IP resulted in partial remission of inhibitor (inhibitor titer: 1 BU); CD20+ testing is pending. He receives daily MMF and FVIII 200 units/kg q.o.d. *Patient 4* is a 6 year old boy with severe hemophilia A due to intron 22 inversion who had failed ITI plus rituximab. ITI + IP resulted in complete remission of inhibitor that persists 1 month later, with no recovery of CD20+ cells. He receives daily MMF and FVIII 40 unit/kg q.o.d. Patient #2 had a single episode of hypersensitivity to FIX, treated with antihistamine. No patient exhibited nephrotic syndrome during therapy.

Summary/Conclusion: ITI + IP resulted in marked improvement in quality of life for all patients. Durability of the remissions is yet to be determined, as only one patient has demonstrated full recovery of CD20+ cells. Successful eradication of inhibitors in these patients, all at high risk of treatment failure, suggests that modulating the interaction of B- and T-cells plays an important role in ITI for select patients. The IP regimen described should be considered for further study in clinical trials.

PB 2.38-3

On-demand treatment costs of Italian haemophilia patients with inhibitors: an exploratory lifetime economic model

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Background: Quantifying the health economic and the health related and quality of life (HRQoL) impact of on-demand (OD) treatment of bleeding episodes has not been done in Italian haemophilia patients with inhibitors.

Aims: this study estimated lifetime OD costs and HRQoL associated with recombinant activated Factor VIIa (rFVIIa) and plasma-derived activated prothrombin complex concentrate (pd-aPCC) from the Italian payer perspective.

Methods: A lifetime semi-Markov cohort model, reflecting 2-year old male haemophilia patients with high-responding inhibitors, was used to calculate outcomes associated with OD treatment of bleeds with rFVIIa or pd-aPCC. Using a 3-month cycle length, the model simulates patient transitions through eight health states: pre-ITI, delayed ITI, low-dose ITI, high-dose ITI, tolerised, partially-tolerised, non-tolerised, and death. Throughout the model, patients can experience mild-to-moderate bleeds (treated initially at home) and major bleeds, which require hospitalisation. Published international studies informed efficacy, utility, and other clinical inputs, while Italian cost schedules informed cost parameter values. Outcomes include life expectancy, quality-adjusted life expectancy, cost per bleeding episode, and direct treatment costs over time. Costs and effects were discounted at 3% in the base case analysis.

Results: Default analysis showed lower lifetime and per-bleed costs with rFVIIa compared with pd-aPCC (lifetime:€4,583,205 vs. €5,366,519 and per-bleed:€23,477 vs. €24,406, respectively). Hospitalisation length of stay is important for non-drug costs as length of stay for less than a day, costs €387 whereas stays of > 1 day costs were €4241. Patients treated with rFVIIa also had slightly higher quality-adjusted life expectancy, by 0.07 QALYs in base case analysis; this value may rise as high from 0.92 to 3.48 QALYs if pain relief from earlier bleed resolution improves underlying utility value by 5–20% compared to pd-aPCC.

Summary/Conclusion: Treatment with rFVIIa can lead to lower costs, with the difference attributable to lower costs of ITI, a less expensive dosing schedule, and improved bleed resolution, which reduces hospitalizations. If faster bleed resolution leads to earlier pain relief, rFVIIa treatment may improve quality-adjusted survival as well.

PB 2.38-4

Multi-centre Australia wide study comparing FVIII assay variation in haemophilia A patients receiving two different recombinant FVIII products using the one stage and chromogenic assays

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Background: In Australia, Xyntha (B domain deleted) and Kogenate (full length) are the two recombinant antihaemophilic factor VIII products available. Two common assays, chromogenic assay (FVIII:Chr) and one stage assay (FVIII:1S) are used to measure Factor VIII replacement levels (FVIII IU/dL) in patients with haemophilia A (HA). Discrepancies in FVIII IU/dL levels have been reported between these assays. Furthermore, it is uncertain how these different recombinant FVIII products could potentially affect the measurement of FVIII IU/dL levels by the FVIII:1S method. This has created an uncertainty in clinical practice for accurate replacement of FVIII in HA patients.

Aims: This multi-centre study aimed to evaluate the (i) inter-laboratory variability in the FVIII:1S assay and (ii) discrepancies between FVIII:1S and FVIII:Chr in severe HA patients receiving either Xyntha or Kogenate.

Methods: Fifteen Australian laboratories and one international laboratory (Sheffield UK) participated in this study. Samples from five patients receiving Xyntha and four patients receiving Kogenate were evaluated. Samples were collected between 1 and 20 h post FVIII treatment. All laboratories performed FVIII assays on the samples provided using their standard reagents and instrumentation. All assays were calibrated with commercially available human plasma standards. Fifteen laboratories performed the FVIII:1S assay and five performed the additional FVIII:Chr assay. A Bland and Altman difference analysis was also performed to assess bias and differences when comparing FVIII IU/dL levels using the FVIII:1S and FVIII:Chr assays.

Results: The mean FVIII level in the nine patient samples ranged from 16 to 124 IU/dL (FVIII:1S) and 22–187 IU/dL (FVIII:Chr). There was good inter-laboratory agreement in the results. The co-efficient of variation (CV%) for FVIII:1S ranged from 14.1% to 20.1% (Xyntha); and 10.4% to 12.5% (Kogenate). The CV% for FVIII:Chr ranged from 8.7% to 14.4% (Xyntha); and 4.2% to 6.9% (Kogenate). The FVIII:Chr method measured higher FVIII results compared to the FVIII:1S with a 1.5 times (range 1.5–1.7) increase in FVIII levels (Xyntha) but only a 1.2 times (range 1.0–1.3) increase in FVIII levels (Kogenate). The degree of agreement between FVIII:1S and FVIII:Chr when assessed using the Bland Altman analysis showed the FVIII:Chr assay had a positive bias of 42, standard deviation (SD) of bias = 31 (Xyntha); and a positive bias of 25, SD of bias = 13 (Kogenate).

Conclusion: There is a wider range of variation (CV%) when measuring FVIII levels in patients on Xyntha replacement compared to patients on Kogenate when using the FVIII:1S method. FVIII levels measured using the FVIII:Chr method resulted in an average 1.5 times higher level compared to FVIII:1S in patients on Xyntha replacement. This study highlights the challenges in measuring FVIII levels in patients on the two available FVIII replacement products available in Australia. Thus, depending upon the assay used, a patient with HA may receive vastly different quantities of FVIII replacement therapy. A product specific concentrate standard may be useful in standardizing FVIII measurements and allow the appropriate dose of FVIII to be administered to HA patients.

PB 2.38-5

Introducing the KAPPA prospective registry in hemophilia A: design, objectives and progress

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Background: A recent Swedish health technology assessment of replacement treatment strategies in moderate and severe haemophilia, highlighted the lack of suitable data available for analyzing comparative effectiveness and cost-effectiveness. As a consequence, studies published to date have been limited by retrospective and incomplete data, small sample size, lack of generalization due to specific and narrow target populations, and sometimes inappropriate design. Notably, there is a paucity of data on outcomes of on-demand treatment, which is the only treatment available for many patients in a global perspective.

Aims: To introduce and present the aims, design and progress of the KAPPA (Key Aspects of medical Practice in Patients with haemophilia A) registry

Methods: Lund University (LU) has initiated the KAPPA project to collect valid evidence on the impact of treatment regiments on Health Related Quality of Life (HRQL) of haemophilia A patients. We will enrol 1000 patients from eight different centres in Algeria, Denmark, Norway, Latvia, Lithuania, Sweden, Tunisia and Turkey between February 2013 and January 2016. We use an online Electronic Data Capturing (EDC) system for data collection and storage. KAPPA database is in English and includes questions encompassing the following: socio-demographics, treatment, bleeds, HRQL, Haemophilia Joint Health Score (HJHS), co-morbidities and adverse reactions. Physicians will consecutively enrol eligible patients as they visit the centre. Eligibility criteria include: a Factor VIII activity at diagnosis equal to or < 5%, the patient must visit the centre at least once a year and provide informed consent. Clinicians have access to their patients' identifying information for treatment's follow-up, however we will transfer anonymous data for the study. The Ethics Committee (EC) of LU approved the study and separate permissions from EC in each participating country will be obtained. In a multivariate model, we will predict the utility of treatment after adjustment for age, socio-economic status and co-morbidities.

Results: As of January 2013, we have assessed and confirmed the feasibility of performing the study in each centre, prepared the EDC, and have submitted ethics proposals in the pilot sites. We will implement the pilot study between February and March 2013 with the aim of testing the database's functions and the study's training package as well as communication process between the centres and study teams. An interim report will be prepared at the end of 2013.

Summary/Conclusion: Prospective registries are the best choice for collecting evidence for research as well as recording patients' data for better clinical decisions. By providing the infra-structure for collecting and summarizing real world patient data, the KAPPA registry will also assist patients, clinicians and health decision makers. It enables clinicians to develop and maintain a comprehensive and secure haemophilia database for use in the clinic and for future research. Better decisions can be made by clinicians through access to patients' treatment history. Finally, the final report of KAPPA will enable health decision makers to be better informed when having to make decisions on resource utilization and budget allocation for the haemophilia A treatment.

PB 2.38-6

Social status of adult haemophilia patients: preliminary results of a comparative single-centre cohort study with thrombophilia patients

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Background: The impact of the disease on the social status of haemophilia patients is not well established. Joint bleeds may lead to arthropathy with potential effects on school education and professional career. Recent studies on social status in adult haemophilia patients showed a higher rate of unemployment and occupational disability compared to the general male population in the Netherlands or to age- and sex-matched controls. With the introduction of prophylactic factor replacement, younger haemophilia patients have less joint damage, but it is unclear how this affects their social status.
Aim: We therefore explored the social status of patients with haemophilia (PWH) from our centre in comparison to an age- and sex-matched group of patients with thrombophilia (PWT).
Methods: PWH routinely visiting the Haemophilia Treatment Centre completed a questionnaire including socio-demographic data, questions regarding social status and a generic quality of life questionnaire (SF-36) as did PWT. Social status is usually defined through education, employment and income; quality of life (QoL) assessment provides insight into social and role functioning. In addition, clinical data were collected from patient files in both groups.
Results: So far, data from 40 PWH (27 severely affected) and 40 PWT have been analysed. The majority of PWH received on-demand treatment (61.5%). PWH had a mean number of 8.03 + 10.3 bleeds, a median number of target joints of 0 (0–3) and a mean orthopaedic joint score of 9.61 + 9.0. Mean age of PWH and PWT was 43.8 + 14.8 and 45.2 + 13.4 years, respectively. There was no significant difference concerning partnership, number of children and school or professional education between the groups. However, there was a trend towards higher graduation in PWT. By contrast, more PWT ($P < 0.02$) were actually working (85.0%) compared to PWH (62.5%); similar proportions of PWT and PWH were retired ($P = 0.057$), while only PWH were either retired due to their disease ($n = 6$) or unemployed ($n = 3$). No difference was found with regard to the amount of working time (full-time vs. part-time) and the type of occupation. Monthly income was lower in PWH ($P < 0.027$). With regard to QoL, PWH reported worse values in the domains 'physical functioning', 'pain' and 'general health' of the SF-36 compared to PWT; the physical summary score (PCS) was significantly worse as well ($P < 0.001$)
Conclusions: Despite the availability of factor concentrate for bleeding prophylaxis and treatment, PWH still have a lower social status with respect to employment and income compared to an age- and sex-matched group of PWT.

PB2.39 – Acquired Coagulation Disorders – II

PB 2.39-1

Heat treatment and immunological testing substantially improves the detection of factor VIII antibodies in acquired haemophilia A

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Background: Acquired haemophilia A is a rare bleeding disorder resulting from the development of auto-antibodies to the factor VIII (FVIII) pro-coagulant protein. Although good response rates are seen to conventional immunosuppressive treatment, it is associated with significant morbidity and mortality. The laboratory monitoring of treatment response is guided by the Bethesda assay despite samples often having significant amounts of residual FVIII present. In congenital haemophilia A, a FVIII ELISA kit (Hologic, previously GTI Diagnostics) and a modification (heat treatment) to the Bethesda assay have been described previously for the detection of FVIII antibodies. These techniques do not form a part of routine laboratory practice in the United Kingdom. We describe the application of these techniques in the setting of acquired haemophilia A.
Aims: To compare the Bethesda assay with a FVIII ELISA and to investigate the effect of heat treatment in the detection of FVIII antibodies in acquired haemophilia A.
Methods: Plasma samples sent for routine inhibitor testing from patients with acquired haemophilia A were tested using the Bethesda assay in parallel with the FVIII ELISA before and after heat treatment at 58 °C for 90 min.
Results: Thirty-nine samples from eight patients were tested in parallel by the Bethesda assay and FVIII ELISA before and after heat treatment. In the untreated samples, only two samples (5.1%) were positive by the Bethesda assay with 11 samples (28.2%) being positive by ELISA. Following heat treatment 20 samples (51.2%) were positive by the Bethesda assay and 16 (41.0%) positive by ELISA. If both tests were used in parallel, 12 samples (30.8%) were positive without heat treatment and 24 samples (61.5%) were positive following heat treatment by one or more technique. All samples that were positive following heat treatment showed significant levels of FVIII in the sample prior to this step (median FVIII:C 61.25 IU/dL, range 24.1–362.4 IU/dL). Of the eight patients tested, a change from negative to positive would have occurred in one or more samples from six of the patients if these tests had been part of routine clinical care.
Conclusions: The results of this study suggest the Bethesda assay in isolation may significantly underestimate the presence of FVIII auto-antibodies in acquired haemophilia A, with potential clinical repercussions. The relevance and clinical utility of FVIII antibody detection using these methods would ideally require further exploration in collaborative trials.

PB 2.39-2

FEIBHAC study: prospective clinical and biological evaluation of antihemorrhagic treatment with aPPCs (Factor eight inhibitor bypassing activity) in Acquired Hemophilia A (AHA)

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Background: AHA is a rare disease due to autoantibodies anti FVIII, characterized by bleeds. Recommended as first line treatment for the severe acute bleed, rFVIIa and aPPCs (FEIBA) have a similar efficacy and tolerability.
Aim: To assess FEIBA posology according to the haemostatic efficacy in patients with AHA and bleed treated by FEIBA in first or second line therapy.
Method: French prospective observational multicenter longitudinal (3 months) registry for 3 years. The bleed characteristics, the physician evaluation of FEIBA efficacy and its consequences on the posology, the reasons for initiating haemostatic treatment and choosing FEIBA, the anamnestic response, the adverse events (AEs) related to FEIBA and fatal outcome were analyzed.

Results: Thirty-three patients were evaluated: median age: 83 years [IQ 76–87]; sex ratio H/F: 1/1.54. AHA had already been known in five patients. At Inclusion: median FVIII: 2.00% (IQ 1.00–3.00), median INH titre: 15 BU/mL (IQ 8–60). Among the 28 patients without AHA known before, the delay in diagnosis after the bleed onset was 3 days (IQ 1.00–14); this delay had a significant impact on the interval between the bleeding onset and the start of FEIBA ($P < 0.001$). Approximately 75% of the haemorrhages were spontaneous and tissular (muscular hematomas with or without ecchymosis in 45%). The severe score of bleeding was independent of the FVIII level and INH titre. In 87.5%, the bleed was the reason for the treatment. In 85%, FEIBA was prescribed in 1st line therapy. The initial median posology was 76 U/kg, every 12 h in 54.5% and 8 h in 33.4%; it was independent of the severity score of bleeding. After 24 h, 10.7% of the haemorrhages were controlled, 75% stabilized or improved. When the physician concluded in stabilization, dosage and frequency weren't modified; in case of aggravation or improvement, the frequency was changed in 27%. The total mean duration was 8.5 days; the mean delay between the start of FEIBA and the disappearance of the active sites (7.6 days) rose with the increase of the duration of the treatment ($P < 0.001$).The delay between the disappearance of the active sites and the stop of FEIBA (2.46 days) rose with the duration of the treatment ($P = 0.084$). In three patients, FEIBA was stopped.

There were no anamnestic response; one non serious AE was related to FEIBA (lack of efficacy), six serious AEs were possibly related to FEIBA (two biological DIC, two venous thrombosis (one deep) in the same patient and two fibrinogen decreased in the same patient); six deaths were reported and one possible related to FEIBA (Acute coronary syndrome). Per protocol, all unrelated serious and non serious AEs (exception of fatal outcome) were not collected.

Conclusion: AHA is still diagnosed with delay and this has a negative impact on the start of the hemostatic treatment. The FEIBA posology was relatively stable during the treatment whatever the physician evaluation, and independent of the severity score of bleeding. FEIBA prescription is often continued for a few days while the active sites have disappeared. FEIBA was efficient and tolerated in this elderly population.

PB 2.39-3

Treatment characteristics and associated costs of factor replacement and immunosuppressive therapy among patients with acquired hemophilia A

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Background: Acquired hemophilia A (AHA) is a rare, potentially life-threatening hemostatic disorder caused by autoantibodies against coagulation factor VIII (FVIII). The management strategy for patients diagnosed with AHA requires a two-step approach, the first directed at controlling the bleeding and the second aimed at inhibitor elimination. Standard therapeutic agents used for bleed treatment include recombinant activated FVII (rFVIIa) and FVIII inhibitory bypassing activity (FEIBA), while treatments for inhibitor eradication are more varied and include the use of different immunosuppressive agents. Due to the rarity and limited number of AHA diagnoses, it is nearly impossible to determine the relative value of each of these different treatment strategies, however the therapeutic agents used to care for persons diagnosed with AHA are incredibly expensive and early detection and prompt diagnosis are essential in ensuring improved health outcomes.

Aims: The primary objectives of this study were to evaluate treatment characteristics and assess the costs associated with factor replacement and immunosuppressive therapy among persons with AHA.

Methods: Eligibility was limited to patients with a clinical diagnosis of AHA, who received care at the Gulf States Hemophilia and Thrombophilia Center between 2002 and 2012. We conducted a retrospective study, and data were obtained from the patients' medical charts, and pharmacy and hospital records. Medicare reimbursement rates were used to calculate the per-unit cost of factor and immunosuppressive therapy as well as associated hospital physician fees.

Results: During the observation period, 15 patients were diagnosed with AHA, however complete data were only available for 12 of these patients. The majority of the sample were women (58%), were > 60 years of age (67%), and were followed for an average of 68.4 weeks. Inhibitor levels ranged from 19 to 11,000 BU (mean 1352 BU). Complete remission was achieved in 50% of the patients. Two deaths occurred (bleeding contributed to one), giving an overall mortality rate of 17%. Conditions associated with AHA were identified at diagnosis or subsequent follow-up and included autoimmune diseases (25%) and malignancies (17%). Roughly 58% of cases were idiopathic. Bleeding episodes were treated with rFVIIa and/or FEIBA (one patient also received FVIII), and all patients received a single agent or a combination of cyclophosphamide, rituximab, and/or prednisolone for inhibitor treatment (42% of patients received combination therapy). A total of $3.3 million USD was spent by 12 patients during the observation period (mean $277,000 per patient). The greatest costs were associated with factor replacement therapy $3,000,000 (over 2 million units dispensed) followed by immunosuppressive treatment, which accounted for approximately $283,000. Patients stayed an average of 24 nights in the hospital due to AHA related complications and the physician fee associated with their stay accounted for roughly $36,000.

Conclusions: While over $3 million was spent to treat 12 AHA patients, this figure only represents the cost of factor, immunosuppressive therapy, and physician fees, and does not take into account any other costs including hospital, laboratory, and pharmacy costs. To the author's knowledge, this is the first study to examine the costs associated with AHA treatment.

PB 2.39-4

Enzyme-linked immunosorbent assay (ELISA) detecting antibodies to von Willebrand factor in patients with Acquired von Willebrand Syndrome (AVWS): clinical significance

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Background: Acquired von Willebrand Syndrome (AVWS) is a rare bleeding disorder, similar to the congenital disease in terms of laboratory findings. However, unlike congenital VWD, it arises in individuals with no personal or family history of bleeding. The disease displays bleeding tendency, with bleeding episodes typically affecting mucocutaneous or gastrointestinal districts. AVWS is usually associated with a variety of underlying conditions, including lynphoproliferative and myeloproliferative disorders (45–60%), solid tumours, immunological and cardiovascular disorders. As regards the lynphoproliferative disorders, monoclonal gammopathy of undetermined significance (MGUS) is the condition most frequently associated with AVWS. The prevalence of autoantibodies against VWF in patients with gammopathy varies from 18% to 66%.

Aim of the Study: To develop a sensitive ELISA assay to detect IgG and IgM antibodies against VWF and to verify the prevalence of autoantibodies in AVWS.

Methods: We developed an ELISA assay using recombinant VWF protein immobilized on polystyrene plates and sheep/goat polyclonal anti-human IgG or IgM labelled with peroxidase. AVWS was defined as follows: VWF below normal range, negative personal or family history

of bleeding diathesis, late onset of bleeding symptoms (spontaneous or after challenge). Twenty-three consecutive patients (12 males, 11 females) affected by AVWS, who referred to the Angelo Bianchi Bonomi Hemophilia and Thrombosis Center (Milan, Italy) between 2006 and 2012, were enrolled. A group of 40 healthy subjects (20 males and 20 females) was also tested in order to calculate the cut point value and the Normalization Factor during pre-study validation. This enabled us, during analytical study, to calculate a floating cut point: samples with OD above the floating cut point were considered positive for antibodies to VWF.

Results: The median age was 66 years (interquartile range [IQR]: 55–72 years). Twenty-one patients (93%) had an associated disease: 13 (57%) monoclonal gammopathy (11 monoclonal gammopathy of undetermined significance, one Waldestrom disease, one chronic lymphocytic leukemia), one Crohn's disease, two cardiovascular disorders, one lung cancer, four thrombocythemia. Two patients presented idiopathic AVWS. With our ELISA assay we detected auto-antibodies in 10 patients out of 23 (43%) (eight patients positive for both IgG and IgM, one patient positive only for IgG and one patient positive only for IgM). Twelve patients (52%) had VWF:RCo < 10%: of these, nine (75%) had IgG or IgM monoclonal component. Four (44%) of these nine patients were positive for autoantibodies to VWF (both IgG and IgM). Three more patients with VWF:RCo < 10% and other associated diseases (Crohn's disease, cardiovascular disease and cancer) were positive for anti-VWF autoantibodies. Three patients with VWF: RCo ≥ 10% were positive for autoantibodies to VWF.

Conclusion: The identification of anti VWF autoantibodies might help to diagnose patients affected by AVWS. With the present ELISA assay 10 patients out of 23 were positive for antibodies to VWF.

PB 2.39-5

Haemostatic changes following military trauma and major blood loss

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Background: Military trauma in Afghanistan is predominantly blast and high velocity gunshot wounds with associated high degree of soft tissue disruption and major haemorrhage.

Haemorrhage is controlled immediately at the point of wounding, often with tourniquets. Evacuation is usually via helicopter where prehospital administration of tranexamic acid and blood products occurs where appropriate before reaching the hospital. The effect of military trauma on haemostasis has not previously been studied and understanding the defect would help improve management.

Aims: To assess haemostatic changes in individuals with major military trauma received at Joint Force Hospital, Camp Bastion, Afghanistan.

Methods: Thirty-three sequential patients with blast and gunshot injuries with major blood loss presenting to the hospital, and who survived into their first surgery, were assessed and blood taken on admission for haemostatic studies. An injury severity score (ISS) of > 15 signifies major trauma.

Samples were collected in a ratio of 9:1 in 0.102M citrate & processed and frozen locally, then shipped back by a cold chain to the United Kingdom where they were stored at −80 °C. For each assay samples were analysed together to prevent interassay variability.

Results: Thirty-two out of 33 patients were male, with a median (range) age of 25 (19–35), On admission, pulse rate 110 (0–176) bpm, pH 7.17 (6.68–7.32), base deficit 7 (27–5), Injury severity score 27 (1–75). In the first 24 h total median (range) PRBC 10 (0–39), FFP 8 (0–36), Platelets 2 (0–12) cryoprecipitate 1 (0–11). Two patients received fresh whole blood (2 and 3 units respectively). Fifty-eight percent had received tranexamic acid prior to admission.

Admission haemostatic values showed median (range) [normal range] values of Platelet count 235 (178–298) [150–450] × 10^9/L; Clauss fibrinogen 1.56 (0.57–2.2) [1.5–4] g/L; PT 19.9 (13.8–30.9) [12–15.9]sec; prothrombin Fragment1 + 2 1382 (129–2380) [200–1200] pM; soluble tissue factor 216 (102–589) [40–300] pg/mL; D-dimer 3106 (536–6001) [< 145] ng/mL; t-PA antigen15 (4–108) [1–15] ng/mL; PAI-1 activity 34 (11–119) [0–33] ng/mL; Plasmin-antiplasmin complexes 6980 (1041–45,200) [150–800] ug/L; prothrombin levels 0.69 (0.37–1.16) [0.5–1.5] iu/mL; factor X 0.68 (0.33–1.09) [0.5–1.5] iu/mL; factor VII 0.66 (0.31–1.63) [0.5–1.5] iu/mL; Factor V 0.51 (0.08–1.06) [0.5–1.5] iu/mL.

Summary/Conclusions: Patients are coagulopathic with markedly reduced fibrinogen and factor V levels, with a lesser reduction of other coagulation factors. Markers of fibrinolysis (t-PA, PAP and D-dimers) are greatly increased with evidence of good thrombin generation (elevated PF 1 + 2). The study is ongoing.

PB 2.39-6

Long term follow-up in patients with acquired hemophilia: the role of immunosuppressive therapy

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Background: Acquired hemophilia is a rare but potentially life-threatening bleeding disorder caused by development of auto-antibodies against FVIII. Recent data of European registry (EACH 2) and from United States (Boles et. al) confirm the efficacy of immunosuppressive therapy with corticosteroid alone or in association with Rituximab at standard regimen (375 mg/m^2 1 dose per week for 4 weeks) to obtain complete remission (CR) of AHA (defined as the proportion of patients who are in CR without relapse during follow-up). However, the reported duration of patients' follow-up (< 2 years) does not allow to make firm conclusions about the long-term effect of such therapy. Now we report data on AHA patients followed-up for a median time of 3.4 years.

Aims: To assess the long-term response after immunosuppressive therapy.

Patients and Methods: We retrospectively analyzed data from 15 consecutive patients diagnosed with idiopathic and secondary AHA, evaluated between June 2001 and December 2012. Primary objective was to assess the role of immunosuppressive drugs (steroids with or without Rituximab) after a follow-up of at least 3 years, by comparing its effectiveness with published data. All patients was treated first with steroids regimen while Rituximab was added in refractory cases. Refractory has been defined as the lack of clinical response, maintenance of severe FVIII deficiency (< 1%) and inhibitor.

Results: Among our population, the median follow-up was 3.4 years; detailed data of main outcomes are reported in Table 1. Two patients (13%) had spontaneously inhibitor disappearance, without immunosuppressive treatment, 5 (33%) were treated with regimens containing rituximab (in combination with steroids), 8 (53%) with steroids alone. Rituximab was administered according to the standard regimen above reported. All Rituximab-based regimens obtained CR as well as steroids-based regimes. However, the average time to obtain the CR was shorter in steroids-based regimen than that in rituximab-based regimes (4.8 vs. 1.5 months, respectively). The rate of relapse of patients treated with rituximab was similar to that of patients treated with steroids (60% vs. 50%, respectively). Seven (46.6%) patients (three treated with Rituximab, four with steroid alone) have experienced relapse after a mean time of 2.6 years. All relapsed patients had idiopathic AHA.

Conclusions: In our population, refractory patients treated with Rituximab based regimes, obtain a CR in all cases. This is clinical relevant since our median follow-up is much longer than previously reported (1232 vs. 262 days of EACH2 registry). However, this response was not maintained in long-term follow-up.

With a median time to relapse of 708 days, 60% of patients treated with rituximab experienced a relapse. The efficacy of subsequent rituximab after the first relapse deserve further and appropriate investigations.

PB2.40 – Heparin-Induced Thrombocytopenia (HIT): Basic

PB 2.40-1

Staphylococcal panton-valentine leukocidin activates platelets via neutrophil secretion products

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Background and Aims: Expression of the toxin Panton-Valentine Leukocidin (PVL) by *Staphylococcus aureus* has been linked to severe life-threatening, necrotizing haemorrhagic pneumonia and fulminant septicemia with poor prognosis especially in young, immunocompetent patients. These complications are often accompanied by thrombotic events. We therefore tested the effect of PVL on human platelets in the presence and absence of isolated human neutrophils.
Methods: Human neutrophils were isolated from citrate anticoagulated whole blood using Percoll gradients. The effect of recombinant PVL on platelet activation and microparticle formation in the presence or absence of autologous neutrophils was measured by flow cytometry. PVL induced lysis of neutrophils was assessed by propidium iodid staining. Release of neutrophil myeloperoxidase and defensins was detected by ELISA technique.
Results: PVL strongly induced platelet activation, but only in the presence of human neutrophils. In the presence of neutrophils, PVL treatment induced fibrinogen binding to human platelets as well as microparticle formation. PVL induced neutrophil lysis and release of defensins and myeloperoxidase, known mediators of platelet activation. PVL-induced platelet activation in the presence of neutrophils was inhibited by known defensin inhibitors as well as by the antioxidants resveratrol and glutathione (GSH).
Conclusions: PVL activated human platelet not directly, but indirectly via human neutrophils. This was elicited by binding to neutrophils, cell lysis and release of defensins and myeloperoxidase. Inhibitors of defensin and myeloperoxidase/HOCl modified protein induced platelet activation inhibited the action of PVL. Platelet activation by PVL-treated neutrophils is therefore likely to depend on HNP release and HOCl formation. These findings might help to combat complications associated with PVL-expressing *S. aureus* strains.

PB 2.40-2

Fibronectin inhibits anti-PF4/heparin antibody induced platelet activation and low fibronectin levels are a potential risk factor for developing clinical HIT

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Background: In heparin-induced thrombocytopenia (HIT) immunoglobulin G (IgG) antibodies against complexes of the positively charged chemokine platelet factor 4 (PF4) and the negatively charged anticoagulant heparin activate platelets which can lead to thrombocytopenia and life-threatening thrombotic disorders. Not all patients with anti-PF4/heparin antibodies develop clinical HIT. As diagnostic assays using washed platelets are more sensitive than assays using platelet-rich plasma (PRP), plasma factors may influence platelet activation by anti-PF4/heparin antibodies.
Aims: The aim was to determine a risk factor contributing to clinical breakthrough of HIT.
Methods: Different plasma fractions were analyzed for inhibiting: (i) PF4 binding of gel-filtered platelets by flow cytometry, (ii) antibody binding to PF4/heparin complexes by ELISA, and (iii) antibody induced platelet activation using the heparin-induced platelet activation test (HIPA). The inhibitory mechanisms were further characterized by surface plasmon resonance (SPR) and photon correlation spectroscopy (PCS). Sera from patients with serologically- and clinically-confirmed HIT and from patients with serologically-confirmed HIT without thrombotic complications were compared concerning the levels of highly potential risk factors.
Results: Plasma and serum had the same inhibitory effect on PF4 binding to platelets. After affinity chromatography the inhibitory activity of serum disappeared. Among others the chromatographic matrix adsorbed IgG, complement factors, alpha-2-macroglobulin, vitronectin and fibronectin. Fibronectin was the only serum factor showing inhibition in all assays tested which was due to direct interaction with PF4/heparin complexes leading to their disruption. Fibronectin serum levels were higher in patients without clinical HIT compared to patients with clinical breakthrough.
Conclusions: Fibronectin protects from platelet activation induced by anti-PF4/heparin antibodies. This might be an explanation for the higher sensitivity of washed platelet assays. As a diagnostic tool eliminating fibronectin from PRP could be a faster and easier alternative to the washing procedure. More importantly, reduced fibronectin plasma levels seem to bear a risk for clinical breakthrough of HIT and might be useful as a prognostic marker.

PB 2.40-3

Usefulness of a murine monoclonal antibody against human platelet factor 4/heparin complexes to select donor platelets in platelet activation assays for diagnosis of heparin- induced thrombocytopenia

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Background: Platelet activation assays are extremely useful for detecting anti-platelet factor 4 (PF4)/heparin complex antibodies (HIT antibodies) that cause heparin-induced thrombocytopenia (HIT). The sensitivity of platelet activation assays depends on the reactivity of donor platelets to HIT antibodies. Therefore, selection of donor platelets is very important. However, it is difficult to evaluate the reactivity of donor platelet to HIT antibodies because sufficient quantities of plasma cannot be collected from HIT patients. We developed a murine monoclonal antibody against human PF4/heparin complexes (HIT-MoAb) and reported that HIT-MoAb-induced platelet activation possesses characteristics comparable to human HIT antibody-induced platelet activation.
Aims: We examined whether the reactivity of HIT-MoAb with donor platelets could be used as an index to select donor platelets, which are highly reactive with HIT antibodies.
Methods: HIT-MoAb was prepared by the ordinal polyethylene glycol method. Human HIT antibody immunoglobulin-G (HIT-IgG) was purified from HIT patient serum using a protein A column. The platelet aggregation test (PAT) was used to investigate platelet activation. HIT-MoAb or HIT-IgG was mixed with donor platelet-rich plasma (PRP) in a 1:1 ratio and incubated at 37 °C for 10 min. Subsequently platelet aggregation was measured by adding heparin (final concentration, 1 U/mL). Both HIT-MoAb and HIT-IgG were used in concentrations of 60, 120, and 200 μg/mL. A maximum aggregation rate of

≥ 20% was considered to be positive aggregation. The area under the curve (AUC) was calculated from the platelet aggregation response curve. The AUC value was used to evaluate the reactivity of donor platelets because AUC is considered to indicate platelet aggregation strength.

Results: PAT with HIT-MoAb (60, 120, and 200 μg/mL) was performed using donor PRPs (n = 11). As a result, 9.1% PRP (1/11) and 63.6% PRP (7/11) showed positive aggregation at ≥ 60 μg/mL and ≥ 120 μg/mL, respectively, whereas, 27.3% PRP (3/11) showed negative aggregation at all concentrations tested. These results indicated that the degree of HIT-MoAb-induced platelet aggregation varied among donor platelets. The total AUC (sum of AUC values at 60, 120, and 200 μg/mL), which is considered as the reactivity of donor platelets, was used to rank donor PRPs from high to low values. The relationship between the reactivity of donor platelets to HIT-MoAb and HIT-IgG was investigated. The ratio of positive aggregation with HIT-IgG was higher when the higher ranked donor PRP was used. Moreover, the total AUCs for HIT-MoAb and HIT-IgG were significantly correlated (Spearman's rank correlation: rs = 0.973; P = 0.0021).

Conclusion: Donor platelets showing high reactivity to HIT-MoAb also exhibited high reactivity to human HIT antibodies, indicating that HIT-MoAb would be useful for evaluating and selecting donor platelets in platelet activation assays for HIT diagnosis.

PB 2.40-4

Evaluation of three new fully automated quantitative immunoassays on diagnosis of heparin-induced thrombocytopenia

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Background: The cause of heparin-induced thrombocytopenia (HIT) is the generation of anti-platelet factor 4 (PF4)-heparin antibodies (HIT antibodies). HIT is a clinicopathologic syndrome, implying that diagnosis of HIT depends on both clinical and pathologic criteria being present. Functional and immunologic assays for detecting HIT antibodies are available. ^{14}C serotonin release assay (SRA) is still considered to be the gold-standard. However, unfortunately, it is done only in a few laboratories. Conversely, the enzyme immunoassays are widely used. Recently, three new fully automated quantitative immunoassays, the HemosIL® AcuStar HIT-IgG$_{(PF4-H)}$, specific for IgG, and the HemosIL® AcuStar HIT-Ab$_{(PF4-H)}$ and HemosIL® HIT-Ab$_{(PF4-H)}$, detecting IgG, IgM and IgA of HIT antibodies were introduced into the market by Instrumentation Laboratory Co Ltd. The HemosIL® AcuStar HIT-IgG$_{(PF4-H)}$ and HemosIL® AcuStar HIT-Ab$_{(PF4-H)}$ are based on a chemiluminescent immunoassay, and HemosIL® HIT-Ab$_{(PF4-H)}$ is based on a latex particle enhanced immnunoturbidimetric assay. In this study, we compared the performance among the SRA, the ELISA kit (PF4 Enhanced®, GTI) and the three new immunological assays.

Subjects/Methods: We measured HIT antibodies in 112 HIT suspected patients and 10 healthy normal subjects by using (A) PF4 Enhanced®, (B) HemosIL® AcuStar HIT-Ab$_{(PF4-H)}$, (C) HemosIL® AcuStar HIT-IgG$_{(PF4-H)}$, and (D) HemosIL® HIT-Ab$_{(PF4-H)}$. We used 0.4 OD units for (A), 1.0 U/mL for (B), (C) and (D), and 50% for SRA as a cut-off value, respectively.

Results: Co-positivity with SRA in four immunological assays (A, B, C, D) was 100%, 100%, 82% and 92%, and that of co-negativity was 63%, 66%, 73% and 66%, respectively. Spearman's rank correlation coefficient of (A) with each of the three immunological assays (B, C, D) was 0.924 (P < 0.001), 0.912 (P < 0.001) and 0.803 (P < 0.001), respectively. Furthermore, the correlation coefficients between (B) and (C), and (B) and (D) were also significant (0.954 and 0.865, respectively). Co-positivity and co-negativity between (A) and (B) were 95% and 98%, respectively. These values were higher than co-positivity (84%) and co-negativity (85%) between (A) and (D).

Conclusion: Co-positivity and co-negativity of the SRA with each of the three new immunological assays were similar to those of the SRA with PF4 Enhanced®, especially the HemosIL® AcuStar HIT-Ab$_{(PF4-H)}$ showed the most agreement among the assays. Furthermore, the PF4 Enhanced®correlated more significantly with HemosIL® AcuStar HIT-Ab$_{(PF4-H)}$ than with HemosIL® HIT-Ab$_{(PF4-H)}$. These results show that the performances of the three new fully automated quantitative immunoassays are comparable to the PF4 Enhanced®. However, further investigation is needed to clarify clinical significance of these assays on HIT diagnosis.

PB 2.40-5

Significance of the measurement of HIT antibodies on diagnosis of heparin-induced thrombocytopenia in the patients with hemodialysis

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Introduction: Heparin-induced thrombocytopenia (HIT) is a side effect of heparin characterized by thrombocytopenia and thrombus formation (blood clotting in the dialysis circuit is frequently observed in the hemodialysis patients with HIT). HIT is caused by the appearance of antibodies to platelet factor 4 (PF4)/heparin complex (HIT antibodies). Therefore, the detection of HIT antibodies as well as clinical findings including thrombocytopenia is an important point for the diagnosis of HIT. The diagnosis of HIT is confirmed if the results of functional and immunological assays are both positive, and excluded if they are both negative. However, the diagnosis is controversial if only one is positive. Therefore, we studied the relationship between HIT antibodies and clinical findings, and evaluated the significance of the measurement of HIT antibodies in the diagnosis of HIT.

Methods: The subjects were 165 patients (96 males and 69 females with a mean age of 67.9 ± 14.0 years) for whom the measurement of HIT antibodies was requested due to suspected HIT in 2003–2008. The HIT antibody level was measured using the ^{14}C-serotonin release assay (SRA) and ELISA (PF-4 Enhanced®, GTI; Waukesha, WI, USA). A release rate of 50% or higher on SRA and an OD value of 0.4 or higher on ELISA were judged to be positive.

Results: The patients were classified on the basis of the results of HIT antibody measurements into four groups: Groups A [SRA(+)/ELISA(+)] (n = 45), B [SRA(+)/ELISA(-)] (n = 9), C [SRA(-)/ELISA(+)] (n = 48), and D [SRA(-)/ELISA(-)] (n = 63), and the rate of reduction in the platelet count, timing of thrombocytopenia, the presence or absence of thrombosis, acute reactions, clotting in the dialysis circuit, and the presence or absence of other causes were evaluated. No significant difference was observed among the four groups in the rate of reduction in the platelet count (A: 62 ± 26%, B: 62 ± 16%, C: 52 ± 25%, D: 54 ± 28%), timing of thrombocytopenia (A: 15 ± 13 days, B: 14 ± 10 days, C: 19 ± 20 days, D: 22 ± 23 days), the presence or absence of thrombosis, or acute reactions. Clotting in the dialysis circuit was observed in 87% (39/45) in Group A, 56% (5/9) in Group B, 81% (39/48) in Group C, and 41% (26/63) in Group D, and its frequency was significantly higher in the ELISA(+) than in ELISA(-) groups. Other causes were noted in 11% of the patients in Group A, 33% in Group B, 13% in Group C, and 43% in Group D, and their frequency was significantly lower in Groups A and C than in Group D. Therefore, the risk of HIT is considered to be high in Group C as well as Group A. In Group B, four patients were positive for another ELISA (antigen: r-PF4/heparin, IgG only), suggesting the presence of

HIT antibodies not detected by the ELISA (antigen: PF4/PVS) used in this study.
Conclusion: The possibility of HIT is considered to be high in hemodialysis patients with clinical findings of HIT (thrombocytopenia, clotting in dialysis circuit, etc.) if HIT antibodies are detected on either functional or immunological assays.

PB 2.40-6
In-vitro inhibition of thrombin generation in heparin induced thrombocytopenia by dabigatran and rivaroxaban

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Background: In heparin induced thrombocytopenia (HIT), platelet activating antibodies cause increased thrombin generation despite the presence of the thrombin inhibitor heparin. Currently approved thrombin inhibtors such as danaparoid or lepirudin were recently shown to decrease thrombin generation in HIT.
Aims: To evaluate the effect the novel coagulation inhibitors dabigatran and rivaroxaban on *in vitro* thrombin generation in HIT.
Methods: To determine degree of thrombin generation inhibition, the final *in vitro* concentration of dabigatran or rivaroxaban was diluted to low (5%), intermediate (50%), or high (95%) levels of the respective drug's *in vivo* concentration. Samples from five patients with platelet activating antibodies were mixed with platelet rich plasma from healthy donors, unfractionated heparin at 0.2 U/mL, and dabigatran or rivaroxaban at the different concentrations. Thrombin generation was measured by Calibrated Automated Thrombinogram with the endogenous thrombin potential (ETP) as primary parameter.
Results: At high and intermediate dabigatran concentrations, ETP was significantly decreased (48 nM [15–51] and 162 [96–360] vs. 511 nM [400–860], $P = 0.002$ and $P = 0.02$, respectively). The low concentrations of dabigatran did not significantly reduce the ETP (265 [183–723] vs. 511 nM [400–860], $P = 0.18$). A high concentration of rivaroxaban significantly decreased median ETP (15 nM [10–133] vs. 511 nM [400–860], $P = 0.004$). The intermediate and low final concentrations of rivaroxaban did not significantly reduce the ETP, but showed a trend towards a lower thrombin generation (128 [35–398], $P = 0.05$ and 239 [176–731], $P = 0.13$, respectively).
Conclusion: Both dabigatran and rivaroxaban decreased *in vitro* thrombin generation in HIT. As the high concentrations of both drugs reduced thrombin generation more efficiently than lower concentrations, high concentrations may be required to treat HIT *in vivo*.

PB2.41 – Rare Bleeding Disorders – III

PB 2.41-1
Investigation of medical indications for PNH screening experiment by flow cytometry

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Background: Paroxysmal Nocturnal Hemoglobinuria (PNH) is an acquired hematopoietic stem cell disorder leading to a partial or absolute deficiency of all glycophosphatidyl-inositol (GPI)-linked proteins. The classical approach to diagnosis of PNH by cytometry involves the loss of at least two GPI-linked antigens on RBCs and neutrophils. Flow cytometry (FCM) is firmly established as the method of choice for screening of PNH. In 2010, technical guidelines were published in order to standardize the diagnosis of PNH by mutiparameter FCM. But there is not a uniform consensus about the medical indications for PNH clone testing by FCM.
Methods: Three thousand and thirty-seven individuals were submitted for diagnostic screening of PNH. CD59 was used for RBC analysis based upon physical parameters. We combined FLAER with CD45, CD24, allowing the simultaneous analysis of FLAER and the GPI-linked CD24 on neutrophil and monocyte lineages by CD45 vs. side scatter.
Results: PNH clone cells were found in 189/3037 (6.2%) cases. Most commonly individuals were screened because of hemoglobinuria (730/3037,24.0%), followed by aplastic anemia (486/3037,16.0%), MDS (448/3037,14.8%), pancytopenia (395/3037,13.0%), hemolytic anemia (298/3037,9.8%), bone marrow failure (211/3037,6.9%), atypical venous thrombosis (64/3037,2.1%), other (405/3037,13.3%). Essentially all patients with classic PNH (95/189,50.3%) report gross hemoglobinuria at some point during the course of their illness. This symptom were absent in patients with PNH-subclincal (e.g. PNH/aplastic anemia or PNH/MDS) (9/189,4.8%) because the clone size is often relatively small. PNH clone cell often were found in PNH combined with another specified bone marrow disorder, including AA (58/189) MDS (23/189) pancytopenia (4/189).
Conclusion: Analysis of the different clinical and biological findings that may contribute to better selection of cases for PNH screening experiment by FCM. The FCM screening of GPI-deficient cells shows that the rate of positive results is higher when cases were tested because of hemoglobinuria, anemia, thrombosis and pancytopenia, and much higher in cases previously diagnosed of aplastic anemia or MDS, and therefore screening for PNH in patients with aplastic anemia, or MDS even in the absence of clinical evidence of hemolysis, is recommended at diagnosis and at least yearly during follow-up.

PB 2.41-2
Genotype and phenotype relationships in 10 unrelated Pakistani patients with inherited FVII deficiency

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Background: Inherited factor VII (FVII) deficiency is one of the commonest rare bleeding disorders (RBD). It is characterized by a wide molecular and clinical heterogeneity and an autosomal recessive pattern of inheritance.
Aims: FVII deficient patients are still scarcely explored in Pakistan albeit rare bleeding disorders became quite common as a result of traditional consanguineous marriages. This study aims to give a first insight of *F7* gene mutations in Pakistani population.
Material and Methods: The study was conducted in 10 unrelated patients with inherited FVII deficiency living in Pakistan. A clinical questionnaire was filled out for each patient who was then graded according to two previously published classifications for RBD (Maraini *et al* 2005, and Peyvandi *et al* 2012) and a bleeding score (Tosetto *et al* 2006). The FVII: C and FVII:Ag levels were determined using a human recombinant thromboplastin (RecombiPlastin 2G, IL) and an ELISA (Asserachrom FVIIAg Diagnostica Stago) respectively. Direct sequencing was performed on the coding regions, intron/exon boundaries and 5′ and 3′ untranslated regions of the *F7* gene.
Results: Ten unrelated FVII-deficient Pakistani patients (median FVII: C = 2%; ranges = 2–37%) were investigated. Molecular analysis allowed us to identify nine different mutations; among these, five were novel: (c.-57 C>T, p.Cys82Tyr, p.Cys322Ser, p.Leu357Phe, p.Thr410Ala). Only one mutation was located within the *F7* promoter (c.-57C>T) and is predicted to alter the binding site of transcription factor HNF-4. Quantitative deficiency is expected, due to decrease in mRNA transcription. Both p.Cys82Tyr and p.Cys322Ser mutations involved essential disulfide bridges either forming a loop in the Gla

domain or linking both heavy and light chains of activated FVII, respectively. The Leu 357 is located in an external loop of the catalytic domain, involved in the interaction between FVII and its substrate, but also in the catalytic conversion of FVII. The remaining novel p.Thr410Ala mutation changed a polar residue into an Alanine with hydrophobic side chain presumably leading to a pathogenic impact on the FVII molecule. Six out of seven patients with FVII:C levels below 10% were homozygous in connection with the high percentage of consanguinity in our series. Comparison of the three previously published classifications showed that both clinical classifications were corroborating in our small series. Conversely the bleeding score appears to better qualify the bleeding tendency when comparing the three patients with the same *F7* genotype.

Conclusion: Molecular analysis of an even small series of 10 FVII-deficient patients allowed us to report five novel mutations. The p.Cys82Arg mutation was the most frequent in our series. Moreover, the use of the Tosetto's bleeding score can be a promising tool to classify patients with rare bleeding disorders.

PB 2.41-3

Mild fibrinogen disorders: correlation between genotype and phenotype

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Background: Fibrinogen is a complex dimeric protein of 340 kD, synthesized in hepatocytes as a hexamer composed of three pairs of non identical polypeptides (Aα, Bβ and γ chains, identified by a different gene *FGA*, *FGB* and *FGG*). Inherited fibrinogen disorders are classified according to a complete (afibrinogenemia) or partial (hypofibrinogenemia) fibrinogen deficiency and to an abnormal circulating molecule (dysfibrinogenemia) in plasma. The risk of bleeding or thrombosis conferred by partial deficiency or qualitative abnormality remains unclear.

Aim of the Study: We evaluated the clinical manifestations and the genotype of 24 families (54 patients) showing laboratory mild fibrinogen abnormalities.

Results: Abnormalities at thrombin-cleavage site in alfa chain (Arg16Cys, Arg16His and Arg19Gly) did not cause bleeding, while thrombotic events can be associated with Bbeta mutations at the same site (Arg14Cys). A significant bleeding tendency with impaired wound healing was reported with a novel Ile105Thr mutation in gamma chain. Overall, bleeding tendency was usually very mild and most patients did not require specific treatment. A woman with partial hypofibrinogenemia (fibrinogen 45 mg/dL) associated with a novel Leu344Phe mutation in gamma chain was treated prophylactically with fibrinogen concentrate during pregnancy because of an early placenta detachment with uterine bleeding. Familial increased risk of venous thromboembolism was associated with a novel Trp208Arg mutation in gamma chain. Four additional novel mutations (Arg57Lys in alfa, IVS5 + 2T>A and Ter462Gln in beta, and Cys339-Ser in gamma chain) were also detected.

Conclusions: Mild disorders of fibrinogen concentration and function are important to establish the structure-function relationships and clinical correlates of different mutations.

PB 2.41-4

Characterization of adult patients with a mild to moderate bleeding phenotype from the Vienna Bleeding Biobank

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Background: Mild bleeding disorders (MBDs) manifest in a variety of clinical symptoms and are a common cause for thorough hemostaseological investigation in adults. Still, no diagnosis can be established in the majority of patients.

Aim: Establishment of a Biobank of clinically extensively characterized patients with MBD for identification of underlying causes.

Methods: Patients aged > 18 years were consecutively enrolled after giving written informed consent. The severity of bleeding was estimated using a standardized bleeding score (Rodeghiero, J.Thromb.Hameost.2005:3:2619). Routine coagulation tests, von Willebrand factor antigen (vWF:Ag) and activity (vWF:RCo), single clotting factors, and platelet function, assessed by platelet function analyzer (PFA-100), aggregometry, and glycoprotein expression were performed.

Von Willebrand syndrom (vWS) was considered as definite when vWF:RCo was < 30%, and as possible vWS when vWF:RCo was between 30% and 50%. When platelet function tests revealed repeatedly abnormal results definite thrombocytopathy was diagnosed; possible thrombocytopathy was diagnosed when results were abnormal in only one available investigation.

Results: From 11 to 2009 until 12 to 2012 215 patients (female = 176, 82%) were enrolled. Seventy-three patients (34%) had a positive family history for bleeding. Median age [Interquartile (IQR) range] was 41 [29–54] years. There was no age difference between female and male patients. Patients with MBDs had a median bleeding score of 4 (IQR 2–6). We observed no difference in the severity of bleeding between male and female patients.

Common bleeding symptoms comprised skin manifestations in 66%, post-surgical bleeds in 51%, of which 53% demanded revision. Further manifestations were bleeding after tooth extraction in 33%, repeated episodes of epistaxis in 28%, small wound bleeding in 28%, and oral cavity bleeding in 11%. Nine percent of patients had gastrointestinal bleeding, one patient reported muscle and two joint bleeding. Twenty-five percent women suffered from hypermenorrhagia, and 13% of the women, who had given birth, reported postpartum bleeding.

In five patients (2%) definite vWS was diagnosed and in 13 patients (6%) vWS was considered possible. A not further characterized definite thrombocytopathy was confirmed in 11 patients (5%), in 27 patients (13%) a platelet function disorder was suspected (possible thrombocytopathy). Six patients (3%, female = 1) had factor VIII deficiency with levels of < 50% (< 30% in three of them), excluding those with vWS type Normandie. In one patient dysfibrinogenemia was diagnosed. Of patients without an established diagnosis, one each had repeated prolongations of APTT and PFA measurements of unknown cause; further six had deficiency of one or two single clotting factors, which could not be attributed to a bleeding tendency. In 151 (female = 129) patients (70%) all tests showed normal results and no diagnosis could be established. The median bleeding score was 4 (IQR 2–6) in patients with a definite or assumed diagnosis and 3 (2–5) in those without any identified abnormality ($P = 0.25$).

Summary: Like in previous studies, a definite or possible diagnosis explaining the bleeding tendency could be established in only 30% of patients with MBDs. The severity of bleeding, however, was almost equal in patients with an established diagnosis as in those without. Further mechanisms responsible for MBDs still have to be identified.

PB 2.41-5

Long-term prophylaxis in patients with severe factor VII deficiency

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Introduction: According to the recent paper by the European Network of Rare Bleeding Disorders, there is a weak association between FVII levels and bleeding manifestations. In patients with a previous episode of intracranial bleeding, prophylaxis is generally initiated to prevent subsequent cerebral bleeding episodes.
Aim of the Study and Methods: To describe two patients on long-term prophylaxis with plasma-derived factor VII concentrate
Results: Case 1: A 53-years-old woman affected by severe congenital FVII deficiency (< 1%) presented a significant bleeding history (easy bruising, severe epistaxis, recurrent spontaneous hemartrosis, bleeding after tooth extractions, severe menorrhagia requiring treatment) from childhood, but the hemorrhagic disorder was diagnosed only when she was 18-years-old. Between 43 and 50 years, she presented several episodes of gastrointestinal bleeding (due to angiodysplasia), that required hospitalization. Long-term prophylaxis was started at the age of 50, because of the gastrointestinal bleeding. She was treated with plasma-derived FVII concentrate (pdFVII) twice a week (600U; 12 U/kg). The patient had no bleeding episodes for 3 years and did not require any additional treatment with pdFVII (except for surgical procedures). After 3 years, prophylaxis was interrupted for 1 month (due to the unavailability of the drug). During this period the patient presented another severe episode of gastrointestinal bleeding requiring hospitalization and treatment with activated recombinant FVII (rFVIIa) and red blood cells transfusion. After this episode the patient started again the prophylaxis with pdFVII (same dosage as before). The molecular analysis of factor VII gene showed compound heterozygosity: C.430 + 1 G/A; VS7 + 7 A>G; Thr419Met.
Case 2: A 23-year-old young woman affected by severe congenital FVII deficiency (< 1%) was diagnosed 4 days after birth, because of melena and extensive bruising that required treatment with fresh frozen plasma. In the first 7 years of life the patient had several episodes of spontaneous and post-traumatic hemartrosis/hematomas and four episodes of intracranial bleeding (the first at the age of 3 months, after which surgery was performed and a cerebral ventriculo-peritoneal shunt was positioned) treated with infusion of pdFVII every 6 h. At the age of 7 years, she started the long-term prophylaxis with daily infusions of pdFVII (50 U/kg; actually 3000 U). Since then (follow-up 16 years) the patient did not present any bleeding episodes that needed any more treatment. The molecular analysis of factor VII gene showed compound heterozygosity: Cys135Arg; 17 bp deletion at nucleotide 10585.
Discussion: Prospective studies that evaluate the efficacy of long-term prophylaxis are not available, as well as data regarding the prevalence of patients on prophylaxis with pdFVII or with rFVIIa. The theoretic advantage of pdFVII should be the longer half-life of the drug (and consequentially the less frequent administrations), but recently patients using successfully only one weekly infusion of rFVIIa were described. In this report we described two patients on long-term prophylaxis with pdFVII; in the two patients, a different dose regimen was used because of the different indication and because no evidence based guidelines are available on the minimal treatment to successfully avoid life-threatening hemorrhages in factor VII deficient patients

PB 2.41-6

Survey of practices: difficulty of venipuncture practice and contribution of a vein locating device in patients requiring multiple peripheral venipunctures

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Background: As part of the treatment or monitoring of their disease, a significant number of patients may have multiple venipunctures. This is the case, in particular, for patients with clotting disorders, who undergo regular punctures as well as self-treatments. These repeated punctures may result in weakening of the veins, making venous access difficult and inducing a negative feeling for the patient (increased pain and stress).
The existence of a peripheral vein locating device 'AccuVein®' in this context could facilitate the practice of venipunctures and provide us with the opportunity to assess the venipuncture practices and the interest of this device in this population.
Aim: To describe practices for venipunctures and their difficulty for patients with multiple peripheral venipunctures and the contribution of a peripheral vein locating device 'AccuVein®'.
Methods: French, pilot, survey of practices carried out in France, in four university hospital units (CHU Caen, Paris, Lille and Reims).
According to the use or not of the device, the nurses collected the numbers of attempted punctures, the pain and the stress of patients before and during the punction and the stress of the nurses as well.
Results: To date 150 patients have been analyzed:For the group without venous difficulty with or without device (AccuVein®): no difference in the number of attempts (0 in median for the two sub-groups), or in the pain and stress of the patient. For the group with venous difficulty with or without device (AccuVein®): median number of puncture attempts −0.5 (the device) and 0 (without the device), a trend in favor of the device.
In the subgroup with AccuVein®: less stress for the patient ($P = 0.055$), a reduction in pain ($P = 0.003$). The main reason for using AccuVein® was to locate the vein immediately.
Conclusion: The device 'AccuVein®' seems to bring an interest to the practice for venipuncture in patients with difficult venous access with a decrease in the number of attempted puncture, pain and stress.

PB2.42 – Von Willebrand Disease: Clinical – III

PB 2.42-1

Molecular characterisation and discrimination of patients with Type 2 Von Willebrand disease

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Background: Diagnosis of von Willebrand disease (VWD) sub-type is important in guiding patient treatment and genetic counselling. VWD discrimination is based on laboratory analysis of VWF levels and function. However mutation analysis of the *VWF* gene complements laboratory measurement and aids in distinguishing qualitative (Type 2) and quantitative (Types 1 and 3) deficiency of VWF. Most mutations associated with types 2A, 2B and 2M VWD occur in *VWF* exon 28 which encodes the functionally important VWF-A1 and A2 domains.

Therefore a targeted approach to mutation analysis allows detection of most mutations associated with these sub-types.
Aims: To screen and identify mutations associated with Type 2 VWD in a cohort of Irish patients with VWD.
Methods: Samples were collected from patients with a diagnosis of VWD and prior VWF:RCo/VWF:Ag ratio of ≤ 0.7. VWF:Ag was measured by an immuno-turbidometric method, VWF collagen binding (VWF:CB) was measured by ELISA, and VWF:RCo levels determined by platelet aggregometry. VWF multimers were analysed by discontinuous slab agarose gel electrophoresis. *VWF* exon 28 was amplified in four separate overlapping PCR reactions and the resulting product sequenced by standard di-deoxysequencing methods. Analysis of *GP1B* was also performed in a single kindred. In-silico structural analysis was also performed.
Results: Twenty-six discrete mutations were detected in *VWF* exon 28 in a total of 42 kindred, comprising 61 patients analysed. Twenty-three of the 26 mutations were associated with Type 2 VWD and a dominant family history. Six different mutations associated with 2A VWD were detected in 10 kindred comprising 14 patients. A novel candidate mutation was detected in three apparently unrelated kindred (p.Gln1541Arg). All patients were heterozygous for mutations that predicted amino-acid substitutions in the A2 domain. Four mutations associated with 2B VWD were detected in five kindred, an additional family had a mutation in *GP1B* (p.Gly249Val) causing platelet-type VWD. Ten different mutations causing 12 amino-acid substitutions were associated with definite 2M VWD. VWF:RCo/Ag ratios varied from 0.24 to 0.64 whereas VWF:CB/Ag ratios varied from 0.67 to 0.96 and HMW multimers were present. Notably two candidate gene conversion events between pseudogene and VWF exon 28 were associated with 2M VWD. One event predicting the amino-acid substitutions p.Pro1266Leu; p.Val1279Ile in the A1 domain was detected in three apparently unrelated kindred. A second candidate gene conversion event that predicted p.Val1229Gly and Asn1231Thr was detected in two unrelated patients. This has been previously associated with Type 1 VWD. Mutations predicting p.Arg1374Cys/His/Leu in the A1 domain were detected in six kindred. Mutation at this residue reduces VWF:Ag (0.14–0.25 IU/mL) and further reduces VWF:RCo (< 0.1–0.12 IU/mL). The mutations also affects VWF:CB (ratio 0.5–0.7) with a modest reduction of HMW multimers, thus classification of these patients is difficult. In additional two novel mutations; resulting in type 1 quantitative deficiency were detected: c.3675-1G>A splice site mutation, and p.Arg1566*. The latter mutation was also present in trans with the modifying polymorphism p.Tyr1584Cys.
Conclusions: Novel candidate mutations with unique molecular mechanisms were identified as causing type 2VWD. Molecular analysis complements laboratory measurement and provides further insight into the basis of VWD.

PB 2.42-2
Use of double virally inactivated FVII/vWF in 30 children and young people with von Willebrands disease – a single centre experience

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Background: Children and young people (CYP) with von Willebrands disease (vWD) have mild to severe bleeding manifestations which appear spontaneously or following trauma or surgical intervention. Treatment is given to prevent or treat bleeding; for some this requires treatment with plasma derived concentrates. UK treatment guidelines recommend using plasma products which have been manufactured to the highest standards to limit exposure to potential blood borne viral disease.
Aims: A double virally inactivated FVIII/vWF concentrate was licensed for use in the UK in September 2009. All children treated at this centre since then have received this new product. A prospective monitoring record documenting safety and efficacy and outcomes of treatment was established, recording dose frequency, response to treatment and adverse events.
Methods: Since introduction of the new product, treatment of CYP, unresponsive to Desmopressin, has been recorded on a central database. All treatments are recorded including reason for treatment, dose, and response to treatment: bleed resolution, breakthrough bleeds for those on prophylaxis, surgical outcomes and adverse events.
The data is analysed regularly to assess results of treatment, adverse events and treatment outcomes.
Results: To date 30 CYP (14 girls:16 boys, 21 type 1, 1 type 2M, 6 type 3 vWD, 2 acquired) aged 1 day to 17.8 years have been treated either on demand for bleeds (four subjects for 23 bleeds, four have had continuous prophylaxis and 27 subjects have undergone 35 surgical procedures).
Surgical procedures varied from minor to major surgery (dental extractions to spinal, cardiac and neurosurgery) treatment was administered for 1–5 days. For major procedures FVIII and vWF levels were monitored to ensure adequate haemostatsis without any evidence of accumulation of vWF postoperatively.
No postoperative bleeding, complications or adverse events were seen. The CYP treated prophylactically have experienced no breakthrough bleeds on a dose regimen of 28–53 iu/kg/dose, administered at home every 48–72 h maintaining a 48 h trough > 1 iu/dL.
CYP treated on demand have responded to a single dose of ~50 iu/kg.
Summary/Conclusion: This study, of a heterogeneous group of CYP with vWD confirms data obtained in clinical trials that this product is efficacious, safe and well tolerated. Bleed resolution or prevention is 100% in this cohort. For CYP on home treatment ease of reconstitution and infusion and absence of breakthrough bleeding promotes treatment concordance.

PB 2.42-3
Results of a prospective, non-interventional clinical study in 170 VWD patients with a new generation of VWF/FVIII concentrate in Germany

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Background: With marketing authorisation in 2005, a non-interventional study (SET = Surveillance of Efficacy and Tolerability) with a double virus inactivated VWF/FVIII concentrate (Wilate) was initiated in Germany. After duration of 7 years, the inclusion of patient documentation was terminated to finally evaluate the study data. At data lock point, 170 patients suffering from von Willebrand's disease (VWD) had been included. Some of the results from the final evaluation regarding safety and efficacy will be presented.
Aim: The presented study was performed to evaluate the haemostatic efficacy and safety of a newly introduced VWF/FVIII product in the treatment of all types of VWD patients in every day clinical setting and to validate the results from pivotal clinical trials.
Methods: Patients of any age suffering from hereditary or acquired VWD requiring replacement therapy were included. Apart from demographic and anamnestic data, details of all injections for treatment of bleeding episodes, surgeries and prophylactic treatment were documented. Clinical efficacy and tolerability were rated by the treating physician using four-point verbal scales. All data underwent a predefined data management process including double data entry and plausibility checks by an independent statistical institute.

Results: Twenty-six treatment centres provided data of 170 patients suffering from VWD reflecting the broad spectrum of disease severity. About two thirds of the patients are female; the age ranges from 1 to 85 years at study entry. Six cases of acquired VWD and 7 type 3 VWD were included. Type 2 of various subtypes accounts for about 30% of the patients and the remainder suffers from type 1 VWD. One hundred and eight surgical procedures in 82 patients were documented. In all rated surgical procedures, the efficacy of the concentrate was assessed to be 'excellent' or 'good'. Efficacy in 156 bleeding episodes was assessed as 'excellent/good' in 95.2% of ratings. Prophylactic treatment of 13 patients resulted in a drop of bleeding frequency of under 1 bleed per month. Five patients had experienced suspected adverse drug reactions – all without sequelae. The ADR rate per injection was as low as 0.1%.
Conclusion: The results, reflecting the experience of routine use in all types of VWD in all types of clinical settings, confirm the excellent efficacy and tolerability which had been demonstrated during the extensive panel of clinical trials.

PB 2.42-4
Thrombin generation in platelet-rich plasma is sensitive for the activation status of Von Willebrand Factor

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Background: Von Willebrand Factor (VWF), a multimeric protein, plays a key role in hemostasis through its interactions with platelet glycoprotein Ib (GPIb), Factor (F) VIII and subendothelial collagen. VWF in plasma is unable to interact with GPIb, unless it is structurally modified by binding for example to subendothelial collagen. More recently, evidence exists that under flow conditions VWF can bind to formed fibrin and subsequently GPIb, thereby activating platelets. Von Willebrand Factor disease (VWD), the most common inherited bleeding disorder in humans, results from deficits in the quantity (Type I; reduced levels of normal functional VWF/Type III; complete absence of VWF) or function (Type II) of VWF. Type IIA VWD is characterized by a loss of high molecular weight VWF, due to faulty assembly or enhanced proteolysis, while in type IIB VWD a mutation in VWF results in its spontaneous binding to GPIb. Type IIN VWD represents an inherent VWF defect causing defective FVIII binding.
Aims: In this study we have investigated the impact of the quantity and activation status of VWF on the thrombin generation in platelet-rich plasma (PRP).
Methods: Thrombin generation (TG) was determined by calibrated automated thrombography in platelet poor vs. rich plasma of healthy controls (HCs; $N = 9$) and of patients with VWD type IIA ($N = 5$), IIB ($N = 5$), IIN ($N = 3$) and type III (simulated by VWF deficient plasma). PRP samples were prepared from platelet-poor plasma (PPP) by adding washed platelets from a HC sample, to a final concentration of 3×10^8 platelets/mL. TG was initiated with 1 pM TF and 4 μM phospholipids (PPP only). Both in PPP and PRP, all results were normalized to the average of the HC samples. Subsequently the ratio of PRP/PPP was determined for the four parameters of the TG (lag time, time to peak (ttP), endogenous thrombin potential (ETP) and peak).
Results: In most samples of VWD patients, thrombin generation in PPP was found to be lower compared to the HCs, probably because of the reduced FVIII levels present in the samples. The ratios for the lag time and ttP were not significantly affected in VWD samples compared to HCs, apart from a small increase in type III VWD. However, a significant increase in the ratio PRP/PPP was observed for both the ETP and peak in type IIB patients compared to HCs. This increase was not found in type IIA or type III samples, and only marginally in type IIN samples. This increase is likely to be explained by the spontaneous interaction of the mutated VWF in type IIB patients with GPIb, inducing the activation of platelets.
Conclusion: By comparing TG in PRP and PPP from different VWD patient samples, we have demonstrated that the TG is sensitive to the activation status of VWF.

PB 2.42-5
Comprehensive classification of patients with von Willebrand Disease after systematic genotypic analysis

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Background: Von Willebrand disease (vWD) is a bleeding disorder caused by abnormalities in Willebrand factor concentration and/or function. The prevalence is 1: 100, but only 1:10,000 cases have a clinical significant bleeding tendency. VWD can be divided in three different subclasses, but classification remains difficult if based on well-standardized laboratory assays.
Aims: Since classification is still a challenge we initiated a national reference centre for von Willebrand factor genomic analysis. The aim of this center is to improve vWF classification. To do so, genetic abnormalities in the vWF gene in patient suspected to suffer from vWD will be correlated to the other vWF parameters.
Methods: DNA of patients suffering from vWD was analyzed by Sanger sequencing the coding regions including the intron-exon boundaries of the vWF gene. Large deletions and duplications in the vWF gene were detected by MLPA.
Results: Up to December 2012, 186 patients were analyzed. One hundred and eight patients had a genetic abnormality. The mutations found were eight nonsense mutations, 105 missense mutations, nine small deletions, four duplications, and four large deletions detected by MLPA. In 78 patients no mutations were found. Of all mutation detected 104 were reported previously and 16 were novel. Thirteen of the novel mutations were missense mutations, one nonsense and two small deletions. Twenty-nine patients were carrying the D1472H polymorphism affecting the von Willebrand Factor Ristocetin cofactor activity as reported by Flood et al, Blood, 2010. The *de novo* mutations were further analyzed by in silico analysis.
Summary/Conclusions: Our systematic genotypic approach resulted in 58% of the cases in a confirmation of von Willebrand disease. Furthermore, we were able to confirm in 90% of the cases a vWD classification. Finally, we expect that our approach will result in the identification of specific haplotypes since the rate of polymorphisms in von Willebrand factor is high. The results of our analyses will be included in the appropriate ISTH/WFH-related registries.

PB 2.42-6
Evaluation and clinical application of von Willebrand factor antigen and ristocetin cofactor by chemioliminiscence in a new analyzer ACL AcuStar in the diagnosis of von Willebrand disease

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Background: Von Willebrand disease (VWD) is the most common congenital bleeding disorder, caused by inherited quantitative and qualitative defects of the von Willebrand factor (VWF). An effective diagnosis of VWD includes assessment of FVIII coagulant activity,

VWF antigen (VWF:Ag) determined usually by ELISA, and VWF ristocetin cofactor (VWF:RCo) by aggregometry. Since its introduction, various modifications of VWF:RCo were proposed due to complexity, performance time, poor reproducibility and poor sensitivity to low levels of VWF. The ACL Acustar is the first analyzer that introduces chemioluminiscence technology in the detection of VWD. This new instrument allows to perform automated quantitative assays of VWF:Ag and VWF:RCo.

Aims: Comparison of the results of VWF:Ag and VWF:RCo obtained by conventional methods with the ones by new automatic analyzer ACL Acustar, in normal subjects and in patients with VWD. Evaluation of the performance of ACL Acustar in terms of imprecision, sensibility, specificity and probability to make a correct VWD diagnosis.

Methods: Citrated plasma samples were obtained from $n = 146$ patients with previously diagnosed congenital VWD (Type 1, $n = 51$; Type2A, $n = 35$; Type 2B, $n = 16$; Type 2M, $n = 30$; Type 2N, $n = 5$; Type 3, $n = 9$) and $n = 30$ healthy normal subjects. The VWF levels were analyzed by ELISA (VWF:Ag-ELISA) (DG-EIA VWF, Diagnostic Grifols), by light transmission aggregometry with lyophilized fixed platelets and ristocetin (VWF:RCo-LTA) (Helena Biosciences), and in the ACL AcuStar were tested reagents HemosIL® Acustar VWF:Ag (VWF:Ag-IL) and HemosIL® Acustar VWF:RCo (VWF:RCo-IL) (Instrumentation Laboratory). VWF:RCo/VWF:Ag ratio and multimeric distribution (SDS-agarose gel) of VWF were determinated in all samples VWD and genetic analysis in 77/146. Statistical analysis performed with Analyse-It v2.30.

Results: Normal ranges were: VWF:Ag-ELISA 41–156 U/dL, VWF:RCo-LTA 50–158 U/dL, VWF:Ag-IL 41–156 U/dL and VWF:RCo-IL 50–158 U/dL. The lower limit level detected of VWF:RCo-LTA in our laboratory is 4U/dL. Diagnosis algorithm considered VWF levels lower than 30 U/dL for the diagnosis of type 1 VWD and a cut-off of ratio VWF:RCo/VWF:Ag 0.7. ACL Acustar methods showed low levels of intra-assay and inter-assay imprecision (CV < 10%). Passing Bablok regression comparing VWF:Ag-ELISA and VWF:Ag-IL methods, yielded a slope of 1.07 (95% CI: 1.04–1.11), intercept of 1.49 (95% CI: 0.58–2.29). Comparing VWF:RCo-LTA and VWF:RCo-IL, slope was 1.18 (95% CI: 1.13–1.24), intercept 0.09 (95% CI: −0.23–1.24). Pearson r was 0.98 (95% CI: 0.94–0.99) comparing VWF:Ag methods and 0.97 (95% CI: 0.96–0.98) comparing VWF:RCo methods. Kappa test between original diagnosis and found diagnosis with both methods was > 0.8, being higher with ACL Acustar methods. A 3.4% (5/146) diagnosis discrepancies between two methods can be explained by limitations in sensitivity of VWF:RCo-LTA. Discrepancies between original and new method based diagnosis were 2.7% (4/146). Multimeric and genetic analysis were of help in clarifying these discrepancies.

Conclusion: New chemioliminiscence methods analyzed in ACL AcuStar can detect VWD and discriminate between type 1, 3, and variant forms. They offer a automated, faster, reproducible, more sensitive and less cumbersome alternative to conventional assays, specially VWF:RCo-LTA. In conjunction with multimer analysis and genotype they are reliable methods for the laboratory diagnosis of VWD.

PB 2.43-1

Dextran sulfate blocks assembly of UL-VWF strings on the surface of endothelial cells

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Background: Vascular endothelial cells (ECs) provide a highly interactive barrier between blood and the underlying tissues and are of vital importance in maintaining vascular homeostasis. ECs contain specific cigar-shaped storage organelles, designated Weibel-Palade bodies (WPBs), which upon stimulation release various components into the bloodstream. The main protein stored in WPBs is von Willebrand factor (VWF), a multimeric glycoprotein which is crucial for binding of platelets under shear stress. Upon exocytosis of WPBs VWF multimers assemble into ultra large VWF (UL-VWF) strings that remain anchored to the surface of endothelial cells. Our current knowledge on the conversion of VWF tubules as stored in WPBs into UL-VWF strings is limited.

Aim: In this study we investigated the ability of different pharmacological compounds to interfere with assembly of UL-VWF strings on the surface of endothelial cells.

Methods: Human umbilical vein endothelial cells were cultured in flowchambers, pre-incubated with various pharmacological inhibitors and stimulated with histamine or ionomycin under flow (2.5 dyn/cm^2) to release UL-VWF strings. UL-VWF strings were visualized by perfusion of beads coated with a monoclonal or polyclonal anti-VWF antibody or washed platelets. Images were taken of 20 randomly chosen optical fields and analyzed for number and length of VWF strings. VWF and VWF propeptide release was measured in medium from statically stimulated cells with or without pharmacological inhibitors by ELISA. Confocal microscopy of histamine-treated HUVEC employing an Alexa488 labeled polyclonal antibody was used to visually monitor the effects of the different inhibitors.

Results: The effect of different pharmacological inhibitors on assembly of UL-VWF strings was evaluated. Increasing concentrations of heparin (50–250 μg/mL) did not prohibit the number and length of UL-VWF strings (ranging from 50 to over 400 μm) on endothelial cells following stimulation with histamine. Also mannan (0.5–5 mg/mL) did not affect UL-VWF string formation, indicating that mannan-sensitive C-type lectins do not contribute to formation of UL-VWF strings. Incubation with the sulfated poly-anion dextran sulfate (at concentrations of 50–250 μg/mL) completely abolished histamine-induced formation of UL-VWF strings on the surface of endothelial cells. Also, ionomycin induced formation of UL-VWF strings was impaired under these conditions indicating that the dextran sulfate did not block the interaction of histamine with its receptor. We subsequently monitored whether dextran sulfate blocked release of WPBs under static conditions. Dextran sulfate showed a marked inhibition of histamine- and ionomycin -induced VWF release. Strikingly, release of VWF propeptide was not affected by dextran sulfate. Confocal microscopy revealed that VWF containing clusters, most likely originating from fused WPBs, were present on the plasma membrane of cells stimulated in the presence of dextran sulfate. In control cells stimulated with histamine in the absence of dextran sulfate these structures were not observed.

Summary/Conclusions: Our findings show that anionic dextran sulfate polymers interfere with assembly of UL-VWF strings on the surface of endothelial cells. Confocal microscopy suggests that dextran sulfate prevents the conversion of newly released VWF multimers into UL-VWF strings following fusion of WPBs with the plasma membrane.

PB 2.43-2

Platelet aggregates developed at high shear rate grow rapidly, are unstable, and incorporate vWF

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Background: At sites of vascular injury, von Willebrand factor (vWF), a large multimeric protein, aids in the arrest and aggregation of platelets. We have demonstrated that vWF can form large fibril aggregates (> 100 μm in length) when perfused over immobilized collagen type I surfaces under elevated shear rate conditions. In platelet free plasma (PFP) we observed a threshold shear rate of 30,000/s, while we found a threshold of 10,000/s in whole blood (WB). We believe that the fibril aggregates consist of many cross-linked vWF molecules based on their long length and width (~300 nm). Here we report that exposure of soluble vWF to high shear rates in whole blood enhances the incorporation of vWF into growing platelet aggregates resulting in rapid and embolic platelet adhesion.

Aim: We sought to investigate the role of soluble vWF in the process of platelet deposition onto a collagen type I surface under elevated shear rates mimicking those found in stenosed coronary arteries.
Methods: Whole blood from healthy donors was collected into 100 μM PPACK to inhibit the generation of thrombin. Samples were perfused through a microfluidic channel via syringe pump over a collagen type I surface. Platelet and vWF deposition was visualized using fluorescently labeled antibodies and epifluorescence microscopy.
Results: Perfusion of WB at 200/s over a collagen type I surface resulted in platelet adhesion which reached steady state after ~20 min and incorporated no detectable vWF. These aggregates represented the balance between the activating forces (ADP and Thromboxane A2) aiding the capture of flowing platelets and shear forces which mechanically remove adhered platelets. Based on our previous efforts to model shear rate as a function of aggregate growth we estimate this value to be ~1000–2000/s. Counterintuitively, we found that platelet adhesion was restored by increasing the local wall shear rate (> 10,000/s) after steady state was achieved. The resulting aggregates grew rapidly (~5-fold faster), were embolic, and incorporated vWF. We believe that the enhanced deposition of vWF onto the aggregate resulted in elongated platelet contact times with the steady state deposit. We have previously demonstrated that fibril aggregates of vWF form on these surfaces of platelets under plasma perfusion. Rapid and embolic platelet adhesion (at > 10,000/s) could also be achieved from surfaces of collagen and required $\alpha_{IIb}\beta_3$ as well as Gp1b. In addition, sharp transitions in the adhesive mechanism (with incorporation of vWF) were observed starting with intermediate bulk shear rates (1000–2000/s). Approximately 50–60% of channel occlusion starting from these conditions resulted in shear rates above the critical value (~10,000/s).
Conclusions Under conditions of high shear rate that mimick coronary stenosis, vWF can form long fibril aggregates on surfaces of collagen and platelets. These aggregates appeared to be the nucleating event for rapid and embolic platelet deposition under WB flow in microfluidic channels. This vWF mediated mechanism of platelet adhesion may underlie ischemic events that result from full vessel occlusion or embolus.

PB 2.43-3
Functional role of von Willebrand factor (VWF) triplet bands in glycoprotein Ib-mediated platelet adhesion and thrombus formation under flow

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Background: Multimeric glycoprotein von Willebrand factor (VWF) exhibits a unique triplet structure of individual oligomers, resulting from ADAMTS13 (a disintegrin and metalloproteinase with thrombospondin type 1 motifs 13) cleavage. The faster and slower migrating triplet bands of a given VWF multimer have one shorter or longer N-terminal peptide sequence, respectively. Within this peptide sequence, the A1 domain regulates interaction of VWF to platelet glycoprotein (GP)Ib.
Aim: Distribution of VWF triplet bands is significantly altered in some types of von Willebrand's disease (VWD). Since the properties of individual VWF triplet bands have not been investigated so far, we analyzed platelet-adhesive function of VWF preparations with different triplet composition with regard to differential functional activities.
Methods: Two VWF preparations with similar multimeric distribution but different triplet composition obtained by size exclusion in addition to heparin affinity chromatography were investigated. Preparation A was enriched in intermediate triplet bands, while preparation B predominantly contained larger triplet bands, which was confirmed by VWF multimer analysis. Collagen- and GPIb-binding was determined by surface plasmon resonance (SPR). Platelet adhesion under flow using either reconstituted blood or blood from a type 2A VWD patient was determined using parallel-plate flow chamber models.
Results: Binding studies revealed that preparation A displayed a reduced affinity for recombinant GPIb, but an unchanged affinity for collagen type III, when compared to preparation B. Under high-shear flow conditions, preparation A was less active in recruiting platelets to collagen type III. Furthermore, when added to blood from patients with (VWD), defective thrombus formation was less restored.
Conclusion: Thus, VWF forms lacking larger size triplet bands appear to have a decreased potential to recruit platelets to collagen-bound VWF under arterial flow conditions. By implication, changes in triplet band distribution observed in patients with VWD may result in altered platelet adhesion at high-shear flow.

PB 2.43-4
Characterization of recombinant VWF73 peptide in different prokaryotic expression systems

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Background: VWF73 peptide, the well known susbtrate of ADAMTS13 (a disintegrin and metalloproteinase with thrombospondin motif 13), has been modified by attachment of fluorescein moities to assay ADAMTS13 activity widely in clinic.
Aims: To probe its production in different prokaryotic expression systems and to compare its FRET(fluorescence resonance energy transfer)effect by modification in different specific amino acid, we set out to commit this experiment.
Methods: The wild sequence of VWF73 was amplified using the pSVHVWF1 plasmid (provided by Professor J. Evan. Sadler) as the template and the primers containing BamHI and Hind III plus stop codon in the 5′and 3′ end respectively. The PCR product was cloned into the pQE30 and PET-20b plasmid.
Regarding to the pQE30 plasmid containing wild VWF73, a pair of cysteine (Q1599C + N1610C/Q1599C + P1611C) was introduced to the VWF73 sequence using site-directed mutagenesis, meanwhile, the BamHI cleavage site was removed by deletion mutagenesis, and the final product expressed by pQE30 plasmid was as follows: MRGSHHHHHH plus the VWF73 sequence (Q1599C + N1610C/Q1599C + P1611C). The modified pQE30 plasmid was transformed to the M15 *E. coli* and the corresponding peptide was induced using 1 mM IPTG overnight at 30 °C, and the induced peptide was confirmed by Western-Blot using anti-His antibody-HRP. The expressed peptide was purified by Ni column and treated by TCEP followed by attachment with M-5-F (fluorecein-5-Maleimide) in pH 7.0 condition. The fluorescein-attached VWF peptide was dialyzed against Tris-HCL buffer (pH8.0) thoroughly to remove unattached M-5-F. And the FRET effects of two fluorescein version of VWF (Q1599C + N1610C/Q1599C + P1611C) were assayed using the sequentially diluted normal plasma as the source of ADAMTS13 in Bis-Tris buffer containing CaCl2.
The PET-20b plasmid containing wild VWF73 sequence was modified by several rounds of mutagenesis and the final product sequence expressed by PET-20b was the same as the peptide expressed by the pQE30 plasmid, i.e., MRGSHHHHHH plus the VWF73 sequence (Q1599C + N1610C/Q1599C + P1611C). The modified PET-20b plasmid was transformed to the *E. coli* B.L21 (DE3) and the corresponding peptide was induced using 1 mM IPTG overnight at 30 °C, and the induced peptide was confirmed by Western-Blot using anti-His antibody-HRP.
Results: The modified pQE30 and PET-20b plasmid containing VWF was confirmed by DNA sequencing. Western-Blot domenstrated an

intact one band existed regarding to the pQE30/M15 expression system. However, there existed two bands regarding to the PET20-b/DE3 expression system, implying that certain enzyme (enzymes) lurked in the *E. coli* B.L21, which could specifically/unspecifically cleave the VWF peptide. the FRET effects of two fluorescein version of VWF (Q1599C + N1610C/Q1599C + P1611C) derived from the pQE30/M15 expression system showed that fluorescence release from the peptide of Q1599C + N1610C was approximately two fold compared to the fluorescence release from the peptide of Q1599C + P1611C when the same amount of ADAMTS13 was used.

Conclusion: PET20-b/DE3 expression system is not suitable for the expression of VWF73 peptide while pQE30/M15 expression system is feasible for this expression purpose. And the VWF peptide modified at Q1599C plus N1610C is better than the peptide modified at Q1599C plus P1611C cause the FRET effect of former is almost two fold compared to the latter.

PB 2.43-5

Von willebrand factor levels are strongly associated with atherosclerosis in patients with ischemic stroke

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Background: Increased von Willebrand Factor (VWF) levels are associated with an increased risk of ischemic stroke. As VWF levels are known to be associated with endothelial dysfunction, atherosclerosis is expected to play a role in this association. It is yet unknown what the association between VWF and atherosclerosis is in ischemic stroke patients.

Aims: We investigated the relationship between atherosclerosis and VWF antigen (VWF:Ag) levels in 925 consecutive patients with transient ischemic attack (TIA) or ischemic stroke, using an advanced measurement of atherosclerosis.

Methods: Calcification volumes (mm^3) were scored in the aortic arch and both carotid arteries using multidetector computed tomography (CT) angiography of the craniocervical vessels. VWF antigen (VWF:Ag) levels were measured using ELISA technique. Underlying ischemic events in the patients were classified according to the TOAST criteria. The associations between the calcium volumes and VWF:Ag levels were assessed using linear regression analysis and adjusted for potential confounders.

Informed consent was obtained and the study was approved by a recognised medical ethics committee.

Results: Mean VWF:Ag levels were significantly higher in the presence of calcification in either the aortic arch (1.54 vs. 1.26 IU/mL [$P < 0.0001$]) or the carotid arteries (1.56 vs. 1.27 IU/mL [$P < 0.0001$]). Levels of VWF:Ag rose with increasing aortic arch ($P = 0.005$ for trend) and carotid calcification volume ($P < 0.0001$ for trend). Patients with a small vessel TIA or ischemic stroke had significantly lower VWF:Ag levels compared with the other TOAST subtypes ($P = 0.003$). High VWF:Ag levels were significantly associated with an unfavorable outcome (modified Rankin Scale > 2 vs. ≤ 2; 1.70 vs. 1.40 IU/mL, [$P < 0.0001$]).

Summary/Conclusion: Our study showed a strong association between atherosclerosis in either the aortic arch or the carotid arteries and high VWF levels in patients with TIA or ischemic stroke. This finding supports the hypothesis of a pathophysiologic role of VWF in ischemic stroke which seems to be influenced by the extent of atherosclerosis and could therefore have a prognostic value.

PB 2.43-6

A comparative evaluation of two new automated assays for von Willebrand factor ristocetin cofactor activity and von Willebrand factor antigen

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Background: von Willebrand's disease (VWD) is the most common congenital bleeding disorder caused by a quantitative or qualitative plasma deficiency of the von Willebrand factor (VWF). The initial laboratory classification of VWD is dependent on the measurement of von Willebrand Factor Antigen (VWF:Ag) and VWF activity, usually by ristocetin cofactor activity assay (VWF:RCo).

Aim: To compare the performance of two new automated VWF:Ag and VWF:RCo assays systems from Instrumentation Laboratory (IL, Bedford, USA).

Methods: One hundred and seventy-one healthy normal subjects and 72 patients with VWD (14 type 1, 52 type 2, 6 type 3) were evaluated. VWD patients have been previously characterized, according to the ISTH-SSC guidelines, using a home-made ELISA VWF:Ag determination and a platelet-based VWF:RCo assay on an ACL-9000 (Lattuada *et al*, 2004), as reference first-level tests. All samples were obtained after informed consent. VWF:Ag and VWF:RCo were performed using fully automated assays that utilised different principles: immunoturbidimetric assays (HemosIL VWF:RCo and HemosIL VWF:Ag) were performed on an ACL TOP analyser and chemiluminescent assays (AcuStar VWF:RCo* and AcuStar VWF:Ag*) were performed on an ACL AcuStar instrument. Both HemosIL VWF:RCo and AcuStar VWF:RCo assays use recombinant GPIb in the presence of ristocetin. Assay imprecision was assessed using commercial control plasmas. All reagents and analysers were from IL.

Results: VWF:Ag and VWF:RCo determination performed on the ACL AcuStar exhibited low levels of intra- and inter-assay imprecision for normal and pathological control plasmas (CV% range 3.3–6.9). The VWF:Ag and VWF:RCo assays performed on ACL TOP also gave low levels of intra- and inter- assay imprecision for normal and pathological control plasmas (CV% range 0.8–4.7). For both normal (observed range: VWF:Ag 44.6–173.9 U/dL; VWF:RCo 43.1–191.5 U/dL) and VWD (observed range: VWF:Ag 0.3–115.1 U/dL; VWF:RCo 0.5–57.2 U/dL) samples there was good correlation between the two VWF:Ag and VWF:RCo Methods: (Pearson correlation coefficient $r = 0.92$ and $r = 0.82$ respectively).

Both new assays systems gave consistent results compared to the previous diagnosis.

AcuStar VWF:RCo and VWF:Ag demonstrated superior lower level of detection compared to HemosIL VWF:RCo and VWF:Ag (0.5 vs. 6 U/dL and 0.3 vs. 12 U/dL respectively).

Conclusions: The VWF:Ag and VWF:RCo assays performed on ACL AcuStar and on ACL Top seem to be reliable and precise. The use of these tests for the VWD diagnosis merits further evaluation in a larger VWD population.

*In development. Not currently saleable.

PB2.44 – Haemophilia: Miscellaneous

PB 2.44-1

Haemophilia and home treatment: optimizing knowledge and practical skills by an e-learning program

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Background: Home treatment was first described in 1974, and is currently the standard of care for patients with severe haemophilia. The advantages of home treatment are evident and well-known, and have been recorded in numerous publications. Home treatment increases the responsibility of the patients for their treatment and care. Mistakes may occur by incorrect diagnosis of a bleeding episode, lack of compliance to the proposed treatment regimen, or under dosing in case of life-threatening bleeding events. With home treatment the responsibilities for correct treatment are in part transferred from the physician to the patient or his parents. Adequate education of the patient and/or parent is therefore required. Hence, it is of utmost importance to attain high level of knowledge and practical skills.
Aim: The primary aim of this study was to obtain insight in the knowledge and skills of haemophilia patients who have treated themselves at home for several years. A second aim was to study whether or not the introduction of an educational program by means of an e-learning module, created especially for this purpose, improved the knowledge and skills in the group of patients that were examined. The e-learning program takes maximum of 20 min.
Methods: We performed a randomised intervention study. Participants who were already on home-treatment and had undergone extensive home-treatment training in the past were recruited at theHaemophiliaTreatmentCenter of the Erasmus University Medical Centre. Patients were divided into two groups: the intervention group who had an e-learning training, and the control group, that had no additional training. Subjects had to complete a test at two occasions, which consisted of the intravenous injection of clotting factor concentrate while being observed, and completed a questionnaire with 77 items. After 1 month, all participants completed the same questionnaire (post-test) and were again observed and scored during self infusion. The intervention group received an e-learning program before the second test.
Results: At base-line, the patients (n = 30) showed a relatively low level of knowledge (24 of 48 questions correct) the practical skills of injection are performed correctly by 75% of the patients. At second measurement the e-learning group (n = 16) showed a higher level of knowledge in comparison with the control group (n = 14) who did not receive an e-learning program (36 of 48 questions correct). In the observation of practical skills a difference between the two groups was found as well (using a 20 items checklist, at baseline the patients scored 11 out of 20, at second observation 16 out of 20). Finally, the survey shows a clear association between self-efficacy of the patients and the level of knowledge.
Conclusion: This study shows that patients with haemophilia, who are on home treatment, have a relatively low level of knowledge and skills. This can be improved by an educational intervention, such as an e-learning program. In our centre the e-learning is already implemented for patients with haemophilia in the transition from children to adult care and in the management of home treatment care (as a follow-up training).

PB 2.44-2

Personalised prophylaxis in haemophilia A: how low can you go?

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Background: The United Kingtom Haemophilia Centre Doctors' Organisation (UKHCDO) recognises that the pharmacokinetics of Factor VIII (FVIII) varies considerably amongst adult patients with severe Haemophilia A (between 8 and 23) hours. This is influenced by a number of physiological and pathological variables. In our haemophilia centre we encourage a strict prophylaxis regimen for all severe haemophilia patients, and encourage regimens that are individualised. This is in line with the UKHCDO which recommends that prophylaxis regimens in adults should be personalised using bleeding phenotype and pharmacokinetics.
Aims: We decided to review all prophylaxis regimens in adult patients with severe Haemophilia A, with the aim of reducing the amount of concentrate to the minimum effective level. To date we have collected data from nine patients (from a possible 38) and data collection is ongoing.
Methods: We have found that using prospectively assessed trough levels and bleed diaries in all evaluable patients with severe Haemophilia A, we have been able, in some cases, to significantly reduce the amount of prescribed FVIII administered, with the aim of improving cost effectiveness.
Results: Prior to this review, doses of FVIII for prophylaxis varied between 10 and 40 IU/kg (mean 27 IU/kg). To date, we have found that the number of units per kilogram of FVIII concentrate required for effective prophylaxis ranges from 6 to 40 IU/kg (mean 24.4 IU/kg). In many cases, through patient education, we have been able to reduce trough levels to < 3 IU/dL. This is an ongoing project and we are continuing to reduce the FVIII usage further in patients whose trough is > 3 IU/dL. Dosing regimens are further influenced by patient lifestyle with some requiring infusions every 24, 48 or 72 h. Collecting trough samples can be challenging for a number of reasons, including missed doses, missed appointments and reluctance to return to the clinic for repeated sampling.
Summary: In conclusion, it is clear that in order to be cost-effective and clinically effective, prophylaxis regimens in severe Haemophilia A need to be individualised. The amount of FVIII required may be significantly lower then previously thought. Bleeding phenotype, as well as half-life are important. We have also found that weight reduction initiatives have also reduced consumption of FVIII in some of these patients. Future work includes correlation of FVIII mutation with minimum prophylaxis dose required, and addressing other factors that may be predictors in detmining the miniumum effective dose of FVIII concentrate. There are further challenges; addressing patients mis-conceptions about their prophylaxis regimens and ensuring they are fully informed and involved in all treatment decisions.

PB 2.44-3

Buprenorphine does not influence the inflammatory response in haemophilia A mice with experimentally induced haemarthrosis

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Background: Haemarthrosis is the most common clinical manifestation of haemophilia and is responsible for major morbidity in haemophilia patients. After an intraarticular bleeding episode blood is removed by absorption to the synovial tissue and with recurrent bleeding episodes degradation products of red blood cells accumulate in the tissue triggering an inflammatory response. The murine experimentally induced knee bleeding model is an important model in hae-

mophilia research and as the inflammatory response is a crucial part of the pathogenesis of haemophilic arthropathy any administration of analgesics should ideally not alter this response. Currently it is unknown if the use of analgesia in this model might impact the inflammatory response.
For animal welfare and ethical reasons however, the full analgesic protocol in this model should be continued and the response of buprenorphine on the inflammatory response evaluated.
Aim: The aim of the present study was to investigate the inflammatory response after a needle induced knee bleed in haemophilia A mice treated with buprenorphine or saline.
Materials and Methods: One hundred and sixty mice were randomized in two groups to blindly receive buprenorphine or saline. All mice were anaesthetized and knee injury was induced by inserting a 30G needle into the right knee joint. At t = 6, 24, 48 and 72 h twenty mice from each group were terminated and the following parameters were assessed: Change in bodyweight, change in joint diameter, Visual Bleeding Score (VBS), white blood counts, haematocrit, platelet concentrations, haemoglobin, plasma haptoglobin and plasma and synovial fluid levels of 23 different cytokines (IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-17, eotaxin, G-CSF, GM-CSF, IFN-γ, KC, MCP-1, MIP-1α, MIP-1β, RANTES and TNF-α). Twenty mice were terminated at t = 0 receiving no injury or treatment to provide baseline measures.
Results: No significant alterations were observed in joint diameter, VBS, white blood counts, haematocrit, platelet concentrations and haemoglobin. Furthermore, 21 cytokines in plasma and 22 cytokines in synovial fluid, were unaffected by the administration of buprenorphine. Slight alterations in bodyweight ($P < 0.01$), of plasma G-CSF ($P < 0.05$) and haptoglobin at t = 48 h ($P < 0.05$), plasma and synovial eotaxin ($P < 0.05$ for both) were found in buprenorphine treated mice.
Conclusion: We have demonstrated that buprenorphine does not overall impact the inflammatory response and the use of buprenorphine in the knee bleeding model in haemophilic mice should be continued.
Perspectives: These data should call for further investigation in other inflammatory *in vivo* models and be included in the ethical and 3R considerations applied when working with these models.

PB 2.44-4

Identification and characterization of an L1 insertion in intron 16 of F8 in a patient with hemophilia A

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Background: A wide variety of genetic abnormalities in the coagulation factor VIII gene (*F8*) have been found to cause hemophilia A. Among these abnormalities, insertion mutations are relatively rare.
Aim: We aimed to investigate a case of hemophilia A in whom the PCR amplification products from a part of *F8* could not be obtained.
Patient and Methods: The patient was a Japanese male with severe hemophilia A. He had a target joint, which had repeated bleeding episodes, and occasionally had serious bleeding such as iliopsoas muscle bleeding or pharyngeal bleeding. No other cases of hemophilia were found in his family. Genomic DNA was extracted from blood cells using the EZ1 DNA Blood 350 μL Kit (Qiagen) on a BioRobot EZ1 workstation (Qiagen). All 26 exons and their flanking intronic regions of *F8* were amplified independently by PCR and the nucleotide sequence was determined. Each amplicon was purified and directly sequenced by the dideoxy method using a BigDye Terminator Kit (Applied Biosystems). Sequencing was performed on a 48-capillary 3730 DNA Analyzer (Applied Biosystems). The nucleotide sequences were compared with reference sequences.
Results: We could not obtain PCR products from exon 16 or 17 amplification. We therefore considered that intron 16, which was common to both amplifications, might include some genetic abnormality. The PCR product amplified from exons 16–18 of the patient's *F8* was apparently 3 kb larger than that of normal control. This result indicated the possibility that the patient had a large insertion in intron 16. The sequencing of the patient's abnormally large PCR product revealed an insertion size of 2134–2137 bp and indicated that the nucleotide sequence was a part of L1 [LINE (long interspersed elements)-1, non-LTR retrotransposons]. L1 was inserted 98 bp 3' from the end of exon 16 and was flanked by a 10–13 bp target site duplication. The insertion contained 3' portions, which comprised most of the latter half of the L1 sequence, including the poly(A) tract. The insertion was divided into two blocks of sequence, each connected by a head-to-head arrangement. We also identified an intron 22 inversion from the patient's *F8*.
Summary/Conclusion: We identified a quite rare L1 insertion from the *F8* of a patient with hemophilia A. Thus far, such insertions have been reported in only three cases. Two of these three insertions, detected in an exon, were thought to be an etiology of hemophilia A. However, it is reported that the remaining insertion detected in an intron was not an etiology. In this patient, the direct etiology of hemophilia A is intron 22 inversion. If the L1 insertion causes hemophilia, abnormal splicing can be presumed because the possibility that the inserted sequence included a potential splice site was confirmed by *in silico* analysis. However, there is no direct evidence that this insertion causes hemophilia A. Further study is required to determine whether this insertion causes hemophilia A.

PB 2.44-5

Haemophilia A with and without historical inhibitors evaluation of T regulatory and memory B lymphocytes populations

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Background: FVIII inhibitor (IN) development is the most feared complication of substitutive therapy in patients with Haemophilia A (HA). Its major clinical impact justifies thorough investigation of factors that may elucidate the underlying pathophysiology and their modulating factors.
CD4+ CD25+ T regulatory cells (Treg) are involved in the induction of tolerance and prevention of the T helper (Th) response which is responsible for IN development. In the immune response to antigen T-dependent, such as FVIII, the immunological memory is generated via CD19+ CD27+ memory B cells.
Tolerance to FVIII in patients with IN can thus be theoretically obtained by two pathways: increased Treg response and/or CD19+ CD27+ B cell anergy.
Could the characteristics of these two lymphocyte populations be predictive of IN development?
Aims: To evaluate circulating Treg and CD19+ CD27+ cells in HA patients with (HAI) and without (HAnI) history of IN and in a group of healthy volunteer controls (Ctl)
Methods: flow citometry analysis of peripheral blood samples from six HAI, mean age 23y (14–42), 10 HAsI, mean age 18y (2–36) and 14 Ctl, mean age 22y (3–40).
Three HAI were low responders, and three high responders, only one had IN > 5UB at the time the study was performed.
Lymphocytes were gated based on size (FSC) and complexity (SSC). Treg were characterised as CD4+ CD25+ CD127low lymphocytes, and memory B cells as CD19+ CD27+ lymphocytes.
Results: Percentage of CD4+ CD25+ CD127low cells: HAI 8.6 ± 1.1, HAnI 8 ± 1.6 and Ctl 7.6 ± 1.7. CD19+ CD27+ cells: HAI 32.6 ± 19.2, HAnI 26.6 ± 14.3 and Ctl 30.1 ± 10.9. We did not document any statistically significant difference between the three groups studied. The proportion of lymphocytes T and B was similar between the groups.

Conclusion: Given its relevance in the suppression of the antibody-mediated response to substitutive therapy, we would expect a decrease of Treg in the HAI group, which was not observed in this sample.
Treg and CD19+ CD27+ frequency were similar between HAI, HAnI and Ctl groups. We hypothesise the results might be different if all HAI patients had high IN titres at the time of analysis, in keeping with what has been described by other research groups who documented a decrease of circulating CD19+ CD27+ in these patients. In our series, the only patient with IN > 5UB was not significantly different from the HAsi or Ctl. Although the number of patients enrolled is still reduced, our results hint to a lack of predictive value of circulating Treg and CD19+ CD27+ percentage in the development of IN in HA patients

PB 2.44-6

Stop codon readthrough with PTC-124 and gentamycin in hemophilia B caused by nonsense mutations

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Background: One of the most important molecular mechanisms in severe hemophilia B, which is caused by the mutations in the factor IX gene, is nonsense mutation, carrying premature termination codon (PTC), which lead to the production of nonfunctional truncated protein. Aminoglycoside antibiotics, such as Gentamycin, could induce readthrough of PTC and increase the production of the protein with nonsense mutation. However, using of high dosage of aminoglycosides for the readthrough therapy, has significant adverse effects including the nephrotoxicity and ototoxicity. PTC-124, a novel and non-toxic chemical compound, could be able to accomplish this task.
Aims: At present, due to lacking of the eradicative therapy, the hemophilia B patients should infusion the expensive blood coagulation factor in their lifetime. So searching for the new drugs has significantly social effects. Through our study, we want to compare the readthrough ability of different concentrations of PTC-124 and Gentamycin in hemophilia B caused by different PTC and provide the basis for the further researches of readthrough therapy.
Methods: The international hemophilia B mutation database was analyzed for finding the hot spots of nonsense mutation sites in FIX gene. The nonsense mutants were constructed by site-directed mutagenesis. Detecting the HEK-293 cells viability after treated with different concentrations of PTC-124 and Gentamycin for 48 h, using CCK-8 (Cell Counting Kit-8). Then, HEK-293 cells were transfected with either the wild-type or mutated constructs by liposome-mediated gene transfer method, then treated with different concentrations of PTC-124 and Gentamycin for 48 h. The mRNA expression levels of FIX gene were detected by real-time PCR. The procoagulant activity (FIX : C) and antigen (FIX : Ag) of FIX in the cell lysate were detected using one stage method and ELISA, respectively.
Results: The hot spots of nonsense mutation sites and our objects in FIX gene were R29X,R116X,W194X,R333X. Except the designed sites, there was no other nucleotide mutations in the sequences of four mutants. The IC50 of PTC124 and Gentamycin in HEK-293 cells were 111.585 ± 9.597 and 4.23 ± 0.32 mM respectively, then select different concentrations of PTC124 and Gentamycin for the readthrough study. Compared with the untreated controls, the mRNA expression levels of FIX gene and the FIX : C and FIX : Ag of FIX in the cell lysate treated with different concentration of PTC124 and Gentamycin were significantly elevated ($P < 0.05$) in those four mutants.
Conclusion: From our data, the PTC124 and gentamycin possessed the similar efficiency of read-through, while with less concentration and toxicity. In hemohilia B, even a small increase in FIX expression level can dramatically ameliorate the clinical phenotype, so the readtrhough approach can be a good therapeutic strategy. Although the aminoglycoside antibiotics, such as Gentamycin, could increase the functional protein prodution, however, these drugs have serious side effects at therapeutically relevant concentrations. PTC124, which is not structurally related to aminoglycosides, could be able to provide a better treatment for hemophilia B caused by nonsense mutation.

PB2.45 – Acquired Coagulation Disorders – III

PB 2.45-1

Hemostasis disorders are indicators of early infectious complications in severe burned

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Introduction: Early infectious complications – sepsis and pneumonia developing in acute burn disease are of serious risk for patients with severe heat injury. These complications are characterized by a fulminant, severe course, complex diagnostics, and high case fatality rate. However, the role of hemostasiological disorders in the development of both generalized, and local infections in severe burned is taken into account insufficiently.
Aim: The aim of the investigation was to study the characteristics of hemostasis disorders in the development of purulent-septic complications in acute burn disease.
Methods and Materials: We studied hemostasis state and biochemical measurements of blood in 167 patients with burn area over 20% of body surface, within the period from the 1st to 12th days after burn. In 33 patients acute burn diseases was complicated by sepsis development, in 67 patients – by pneumonia, and 67 patients had no complications in the form of sepsis and pneumonia. The groups under study were balanced in age, damage area and severity. Sepsis and pneumonia were revealed in clinical picture in the period from the 4th to 12th days after burn. DIC syndrome diagnosis was made according to standard criteria: the presence of an etiological factor, hemostasis system imbalance, and dysfunction of shock-organs.
Results: The comparative analysis of findings showed the development of infectious complications in early burn diseases to be preceded by the increase of hemostasis system disregulation. The suppression of endogenous anticoagulants, fibrinolysis depression, and thrombocytopenia were accompanied by the enhancement of procoagulant link of hemostasis system and the decreased functional activity of detoxication organs – the kidneys and the liver. Throughout the study period the hemostasiological indices reflecting the DIC syndrome severity correlated with biochemical blood metabolites indicating hepatorenal failure progression ($r = 0.55–0.85$; $P < 0.05$).
The patients with advanced sepsis and pneumonia had reduced antithrombin III that characterized the functional activity of anticoagulant element, and exhibited prognostic characteristics in terms of poor outcome of burn disease and the development of both generalized ($\chi^2 = 10.6$; df = 2; $P = 0.004$), and local ($\chi^2 = 14.3$; df = 2; $P = 0.0008$) infectious processes. We found the following causes of antithrombin III reduced activity in acute burn disease: elastase inhibition of polymorphonuclear leukocytes on day 1–2 after burn ($r = -0.49$; $P = 0.02$), the use of protein synthesis function of the liver due to blood coagulation activation on day 1–3 ($r = 0.44–0.60$; $P < 0.05$) and the decrease of this function on day 3–12 ($r = 0.34–0.85$; $P < 0.05$).
Retrospective chat analysis of severe burned with verified sepsis and pneumonia showed the changes of hemostasiological indices corresponding to acute DIC syndrome to appear 1–8 days earlier than sepsis and pneumonia manifestations.
Conclusion: 'Early' infectious complications of burn disease develop against the background of profound disorders of hemostasis system corresponding to acute DIC syndrome. Disregulation of hemostasis system is a favourable condition for sepsis and pneumonia, and can be the predictor of their development.

PB 2.45-2

Haemostatic markers predict survival in African patients with burn injuries

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Background: Burn injuries remain a major problem in the developing countries of Africa. They rank among the leading cause of trauma deaths seen in Nigeria due to inadequate health care facilities. Hemorrhage and thrombosis constitute two of the most serious complications of thermal injuries. Significant haemostatic abnormalities can be detected in majority of patients with thermal injuries and these abnormalities underlie the characteristic thrombotic and hemorrhagic diathesis seen in these patients.
Aims: We therefore hypothesized that some molecular markers of coagulation and haemostatic activation may be of clinical predictive relevance in burn patients.
Methods: We studied a total of thirty five patients (mean age 33.7 ± 15. 1; 22 males, 13 females) admitted to the Burns intensive care unit within 24 h of injury and followed up during the period of the first week of burns along with twenty healthy control individuals. Blood was withdrawn on days 1, 2, 3, 5 and 7 or until discharge if < 7 days, and analyzed for hemoglobin (Hb), erythrocyte sedimentation rate (ESR), prothrombin time (PT), activated partial thromboplastin time (APTT), plasma fibrinogen (PFC), and D-dimer (DD) using standard techniques. Clinical variables evaluated include percentage total body surface area burns, percentage full thickness burns, presence or absence of inhalational injury. Results were statistically analyzed using student's t-test and Pearson correlation coefficient. Probability values less or equal to 0.05 were significant.
Results: Showed that there is no significant difference in PT, APTT, PFC and DD in patients with < 5% total body surface area burns compared to controls ($P > 0.05$). However PT, APTT and DD were significantly increased in patients with Burns > 5%, compared to controls ($P < 0.05$), and therefore suggestive of significant hyper coagulation in early phase of burns. There were significant negative correlations between PFC and the percentage of the total body surface area burnt and the percentage of the full thickness burns size. ($r = -0.63$; $r = -0.59$ respectively $P < 0.05$). Severe burns > 45% was associated with a significantly higher frequency of consumptive coagulopathy compared to those with lesser percentage of burns ($P < 0.05$).
Conclusion: PFC may be useful as a marker for predicting survival in burns patients and therefore represent a suitable tool for risk stratification.

PB 2.45-3

Traditional and whole blood coagulative profiles in patients with left ventricle assist devices

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Background: Left ventricle assist devices (LVAD) are increasingly adopted when treating refractory heart failure patients with the indication of either bridge-to-transplant or destination therapy, which is an alternative to cardiac transplantation. According to the flow they produce, LVADs are divided in pulsatile- and continuous-flow devices; the latter – nowadays the most frequently used – are further divided into axial and centrifugal pumps. Antithrombotic prophylaxis is an invaluable mainstay of postoperative LVAD patients' management because of the intrinsic device thrombogenicity and the consequent thromboembolic risk of those patients.
Aims: To compare the impact of two different continuous-flow devices on the traditional and whole blood coagulative profiles: Jarvik 2000, axial flow pump vs. HeartWare HVAD, centrifugal device based on magnetic levitation.
Methods: Data of traditional and whole blood coagulative profiles, and namely thromboelasometric (performed by ROTEM) and aggregometric (performed by Multyplate) evaluations, were reviewed and a comparison between 13 patients with Jarvik 2000 and eight patients supported with HeartWare HVAD was performed.
Results: Platelets count were significantly higher in the HeartWare group than in the Jarvik group (D7 $P = 0.01$; D14 $P < 0.0001$; D21 $P = 0.0002$, D28 $P = 0.003$, D60 $P = 0.002$). Maximum clot firmness (MCF) was significantly higher in the HeartWare group than in the Jarvik group (INTEM: D5 $P = 0.007$, D13 $P = 0.0104$, D21 $P = 0.0004$, D28 $P < 0.0001$; EXTEM: D5 $P = 0.04$, D13 $P = 0.02$, D21 $P < 0.0001$, D28 $P = 0.0001$; FIBTEM: D5 $P = 0.03$, D13 $P = 0.02$, D21 $P = 0.09$). The Area Under the Curve (AUC) was higher in the HeartWare group than in the Jarvik group (TRAPtest: D5 $P = 0.005$, D21 0.04, D28 $P = 0.01$, D13 $P = 0.05$; COLtest D5 $P = 0.004$, D21 $P = 0.01$, D28 $P = 0.02$).
Comparison of the two groups showed significantly higher levels of platelets count, Intem-Extem-Fibtem MCF, TRAP e COL tests in the HeartWare than in the Jarvik group.
Conclusions: Comparison between Jarvik 2000 and HeartWare HVAD proved that the two pumps have almost different effects on coagulative system. Because of the direct implication of ventricular assist devices in the development of acquired von Willebrand syndrome we speculate on the possible different role of each of the two pumps in order to modulate von Willebrand factor plasma levels causing different impairment of the coagulative profile. Larger studies are needed to confirm our findings and to evaluate the possible causative role of von Willebrand factor in the imbalance of coagulation cascade.

PB 2.45-4

Comparison between defects of hemostasis and ROTEM parameters in children with dengue infection

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Background: Dengue virus causes a febrile illness, dengue fever (DF), and less frequently a life-threatening illness, dengue hemorrhagic fever (DHF). Bleeding in dengue patients is caused by vasculopathy, thrombocytopenia, platelet dysfunction and coagulopathy.
Aims: To evaluate whether ROTEM, a new global hemostsis analyser, correlate with standard coagulation tests to detect severity of bleeding diathesis in these patients.
Methods: A prospective observational study. Blood was obtained from 70 children: 41children with dengue infection on day 4–6 of the illness (22 with DF,19 with DHF) and 30 age-matched healthy children as controls. Standard coagulation tests and ROTEM parameters were analyzed and compared.
Results: DHF patients had significant higher in PT, aPTT than DF patients but there is no significant difference in fibrinogen level between two groups. DHF patients had a significant longer time in clotting time (CT), clot formation time (CFT) in EXTEM and INTEM than DF patients. In addition, DHF patients had significant lesser maximum clot firmness (MCF) and lower amplitude (A10,A20) than DF patients. We observed a good correlation between platelet count and both INTEM-MCF ($r = 0.82$, $P < 0.001$) and EXTEM-MCF ($r = 0.83$, $P < 0.001$), a moderate correlation between PT and EXTEM-MCF ($r = -0.51$, $P < 0.001$), aPTT and INTEM-MCF ($r = -0.58$, $P < 0.001$) and a weak correlation between fibrinogen and FIBTEM-MCF ($r = 0.34$, $P < 0.001$).
Conclusions: The abnormalities of hemastasis in dengue infection were significant different between disease severity (DF and DHF) as determined by both standard coagulation tests and ROTEM analysis. We also found a strong correlation between platelet count and both INTEM-MCF and EXTEM-MCF.

PB 2.45-5

The ratios of pro to anticoagulant factors: index of hemostatic imbalance in cirrhotic patients

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Background: Patients with liver cirrhosis are characterized by decreased levels of most pro- and anti-coagulant factors. This state result in an unstable balance. Therefore, patients are prone to hemorrhagic and thromboembolic events particularly in advanced stages.
Aim: The aim of this study was to evaluate the ratios of procoagulant to inhibitor coagulation factors in cirrhotic patients according to disease severity.
Methods: A case control study including 51 cirrhotic patients and 51 healthy subjects matched by age and sex was conducted. Patients were stratified according to Child Pugh classification. Procoagulant factor activity (factor VII, II, V, VIII, XII) and inhibitor factor activity: Protein C (STACLOT PC), protein S (STACLOT PS, antithrombin (STACHROM AT) were determined. All tests were performed on STA COMPACT STAGO. Mean value of procoagulant to inhibitor coagulation factor ratios were compared to those in controls and investigated in patients according to Child Pugh classification. Statistical analysis was performed with SPSS software version 19.0.
Results: Mean age was 56.8 years old [range16–86]. Sex ratio was 0.9. Patients were classified in Child Pugh A in 13 patients (25.5%), B in 23 patients (45.1%), C in 15 patients (29.4%). Among ratios, II/PC, V/PC, VII/PC, XII/PC were significantly higher in cirrhotic patients than in controls (respectively, $P = 0.001$, $P = 0.002$, $P = 0.001$, $P = 0.001$) but not between Child Pugh classes. Likewise, VIII/PC, VIII/PS and VIII/AT were significantly higher in cirrhotic patients than in controls ($P < 0.001$) and increased significantly from class A to C ($P < 0.001$), reaching a value of 5. On the other hand, II/PS is lower in cirrhotic patients than in controls showing marginal significance ($P = 0.04$). However, II/PS,V/PS, VII/PS decreased significantly from class A to C.
Conclusion: The ratios of pro- to anti-coagulant factors can be considered as indexes of the coagulation imbalance. This hypothesis was supported by some authors hypothesizing that hypercoagulability was due to high factor VIII combined with low PC. Moreover it should be evaluated in larger studies in cirrhotic patients particularly with thromboembolism events.

PB 2.45-6

Shortened blood coagulation times in two distinct visceral obesity models, WBN/Kob-Lepr(fa) rats and diet-induced obese mice

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Background: Visceral obesity is associated with a high incidence of ischemic heart disease, and many cases are complicated by impaired glucose tolerance, hyperlipidemia, and high blood pressure. The blood coagulation system plays a major role in thrombus formation. However, little is known about the relationship between visceral obesity and blood coagulation.
Aims: The aim of this study was to investigate blood coagulation times in genetically obese rats and diet-induced obese (DIO) mice in order to clarify the relationship between visceral obesity and blood coagulation.
Methods: In this study, we used two distinct obese animal models, genetically obese rats and diet-induced obese mice. In both animal models after body weight had plateaued, blood coagulation times were compared with those of respective control animals. In blood coagulation assays, activated partial prothrombin time (aPTT), prothrombin time (PT), and thrombin time (TT) were measured in citrated plasma. The homeostasis model assessment of insulin resistance (HOMA-IR) was used as an indicator of insulin resistance.
Results: The WBN/Kob-Lepr*fa* (*fa*/*fa*) rat is a new congenic rat created by introducing the Lepr*fa* gene (a mutant gene derived from the Zucker fatty rat) into the WBN/Kob rat. The *fa*/*fa* rats at 10 weeks of age exhibited a significantly ($P < 0.01$) shorter aPTT and PT than age-matched Wistar rats. C57BL/6J mice fed a high-fat diet (60%) for 10 weeks, a DIO model, exhibited significantly ($P < 0.01$) shorter aPTT, PT, and thrombin time than lean mice fed a standard diet. Significantly higher body weight, visceral fat weight, and insulin resistance were also shared by *fa*/*fa* rats and DIO mice.
Summary/Conclusions: These results suggest that visceral obesity is related to accelerated blood coagulation in addition to disrupted metabolism of glucose and lipids. This study showed the existence of accelerated blood coagulation in two distinct visceral obese animal models. To the best of our knowledge, this is the first report on shortened blood coagulation times in *fa*/*fa* rats and DIO mice. In addition to visceral obesity, both models exhibited hyperlipidemia and increased insulin resistance. The *fa*/*fa* rat and DIO mouse can be considered useful obesity models for research on the biomarkers of cardiovascular disease.

PB2.46 – Anticoagulant Agents – VIII

PB 2.46-1

Inhibition of coagulation by the low molecular mass metalloprotease from Indian cobra (Naja naja) venom

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Background: Snake venom metalloproteases (SVMPs) are an interesting class of enzymes known to play key role in snake venom induced pharmacological effects especially, vascular damage and platelet functions. In contrast to P-III class SVMPs which are known for vascular damage causing massive hemorrhage, P-I and P-II class SVMPs are less destructive. SVMPs with fibri(noge)nolytic and platelet aggregation inhibitory properties may serve as therapeutic lead molecules in the management of cardiovascular diseases. In this study, a non-toxic P-I class SVMP which is basic in property that inhibit plasma coagulation and collagen induced platelet aggregation has been isolated and studied from Indian cobra (*Naja naja*) venom.
Aims: To understand the molecular mechanisms of anticoagulant, and antiplatelet properties of the isolated P-I class of SVMP.
Methods: CM-Sephadex C-25 column chromatography, SDS-PAGE, MALDI-TOF mass spectrometry, caseinolytic, plasma re-calcification time, APTT, PT, TCT, fibrinogenolytic, fibrinolytic, fibronectinolytic and collagenolytic activities have been assayed. Preparation of platelet rich and poor plasma, and washed platelets from human and agonists induced platelet aggregation studies have been performed.
Results: In this study a relatively simple and an efficient protocol has been adopted to isolate a low molecular mass metalloprotease which is basic in property from cobra venom. A tandem two step chromatography on a CM-Sephadex C-25 column results in a pure metalloprotease sample that revealed single sharp peak with the molecular mass of 13.87 kD in MALDI-TOF mass spectrometry. Both EDTA and 1,10-phenanthrolene abolished casein, fibrinogen, fibrin, and fibronectin degrading properties and as well as collagen induced aggregation of human washed platelets by the enzyme. The enzyme is devoid of lethality, edema, hemorrhage, dermo- and myonecrosis. Collagens type-I and IV are resistant to proteolytic digestion. But, the enzyme strongly inhibited coagulation of citrated human plasma with a prolonged APTT, PT and TCT responses. It is an α-fibrinogenase with a selective specificity on the Aα-chain of fibrinogen, in addition it hydrolyzed the α-polymer and α-chains of fibrin clot. Of the several agonists

tested, the enzyme specifically inhibited the collagen induced aggregation of human washed platelets with an IC_{50} of 147 ± 10 nM.
Summary/Conclusion: A non-toxic low molecular mass metalloprotease that inhibits plasma coagulation and collagen induced platelet aggregation has been isolated and characterized. Further, studies on the possible mechanisms of inhibition are in progress.

PB 2.46-2

Long-term oral anticoagulant management associated with routine medical care (RMC) in patients with non-valvular atrial fibrillation (NVAF) in Canada

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Background: There are no prospective, long-term studies describing the process and quality of care (including time in therapeutic range), resource utilization (RU), and quality of life (QoL) associated with being on warfarin for non-valvular atrial fibrillation (NVAF) patients managed through routine medical care (RMC) in Canada.
Methods: Resource utilization associated with Oral Anticoagulant Management (ROAM) is a prospective cohort study conducted across nine Canadian provinces whose objective is to describe the process and quality of care, RU, clinical events and utility scores associated with long-term oral anticoagulant (warfarin) therapy in patients with NVAF. Eligible, consenting patients are followed for 48 weeks and complete a weekly diary providing data on international normalized ratio (INR) test dates and values, RU associated with warfarin monitoring, and all physician visits, procedures and hospitalizations. INR values, test dates and source documentation related to patient-reported clinical events are also collected from the participating physicians. Utility scores, representing the patient's health state, measured using the EuroQol-5D (EQ-5D) standardized QoL instrument are collected every 4 weeks.
Results: Five hundred and fifty-one patients were recruited from April 2008 to July 2012, 92% from RMC (primary care physicians and specialists) and 8% from anticoagulant clinics. Median age [range] was 74 [66, 80] years with 218 females (39.5%). Four hundred eighty-two patients who completed the study and had physician provided INR results are included in this preliminary analysis. Comorbid conditions included hypertension (67%), diabetes (26%) and ischemic heart disease (30%). Physicians reported a total of 6705 INR tests over the study duration (mean ± SD: 15.3 ± 8.3 tests per patient) while patients recorded 7260 tests being completed in their diaries over the same time period (16.8 ± 9.5 tests per patient). Of the patient reported tests 5781 INR results (80%) were communicated to patients, most often by telephone (55%) or in person (21%) within a mean of 1.4 ± 2 days. Average time in therapeutic range (TTR) using the Rosendaal method was 70% using physician reported values and 69% based on patient reported values. Mean INR testing frequency was 28.4 days following an INR in the therapeutic range, 22.9 days following an INR < 2, and 19.3 days, following an INR > 3. The mean baseline [95% CI] utility score was 0.85 [0.84, 0.87]. Mean [95% CI] change in utility scores per 6 months was −0.007 [−0.013, −0.001].
Conclusions: This is the first prospective cohort study of NVAF patients on long-term warfarin therapy being monitored primarily through RMC in Canada. The majority of patients are monitored by telephone with 80% receiving INR results and discrepancies between patient and physician recorded INR test dates. TTR is higher than previously reported for RMC, which may suggest a self-selection bias for better quality physician-patient combinations. Although frequency of testing was appropriate for in-range INRs, the times to testing after out-of-range results appear delayed. Analysis of utility scores showed generally high scores for this community dwelling population, stable over the duration of the study.

PB 2.46-3

Edoxaban, an oral direct factor Xa inhibitor, inhibits tissue-factor induced human platelet aggregation and clot-bound factor Xa *in vitro*

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Background: Tissue factor-induced platelet aggregation and factor Xa (FXa) bound to clot contribute to the formation and growth of thrombus. Edoxaban is an oral, direct, once-daily and selective FXa inhibitor in late-stage clinical development. Fondaparinux is a parenteral indirect (antithrombin-dependent) FXa inhibitor.
Aims: To determine the effects of edoxaban on human platelet aggregation induced by tissue factor and clot-bound FXa *in vitro* and to compare the effects of edoxaban, a direct FXa inhibitor, with an indirect FXa inhibitor (fondaparinux).
Methods: Citrated human platelet-rich plasma (PRP) from healthy subjects was treated with 3 mM H-Gly-Pro-Arg-Pro-OH AcOH to prevent fibrin polymerization, and spiked with edoxaban or fondaparinux. Human tissue factor (Innovin or RecombiPlasTin) plus 7.5 mM $CaCl_2$ were added to PRP and platelet aggregation was measured with an optical aggregometry. The area under the curve of 5 min-aggregation response was measured. Whole blood clot was formed by incubating human blood with 25 mM $CaCl_2$ for 60 min at 37 °C and washed with ice-cold buffer for four times. The clot was incubated with 0.9 μM prothrombin in the absence or presence of FXa inhibitors for 10 min at 37 °C. For fondaparinux, 2 μM antithrombin was added. The amount of prothrombin fragment F1 + 2 (F1 + 2) was measured with an ELISA. Activity of free FXa was measured using human FXa and its chromogenic substrate S-2222.
Results: Both types of tissue factor (Innovin and RecombiPlasTin) activated human platelets and evoked aggregation. Edoxaban inhibited tissue factor-induced platelet aggregation in a concentration-dependent manner. The IC_{50} values for Innovin and RecombiPlasTin-induced aggregation were 150 and 110 nM, respectively. At 300 nM, edoxaban completely inhibited the aggregation response. Fondaparinux inhibited RecombiPlasTin-induced aggregation with IC_{50} of 9.3 μM, but did not show complete inhibition up to 30 μM. Fondaparinux had no effect on Innovin-induced platelet aggregation. Incubation of clot with prothrombin generated F1 + 2, indicating the activity of FXa in clot. Edoxaban concentration dependently inhibited both free and clot-bound FXa. The IC_{50} values were 2.3 and 8.2 nM, respectively. Fondaparinux inhibited free FXa with IC_{50} of 5.4 nM, but concentrations 40-times higher were required to inhibit clot-bound FXa (IC_{50} = 217 nM).
Conclusions: In this study, edoxaban, a direct FXa inhibitor, was determined to be a more potent inhibitor of tissue factor-induced platelet aggregation and clot-bound FXa than fondaparinux, an indirect FXa inhibitor. We previously reported that edoxaban is superior to fondaparinux in preventing arterial thrombosis (1). The present findings may explain the difference in the effects on arterial thrombosis between these FXa inhibitors and suggest the benefits of direct inhibition of FXa.
Reference:
1. Fukuda T. et al. Comparison of antithrombotic efficacy between edoxaban, a direct factor Xa inhibitor, and fondaparinux, an indirect factor Xa inhibitor under low and high shear rates. Thromb Haemost. 2011;106:1062–8.

PB 2.46-4

Antithrombotic effect of direct oral anticoagulants as compared to warfarin evaluated experimentally in treated patients

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Background: Patients undergoing total knee replacement develop deep vein thrombosis if not given thromboprophylaxis; 0.1–1.7% will suffer fatal pulmonary embolism (PE). Oral anti-vitamin K anticoagulants (such as warfarin) are effective for preventing venous thrombosis, but require laboratory monitoring to maintain a therapeutic range as defined with the International Normalized Ratio (INR). New oral anticoagulants directly inhibiting coagulation factor (F) Xa have proven similarly effective and are currently administered in a fixed 'one fits all' dose without laboratory monitoring.
Aims: Our goal was to compare the antithrombotic effect of a FXa inhibitor (rivaroxaban) and warfarin, each administered according to guidelines, measuring platelet aggregation and fibrin deposition in blood perfused over fibrillar collagen type I at two different shear rates.
Methods: Blood containing 0.011 M trisodium citrate was recalcified with 5 mM calcium chloride and perfused over a surface coated with fibrillar collagen type I at the wall shear rate of 300 or 1500/s. Platelets and fibrin were detected in situ through distinct fluorochromes. We tested 10 normal controls, 12 patients treated with warfarin (INR between 1.94 and 2.90) and 10 patients treated with rivaroxaban between 8 and 16 days from the initiation of therapy. The volume of platelet aggregates and fibrin deposited onto collagen was measured from stacks of confocal optical sections separated by 1.5 μm in height. Results are reported here as mean ± standard error of the mean; statistical analysis was performed by one-way analysis of variance.
Results: There was no significant difference in the volume of platelet aggregates formed in blood of normal controls and patients treated with either warfarin or rivaroxaban perfused over collagen at 300 or 1500/s. In contrast, the volume of fibrin formed around and between platelet aggregates was significantly reduced in the warfarin-treated patients both in blood perfused at 300/s (controls: 286749 ± 53104 μm^3; patients: 62105 ± 44282 μm^3; $P < 0.001$) and 1500/s (controls: 231,999 ± 142,998 μm^3; patients: 6724 ± 4131 μm^3; $P < 0.001$). The results in rivaroxaban treated patients were different because the volume of deposited fibrin (215,472 ± 115,956 μm^3) was not significantly different from control in blood perfused at 300/s but was significantly decreased (54,626 ± 66,988 μm^3; $P < 0.001$) in blood perfused at 1500/s. Of note, in warfarin treated patients all individual fibrin volumes were below the lower limit of the normal range; of the rivaroxaban treated patients, 7/10 had normal fibrin volume in blood perfused at the lower shear rate, and 2/10 remained normal even at the higher shear rate.
Summary/Conclusions: Treatment with the direct FXa inhibitor, rivaroxaban, at the dose prescribed according to current guidelines results in a lesser *in vitro* anticoagulant effect than caused by warfarin administration controlled by individual INR testing. The significance of these findings using ex vivo blood perfusion relative to the results of present and future clinical trials remains to be established.

PB 2.46-5

The relationship between anti-Xa activity and complications in orthopaedic patients receiving prophylactic fondaparinux

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Introduction: Preventing the development of deep vein thrombosis (DVT) is clinically important, because pulmonary embolisms (PEs) caused by DVTs are often fatal. Orthopaedic surgery is associated with a very high rate of postoperative venous thromboembolism (VTE). The thromboprophylaxis by fondaparinux is useful in patients after orthopaedic surgery. This study evaluated anti-Xa activity and hemostatic markers in patients who underwent major orthopaedic surgery, and were treated for the prophylaxis of deep vein thrombosis (DVT) with fondaparinux to examine the relationship between anti-Xa activity and DVT or decrease of haemoglobin (Hb).
Methods: The relationship between anti-Xa activity and complications was examined in 176 patients with major orthopaedic surgery treated with 1.5 mg of fondaparinux once a day for 14 days. One hundred forty-two patients were treated with Fondaparinux for 14 days (complete administration; CA group) but 34 patients discontinued prophylaxis due to low Hb level by physician in charge (withdrawal group). All patients also were intermittent pneumatic compression stockings from the day of surgery until postoperative day 2, and they also wore graduated compression stockings continuously. Anti-Xa activity, fibrin and fibrinogen degradation products (FDP), D-dimer, soluble fibrin (SF) and AT activity were measured in 176 patients after THA or TKA before, and on day 1, day 4, day 8, and day 15 of the administration of fondaparinux. The decrease of Hb was calculated from the value obtained on day 1 to the lowest value obtained during 14 days.
Results: The frequency of deep vein thrombosis DVT and Hb decrease were significantly higher in the withdrawal group. The frequency of DVT tended to be higher in the group treated with < 0.2 mg/L of anti-Xa activity and the frequency of > 2 g/dL of Hb decrease was significantly higher in the group with > 0.4 mg/L of anti-Xa activity on day 1. The weight, body mass index and body surface index were the highest in the group with 0–0.2 mg/L of anti-Xa activity, and the Hb decreasing was the highest in the group with > 0.4 mg/L of anti-Xa activity.
Conclusion: The continuous administration of fondaparinux is recommended after major orthopaedic surgery, and anti-Xa activity should be controlled < 0.4 mg/L on day 1.

PB 2.46-6

Association of warfarin therapy after prosthetic heart valve replacement with risk of thromboembolic complications, bleeding and mortality

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Background: Bioprosthetic valves are more commonly used because many of these patients are elderly and/or have comorbid conditions that render them at increased risk of bleeding with the prolonged anticoagulation therapy that would be required with implantation of a mechanical valve.
Especially in patients with hemodialysis, long-term anticoagulant therapy thought to be controversial for the patients with atrial fibrillation (AF) alone. If these patients have bioprosthetic valve, this may be more complex and difficult problem because with the limited evidence to date based on limited data, the trade-off between reduced thromboembolic events with warfarin and increased bleeding remains uncertain.

Aims: To clarify the association of warfarin treatment with the risk of thromboembolic events (TEs), bleeding events (BEs), and cardiovascular deaths after heart valve replacement surgery in patients with hemodialysis.
Methods: Between January 1, 2004, and May 31, 2011, total number of patients having heart valve replacement in this institute was 501 patients. Of these patients, hemodialysis patients were identified 33 patients. Target PT-INR was set 1.5–2.5 according to Japanese guidelines for high risk of bleeding.
Primary events studied included strokes, thromboembolic events, cardiovascular deaths, bleeding events and all cause of death. Valve types, valve position, ejection fraction, atrial fibrillation, other comorbid disease and risk factors for thromboembolism and bleeding were also evaluated.
Results: Two people were excluded because of perioperative death. Patients were followed up for a mean of 2.2 years. A total of 14 patients were continuing on warfarin (group A) treatment after surgery. TTR of this group was 80.9% throughout study periods.
Seventeen patients discontinued warfarin treatment (group B) at the first 3 month after surgery. Baseline characteristics were not different from two groups. There were nine TEs, nine BEs and seven deaths (cardiovascular death 3)
There was no thromboembolic event in group A. On the other hand, group B have nine TEs (Ischemic stroke 4, Myocardial Infarction 4). However, there was no significant increase of BEs (Group A 4cases, Group B five cases, $P = 0.959$) between both groups. All cause of death and cardiovascular death were also no significant difference between two groups
Conclusion: In this well-controlled and low anticoagulant intensity, valve replacement patients with hemodialysis have similar risk for BEs despite warfarin therapy, but have significantly increased risk of TEs without warfarin therapy. Close and careful anticoagulant control is useful for those hemodialysis patients with heart valve replacement.

PB2.47 – Anticoagulant Agents – IX

PB 2.47-1

Biochemical and pharmacological differentiation of dabigatran, apixaban and rivaroxaban

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Introduction: New oral anticoagulants represent synthetic low molecular weight (< 500 daltons) peptidomimetic inhibitors of thrombin and factor-Xa. To date, limited data to compare the active form of dabigatran with apixaban and rivaroxaban in biochemical and pharmacological studies is available. The purpose of this study is to compare the three drugs in various biochemical and pharmacological studies to establish differences, which may predict their safety and efficacy profile in different indications.
Materials and Methods: The active form of dabigatran was purchased from Selleckcham (Houston, TX). Apixaban and rivaroxaban were obtained commercially and were of synthetic origin. The whole blood anticoagulant activities of these agents were measured using celite ACT by supplementing in native human whole blood (n = 10–12) at a concentration of 1 μg/mL. The TEG profile was also determined at concentrations < 250 ng/mL. The anticoagulant studies were carried out at a range 0–2.5 μg/mL utilizing various aPTT, PT, Heptest and PICT test. Thromboplastin induced thrombin generation was measured using a fluorimetric method (Technoclone Austria) in a concentration range of 0-1 μg/mL in PPP and PCCs. Proteomic analysis of thromboplastin activated PCCs and its inhibition by the three agents was also studied, followed by western blotting analysis to characterize cleavage products. Platelet aggregation studies were carried out in human PRP (n = 15–20) using ADP, epinephrine, collagen, arachidonic acid and 2.5U thrombin reagent. The bleeding profile of each agent was measured in a modified rat-tail bleeding time.
Results: In the ACT studies at 1 μg/mL, dabigatran produced significantly higher anticoagulant effects (306 ± 16) vs. saline (124 ± 6), in contrast to apixaban (142 ± 5) and rivaroxaban (149 ± 6.8). In the TEG analysis, dabigatran produced marked alterations of such TEG parameters as r-time, k-time, ma and angle in contrast to both rivaroxaban and apixaban, which exhibited weaker effects. In the PT, aPTT, and Heptest, dabigatran produced relatively stronger anticoagulant effects in comparison to rivaroxaban and apixaban. In the PICT test, dabigatran produced relatively stronger anticoagulant effects, whereas, rivaroxaban exhibited somewhat weaker effects and apixaban showed the weakest effects. In PPP, dabigatran produced stronger thrombin generation inhibition (IC-50 = 0.19–0.23 μg/mL) in comparison to apixaban (IC-50 = 0.22–0.39 μg/mL) and rivaroxaban (IC-50 = 0.43–0.90 μg/mL); however, in the PCCs (Octaplex and Beriplex), dabigatran (IC-50 = 0.41–1.12 μg/mL), apixaban (IC-50 = 0.05–0.06 μg/mL) and Rivaroxaban (IC-50 = 0.05–0.06 μg/mL). In the proteomic analysis in PCCs, while both apixaban and rivaroxaban inhibited the generation of thrombin as evident by the absence of 36 kDa peak, dabigatran did not inhibit the generation of the 36 kDa peak. All agents did not have any effect on agonist induced platelet aggregation, except dabigatran, which inhibited thrombin induced platelet aggregation. In the rat-tail bleeding studies, at 100 μg/mL, dabigatran produced the strongest effects (32 ± 6 min) followed by rivaroxaban (21 ± 4 min) followed by apixaban (7 ± 2 min).
Conclusions: These studies demonstrate that the oral anti-Xa and IIa inhibitors not only inhibit their target enzymes but produce their anticoagulant, anti-protease and thrombin generation inhibition effects by multiple mechanisms. The inhibitory profile of each agent exhibited a different spectrum and is matrix dependent. Dabigatran appears to produce stronger anticoagulant effects in comparison to rivaroxaban and apixaban. Differences in thrombin generation inhibitory profiles among these agents were also noted.

PB 2.47-2

Bone density in patients with VTE treated with VKA. Follow-up of 25 patients over 10 years

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Background: There are still areas of uncertainty as regards the correlation between reduction in bone density and long-term anticoagulant prophylaxis with vitamin K antagonists (VKA).
Aims: To estimate the reduction in bone mineral density (BMD) in venous thromboembolism (VTE) patients anticoagulated long-term with VKA and receiving or not osteoporosis prophylaxis (OP).
Methods: In the period from 1998 to 2011 25 VTE patients were permanently treated with VKA. (INR target = 2.5). Among them nine patients received additionally OP (OP included Alfacalcidol 0.25 μg and Calcium Carbonate 1500 mg daily). During follow-up in every patient there was assessed three times BMD – at the beginning of the study and after 5 and 10 years of observation. Two different bones (femoral neck and lumbar spine 2–4) were studied.
Results: In 15 patients without OP after 5 and 10 years the mean reduction in femoral neck BMD was 0.087 g/cm^2 (min. −0.008 g/cm^2, max. 0.212 g/cm^2, median 0.093, SD 0.066) and 0.217 g/cm^2 (min. 0.048 g/cm^2, max. 0.437 g/cm^2, median 0.207, SD 0.106) respectively. The reduction in lumbar spine BMD after 5 and 10 years was 0.060 g/cm^2 (min. −0.205 g/cm^2, max. 0.391 g/cm^2, median 0.047, SD 0.141) and 0.205 g/cm^2 (min. 0.003 g/cm^2, max. 0.539 g/cm^2, median 0.190, SD 0.125) respectively. In nine patients with OP after 5 and 10 years the mean reduction in femoral neck BMD was 0.060 g/cm^2 (min. −0.091 g/cm^2, max. 0.232 g/cm^2, median 0.503, SD 0.102) and 0.122 g/cm^2 (min. −0.133 g/cm^2, max. 0.402 g/cm^2, median 0.148, SD 0.167) respectively. The reduction in lumbar spine BMD after 5 and

10 years was 0.056 g/cm^2 (min. −0.190 g/cm^2, max. 0.381 g/cm^2, median 0.075, SD 0.154) and 0.092 g/cm^2 (min. −0.181 g/cm^2, max. 0.239 g/cm^2, median 0.144, SD 0.136) respectively.
Conclusions: In VTE patients treated with VKA without OP the reduction in BMD was noted earlier and was more profound (especially between 5 and 10 years of observation) in comparison with those with OP.

PB 2.47-3
Effect of Dabigatran and Rivaroxiban on thrombomodulin mediated activation of protein C and thrombin activated fibrinolysis inhibitor (TAFI)

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Introduction: The new oral anticoagulant agents Dabigatran and Rivaroxiban target thrombin and factor Xa to mediate their anticoagulant effects perspectively. This study was designed to investigate the effects of active form Dabigatran and Rivaroxiban on the activation process of protein C and TAFI by thrombin-thrombomodulin complex.
Materials and Methods: The active form of Dabigatran was synthesized. While Rivaroxiban was extracted from commercially available tablets. Both agents were dissolved in appropriate solution matrices at a stock concentration of 100 μg/mL. Thrombin-thrombomodulin mediated activation of protein C and TAFI were measured using specific chromogenic substrate based methods at a concentration of 0–10 μg/mL in different matrices. The activation of Protein C and TAFI was laso measured using mass spectrometric and immunoblotting methods.
Results: Dabigatran produced a strong inhibition of the generation of both the activated protein C and TAFI (IC_{50} < 1.0 μg/mL) whereas Rivaroxiban did not produce any inhibition of the activation of either of these proteases. Dabigatran also inhibited the amidolytic actions of thrombin-thrombomodulin complex whereas Rivaroxiban did not produce any effect. Neither Dabigatran nor Rivaroxiban produced a direct inhibition of activated protein C or TAFI at concentrations of up to 10 μg/mL. Mass spectrometric and immunoblotting methods showed that Dabigatran blocked the activation of both TAFI and Activation C by thrombin-thrombomodulin complex.
Conclusions: The persistant inhibition of thrombin and its regulatory effects by Dabigatran may differentiate its pharmacologic profile from Rivaroxiban. Since thrombin plays several regulatory functions, its persistent inhibition may compromise hemostatic regulation. The observed adverse events such as the reported myocardial infarction signal may be related to the indiscriminate inhibition of thrombin.

PB 2.47-4
Are global hemostasis assays superior to prothrombin time (INR) for the assessment of hemostatic correction after warfarin withdrawal before invasive procedures?

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Background: In patients on chronic warfarin therapy who require invasive procedures (e.g. endoscopy, biopsy, major surgery etc.), warfarin is temporarily withdrawn. For monitoring of hemostatic correction after warfarin withdrawal prothrombin time (INR) is currently the test of choice, although there is only a weak association between INR and bleeding after these procedures.
Aim: To establish whether global hemostasis assays are superior to INR for establishing the risk of bleeding after invasive procedures.
Methods: Forty-two patients (68 ± 12 years, 12 women) were included after informed consent was obtained and followed for 30 days after the invasive procedure. The study was approved by the Slovene Ethics Committee. INR, thrombin generation (Technothrombin®), overall hemostasis potential (OHP), overall coagulation potential (OCP) and overall fibrinolytic potential (OFP) were determined in venous blood obtained during warfarin treatment and after warfarin withdrawal on the day of invasive procedure.
Results: During warfarin treatment median INR value of 2.5 (2.1–2.8) decreased to 1.3 (1.2–1.4, both values inter-quartile range) on the day of the procedure. Comparing values during warfarin treatment with values after warfarin withdrawal enhanced thrombin generation was observed (lag time: 19.2 vs. 14.0 min, $P < 0.001$; peak thrombin: 73.0 vs. 182.0 nM, $P < 0.001$; time to peak: 23.7 vs. 18.5 min, $P < 0.001$; endogenous thrombin potential: 955.5 nM × min vs. 2163.4, nM x min, $P < 0.001$, respectively) and global hemostasis was increased (OHP: 17.4 Abs-sum vs. 21.9 Abs-sum, $P = 0.002$; OCP: 23.2 Abs-sum vs. 26.2 Abs-sum, $P = 0.002$; OFP 22.7% vs. 17.9%, $P < 0.001$, respectively, all values medians).
During warfarin treatment INR values negatively correlated with endogenous thrombin potential ($R = -0.38$, $P < 0.5$), but no association was observed between INR and OHP, OCP or OFP. On the other hand significant associations were observed between thrombin generation variables (lag time: $R = 0.55$, $P < 0.001$; peak thrombin: $R = 0.44$, $P < 0.01$; time to peak: $R = 0.58$, $P < 0.001$; endogenous thrombin potential: $R = 0.35$, $P < 0.05$), OHP ($R = 0.27$, $P = 0.09$), OCP ($R = 0.62$, $P < 0.001$), and OFP ($R = 0.47$, $P < 0.01$) determined during warfarin treatment with those determined after warfarin withdrawal.
Eight out of 42 patients (19%) suffered one major (after prostatectomy) and seven minor bleedings (after colon dilatation, colonoscopy, revision after prostatectomy, prostate biopsy, coronary bypass surgery) in the follow-up. Patients who bled did not differ significantly from patients with no bleeding in any of the measured variables.
Conclusions: Global hemostasis assays were not superior to prothrombin time (INR) for the assessment of hemostatic correction after warfarin withdrawal, since with these assays we were not able to differentiate between patients who bled after invasive procedure from those who did not. The most important predictors of haemostatic profile in an individual patient after warfarin withdrawal were her/his parameters during warfarin treatment.
The study was supported by the Slovenian Research Agency, Grant no. J3-2128.

PB 2.47-5
Off-label use of recombinant factor VIIa: results from a 10-year university hospital study

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Background: Recombinant factor VIIa (rFVIIa; NovoSeven, Novo Nordisk, Denmark) was first approved for use by the Food and Drug Administration roughly 15 years ago to treat patients with congenital or acquired hemophilia and inhibitors. Despite concerns regarding the effectiveness and safety of its off-license indications, rFVIIa has increasingly been used to prevent and treat excessive bleeding due to trauma and/or surgery in clinical settings.
Aims: The primary objectives of this study were to examine off-label rFVIIa use in non-hemophilic patients by ordering service, dose, and patient survival. Survival was determined by the patient's discharge status either to their home, or transfer to an outside facility including a nursing home, long-term care facility or hospice.
Methods: We performed a retrospective evaluation among patients who received rFVIIa and were admitted to Memorial Hermann Hospital – Texas Medical Center (MHH-TMC) between August 2002 and December 2012. The Medication Use Evaluation (MUE) was utilized to track rFVIIa use, and all data for this study were obtained from clinical pharmacy records. Administration of rFVIIa was based on a stringent protocol and required approval by the attending hematolo-

gist as well as clinical pharmacist. Recombinant FVIIa was only utilized as an adjunct therapy to standard of care procedures and conventional methods of hemostasis were used prior to rFVIIa consideration, which included the use of blood products, surgical therapy, topical hemostatic agents, and/or interventional radiology. Patients were eligible to receive the drug based on the following criteria: (i) the patient had received at least 10 units of packed red blood cells and fresh frozen plasma, (ii) the patient had a fibrinogen level > 200 mg/dL, and (iii) the patient had a platelet count > 50,000/mm^3.

Results: During the 10-year study period, a total of 889 patients received off-label rFVIIa at MHH-TMC. Between 2002 and 2007, there was greater than a five-fold increase in the number of patients receiving rFVIIa for off-label indications, after which utilization was consistently steady. Cardiovascular surgery (n = 212) was the most frequent context for use of rFVIIa followed by trauma (n = 169), pediatric services (n = 117) and neurosurgery (n = 117), and neurology (n = 102). Doses ranged from 20 to 90 μg/kg of body weight (each patient received an average of 4000 μg per dose) with an average of 1.2 doses given per patient, which is well below the allowable two doses allotted by the protocol. Roughly 56% of patients receiving off-label rFVIIa were discharged from the hospital during the reporting period. In addition, the ratio of off-label to label use of rFVIIa at MHH-TMC was 87% and 13%, compared with the national ratio of 97% and 3%.

Conclusions: Recombinant FVIIa continues to be used for off-license indications in hospital settings with varying patient outcomes. Further research regarding the efficacy and safety associated with the drug's off-label use is necessary.

PB 2.47-6

Measurement of dabigatran and rivaroxaban in primary prevention of venous thromboembolism in 106 patients, who have undergone major orthopedic surgery. An observational study

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Background: No routine coagulation laboratory test is recommended during rivaroxaban or dabigatran treatment. However measuring drug concentration and/or anticoagulant activity can be helpful in some special clinical settings, such as bleeding, thrombosis recurrence, reversal monitoring or emergency surgery.

Aims: The effects of dabigatran etexilate and rivaroxaban on various coagulation assays have been previously studied in normal plasma spiked with increasing concentrations of the drug. In contrast, few data are available in routinely treated patients. In order to perform and to interpretate the results of these tests, it is necessary to determine the usual responses of patient's plasma.

Methods: We have used several coagulation tests in a prospective study including 106 patients receiving thromboprophylactic treatment with dabigatran 150 or 220 mg od and rivaroxaban 10 mg od for major orthopaedic surgery. The most common tests – prothrombin time (PT) and activated partial thromboplastin time (aPTT) – give results, which vary according to the reagent used. To overcome this limitation, we advocate the use of plasma calibrators, which suppresses the inter-laboratory heterogeneity of results. Anti-Xa measurement and Hemo-Clot, a thrombin diluted clotting assay, are specific assays which have been proposed for rivaroxaban and dabigatran respectively. These tests, conventional PT, aPTT and thrombin generation (TG) have been performed.

Results: We demonstrated that measurements of both drugs can determine reliably the drug concentration in patients' plasmas. PT is more prolonged with rivaroxaban than with dabigatran. Interestingly, the pattern of TG was clearly different in relation to the difference in the mechanism of action of the two new anticoagulants.

Conclusion: A significant inter-individual variability of response is demonstrated. The mean Cmax of rivaroxaban is 140 ng/mL (extremes 0–412). This result is in good correlation with the previous French similar study (Freyburger G. et al. 2011) showing the feasibility of this measurement. Rivaroxaban induces a greater increase of PT than dabigatran. aPTT is sensitive to dabigatran. Rivaroxaban concentrations were in good agreement with two other studies while unexplained lower concentrations than expected were found in dabigatran patients receiving 220 mg once a day [mean Cmax 60 ng/mL (extremes 0–320)]. An interference by pantoprazole, which was given to each patient and which reduces dabigatran absorption, could explain the observed lower than expected results. A complementary study is in progress to confirm this hypothesis.

PB2.48 – Anticoagulant Agents – X

PB 2.48-1

Ambulatory treatment of venous thrombosis in patients older than 85 years

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Background: Incidence of venous thrombosis (VT) increases markedly with age. Treatment of VT in very old patients presents unique problems related to aging such as renal insufficiency, low body weight and numerous co-morbid conditions. Age is also an independent risk factor for major bleeding during anticoagulant therapy. Since elderly patients are often excluded from clinical trials, treatment regimens based on results from clinical trials might not be suitable for very old patients with VT. Furthermore, data on outpatient management and outcome in very old patients treated for VT is scarce.

Aim: The aim of our study was to analyse clinical characteristics and outcomes of patients with VT who were older than 85 years and were treated in outpatient ambulatory setting.

Methods: In the retrospective study, data of consecutive patients treated for VT at our centre from January 2010 to December 2012 were analysed. Only patients aged ≥ 85 years were included in further analysis. Clinical characteristics and outcome (bleeding, VT recurrence and death) within 30 days of therapy were identified by manually reviewing the patients' records. Also, the patients and/or their caregivers were contacted by telephone. Categorical data were compared using c^2 test.

Results: Among 1100 patients with objectively confirmed VT of lower legs, 102 (9.3%) patients were older than 85 years. Ninety-five (93.1%) were eligible for ambulatory treatment (mean age 88.7 (85.1–97.9) years, 71.6% women, 96.8% proximal VT, 82.1% first event). 40.0% of ambulatory treated patients were residents of nursing homes and 44.2% had chronic kidney disease or heart failure. On average, the number of concomitant drugs in these patients was 5.3. VT was provoked in 58% of patients, the most common risk factor being poor mobility (32%) followed by cancer (15.8%). The latter was newly diagnosed in 14/15 patients. All cancer patients were treated with low molecular weight heparin (LMWH). Non-cancer patients were initially treated with once daily LWMH followed by warfarin (INR 2–3). Average time to therapeutic INR was 8.8 days. Self-injection of LMWH was performed in 42% of patients. At 30 days, major non-fatal bleeding events were registered in three (3.2%) patients (two gastrointestinal, one haematoma in abdominal wall). There were two (2.1%) recurrences of VT, both in patients on warfarin at INR values in therapeutic range. Two (2.1%) patients died; one from cancer, for the other the cause of death could not be established. Among several variables we examined for possible association with adverse outcomes (self-injection of LMWH vs. injection by professional, kidney disease or heart failure, warfarin vs. LMWH, nursing home vs. home treatment), none proved statistically significant.

Summary: The incidence of major bleeding in our patients exceeds the incidence of VTE recurrence, but neither incidence is higher than those

already reported in a cohort of patients older than 65 years. Therefore, even in very old patients with several concomitant diseases treatment of VT on outpatient basis seems safe and reasonable.

PB 2.48-2

Self-reported adherence with warfarin and new oral anticoagulants in patients on chronic oral anticoagulant therapy

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Background: Non-adherence to prescribed medications is a common clinical problem among patients with chronic conditions. The efficacy and safety of anticoagulation therapy might be significantly decreased in patients with poor adherence. Recently approved new oral anticoagulants (NOAC) have a shorter half-life compared to vitamin-K antagonists (VKA) and therefore there are theoretical concerns that patients with non-adherence on NOACs might be at higher risk of adverse events (recurrent venous thromboembolism or stroke/systemic embolism). Furthermore, poor dose timing (i.e. delay between the time taking during the day) might also be a concern among patients taking NOACs, especially if taken once daily. Self-reported adherence of patients on anticoagulant therapy (warfarin and NOACs) is seldom in the literature.

Aims: To assess the self-reported adherence of patients on oral anticoagulant therapy and compare adherence between patients on VKA and NOAC (rivaroxaban and dabigatran).

Methods: A cross-sectional study, of consecutive patients followed at our oral anticoagulants clinic was performed. All patients completed an anonymous survey (22 questions) including the 4-item Morisky score to assess self-reported adherence. The Morisky score has been previously validated to assess non-adherence and its reliability is high (Chronbach alpha 0.61). Non-adherence was defined as patients with one or more positive answers to one of the four questions from the Morisky score. Timing adherence and a preference towards reminders were also investigated.

Results: A total of 273 patients on anticoagulant therapy that completed the survey. The median age was 72 (range 19–92). Fifty one (18.7%) patients were using NOAC (13.6% rivaroxaban and 5.1% dabigatran). Non-adherence was reported by 40.5% (95% CI: 34.0–47.3%) of patients in the VKA group and 43.1% (95% CI: 29.3–57.8%) of patients in the NOAC group (38.4% and 53.8% for rivaroxaban and dabigatran respectively). Dose timing adherences were 90% for both groups (90.2% for NOAC and 93.2% for VKA group). Finally, 25.8% of the total patients agreed that a regular reminder would be beneficial. The preference for frequency was either weekly or daily and e-mail was the most popular reminder tool.

Summary/Conclusions: Rates of non-adherence to anticoagulants are high among patients requiring long-term anticoagulotherapy. There are no major differences in self-reported rates of adherence between VKA and NOAC. Given the short half-life of NOACs, future trials evaluating the risk of arterial or venous events in patients without adequate adherence are needed.

PB 2.48-3

Non-clinical safety and efficacy of prothrombin complex concentrates (PCC) for the reversal of dabigatran mediated anticoagulation

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Background: Prothrombin complex concentrates (PCC) have been successfully used for the acute treatment and perioperative prophylaxis of bleeding in acquired deficiency of the prothrombin complex coagulation factors in case of vitamin K antagonist overdose. Recently, PCC mediated reversal of bleeding could also been shown following treatment of rabbits with high doses of the direct thrombin inhibitor dabigatran [Pragst et al., 2012].

Aims: It was the aim of the present study to evaluate the potential procoagulant risk associated with the use of PCC for anticoagulation reversal.

Methods: Rabbits were anesthetized and an arterial-venous shunt (AV-shunt) was created by connecting the right jugular vein and left carotid artery via a catheter both jointed by a glass body. An ear artery catheter served to monitor hemodynamic, coagulation and hematological parameters. Following baseline measurements, animals received an intravenous (IV) loading dose (t = 0 min) followed by a continuous IV infusion of dabigatran (BIBR-953), the active metabolite of dabigatran etexilate (Boehringer Ingelheim, Germany) via the ear vein (t = 1–90 min) to maintain stable plasma levels exceeding clinically relevant doses. At 5 min prior to opening of the AV-shunt, PCC (Beriplex P/N, CSL Behring) was administered (t = 15 min). The AV-shunt was temporarily opened, and blood flow, thrombotic occlusion time and thrombus wet weight determined over 30 min (t = 20–50 min). Afterwards, anticoagulation-induced bleeding and reversal was assessed following a standardized kidney injury. Total blood loss and time to hemostasis were monitored over a 30 min observation period (t = 60–90 min). Furthermore, blood samples were collected to determine thrombin inhibition levels (HTI assay), thrombin generation (TGA), prothrombin time (PT), D-Dimers, thrombin-antithrombin (TAT) complexes and F1 + F2 fragments. Finally, kidney, heart, lung and brain tissue were prepared for histopathological evaluation with special focus on possible procoagulant changes.

Results: Dabigatran anticoagulation was observed following loading doses of ≥ 75 μg/kg as indicated by reduced incidence of thrombotic occlusion, increased thrombotic occlusion time and reduced thrombus wet weight after opening of the AV-shunt. At doses ≥ 200 μg/kg, even the addition of PCC at high doses of 300 U/kg could not fully reverse dabigatran induced anticoagulation within the AV-shunt model. At the same dabigatran doses (≥ 200 μg/kg), marked bleeding signals were obtained following kidney incision compared to control, which were reversed dose-dependently by IV infusion of PCC. Treatment with 300 U/kg of PCC resulted in the formation of lung thrombi (grade 1 = minimal). However, the incidence of lung thrombi was reduced dose-dependently with increasing plasma levels of dabigatran measured.

Summary/Conclusion: Overall, the study confirmed the effective reversal of dabigatran induced bleeding by PCC while maintaining dabigatran's expected anticoagulant effects. Furthermore, the potential procoagulant effects of high dose PCC could be markedly reduced or even completely inhibited in the presence of dabigatran.

PB 2.48-4

Rivaroxaban for the treatment of symptomatic deep vein thrombosis and/or pulmonary embolism in Chinese patients: a subgroup analysis of the EINSTEIN DVT and PE studies

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Background: The worldwide EINSTEIN DVT and EINSTEIN PE phase III programme included 8282 patients with acute symptomatic deep vein thrombosis (DVT) and/or pulmonary embolism (PE) and showed that a single-drug approach with fixed-dose rivaroxaban, an oral Factor Xa inhibitor, was as effective as dual-drug combination therapy with enoxaparin, overlapping with and followed by international normalized ratio-guided vitamin K antagonist (VKA) therapy, in reducing the risk of symptomatic recurrent venous thromboembolism (VTE). In a pooled analysis of these studies, rivaroxaban was also associated with a significant reduction in the occurrence of major bleeding ($P = 0.002$). The EINSTEIN programme is the first time that Chinese hospitals have participated in a worldwide programme for the treatment of DVT and PE.

Aim: To assess the efficacy and safety profile of rivaroxaban for the treatment of VTE in a subgroup of patients from China.

Methods: A total of 439 Chinese patients who had acute symptomatic DVT ($n = 211$), or PE with or without DVT ($n = 228$), were randomized to receive rivaroxaban (15 mg twice daily for 3 weeks, followed by 20 mg once daily) or standard therapy with enoxaparin (1 mg/kg twice daily) overlapping with and followed by an adjusted-dose VKA for 3, 6 or 12 months. The primary efficacy outcome was symptomatic recurrent VTE. The principal safety outcome was major or non-major clinically relevant bleeding. Major bleeding was a prespecified secondary outcome.

Results: The primary efficacy outcome occurred in 7 (3.2%) of 220 patients in the rivaroxaban group and in 7 (3.2%) of 219 patients in the enoxaparin/VKA group (hazard ratio, 1.04; 95% confidence interval, 0.36–3.0; $P = 0.94$). The principal safety outcome occurred in 13 (5.9%) of 219 patients in the rivaroxaban group and in 20 (9.2%) of 218 patients in the enoxaparin/VKA group (hazard ratio, 0.63; 95% confidence interval, 0.31–1.26; $P = 0.19$). No major bleeding was seen in patients receiving rivaroxaban, while major bleeding was observed in 5 (2.3%) patients receiving enoxaparin/VKA therapy. In frail patients (defined as age > 75 years and/or creatinine clearance < 50 mL/min and/or body weight ≤ 50 kg), major or non-major clinically relevant bleeding occurred in 4 (8.9%) of 45 patients receiving rivaroxaban compared with 7 (15.2%) of 46 patients receiving enoxaparin/VKA therapy.

Summary/Conclusions: In Chinese patients with acute symptomatic DVT and/or PE, rivaroxaban appeared to be as efficacious as standard enoxaparin/VKA therapy. Consistent with the results in the overall analysis, Chinese patients who received rivaroxaban were observed to have fewer major bleeding events than Chinese patients who received enoxaparin/VKA. The results obtained with rivaroxaban in Chinese patients are consistent with those seen in the larger worldwide programme.

PB 2.48-5

Comparison of use of prothrombin complex concentrates vs. recombinant factor VIIa (rVIIa) in warfarin related intracranial hemorrhage

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Background: Intracranial hemorrhage (ICH) related to warfarin is a devastating occurrence with high rates of morbidity and mortality. It is thought that prompt reversal of the warfarin effect is crucial for improved outcomes. Although there is data that outcomes are improved with the use of prothrombin complex concentrates (PCC), in the United States the four-factor PCC are not available. For years after its introduction rVIIa was used to reverse warfarin and was recommend for this purpose in several guidelines. Recent data has been published showing effectiveness of a 3-factor PCC – low dose rVIIa (PCC-rVIIa) combination in warfarin related ICH.

Aims: This abstract reviews our 4 year sequential experience with first using rVIIa and then PCC-rVIIa for warfarin-ICH.

Methods: Permission was obtained from the IRB to review 67 charts of patients with warfarin -ICH who received either PCC or rVIIa. Patients either had spontaneous ICH or ICH related to a ground level fall (GLF). Patients on warfarin with ICH due to more extensive trauma were not analyzed. The standard warfarin-ICH reversal protocol from 2009 to 2011 was 40 μg/kg of rVIIa. In 2011 this was changed to 4000 units of Profilnine plus 1 mg of rVIIa to provide VII activity. (Sarode R, et al. J Neurosurg. 2012;116(3):491–7.)

Results: Sixty-seven patients were identified during this time period – 23 received rVIIa and 44 received PCC-rVIIa. Age, clinical indications for anticoagulation, and type of ICH were well matched in the two groups.

	PCC-rVIIa	rVIIa
Mean age	72.6 ± 2.0	75.5 ± 2.6
Reason for anticoagulation (%)		
Atrial fibrillation	28 (63)	16 (69)
Cardiac valves	6 (14)	4 (17)
Deep venous thrombosis	8 (18)	3 (13)
Spontaneous	22 (50)	10 (43.5)
GLF	22 (50)	13 (56.5)
Intracerebral hemorrhage	25 (57)	13 (56.5)
Subdural hematoma	15 (34)	10 (43.5)
INR before treatment	3.6 ± 0.4	2.9 ± 0.3
INR after treatment	0.8 ± 0.02	0.93 ± 0.17
Change in INR	2.8 ± 0.4	2.0 ± 0.3
FFP units	0.8 ± 0.2	2.1 ± 0.4*
Died	13 (29)	8 (34)
Discharged to home	18 (40)	3 (13)*

Conclusion: Effectiveness of warfarin reversal as measured by INR was the same in both groups. Despite similar death rates more patients who receive PCC-rVIIa were able to be discharge to home rather than to a care facility. In conclusion while it appears that PCC-VIIa may be associated with better functional outcomes, ICH in warfarin patients has a considerable mortality rate no matter what agent is used for reversal.

PB 2.48-6

Is clot structure impaired in patients on warfarin?

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Background: Global assays for monitoring patients on warfarin have been proposed in cases where conventional INR fail to correlate the clinical phenotype.

Aim: We investigated the anticoagulant effect of warfarin on clot strength by means of whole blood coagulation assays.
Methods: We enrolled in the study 56 patients (27 male/29 female) with a median age of 66 (18–89) years. They were on long term anticoagulation for a median period of 3 (0.5–16) years. Twenty nine were on warfarin for primary prophylaxis (22 atrial fibrilation-7 valve replacement) and 27 for secondary prevention (thrombotic events). All patients were in steady state at the time of sampling with INR of 2.5 (1.5–4.1) and on no other medication affecting haemostasis. Platelet counts were within normal. Changes in clot structure were assessed by HAS (Hemodyne Hemostasis Analysis System) measuring clot elastic modulus (CEM), TEG (Tromboelastography) measuring maximum amplitude (MA) and ROTEM (Thromboelastometry) expressed by maximum clot firmness (MCF). Citrated whole blood containing corn trypsin inhibitor (20 μg/mL) was clotted in the presence of $CaCl_2$ and low tissue factor (0.35 pM) and was evaluated using TEG and ROTEM whereas for HAS we used native recalcified whole blood. Reference ranges were based on measurements of 20 normal individuals under the same experimental conditions and internal quality control was run in parallel. Plasma levels of clotting factors (F) FII, FVII, FX, FIX and Clauss fibrinogen were also measured.
Results: We found significant reduction in CEM on patients receiving warfarin compared to normals (mean 12.5 ± 1.1 vs. 28.3 ± 2.8) Kdynes/cm^2 but no changes for MCF (mean 56.2 ± 2.2 vs. 55.2 ± 0.8) mm and MA (mean 58.5 ± 1.4 vs. 60 ± 0.9) mm respectively. Fibrinogen levels 3.3 (2.2–8.1) g/L correlated strongly with all clot strength parameters: CEM (r 0.7), MA (r 0.6), MCF (r 0.5) ($P < 0.001$). There was no correlation between INR and any of the markers used to assess clot stiffness. MCF and MA were strongly correlated with each other (r 0.8) with no difference in absolute values ($P < 0.001$).
Conclusions: The dose effect of warfarin expressed by INR was not found on any of the clot strength markers. Fibrinogen as expected was the only factor of those examined related to clot stiffness. Nevertheless we noticed a discrepancy among the tests applied in this setting. The big reduction found with HAS was not detectable with neither ROTEM or TEG. One explanation for our findings could be the different principle of function between the machines used leading to different sensitivity towards factors affecting clot strength. The possible impairment of clot elastic properties as reflected in HAS may be through platelet interactions and could offer an explanation for the diversity in clinical phenotype often found in patients anticoagulated with warfarin sharing the same INR.

PB2.49 – Anticoagulant Agents – XI

PB 2.49-1

Quality of anticoagulation and bleeding and thrombotic risk in relation to CHADS2 score: analysis AF cohort of Epica study

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Background: The quality of anticoagulation expressed as time in therapeutic range (TTR) is associated to both bleeding and thrombotic events during vitamin K antagonists treatment. It is unknown if TTR in patients with Atrial Fibrillation (AF) is related to their baseline stroke risk.
Aim: Aim of this study is to evaluate TTR in relation to $CHADS_2$ score in the AF cohort of EPICA Study (1).
Methods: We defined patients with TTR ≥ 60% as adequately anticoagulated. We calculated the distribution of patients with TTR < 60% and the rate of bleeding and thrombotic events in relation to $CHADS_2$ score.
Results: We studied 3015 AF patients (males 45%; median age 83 years, range 80–102; 7620 patient-years). The total quality of anticoagulation measured as time spent within, above and below the TTR was 63%, 14% and 23%, respectively (IQR for TTR = 50–75). During follow-up 132 major bleeding and 112 thrombotic events were recorded. The distribution of patients with TTR < 60% and bleeding and thrombotic events in relation to $CHADS_2$ score is reported in table.
Conclusion: As expected, the rate of major bleedings and of thrombotic events increase with $CHADS_2$ score. Instead, no difference in the quality of anticoagulation is found in relation to $CHADS_2$ score.
Reference:
1. Poli D, Antonucci E, Testa S, Tosetto A, Ageno W, Palareti G; Italian Federation of Anticoagulation Clinics. Bleeding risk in very old patients on vitamin K antagonist treatment: results of a prospective collaborative study on elderly patients followed by Italian Centres for Anticoagulation. Circulation. 2011;124:824–9.

PB 2.49-2

Fixed vs. variable dosing protocols for prothrombin complex concentrate (PCC) for the emergency reversal of warfarin anticoagulation at two university teaching hospitals

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Background: Four factor PCC is recommended for the emergency reversal of warfarin anticoagulation but the optimal dosing strategy remains unclear.
University Hospital Limerick (UHL) employs a fixed dosing protocol of 25 iu/kg of PCC. The Adelaide, Meath and National Childrens Hospital (AMNCH) protocol advises 25 – 50 iu/kg of PCC depending on INR at baseline.
Our study compares the performance of two dosing protocols for the emergency reversal of anticoagulation in a real world clinical setting.
Methods: Consecutive patients treated with PCC for reversal of warfarin anticoagulation at two university teaching hospitals over a 3 year period were identified from hospital transfusion records. Indication for anticoagulation, indication for and dose of PCC, survival and incidence of thrombosis for 30 days following PCC were recorded. INR at 1 and 4 h following PCC infusion were recorded.
The primary outcome was the proportion of patients achieving an INR ≤ 1.5 at 1 and 4 h post infusion of PCC. Secondary outcomes were survival and incidence of thrombosis.
Results: One hundred and fifty-three patients (84 at UHL and 69 at AMNCH) received PCC for the reversal of warfarin anticoagulation from 1st January 2010 to 31st December 2012. The median age, indication for anticoagulation, reason for emergency reversal and baseline INR were similar between the two groups.
All patients at UHL received PCC at 25 iu/kg.
At AMNCH, 44.9% of patients received PCC at 25 iu/kg, 18.1% at 35 iu/kg and 25.4% at 50 iu/kg.
The median INR was similar between the fixed dose and the variable dose group at 1 h (INR 1.5 vs. INR 1.3) and at 4 h (INR 1.5 vs. INR 1.2) post infusion of PCC.
The proportion of patients achieving an INR ≤ 1.5 at 1 h was not significantly different between the fixed dose and the variable dose (53.3% vs. 70%, $P = 0.37$). There was no significant difference in the percentage of patients achieving INR ≤ 1.5 between the two groups at 4 h post infusion (73.3% vs. 81.4%, $P = 0.069$).
In the subgroup of patients with INR ≥ 5.0 at baseline, the median INR between the fixed and variable dosage groups was similar at 1 h (INR 1.8 vs. INR 1.5) and at 4 h (INR 1.6 vs. INR 1.4). At 1 h,

46.2% of patients in the fixed dose group and 50% of patients in the variable dose group achieved an INR ≤ 1.5.
There were 18 deaths within 24 h of PCC infusion. This subgroup had a higher median INR at baseline (median INR 5.0) but responded to PCC, achieving a median INR 1.2 at 4 h post infusion. The most frequent cause of death was intracranial bleeding.
One pulmonary embolism occurred 28 days post PCC infusion.
Conclusion: Prothrombin complex concentrate prescribed with fixed dosing protocol performs as well as a variable dosing protocol for emergency reversal of warfarin anticoagulation in a real world clinical setting.

PB 2.49-3
Chronic anticoagulation: how the population is changing

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Background: In the last 20 years we observed a huge increase of patient on life-saving anticoagulant therapies for both treatment of venous thromboembolism and cardioembolic stroke prevention related to atrial fibrillation and heart valve replacements.
At present inItaly, Anticoagulation Clinics (ACs) manage patients chiefly on antivitamin K antagonists (AVKs). Even before the introduction of the direct oral anticoagulants (DOA), in the last 10 years patient population has already changed because of an increase of chronic treatments with other anticoagulant drugs, such as low molecular weight heparin (LMWH) or pentasaccharides, which require different management systems.
Aims: The aim of the present study was to analyse patient characteristics, clinical and laboratory controls, considering patient management in an AC in 2002 compared to 2012.
Besides, suitability of our net-telemedicine system to support patient management was evaluated.
Methods: We analyzed laboratory and clinical data stored in our centralized net supported program -TAOnet® Roche – based on a telemedicine system activated from 2001, bi-directional connected with peripheral health care units (Nursing Homes, Groups of General Practitioners and other hospitals of the area).
Each patient has an informatic record, updated in real time, including information on: general clinical conditions, complications, diet intake and drug co-medication, general compliance and drug adherence, significant clinical events such as hospitalizations, surgery and invasive procedures, laboratory test like PT INR (venous or capillary) for AVK patients and aXa activity for LMWH and fondaparinux patients, blood cell count, renal function.
Results: The 10 years activity comparison (2002–2012) showed a significant rise of the patient population from 2122 to 4352 pts, with a mean age that increased from 74 years (range 21–94 year) to 78 (range 14–103 year). Atrial fibrillation was the most frequent clinical condition that increased from 43% of the total population in 2002 to 52% in 2012; venous thromboembolism passed from 17% to 15.4%, while cardiac prosthesis from 20.7 to 12%. Within anticoagulant drugs warfarin is the most frequent used (89% of the total patient population in 2012), while acenocumarol showed a significant decrease (−22%) compared with 2002. We observed a more frequent use of LMWH on long term treatments, from 0.4% in 2002 to 3.6% in 2012. Also clinical and laboratory management has changed in relation to type of drug used, as confirmed by frequency of visits, increase of anti-Xa activity and blood cell count testing and creatinine determination. Our telemedicine system gave the opportunity to re-modulate management in relation to different molecules, assuring a good standardization of clinical control for each patient.
Conclusion: This evaluation gave us some important indications:

1 The population of anticoagulated patients has changed in the last 10 years also for type of drug used and management, even before the introduction of the direct oral anticoagulants in our country
2 Patient management has been adapted for each type of anticoagulant
3 Our clinical-lab program, through a telemedicine system, has given the possibility to modulate management in relation to patient-drug characteristics

PB 2.49-4
Pilot-scale production and purification of snake venom-derived antiplatelet agents

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Background: Snake venom toxins have been reportedly used as a rich source of a number of proteins of biotechnological interest due to their wide range of effects on haemostasis. These effects vary greatly: coagulant, anticoagulant, platelet-activating, anti-platelet, fibrinolytic and hemorrhagic, in either enzymatic or non-enzymatic pathways. *Gloydius* venom contains a variety of proteins that possess antiplatelet activities.
Methods: This study presents recent development in our laboratory to produce and purify antiplatelet proteins/peptides derived from *G. blomhoffii brevicaudus* snake venom. Different matrices of HPLC (size exclusion, ion exchange, affinity and reverse phase chromatography) were employed for purifying the enzymes and their biological and biochemical properties were characterized by using thrombin time assay, SDS-PAGE, 2-D electrophoresis, enzyme electrophoresis, platelet aggregation assay, coagulation assay and MALDI-TOF mass spectrometry.
Results: A purified phospholipase A2 (PLA2) was an acidic protein with Mr of 13 kDa, pI 4.17 and pH optimum 8.5. The PLA2 exhibits 90% ADP-induced platelet aggregation inhibition with a specific activity of 240 U/mg. The fibrino(geno)lytic enzyme (Mr 20 kDa, pH optimum 6.0, pI 7.0) inhibits platelet aggregation 85% and delays thrombin time test by more than 6 min. The protein C activator (Mr 30 kDa, pH optimum 8.0, pI 5.0) also shows an antithrombotic effect (60–70%) in rabbits. Moreover, the isolated plasminogen activator (Mr 27 kDa, pI 6.0) did not activate nor degrade phrothrombin and factor X, serine protease.
Conclusion: Bioprocesses to produce and purify active antiplatelet agents from *G. blomhoffii brevicaudus* venom have been developed, using modern liquid chromatography matrices and, as a result, the patents of novel purification processes were obtained. The satisfactory activities comparable to similar commercially available enzymes have been demonstrated. Ongoing work to optimize large-scale production process is being undertaken.

PB 2.49-5
Telemedicine via CareOnline-system in patients with vitamin-K-antagonist-therapy

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Background: The thrombosis service at the University Hospital Mainz (TDM) was established in 2008 along the lines of the model in the Netherlands to optimized the VKA-therapy. Good INR adherence is essential for the safety and efficacy of vitamin K antagonist (VKA) therapy. In order to care patients living far away from the center the CareOnline-system was established.
Aims: The main objective was to evaluate the impact of specific medico-telemedicine care on the effectiveness of VKA-therapy in patients who could not reach the center.

Methods: Twenty-four patients were attended in the therapeutic drug monitoring (TDM) via the CareOnline-system. The patients or members of the family or local nurses received an extensive consultation on the anticoagulation therapy, the CareOnline-system and were educated in INR-self-testing. The INR-values, the health-data e.g. medical changes, acute illness, bleeding complications, thromboembolism, changes in comedication were documented in a standardized schedule. The data were transfered via scanner and telephone-adapter to the computer of the center. The INR-management (dosage recommendation, INR-monitoring) was performed by special educated staff via telefone and in addition via a letter on the same day. The duration of the pilot study was 12 month. The endpoint was time in therapeutic range (TTR – Rosendaal-method) after stable INR-adjustment (three consecutive INR values within the therapeutic range and VKA intake for a minimal period of 4 weeks). The ideal INR-range was 2–3, the extended INR-range was 1.8–3.2. The INR was routinely measured every 7 days.
Results: A total of 1086 INR-value were measured during the the observation time. The median of the TTR in ideal INR-range (2.0–3.0) was 80.6%. In the extended INR-range (1.8–3.2) the TTR was reached by 88.3%. Neither severely bleeding complications nor thromboembolic events were observed.
Conclusion: Patients with VKA-therapy living far away from coagulation-centers could be treated with a specialized medico-telemedicine care-system. The first results of the pilot study are encouraging. Medical care out of towns/centers will become more difficult in the near future (lack of doctors countryside). Therefore new structure are necessary to quarantee treating patients with high qualty.

PB 2.49-6

First experience of structured introduction of new oral anticoagulants in a Swedish health care district: dabigatran as an alternative to warfarin in atrial fibrillation

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Background: The number of patients on oral anticoagulants (OAC) in Sweden is approaching 200,000 and the treatment is still almost exclusively warfarin. Roughly half of these patients are treated with OAC for stroke prevention in atrial fibrillation.
Dabigatran was the first new oral anticoagulant (NOAC) for stroke prevention in non-valvular atrial fibrillation on the Swedish market.
Even though Swedish warfarin treatment is of very high quality with an excellent time in therapeutic range even in routine care, and warfarin still is the drug of choice for many patients, the NOACs have the potential of increasing the number of atrial fibrillation patients on OAC. On the other hand, the cost of dabigatran treatment is expected to be higher than warfarin treatment.
Choosing the patients that will benefit the most from dabigatran treatment (as an alternative to warfarin) will be the best possible use of economical resources. The Anticoagulation clinics (ACCs) provide an organization possible to use for a follow-up program to gain experience of the drug under Swedish 'real life' conditions.
Aims: To optimize medical benefit and resource utilization by a structured introduction of new oral anticoagulants in our catchment area.
Methods: In the Östergötland County Council area (approx. 430,000 inhabitants) a multi professional group developed an introduction and follow-up program for NOAC treatment. Dabigatran was the first NOAC drug to be introduced.
The program consists of: A physician check list with inclusion and exclusion criteria for dabigatran treatment as in the RELY study as well a local additional criterion (warfarin treatment not suitable, e g difficulties in achieving a stable INR)
A referral procedure to the ACC. If deemed eligible for dabigatran treatment by the ACC physician, the patient is started on dabigatran, otherwise warfarin treatment is initiated or continued if possible.
A follow-up program for at least 12 months.
A reimbursement model where the referring unit has no cost for dabigatran treatment when prescribed within the introduction program, but is charged the full cost for prescriptions outside the program.
Results: Until now approximately 100 patients have been referred to the introduction program, and approximately half of these patients were started on dabigatran treatment in the central health care district of Östergötland. Medical contraindications as well as referring physician and patient misconceptions were reasons not to start dabigatran. Almost no patients were started on dabigatran treatment outside the introduction program.
Summary/Conclusion: We propose that a structured introduction, as described here, may assist the referring physician and the patient in choosing the optimal OAC for the individual patient, while at the same time achieving optimal use of economical resources. In Östergötland introduction programs will be designed for upcoming NOACs, next rivaroxaban and apixaban, as well.

PB2.50 – Blood Coagulation System – II

PB 2.50-1

Identification of hypercoagulability in the rat model of microvascular thrombosis using spatial clot growth dynamics

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Background: Thrombotic complications of vascular diseases are the leading cause of mortality worldwide. Conventional diagnostic assays are often poorly sensitive to thrombotic complications and hypercoagulability (i.e. a tendency to the occurrence of thrombosis or abnormally high blood clotting). Evolving techniques that allow imaging of clot growth during thrombus formation and evaluation of sensitivity of such techniques are of great interest.
Aims: The aim of the study was to investigate the ability of the spatial clot growth assay (thrombodynamics) to detect hypercoagulability and thrombotic complications in the rat model of systemic collagen/epinephrine-induced thrombosis.
Methods: Microvascular thrombosis was provoked in Wistar male rats by administration of epinephrine/collagen solution (60 and 0.5 mg/kg, respectively) via the femoral artery. Blood samples for analysis of coagulation were withdrawn at the baseline and 5, 15, 30, 60, 120 min after administration. The performed thrombodynamics assay is based on videomicroscopic observation of fibrin clot propagation in a non-stirred layer of platelet-free plasma activated by the immobilized tissue factor. The following parameters of clot growth were determined on the basis of the image series: lag time (t_{lag}), initial (V_{in}) and stationary (V_{st}) clot growth velocity, presence of activator-independent clotting (characteristic for hyper-coagulation). The aPTT was measured using the standard laboratory assay following the manufacturer's protocol. Biopsy samples (heart, lung, kidney, liver) were retrieved and processed for hystopathological examination using Martius Scarlet Blue fibrin staining to identify microvascular thrombi.
Results: Rats undergoing microvascular thrombosis showed evidence of hypercoagulability indicated by the thrombodynamics assay, that is: presence of spontaneous clots of different intensity at 5 min and later after administration of collagen/epinephrine which indicated the severity of thrombosis and did not allow to detect V_{in} and V_{st}; increase of both V_{in} and V_{st} (if these parameters are detectable after induced

thrombosis) with the maximum at 15 min after administration – V_{in} and V_{st} increased from 49 ± 7 and 25 ± 3 μm/min at the baseline to 74 ± 7 and 34 ± 3 μm/min, respectively. Although severity and duration of thrombotic complications measured with thrombodynamics are various and have individual differences, hypercoagulability is clearly visible by this method as opposed to the aPTT test which does not indicate the hypercoagulable state. aPTT was only shortened once (from 19.7 ± 0.1 to 15.1 ± 0.1 s) in case of severe thrombosis which led to death. A slight hypercoagulability was detected in the control group with the maximum at 30 min (spontaneous clots did not appear here), this can be explained by surgical intervention and trauma while inserting the femoral artery catheter. Microvascular thrombi of different intensity were found in all organs in the experimental group, at the same time single thrombi of minimal intensity were found in the control group.

Summary/Conclusions: Spatial clot growth assay (thrombodynamics) is sensitive to a hypercoagulable state in the rat model of microvascular thrombosis indicating its diagnostic utility for the evaluation of hypercoagulability and thrombotic risk.

PB 2.50-2

Effects of shear flow on the microstructure and elasticity of incipient clots in whole blood and fibrin-thrombin gels

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Background and Aims: The effect of flow on the rate of blood coagulation has been extensively studied, both *in vitro* and *in vivo*, but little is known about the effects of shear flow on the underlying clot microstructure. The aim of the work presented herein was to investigate the influence of an imposed shear stress on the incipient microstructure of clots formed in both whole blood and fibrin gel systems. The incipient clot is defined herein as the clot that exists when the sample undergoes a transition from viscoelastic liquid to viscoelastic solid like behaviour and hence marks the point at which the rudimentary clot forms.

Methods: Incipient clot microstructure has been characterised by studying the response of the forming clot to small amplitude oscillatory shear stress waves, a technique known as small amplitude oscillatory shear (SAOS). In order to investigate the effect of an imposed shear field a steady stress has been superimposed onto the small amplitude oscillatory waveform. Hence it has been possible to probe changes in incipient clot microstructure induced by the presence of the steady shear field. Visualisation of mature fibrin clot microstructure has also been carried out using laser scanning confocal microscopy (LSCM) and SEM.

Results: In both fibrin gels and whole blood, fractal dimension, D_f (which characterises the complexity of the incipient clot microstructure in the range $1 < D_f < 3$) increased significantly, from a range of 1.99 ± 0.01 (fibrin clots) and 1.74 ± 0.12 (whole blood) at conditions of zero superimposed shear, to a maximum of 2.34 ± 0.04 (fibrin clots) and 2.2 ± 0.09 (whole blood) at the maximum level of superimposed steady shear stress studied herein, suggesting that the clots become more dense and compact under shear. These results are in good agreement with both LSCM and SEM images, which show dramatic changes in fibrin clot morphology with increasing steady shear stress. Increasing applied steady stress resulted in compact stretched bundles of fibrin fibers with few empty spaces.

Summary and conclusions: Significant compaction of incipient blood clots and fibrin gels has been observed as a consequence of applied steady shear stress during coagulation/gelation. The clinical significance of this work can be explained in terms of the potential consequences for fibrinolysis; the nature of clot microstructure under *in-vivo* conditions; and to aid development of thrombolytic or 'clot busting' treatments.

PB 2.50-3

The alterations of hemostasis and thromboelastometry in children after hematopoietic stem cell transplantation

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Background: Hematopoietic stem cell transplantation (HSCT) is currently a curative therapy for malignant and non-malignant hematologic disorders. The disturbance of hemostasis, demonstrated by increased coagulation markers and decreased coagulation and anticoagulation proteins, was reported during the early period post-HSCT. Thromboelastometry, a point-of-care measurement, can assess global hemostasis involving the interaction between coagulation and anticoagulation proteins, fibrinolysis and platelets. Therefore, it may be an alternative method for predicting of an abnormal hemostasis and deciding on the appropriate treatment.

Aims: To demonstrate the changes of hemostasis and thromboelastometry in children post-HSCT and to demonstrate hemostasis complications in children post-HSCT.

Methods: A prospective cohort study was conducted on children who had undergone HSCT. Following informed consent, blood was collected for thromboelastrometry, coagulation markers[thrombin antithrombin complex (TAT), D-dimer, prothrombin fragment (F1 + 2) and anticoagulation proteins (protein C, protein S and antithrombin activities) at pre-HSCT, day 0 (day of stem cell infusion), 14, 30, 60, 90 and 180 post-HSCT.

Results: Twenty patients (six autologous and 14 allogeneic HSCT) and 40 controls were enrolled. Mean + SD for the age of patients (9.0 + 4.8) and the controls (8.3 + 4.6) were similar. There were 10 patients with severe Thalassemia, eight with malignancy and two with other diseases.

At pre-HSCT, protein C and AT activities were significantly lower in patients compared to the levels in the controls. Thromboelastrometry parameters included clotting time (CT) in extem, was significantly longer in patients compared to the level in the controls. Maximum clot firmness (MCF) in extem and fibtem, and lysis index at 60 min (intem) were lower in patients compared to the levels in the control group.

During SCT period, platelet counts were significantly lower on during day 0 until day 180 post-HSCT when compared to the level at pre-HSCT. F1 + 2 levels were significantly higher on day 14, 30 and 90 post-HSCT when compared to the levels at pre-HSCT. On day 90, post-HSCT Protein C and AT activities returned to control levels. The thromboelastometry parameters included CT (intem), lysis index at 60 min (intem) significantly increased on day 14, and day 0 and 14, respectively while MCF (extem) significantly decreased on day 0, 14, 30 and 60 post-HSCT.

Nine patients developed 11 bleeding episodes. Fifty-five percent of the total bleeding episodes occurred on day 0 and 14. Veno-occlusive disease (VOD) was found in four patients between days 8 and 22 post-HSCT. Three significant differences, the platelet count at day 60, AT activity at day 30, MCF (extem) at day 14 and 30, were found in VOD group when compared to the levels of the patients without complication. MCF (extem) on day 14 was significant difference in VOD group compared to the level in bleeding group.

Conclusions: To our knowledge, this study was the longest prospective study and the first to demonstrate the changes of thromboelastometry in HSCT. The abnormal hemostasis was demonstrated at pre-HSCT and post-HSCT, and returned to normal values on day 180 post-HSCT. The abnormalities of thromboelastrometry may result from the decreased clotting factors, low platelet counts and hypofibrinolysis. In the future, thromboelastometry may be used to predict VOD and bleeding complications and to guide the treatment of these complications.

PB 2.50-4

Self-assessed Villalta score in DVT patients and in matched venous thrombosis-free controls

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Background: A substantial number of patients with deep vein thrombosis (DVT) subsequently develops post-thrombotic syndrome (PTS). The Villalta score has been recommended by the International Society of Thrombosis and Haemostasis for diagnosing PTS. The score consists of five symptoms and six signs, none of which are disease specific.
Aims: (i) to assess the rate of PTS after DVT using a modified and self-assessed Villalta score; (ii) to estimate Villalta score in a non-DVT population.
Methods: This cross-sectional study included consecutive patients treated for DVT at Oestfold Hospital, Norway, 3–9 years prior to the inclusion. Villalta score was assessed in the ipsi- and contralateral extremities and in controls using adapted scheme for self-assessment of the Villalta score previously developed and used by our group (correlation coefficient = 0.84 when compared to conventional Villalta score). The study subjects were requested to find two sex and age-matched, venous thrombosis free controls, so called buddy controls. PTS was classified into four categories: severe (> 14), moderate (10–14), mild (5–9) and absent (< 5). The study was approved by the Regional Ethics Committee and written informed consent was obtained from all patients.
Results: Median duration of observation following DVT in 114 patients [median age 61 years; males 76 (66%)] was 5.5 years (range 3.5–8.7). Twenty-one patients had greater than one DVT in the ipsilateral extremity, but none had DVT in the contralateral extremity.
Mean (SD) of Villalta scores in ipsilateral extremities was 7.3 (7.2), in contralateral 3.7 (4.7), and in right and left of controls (n = 83) 2.7 (3.7)/2.4 (3.7), respectively. There was a mediocre correlation between Villalta in ipsi- and contralateral extremities (r = 0.5; P = 0.001). In Ipsilateral extremity 48 patients (42%) had no PTS, 40 (45%) mild, 11 (10%) moderate, and 15 (23%) had severe PTS. Corresponding distribution of Villalta scores [n (%)] in contralateral extremity were 83 (73%), 16 (14%), 8 (7%) and 7 (6%), and in controls, right and left extremities, were 63 and 70 (76–85%), 17 and 10 (21–12%), 2 (2%) and 1 (1%), respectively
Summary/Conclusions: The rate of PTS measured by self-assessed Villalta score in the ipsilateral extremity (59%) was comparable to other studies in which Villalta was assessed by qualified personnel. Villata score of > 4 points was found in 27% of the contralateral extremities and in up to 24% of the controls. Villalta score > 9, indicating moderate-severe PTS was mainly found in ipsilateral (23%) extremity; equivalent scores were substantially less in the contralateral (13%) extremities and in controls (3%). Mean score and distribution of Villalta grades were comparable in contralateral and in controls. To our knowledge, this is the first study to determine Villalta score in a control population with no previous DVT. Self-assessment of the Villalta score may represent a limitation of this study.
In conclusion, our results indicate the low specificity of Villalta score particularly at scores < 10. Increasing the threshold of the Villalta scores for diagnosing PTS may thus be required to improve the accuracy of Villalta score.

PB 2.50-5

Oxidized phosphatidylcholine and ethanolamine from 12- and 15-lipoxygenase significantly enhance tissue factor dependent thrombin generation *in vitro*

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Background: On activation, platelets generate a family of six oxidized phospholipids (PL) via 12-lipoxygenase activity. These comprise 12-hydroxyeicosatetraenoic acids (HETE) attached to either phosphatidylethanolamine (PE) or phosphatidylcholine (PC). Macrophage and neutrophils can generate 15- and 5-HETE PLs respectively.
Aim: To characterise the effects of HETE-PE and HETE-PC on coagulation reactions.
Methods: Liposomes (5% unoxidised PS, 55–65% unoxidised-PC, 20–30% unoxidised-PE and 0–10% either HETE-PE or HETE-PC) were generated incorporating recombinant tissue factor (rTF, 10pM). The rTF/liposomes (final PL concentration 4 μmol) were used to trigger coagulation (TG) in normal plasma, or FVIII deficient plasma and thrombin generation (TG) measured by use of a Thrombinoscope. Separately, the liposomes (without rTF) were tested for their ability to support the tenase, prothrombinase and TF/FVIIa complexes in purified protein reactions.
Results: TG in the presence of liposomes made from unoxidised (control) PLs and rTF resulted in a peak thrombin (PT) of 3 nM. When liposomes were composed of 10% HETE-PL, PT rose to 88 nM with 12-HETE-PE, 72 nM with 15-HETE-PE, and 11 nM with 5-HETE-PE added. Of note, oxidised PC also enhanced TG. PT rose to 133 nM with 15-HETE-PC, 87 nM with 12-HETE-PC and 29 nM with 5-HETE-PC added. The HETEs retained their effects in FVIII deficient plasma. PT in FVIII deficient controls was zero, but in 10% HETE-PL, this rose to 21 nM with 12-HETE-PE or 15-HETE-PE, 5 nM with 5-HETE-PE, 38 nM with 15-HETE-PC, 32 nM with 12-HETE-PC, or 16 nM with 5-HETE-PC added. Increased PT correlated with a reduced lag time. Differences between HETE isomers were statistically significant (P < 0.01) except that between 12- and 15-HETE-PE in normal plasma, and values were taken from a representative experiment of two. A concentration dependent relationship was demonstrated for 12- and 15-HETE-PE, with incremental increases from 0% to 10% of the total phospholipid content causing equally incremental effects on PT (n = 2 experiments). The lowest quantities of oxidised lipid that showed a statistically significant effect (P < 0.01) was 1% 12-HETE PE with a PT of 17 nM, and 3% 15-HETE-PE which had a PT of 18 nM. Concentration dependency is being investigated for the HETE-PCs.
15-HETE-PE did not affect the tenase, prothrombinase, TF/FVIIa, or soluble sTF/FVIIa reactions (n = 5–8 experiments).
Conclusion: HETE-PEs and HETE-PCs significantly elevate thrombin generation even in factor VIII deficient plasma. The mechanism(s) involved are under investigation.

PB 2.50-6

A case with reduced coagulation factor VII level caused by novel compound heterozygous mutations

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Background/Aims: Coagulation factor VII is a plasma serine protease precursor with a critical role in initiation of the blood coagulation process. VII deficiency is a rare coagulation disease. We investigated the molecular mechanisms of a FVII deficiency in a patient with compound heterozygous mutations.

Clinical history: A 50-year-old Japanese male was referred to our hospital because of resection of thyroid tumor. He had no bleeding history. However, by preoperative screening test, prothrombin time (PT) was prolonged 31sec). The activated partial thromboplastin time (APTT) was in normal range. The VII activity and antigen were greatly reduced (activity, 5.0%; antigen, 6.5%). Other hemostatic data were normal. VII deficiency was diagnosed. The parents were not consanguineous, although family examination could not be done.
Methods: Genomic DNA was extracted from peripheral blood leukocytes by a standard method. All exons, exon-intron boundaries and the 5' promoter regions were amplified by PCR. Cyclic sequencing was performed by using an Applied Biosystems DNA sequencing analyzer 310.
The electrophoretic mobility shift assay (EMSA) was analyzed by incubation of PCR products with wild-typed and mutated promotor sequences and nuclear extracts of HepG2 cells.
Results: Sequence analysis revealed that the patient was identified to be a compound heterozygote. One allele showed a point mutation (L26->P) in the central hydrophobic core of the signal peptide, which was previously reported inJapan. Another allele had a novel point mutation of a −58G to C substitution before the translation start site in the hepatocyte nuclear factor 4 (HNF-4) binding site. A moderate reduction in binding affinity of a specific nuclear protein to the −58C-containing oligonucleotide was found by EMSA assays.
Discussion: Several promoter mutations cause VII deficiency due to disruption of specific binding of nuclear protein to promoter elements. It is known that −61T->G and −55C->T show severe bleeding phenotype, in the other hand, asymptomatic or mild bleeding in −60T->C and −59 T->G. In this study, a mutation with −58 G->C was found to retain the mild binding ability with HNF-4. Kavlie et al. reported that the results from EMSA competition comparison are also in agreement with the severity of the clinical symptoms found in the affected patients. A L26->P mutation have been reported inJapanas a VII Morioka that caused an impaired secretion through translocation to and retention in ER with extensive intracellular degradation.
Summary/Conclusion: We reported an asymptomatic compound heterozygous VII deficiency that was identified to be a L26->P and a novel mutation of −58 G->C.

PB2.51 – Blood Coagulation Tests – VII

PB 2.51-1

Investigation of methodological sources of bias in the measurement of vitamin K1 (phylloquinone) in human serum at endogenous concentrations

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Background: Vitamin K is an essential micronutrient involved in the posttranslational modification of various Gla proteins including coagulation factors II, VII, IX and X, proteins C and S, osteocalcin and matrix Gla protein. Serum or plasma vitamin K_1 (phylloquinone) concentrations correlate with dietary intake and functional markers of vitamin K status (e.g. undercarboxylated factor II (PIVKA II)) and provide a useful guide to tissue stores. The Vitamin K External Quality Assurance Scheme (KEQAS) is dedicated to the harmonisation of vitamin K measurement, enabling more reliable cross-comparison of studies of vitamin K status. Previous data has shown that assay bias contributes to about 30% variance seen in comparison of inter-laboratory vitamin K_1 analysis.
Aims: The aim of this study was to quantify the assay bias for each KEQAS participant and to attempt to link the data to specific methodological variations.
Methods: Sources of bias were investigated by distributing a questionnaire to all KEQAS participants (n = 25, response rate = 100%) specifically relating to key methodological information. Bias was calculated for vitamin K_1 results returned between February 2009 and October 2012 generated from serum (n = 22) and ethanolic (n = 10) samples. During this period the majority of KEQAS participants employed HPLC with in-line chemical reduction and fluorescence detection, however two laboratories employed HPLC with mass spectrometry (HPLC-MS).
Results: Mean individual laboratory bias ranged from −28 to 30%. Laboratory bias ranking altered very little when results from ethanolic samples were removed indicating bias did not originate from chromatographic interferences in the serum. The two HPLC- methods returned the two most negatively biased values of −22 and −28% respectively. A major finding was that seven groups (28%) using a commercially available calibrator, returned results with positive bias (range = 13–23%, mean = 19%).
Conclusion: The KEQAS Steering Committee is currently in talks with the manufacturer of the calibrator in order to investigate the reasons for the disparity between KEQAS participants using this commercial calibrator and participants using calibrators prepared in-house. Currently it is not possible to determine the significance of the negative bias seen in results from the two HPLC-MS methods. This may reflect their status as an improved method, free from interferences or may be a result of other confounding methodological factors. Measurement of vitamin K_1 by HPLC-MS represents a potential reference method although further evidence is required to establish this.

PB 2.51-2

The effect of dabigatran on the PTT, thrombin time and clauss fibrinogen assays

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Background: Dabigatran is the only oral Direct Thrombin Inhibitor (DTI) in clinical use. While it does not require routine coagulation monitoring, testing may be necessary in patients with complications such as bleeding. The sensitivity of the PT and PTT assays is limited, and the Thrombin Time (TT) has been suggested as a simple and widely available alternative. However, the TT may be also be affected by changes in fibrinogen level. Further, the most common method of measuring the fibrinogen level, the Clauss assay, is essentially a calibrated dilute TT and may therefore show interference by Dabigatran.
Aims: To compare the sensitivity of the PTT and TT to Dabigatran in patients with acute bleeding or thrombosis. To assess the potential for using TT combined with the Clauss fibrinogen assay as a method of Dabigatran evaluation.
Methods: Data and blood samples were collected between September 1, 2011 and November 1, 2012 on patients taking Dabigatran and presenting to the Emergency Department with an acute complication. PT, PTT, TT, and the Clauss fibrinogen test were performed and compared to the results of the Hemoclot Direct Thrombin Inhibitors (Hyphen Biomed/Aniara) assay calibrated for Dabigatran level measurement. In addition, plasma samples with various fibrinogen levels were spiked *in vitro* with Dabigatran ranging from low trough (10 ng/mL) to the highest supratherapeutic concentrations reported *in vivo* in overdosed patients (5000 ng/mL). Interference in the Clauss fibrinogen assay was assessed using the following commercial reagents: STA-Fibrinogen 5 (Diagnostica Stago), HemosIL Fibrinogen-C (Instrumentation Laboratory Company), HemosIL QFA Thrombin (Instrumentation Laboratory Company) and Dade Thrombin (Siemens Healthcare Diagnostics Products GmbH).
Results: Seventeen patients were captured, 14 of which presented with bleeding, one of which had an ischemic stroke and two in which the presenting complaint was not identified. These patients demonstrated a significant negative bias in PTT results for Dabigatran level compared to normal plasma. TT remained highly sensitive (at least 10 ng/

mL) to the presence of Dabigatran under all clinical circumstances investigated. There was wide variation in the sensitivity of commercial fibrinogen assays to Dabigatran. One assay (HemosIL Fibrinogen-C) showed interference at supratherapeutic as well as therapeutic levels resulting in falsely low fibrinogen levels. Interference could however be mitigated by re-testing at a higher plasma dilution factor. Conclusions The utility of the PTT is compromised in patients with acute complications while on Dabigatran therapy and is not recommended for measurement of drug clearance. The TT assay is a simple and reliable alternative, particularly when used in combination with the fibrinogen level. However, there is wide variation in commercial fibrinogen assay sensitivity to Dabigatran and we would recommend against the use of assays that demonstrate interference in the therapeutic range.

PB 2.51-3

Prediction of unfavourable outcome with rotational thromboelastometry in patients with acute liver injury/failure

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Background: Patients with liver disease often show profound haemostatic abnormalities which may be better characterised by global coagulation assays such as Rotational Thromboelastometry (ROTEM).

Aim: We used ROTEM, which measures several parameters of clotting whole blood, to define the haemostatic profile in patients with acute liver injury/failure (ALI/F), defined as acute liver dysfunction with coagulopathy ± hepatic encephalopathy (HE), in comparison with healthy controls.

Methods: We recruited 35 patients with ALI/F and 40 matched controls between July 2011 and September 2012. Standard coagulation assays (PT, APTT) and detailed haemostatic profiling including platelet count, fibrinogen, D-dimer, coagulation factors (FII, FV, FVII, FVIII) in addition to ROTEM were performed. Parameters assessed by ROTEM were Clotting time (CT, seconds), Clot firmness time (CFT, seconds) Alpha-angle (degrees) and Maximum clot firmness (MCF, mm) using EXTEM, INTEM and FIBTEM to initiate clot formation.

Results: Mean patient age was 34 years, with 63% male. Aetiology was acetaminophen hepatotoxicity in 60%. Fifty-four percent of patients had ALF, 40% with HE grade ≥ 3; median bilirubin, AST and creatinine were 88 μM, 3131 IU/L and 159 μM respectively. Five (14%) patients had unfavourable outcome; 4 (11%) underwent liver transplantation and 1 (3%) died, while 30 (86%) had transplant-free survival (TFS). Five patients had minor bleeding.

All patients had abnormal coagulation profiles with median INR 3.22 (IQR 2.46–6.93), APTR 1.56 (1.28–1.95), platelet count 110 × 10^9/L (66–167), D-dimer 8000 ng/mL (5950–8000) and decreased coagulation factors compared to controls ($P < 0.001$ for all comparisons), except for FVIII which was increased 247 u/dL (214–356) compared to 123 u/dL (97–139; $P < 0.001$).

For both Extem and Intem Median CT, CFT were significantly longer in patients than controls while median MCF and α-angle were significantly lower ($P < 0.001$; for all comparisons). Patients also had longer Fibtem CT with lower MCF than controls ($P < 0.001$).

CT correlated directly to lactate, AST, INR and APTR and inversely to Fibrinogen, FII, FV and FVII while CFT correlated inversely to platelet count, fibrinogen and FVIII. In contrast MCF correlated directly to platelet count, fibrinogen and FVIII as did α-angle to platelet count and FVIII.

Patients with unfavourable outcome had a significantly more hypocoagulable ROTEM profile with a significantly longer Extem_CT 110 (89–243), Intem_CT 244 (212–352), Fibtem CT 94 (71–571), Extem_CFT 301 (160–598) and Intem_CFT 385 (149–717) and lower Extem_MCF 38 (33–54), Intem_MCF 38 (28–53), Extem_α-angle 47 (33–67) and Intem_α-angle 45 (31–67) when compared to those with TFS Extem_CT 68 (557–81; $P = 0.003$), Intem_CT 207 (177–224; $P = 0.025$), Fibtem CT 60 (53–81; $P = 0.022$), Extem_CFT 146 (126–184; $P = 0.034$) and Intem_CFT 121 (105–159; $P = 0.034$) Extem_MCF 51 (47–54; $P = 0.04$), Intem_MCF 51 (47–55; $P = 0.038$), Extem_ α-angle 63 (59–68; $P = 0.034$) and Intem_α-angle 70 (65–72; $P = 0.033$). In identification of 'non-survivors' ROC analysis reveals Extem-CT (0.92) to be a better predictor of poor outcome than other clotting parameters though numbers are small. There was no significant difference in standard coagulation assays.

Conclusion: All patients with ALI/F showed hypocoagulable ROTEM profiles without major bleeding. Patients with unfavourable outcome had abnormal ROTEM profiles, with Extem-CT potentially of use in identifying patients at risk for unfavourable outcome.

PB 2.51-4

Impact of dabigatran on routine and specific coagulation assays in patients treated by dabigatran.

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Background: Dabigatran, as a new oral anti coagulant (NOAC), is a direct thrombin inhibitor which is used in patients with non-valvular atrial fibrillation, to reduce the risk of stroke. With a half-life-time of 14–17 h and a renal clearance of 80%, no need of follow up of dabigatran plasma concentrations is necessary in normal situations. In case of bleeding, overdose and before (urgent) surgery, it is recommended to investigate plasma levels of dabigatran and activated partial thromboplastin time (aPTT). For other purpose coagulation tests are required and the clinician and the laboratory specialist must be aware of the impact of the NOAC's on the coagulation assays to avoid misinterpreting test results.

Aim: The aim of our study was to investigate the effect of dabigatran on the thrombin enzyme or -substrate depended routine and specific coagulation assays in patients treated by dabigatran. Until now, most of the studies are *in vitro* studies with dabigatran spiked plasma.

Methods: Nineteen patients with non-valvular atrial fibrillation treated by dabigatran (> 5 years) were included. The daily oral dose of dabigatran was twice 110 or 150 mg. We measured in citrated platelet poor plasma the following coagulation assays/reagens: international normalized ratio (INR)/STA Hepato Quick®, prothrombin time (PT)/STA Neoplastin plus®, activated partial thromboplastin time (aPTT)/STA APTT kaolin®, fibrinogen/STA fibrinogen®, antithrombin III (ATIII)/STA antithrombin III®, lupus anticoagulant (LAC)/STA CLOT DRVV® screen and confirm and PTT-LA®, and the extrinsic and intrinsic pathways factors II-V-VII-X-VIII-XI-XI-XII/STA factors® on a STA-R Evolution® coagulation analyser (Diagnostica Stago, France). Although we measured activated protein C resistance (APC)/Pefakit APC-R factor V Leiden® (Pentapharm, Basel, Swiss) and dabigatran concentration with Hemoclot thrombin inhibitors® (Hyphen Biomed, Neuville-sur-Oise, France) on the STA-R Evolution®.

Our patients have no coagulation disorders before entering the study. Ethical approval was obtained for conducting the study at the Jeroen Bosch Hospital.

Results: In 18 patients with dabigatran levels ranged from 16 to 240 ng/mL, we found the following results. aPTT rises to abnormal values (range 30.6–67.3 s) as dabigatran concentration increases. As expected, factors of the intrinsic pathway (VIII-IX-XI-XII) were strongly underestimated as dabigatran concentration increases. PT was less affected. Extrinsic factors II-V-VII-X were less underestimated. LAC diagnostic tests were strongly prolonged. APC-R was extremely influenced and was not measurable in our patients. All affected assays seemed to respond in a dose-dependent manner. INR, fibrinogen (Clauss method) and ATIII (based on inhibition of thrombin) were not affected.

One patient showed a dabigatran level of 639 ng/mL. In this patient all assays were strongly affected, except INR and fibrinogen.
Summary/Conclusion: Our results are in accordance with previous results reported in studies with dabigatran spiked plasma. We showed the effect of dabigatran on routine and specific coagulation assays. Most of the assays are influenced, sometimes strongly, which makes the daily interpretation of routine and specific coagulation results a challenge.

PB 2.51-5

The prothrombin time is a poor indicator of plasma rivaroxaban levels in *ex-vivo* samples from patients taking rivaroxaban

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Background: Plasma rivaroxaban levels can be measured by prothrombin time (PT)-based or anti-Xa-based assays calibrated with sets of plasma samples spiked with rivaroxaban. Studies have shown linear dose-responses of PT to rivaroxaban levels when normal pooled plasma is spiked with rivaroxaban over a wide range of concentrations, and that the slope of the dose-response line varies with the type of thromboplastin reagent used. There is limited data on the effect of rivaroxaban on the PT of plasma in patients taking rivaroxaban.
Aims: To assess the degree of variability between PT and plasma rivaroxaban concentration in samples from patients receiving rivaroxaban.
Methods: Double-spun citrated plasma samples (n = 46) from outpatients and in-patients (n = 33) taking prophylactic doses of rivaroxaban were stored deep-frozen until tested. The plasma rivaroxaban levels were determined by anti-Xa based commercial chromogenic assay (Diagnostica Stago). PTs were determined with three recombinant thromboplastins (Innovin, Recombiplastin, Neoplastine R), one rabbit brain thromboplastin (Neoplastine CI Plus) and one human placenta thromboplastin (Thromborel S). All PTs were run on two analysers employing different clot detection principles: optical (Behring coagulation system) and mechanical (STA-R Evolution).
Results: Rivaroxaban levels ranged from 8 to 508 ng/mL, with a median of 119 ng/mL. For all 10 thromboplastin-analyser combinations there was a dose-dependent increase in PT with increasing rivaroxaban level ($P < 0.0001$ for all). The increase in PT with rivaroxaban levels was more consistent with use of recombinant thromboplastins compared to tissue-derived reagents. Values for r^2 for the five thromboplastins were, for BCS and STA-R analysers, respectively: Neoplastine R 0.839 and 0.856; Recombiplastin 0.776 and 0.738; Innovin 0.638 and 0.628; Neoplastine CI Plus 0.491 and 0.572; Thromborel S 0.409 and 0.508. These r^2 values were only marginally different, and not always improved, when PT values were converted to INR. Inspection of individual scatterplots shows a wide range of rivaroxaban concentrations between patients with the same PT. These differences in rivaroxaban levels were 2-fold to 4-fold in many instances. For the reagent with the highest r^2 values, Neoplastine R, a PT of > 30 s on the STA-R and > 35 s on the BCS was indicative of rivaroxaban levels > 400 ng/mL.
Summary/Conclusions: There is wide variation between different thromboplastins in their sensitivity to plasma rivaroxaban concentration as measured by the PT. In none of the 10 reagent-analyser combinations were these relationships close enough to allow the PT to reliably predict the plasma rivaroxaban level. Therefore assessment of plasma rivaroxaban requires a specific assay.

PB2.51 – Blood Coagulation Tests – VII

PB 2.51-6

Impact of sample preparation procedure in the spatial clot growth assay

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Thrombodynamics is a new global coagulation assay based on spatial separation of coagulation activation and propagation phases. Coagulation is activated by immobilized tissue factor and propagates in thin layer of non-stirred plasma. Clot growth is monitored by light-scattering videomicroscopy system and the rate of clot growth is measured.
To be used as a diagnostic tool Thrombodynamics assay requires standardization and validation. In this study we examined the key stages of the sample preparation procedure to minimize variability due to pre-analytical conditions.
Blood was collected into sodium citrate tubes at a 9:1 volume ratio. It was processed by centrifugation at 1600 g during 15 min to obtain platelet-poor plasma (PPP). To obtain platelet free plasma (PFP), the supernatant was centrifuged 5 min at 10,000 g. To avoid contact activation 200 μg/mL of Corn Trypsin Inhibitor was used.
Eight types of citrate blood collection tubes of four manufactures were tested: Sarstedt Monovette, BD Vacutainer (glass and plastic tubes), Greiner Bio-One Vacuette (3.2%, 3.8% of sodium citrate and CTAD), Terumo Venosafe (3.2% and 3.8% of sodium citrate). All the tubes were tested for ≥ 10 apparently healthy volunteers. Rate of clot growth in PFP was not significantly different (P = 0.05) for seven types of the tubes (all of them were plastic). Values obtained with glass Vacutainer tubes were significantly higher (P = 0.05). Mean values of clot growth rate for all the plastic tubes ranged from 25.5 ± 1.9 to 27.6 ± 2.1 μm/min. The mean value for Vacutainer glass tubes was 30.8 ± 3.8 μm/min. There was no significant correlation for clot growth rate for different types of the tubes showing random error introduced by the tubes choice.
APTT values were not significantly different for all the tubes tested (P = 0.05).
For 44 healthy individuals we compared the results with plasma obtained by single blood centrifugation for 15 min at 1600 g (PPP) and plasma that was additionally centrifuged 5 min at 10,000 g (PFP). The significant increase in clot growth rate (P = 0.05) was observed in PPP (28.5 ± 4.2 μm/min) compared to PFP (24.3 ± 2.9 μm/min). There was a significant correlation for the clot growth rate (r = 0.77, P = 0.05) that shows a systematic shift of values obtained with a single centrifugation.
Different centrifugation protocols were studied: the second centrifugation was performed at 10,000 g for 5 min and at 1600 g for 20 min. No significant difference was observed for these methods (n = 7, P = 0.05). If the second centrifugation was performed there was no difference between 1600 and 2100 g for the first 15 min centrifugation (n = 8, P = 0.05).
Twelve samples of fresh and frozen-thawed PFP were compared. Clot growth rates significantly increased for frozen plasma (27.4 ± 2.8 μm/min) compared to non-frozen samples (25.3 ± 1.9 μm/min) (P = 0.05). There was a significant correlation for the clot growth rate (r = 0.65, P = 0.05)
In summary, we showed that glass blood collection tubes cannot be used for the Thrombodynamics assay probably due to increased contact activation, but most of the plastic tubes are suitable. Plasma obtained with one centrifugation and frozen plasma can be used but it requires the corresponding reference values.

PB 2.52-1

Validation of methods for determination of procoagulant activities in immunoglobulins using the NIBSC reference reagent for FXIa

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Background: An increased rate of thromboembolic adverse event reports associated with the use of an intravenous immunoglobulins (IVIG) from a specific manufacturer has been ascribed to increased residual activated coagulation factor XI (FXIa) and possibly other impurities. Since no reference material was available, the National Institute for Biological Standards and Control (NIBSC) developed a FXIa reference reagent prior to the development of a International Standard to promote standardization of analytical methods.
Aims: To validate the methods for testing procoagulant activities in IVIG using the NIBSC Reference Reagent for Activated Blood Coagulation Factor XI (FXIa) code 11/236.
Methods: The following activation markers were validated: Non-activated partial thromboplastin time (NaPTT) and thrombin generation (TGT) test (both using normal platelet poor plasma -PPP- and FXI deficient plasma), FXI antigen -Ag- using an enzyme immunoassay and FXIa by chromogenic assay. The NaPTT was tested at 1/5 and 1/10 dilutions. In all cases, a standard curve with the NIBSC reference reagent that contains 10 arbitrary units (U) per mL was used. Using this reagent all methods were validated in terms of linearity of FXIa and limit of detection (LOD) and limit of quantitation (LOQ) of FXIa. The validation parameters of specificity, accuracy and robustness were also established.
Results: The NaPTT-PPP showed a LOD of 0.018 U/mL and the LOQ for its technique was 0.055 U/mL. The NaPTT-FXI has a LOD of 0.003 U/mL and the LOQ was established at 0.008 U/mL. For both TGT (TGT-PPP and TGT-FXI), the LOD and LOQ were 0.001 and 0.002 U/mL, respectively. In case of FXI assays, the LOD and LOQ for FXI:Ag were 0.35 and 1.06 U/mL, respectively. With the chromogenic FXIa assay, the LOD was 0.0005 U/mL and the LOQ was 0.0015 U/mL. The linearity FXIa range was established in all cases.
Summary: In order to standardize homogenize the procoagulant test applied to discard the thromboembolic risk of IGIV, NaPTT and TGT tests, FXI:Ag and chromogenic FXIa assay have been validated using the NIBSC Reference Reagent for Activated Blood Coagulation Factor XI (FXIa) code 11/236 with satisfactory results. The validated methods were specific, accurate, precise and robust as well as applicable to final product and production intermediate materials.

PB 2.52-2

Human thrombin liquid reagent for fibrinogen determination in samples containing dabigatran

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Background: New oral anticoagulant drugs have recently emerged in the pharmaceutical market. Dabigatran is an oral direct thrombin inhibitor that reaches peak plasma concentrations in the range between 100 and 400 ng/mL, and trough concentrations in the range between 20 and 150 ng/mL. DG-FIB L Human is a liquid reagent from Grifols for the quantitative determination of fibrinogen using the Clauss method, characterized by a broad measurement range, which avoids the need of retesting samples with abnormally high or low levels of fibrinogen. It has been reported that dabigatran can interfere with fibrinogen Clauss tests, leading to erroneous results. A slight modification of the current procedure set for DG-FIB L human in Q Hemostasis Analyzer (Grifols) has been tested in order to avoid the dabigatran interference in fibrinogen determination.
Aims: To study the performance of the reagent DG-FIB L Human in Q Hemostasis Analyzer for the quantitative determination of fibrinogen in human plasma that contains dabigatran.
Methods: All tests were performed in the optical coagulometer Q Hemostasis Analyzer (Grifols). The modified procedure involves a modification of the ratio between plasma and thrombin concentration in the reaction. Linearity and limits of detection and quantification were determined using DG-Ref (Grifols) as calibrator plasma. Precision and accuracy were studied at different levels of fibrinogen with or without spiked dabigatran. The performance of the current and the new procedures was compared in 28 normal and 48 abnormal fibrinogen samples from patients not treated with dabigatran. The effect of dabigatran on the new procedure was studied determining the fibrinogen Clauss of high, normal and low fibrinogen samples (n = 31) at three concentrations of dabigatran (0, 200 and 400 ng/mL).
Results: Both procedures, the current and the new, showed similar performance in terms of precision, accuracy and linearity in samples without dabigatran. With the new procedure the measurement range was 85–1300 mg/dL of fibrinogen, which is an improvement compared to the current method (70–950 mg/dL). The new procedure showed comparable precision results between the samples with or without dabigatran at 200 ng/mL, with coefficients of variation of 4.25% and 4.19% for the normal and 7.14% and 7.12% for the abnormally low fibrinogen level, respectively. The addition of 400 ng/mL of dabigatran to the samples did not affect the accuracy (maximum bias of 9.0%). In the method comparison study using samples without dabigatran, a good correlation of results using the current and the new procedure was observed and no significant differences were detected in a Passing Bablok fit and a difference plot (allowable bias of 15%). The addition of dabigatran to fresh frozen samples at concentrations of 200 and 400 ng/mL produced no significant difference (allowable bias of 15%) in fibrinogen results obtained with the new procedure.
Conclusions: DG-FIB L Human reagent is suitable for routine determination of fibrinogen Clauss. A new procedure is available for quantification of fibrinogen in human plasma samples, including those from dabigatran treated patients containing a dabigatran concentration up to 400 ng/mL.

PB 2.52-3

The rate of reduction in D-dimer level for patients with venous thromboembolism responding to antithrombotic therapy

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Background: D-dimer is a degradation product from the breakdown of cross-linked fibrin. The absence of D-dimer was used to rule out the diagnosis of venous thromboembolism (VTE). Newer D-dimer assay allows quantitative measurement of D-dimer levels. It has been shown that elevated D-dimer level may be associated with higher risk of recurrence when antithrombotic therapy is discontinued. Yet, when patients with venous thrombosis responding to anticoagulant treatment, the dynamic profile of D-dimer is still obscure.
Aim: This study was to evaluate the rate of reduction in D-dimer levels when venous thrombosis was resolving after anticoagulant treatments in patients without other confounding conditions causing high D-dimer levels.
Methods: A retrospective chart review was performed for consecutive patients with newly diagnosed VTE and was treated in a regular fashion with low-molecular-weight heparin (LMWH), bridged to warfarin with a target international normalized ratio (INR) of 2.0–3.0. Patients were excluded if falsely elevated D-dimer level might occur in the following conditions: concurrent bleeding, after resuscitation or trauma, post-thrombolysis, recurrence or progression of venous thromboembolism within 3 months from the first day of diagnosis, disseminated intravascular coagulation, hemolysis, hepatic or renal failure. Quantitative immunoturbidimetric assay was used to determine D-dimer level

every 1–2 weeks. Patients were followed regularly in the clinic for at least 3 months. In this study, patients were defined as responders if no recurrence was detected, either clinically or with radiological imaging, within 3 months. Correlation of coefficient (R^2-value) was used to evaluate the measurable D-dimer level (500–4000 mg/L FEU) against time interval. Student's *t*-test was used to compare the rate of changes in D-dimer levels among between patient groups. A *P*-value of < 0.05 was considered statistically significant.

Results: D-dimer level were evaluated in 13 patients, nine with DVT only and four were complicated by PE. There was a linear decrease in the D-dimer levels when patients with venous thromboembolism were treated with anti-thrombotic drugs. The R^2-value was 0.949, suggesting D-dimer level decreased at the average rate of 119.4 mg/L FEU/day with the median rate at 96.9 mg/L FEU/day and the 25th and 75th percentile were 85.4 and 130 mg/L FEU/day respectively. There was no statistical difference when comparing patients with deep vein thrombosis (DVT) only and those complicated by pulmonary embolism (PE). The rate of reduction in D-dimer level was not associated with the duration to achieve target INR when warfarin therapy was first initiated.

Summary/Conclusion: For patients with newly diagnosed VTE responding to antithrombotic therapy, D-dimer level decreased at a constant rate when it is in the measurable range. Consequently, in the absence of other comorbid conditions causing falsely elevated D-dimer levels, managing physicians may use serial quantitative measurements of D-dimer to monitor the response of therapy, thus allowing early intervention in case of treatment failure. In the absence of other confounding factors, reduction in D-dimer levels appears to be a good surrogate marker for the resolution of thrombus. This is potentially an informative tool for comparing the efficacy of various antithrombotic therapies.

PB 2.52-4

Specific and global coagulation assays in the diagnosis of mild haemophilia A

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Background: FVIII activity (FVIII:C) can be measured by three methods; one (FVIII:C1) or two-stage clotting (FVIII:C2) and chromogenic (FVIII:CR). The FVIII:C of most individuals with mild haemophilia A (MHA) is the same regardless of the method employed. Approximately 30% of MHA patients demonstrate marked discrepancy in FVIII:C activity measured with the different methods. There are two types of assay discrepancy; the lower two-stage form (reduced FVIII:C2 or FVIII:CR with higher FVIII:C1) which is linked to a bleeding diathesis and the lower one-stage form (reduced FVIII:C1 and higher FVIII:C2 or FVIII:CR) where the bleeding risk is minimal or unclear. The current ISTH definition does not define the type of assay to be used in diagnosis and misclassification of some patients with MHA and normals can occur.

Objective: To investigate the incidence of assay discrepancy, assess the impact of alternative reagents on FVIII:C activity assays and determine the usefulness of global assays of haemostasis in MHA.

Methods: The APTT, FVIII:C1, FVIII:C2 and FVIII:CR were measured in 84 individuals with MHA using different reagents. Assay discrepancy was defined as two-fold or greater differences between FVIII:C1 and FVIII:C2. Rotational thromboelastometry (ROTEM) and calibrated automated thrombography (CAT) were performed in discrepant and non-discrepant patients.

Results: Two-fold or greater assay discrepancy was observed in 31% of individuals; 12% with the lower FVIII:C2 form and 19% with the lower FVIII:C1 form. An individual's genotype did not always predict their phenotype. A normal APTT was demonstrated in 11% patients. No statistically significant difference was noted between FVIII:C2 and three FVIII:CR kits ($P < 0.05$). The ROTEM did not discriminate well between MHA and normal subjects. The CAT parameters, ETP and peak thrombin, correlated well with FVIII:C1 and FVIII:CR levels ($r > 0.75$) in our cohort. We showed the meanETPto be 66% of normal in the lower two-stage group and 69% of normal in the non-discrepant group. Mean peak thrombin was 33% and 43% of normal respectively. Patients and carriers of p.Tyr365Cys which is linked to lower one-stage discrepancy and, in our cases no bleeding, had normal ETP and normal or only mildly reduced peak thrombin.

Conclusions: Thirty-one percent of MHA patients exhibited significant (two-fold or greater) assay discrepancy. In our study, 4% of MHA patients would not be diagnosed by laboratories employing only FVIII:C1 assays. Laboratories should utilise both one stage and two-stage (or chromogenic) assays in the diagnosis of patients with possible mild haemophilia A. The APTT cannot be relied on as a screening test where FVIII:C is > 30 IU/dL and in some patients with the lower two-stage form of assay discrepancy where the APTT is within normal limits. Chromogenic assays are a suitable alternative to FVIII:C2. The clinical utility of global assays of coagulation still remains to be defined although some CAT parameters did correlate with phenotype in this small study of assay discrepant patients.

PB 2.52-5

An ECT based assay for thrombelastometry using solid lyophilized pellet reagents

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Background: Along with the development of new anticoagulant drugs the Ecarin clotting time (ECT) became an important method to measure their activity in plasma. The ECT is insensitive for both heparin and classical oral anticoagulants but sensitive for direct thrombin inhibitors (DTI). Unlike aPTT the ECT increases linearly with plasma DTI concentration and therefore provides a method to monitor DTI plasma levels.

Aims: In solution ecarin is stable for 24 h at room temperature. The aim of this work is to develop a stable point – of – care system for thrombelastometry using lyophilized ecarin pellets. These pellets were designed to dissolve immediately in order to add them directly to the whole blood sample right before measurement.

Methods: Droplets were generated using a micro annular gear pump (HNP Mikrosysteme) connected to a Sterican® needle (Braun) and were frozen in liquid nitrogen. The lyophilization process was conducted using a Vertis Freeze dryer (SP Scientific). Whole blood (WB) coagulation profiles were determined using a thrombelastometry device (ROTEM®; Tem International GmbH). Citrated WB was received from the university medical center's blood donation service. Ecarin activity after freeze drying was determined by measuring the ECT and using the respective volume of the initial solution (stored at −80 °C) as a reference. The pellet system was also compared to the standard ROTEM® ECATEM procedure using 20 µL of an ecarin solution (c = 21.5U/mL). The references were recalcified using 20 µL of a 0.2 M $CaCl_2$ solution. The pellet system was recalcified using $CaCl_2$ pellets which were also produced by lyophilization and contained the respective amount of Ca^{2+}.

Results: The liquid pellet formulation contained trehalose as a bulking agent and 125 U/mL ecarin. The lyophilized ecarin pellets (diameter: 1.67 ± 0.03 mm) had a mean mass of 0.72 ± 0.02 mg corresponding to an initial droplet volume of 3.45 ± 0.04 µL. The activity of ecarin after FD was 102.18 ± 7.52%. The results for ECT obtained with the pellet system did not differ from the results obtained using the standard liquid system (CT_{pellet} = 73.0 ± 4.7s, CT_{liquid} = 73.8 ± 1.0s; $n = 10$; $P < 0.05$). Melagatran served as a DTI and was added to the whole blood sample covering concentrations from 0 up to 1.6 mg/mL. The ECT for the liquid system as well as for the pellet system increased linearly with increasing Melagatran concentration. Since stability is

another key aspect ecarin pellets were placed under controlled conditions (40 °C; 50% ambient humidity) in a climate chamber. The activity of ecarin did not significantly ($P < 0.05$) decrease during a 4 week storage period.

Conclusion: In summary it is possible to avoid the stability issue of ecarin solutions and preserve activity by using lyophilized pellets instead of liquid reagents. Therefore the pellet system might become a ready – to – use tool to determine DTI levels in clinical practice by measuring ECT. In addition other coagulation assays could be manufactured as lyophilized pellets as well to avoid additional dilution of the sample.

PB 2.52-6

The effect of platelet poor plasma storage time on thrombin generation inter-assay variability using calibrated automated thrombography

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Background: There is increasing use of calibrated automated thrombography (CAT) in the investigation of haemostatic disorders. To be a clinically relevant assay methods must be standardised and results reproducible. Sample storage time may influence results, particularly if collection takes place over a prolonged period. The acceptable storage time of PPP for thrombin generation testing is currently undefined.

Aims: To assess the effect of PPP storage time on the inter- assay variability obtained in a single centre on the most commonly reported parameters; lag time (LT), time to peak (ttP), peak thrombin (peak) and estimated thrombin potential (ETP).

Methods: Venous blood was collected from five healthy male volunteers with minimal stasis using 21-gauge butterfly needles. The first 5 mL was discarded and the second draw divided into BD vacutainers containing 0.109M trisodium citrate; corn trypsin inhibitor (CTI) at a final concentration of 18.3 μg/mL was added to half the samples. PPP prepared following double centrifugation at 4750 *g* for 10 min was pooled prior to freezing at −40 °C. Hemker's thrombin generation method was carried out by a single user with the Thrombinoscope™ assay, utilising reagents obtained from the manufacturer (Thrombinoscope BV, Maastrichts, Netherlands). Pooled male plasma was tested at 4, 5, 6, 7, 8, 9, 13, 14, 18, 20, 21 and 52 weeks using three thrombin generation assays. The first involved 5pM tissue factor (TF) and 4 μM phospholipids (PL). The second incorporated thrombomodulin (TM), a protein C pathway sensitising agent, at a final concentration of 6 nM TM, found in pre-testing to correspond to suppression of ETP by approximately 50%. The third (1pM TF and 4 μM PL) tested PPP in the presence of CTI. All samples were run in triplicate with calibration wells. Intra-assay variability was tested on PPP derived from one healthy female volunteer, collected and prepared as above. Four weeks after freezing, all three assays were performed, each consisting of 16 samples run in triplicate with a calibrator.

Results: Inter-assay variability for the standard 5pM TF assay was excellent, with coefficients of variation (CV) < 6% for all parameters over 52 weeks. In the presence of TM, CV was < 10% for all parameters up to 13 weeks but increased over time; peak thrombin and ETP were predominantly affected reaching 11.8% and 13.6% at 52 weeks respectively. The 1pM TF assay produced CV that were still acceptable at < 8.5% across 21 weeks for all parameters. Intra-assay CV was excellent throughout (LT 4.8%, 4.2%, 3.1%; ttP 3.3%, 2.5%, 2.4%; Peak thrombin 2.8%, 3.1%, 3.7% and ETP 2.5%, 5.4% and 3%) for the three assays; 5pM TF without TM, 5pM TF with TM, 1pM TF in the presence of CTI respectively.

Summary: Reproducibility of the method is demonstrated, with excellent intra-assay variability. Inter-assay CV's were acceptable (< 10%) for all assays using PPP stored for up to 3 months. These results demonstrate that study samples should be analysed at a consistent time point following their collection, and that batch testing at the end of studies may lead to less reliable data.

PB2.53 – Blood Coagulation Tests – IX

PB 2.53-1

Effect of clot detection method on total error calculated by CLSI protocol EP-10

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Nowdays, the most accepted approach for analytical performance goals is based on the biological variation (BV). In haemostasis laboratory, there are no reliable data on BV of all the investigated analytes. On the other hand, required optimal analytical quality set by biological variation is difficult to achieve.

The aim of this study is to compare the total error (TE) calculated by CLSI protocol EP-10 (preliminary evaluation of quantitative clinical laboratory measurement procedures) with three different detection method in automatic coagulometers: optic, nephelometric and viscosity. The performed tests were PT, FVII and FVIII in% (one stage clotting assay with two dilutions) and fibrinogen (FBG) in mg% by Clauss method. The used thromboplastin ISI was instrument specific: ISI = 1.15 for optic, ISI = 1.11 for nephelometric detection and an ISI = 1.34 for viscosity detection.

The TE values were compared with allowed TE (TEa) fixed by BV and CLIA requirements. Normal and low commercial controls (NC and LC) with target value were used; the control medium levels (MC) was prepared mixing 3.5 vol of NC and 1.5 vol of LC for PT and equal vol of each control for factors and fibrinogen.

Results: The TE determined for PT were: nephelometric 8.9%, optic: 5.8% and viscosity 6.2%. For fibrinogen, TE was: nephelometric 10.5%, optic 10.4% and viscosity 10.8%. For FVII TE were: nephelometric 10.6%, optic 9.5% and viscosity. 10.2% and for FVIII the TE were: nephelometric 12%, optic 10 .5% and viscosity 11.3%.

None of the TE determined in our laboratory achieved the optimum TEa fixed by BV, but the obtained FBG, FVII and FVIII Total Error were within desirable limits fixed by BV by all detection methods. The PT -TE determined with optic and viscosity method were within TE desirable limits fixed by BV but the nephelometric method only achieved the minimum level of quality requirement established by VB. Quality requirement fixed by CLIA for TP and Fibrinogen were reach by the three detection methods.

Conclusions:

1 There were no differences in TE calculated by EP-10 between different methods of clot detection
2 Although our laboratory is participating successfully in an external quality program and also use internal quality control, it is very difficult for clot detection methods to reach the optimum quality requirements fixed by BV. It is particularly true for PT, a global test, whose result depends on the interaction of several factors. The question is: which is the desirable quality for a reliable application of these tests as screening tests for patients with blood coagulation abnormalities or for monitoring anticoagulated patients with warfarin.

PB 2.53-2

INR portable monitors: efficacy of a quality control system

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Background: Since 2001 our Haemostasis andThrombosisCenter decentralized anticoagulation patient management through using portable monitors (PM) in a telemedicine system, bi-directional connected with peripheral health care units (PHU) such as Nursing Homes, Groups of General Practitioners and other hospitals of the area.
One of the main problem in decentralization management is the assurance of the quality provided by systems used.
Aims: The aims of the project were: (i) define a quality control (QC) analytical system for coagulation portable monitors used in the web organization; (ii) measure precision and accuracy in each single peripheral site to ensure good global analytical quality
Methods: Central laboratory coagulometer (STA-R; Roche) was considered as reference system. In PHU were used Coaguchek XS (Roche). Based on current guidelines for laboratory QC, the following criteria were defined:

1 PM suitabily: (i) precision calculated on normal plasmas pool, repeated 10 times, acceptable if CV < 5%; (ii) accuracy, evaluated on 10 pathological specimens with INR < 4.0, accettable if INR differences < ± 0.5.
2 Intra-assay precision: each PHU elaborates monthly the Lewej-Jennings cards. Internal QC, provided by the company, was performed at the beginning of each session and every 20 samples. A CV between ± 20% was considered acceptable.
3 Quaterly accuracy to assess the agreement between analytical instruments: three samples at different therapeutic range were analyzed in duplicate. Differences £ 0.5 INR was considered acceptable.
4 External quality assessment (NEQAS): it considers both laboratory data and clinical treatment, to assess the accuracy of the global therapeutic management.

Results: In the nine PHU, 18 portable monitors were used to perform 18,210 test/year. Analytical precision was very good with a CV always < 5%. Control system showed only two controls out of range (on 360 total controls = 0.55%), giving practical indication for immediate instrument replacement. The external QC NEQAS was optimal.
Conclusions: The adopted QC protocol give an accurate and precise control of PM in use, ensuring the quality of analytical data and, by consequence, optimal patient therapeutic management. QC is a mean to ensure good results and we think that national authorities should garantee the application of correct protocols to allow PM use.

PB 2.53-3

Conventional and new global haemostasis laboratory test reveal hypercoagulation in primary multiple myeloma patients

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Background: During diagnosis and induction polychemotherapy patients with Multiple Myeloma (MM) have an increased thrombotic risk so anticoagulant treatment is prescribed. In some cases standard preventive anticoagulant therapy is insufficient and thrombosis occurs.
Aims: The aim of this study is to reveal laboratory tests that can detect increased thrombotic risk.
Methods: The study included 18 primary MM patients. The following assays were performed: activated partial thromboplastin time (aPTT), thrombin time (TT), International normalized ratio (INR), XIIa dependent fibrinolysis, D-dimers, thrombin generation test (TGT), thromboelastography (TEG), Thrombodynamics (a new method based on a spatial fibrin clot growth registration). Thrombodynamics is characterized by initially clot growth rate (V_i) and spontaneous clotting (indicator of prothrombotic risk).
Results: TGT indicated hypercoagulation in 15 patients (83%): A max was increased in 14 whereas ETP was increased in four patients. Among this group hypercoagulation was confirmed by Thrombodynamics in nine patients (increased Vi and/or spontaneous clotting), aPTT in five patients, D-dimers in seven patients, TEG in four patients. There were three cases with hypercoagulation by TGT where all other performed tests detected normal or hypocoagulation state.
In a group with normal TGT (three patients) Vi was in norm, there were no spontaneous clotting; aPTT was extended in two cases and normal in one case, D-dimers were increased in one patient and in norm in two cases, TEG was in norm.
TT was extended in seven patients, others in normal region. INR was increased in four patients, others in normal region. Correlation between D-dimers and XIIa dependent fibrinolysis time was detected ($R = 0.94$, $P < 0.05$).
One patient had iliofemoral thrombosis in 16 days after the survey. The following results during the primary assay had been registered: increased peak $A_{max} = 225$ nM (normal range 50–170 nM), $V_i = 62$ μm/min (normal range 36–56 μm/min), no spontaneous clotting, shortened aPTT = 30 s (normal range 32–37 s), extended D-dimers level 8.1 mg/L (normal range 0–0.5 mg/L) and all TEG parameters in normal range.
Conclusion: In primary MM patients hypercoagulation state was revealed in nine cases (50%) at least by two tests: TGT and Thrombodynamics. In patient with occurred thrombosis aPTT, D-dimers, TGT and Thrombodynamics revealed increased thrombotic risk.

PB 2.53-4

Thrombin generation test: a new and simplified expression of results

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Background: Thrombin generation (TG) is a global test which describes the haemostatic patient assessment. Because of poor standardization and automation, and difficulties in assigning an individual clinical value TG is not currently available in routine clinical practice. Another actual limitation of TG use is represented by difficulties in finding methodologies able to express synthetic results useful to practical and comprehensive interpretation.
Aims: Aims of the present study were: (i) evaluate haemostatic assessment using TG in four populations: normal patients, congenital/acquired haemophilia, congenital thrombophilia, patients with prolonged aPTT, afferring to emergency department; (ii) evaluate a methodology to simplify the expression of TG results
Methods: A total of 100 patients were studied: 40 healthy donors (I); 20 congenital/acquired haemophilias (II), 20 congenital thrombophilia patients (III) and 20 with prolonged aPTT (IV). TG was performed on Calibrated Automated Thrombogram (CAT-Stago, France) a semi-automatic instrument using fluorogenic substrate. The activation of coagulation was obtained by adding small amounts of tissue factor and phospholipids using PPP reagent (CAT- Stago, France). Blood samples for TG study were collected, immediate centrifuged and poor platelet plasma stored at −80 °C. The results calculated were: lag time (min), peak (nM), time to peak (min), velocity index (nM/min), endogenous thrombin potential-ETP (nM*min).
To evaluate the possibility to describe a thrombin potential as expression of the activation phase related to the total amount of thrombin

generated in relation to the peak, we used the following formula: Peak related to Thrombin Generation – PTG (nM) = area under the curve from t0 to time to peak (activation phase ETP) (nM*min)/total ETP (nM*min) × peak (nM).

Results: The four populations showed the following results expressed as mean ± 1DS:

1 Normal subjects: ETP (nM*min) = 1494.4 ± 307.2; PTG (nM) = 91.6 ± 16.0
2 Haemophilia: ETP (nM*min) = 507.1 ± 256.3; PTG (nM) = 11.2 ± 8.3
3 Thrombophilia: ETP (nM*min) = 1692.8 ± 269.2; PTG (nM) = 104.7 ± 15.5
4 Prolonged aPTT: ETP (nM*min) = 1207 ± 515.3; PTG (nM) = 64.9 ± 41.3

Results obtained in the fourth group, selected on the base of a prolonged aPTT in patients afferring to the emergency department, are expression of the differences in haemostatic profiles, both hyper- and hypo-coagulable, as shown by standard deviation

Conclusion: Thrombin generation, as expression of the balance between hyper- and hypo-coagulability, has well defined the four population studied, both through ETP results than PTG. In our opinion PTG has the advantage to better describe the haemostatic assessment, including the three main parameters of TG test. Further studies are required to validate its value as screening test in daily clinical practice.

PB 2.53-5

Procoagulant changes in blood plasma as a result of plasmapheresis

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Background: Although plasmapheresis donation is a relatively safe technique commonly used today, there are reported cases of severe, sometimes lethal complications if it is applied in donors with thrombophilia or other thrombotic risk factors. Presently, insufficient attention is paid to either donor coagulation state before plasmapheresis and coagulation parameters of collected plasma.

Aims: The aim of the study was to evaluate the donors' coagulation status prior to plasmapheresis and to compare it with that of collected plasma using spatial clot growth assay (thrombodynamics) previously reported to be sensitive to procoagulant changes in plasma.

Methods: The study involved 50 male subjects aged 20–45 with plasma donation experience of not less than five times. Analysis of coagulation was carried out at different points: (i) 1 min prior to plasmapheresis; (ii) in the collected plasma product; (iii) in the thawed collected plasma product (fresh frozen plasma). At every point, different types of plasma were examined. They were: (i) platelet-free plasma (Sample PFP) and frozen platelet-poor plasma (Sample fPPP); (ii) product platelet-free plasma (Sample pPFP); (iii) product fresh frozen platelet-poor plasma (Sample fPPP*). The method used, thrombodynamics, is based on a videomicroscopic observation of fibrin clot propagation in a non-stirred layer of plasma activated by the immobilized tissue factor. The following parameters of clot growth were determined on the basis of the image series: lag time (t_{lag}), initial (V_{in}) and stationary (V_{st}) clot growth velocity, presence of activator-independent clotting.

Results: Compared with PFP, sample pPFP had evidence of hypercoagulability as indicated by a significantly higher median [range] of V_{in} and V_{st} (58 μm/min [44–72 μm/min] vs. 46 μm/min [36–55 μm/min] and 30 μm/min [21–41 μm/min] vs. 25 μm/min [20–29 μm/min], respectively. $P < 0.05$). Compared with fPPP, sample fPPP* had also evidence of hypercoagulability as indicated by a significantly ($P < 0.05$) higher median [range] of V_{in} and V_{st} (64 μm/min [56–74 μm/min] vs. 60 μm/min [50–69 μm/min] and 37 μm/min [29–48 μm/min] vs. 35 μm/min [27–43 μm/min], respectively. $P < 0.05$). Moreover, there were four cases when plasmapheresis led to activator-independent spontaneous clotting in product plasma, which indicates the high rate of hypercoagulability. Two of them showed slight hypocoagulability prior to plasmapheresis as well.

Summary/Conclusions: Plasmapheresis procedure shifts coagulation parameters from normo- to hypercoagulable state, as shown by thrombodynamics assay. Thrombodynamics can have a potential utility for the evaluation of both donors' coagulation status prior to plasmapheresis and product plasma collected using plasmapheresis.

PB 2.53-6

Thrombin Dynamics Test: a new global coagulation assay for the evaluation of the propagation phase of thrombin generation

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Introduction: Prothrombin Time and aPTT are routinely used for the evaluation of coagulation but they ignore thrombin generation (TG) process. The rate of the propagation phase of TG, which is governed by the formation of the enzymatic complexes prothrombinase and intrinsic tenase, is related to the presence of hypercoagulability and is decreased by the anticoagulant treatment. Thrombin Dynamics Test (TDT) is a new assay that measures the rate of the propagation phase of thrombin generation. In the present study we evaluated the performance of the assay.

Materials and methods: TDT was performed as follows: 60 μL of platelet poor plasma (PPP) are mixed with 40 μL of NaCl (0.9%) and 20 μL In-TDT activator. After 3 min incubation at 37 °C 60 μL of In-TDT Reagent were added. Thrombin generation, triggered by aPTT/elagic acid based reagent, is detected by fast thrombin-based conversion of a chromogenic substrate and release of p-nitro-aniline which is continuously recorded at 405 nm (ThermoScientificMultiskan FC). Fibrin formation in the sample is inhibited by the addition of fibrin polymerization inhibitor (Pefabloc). All reagents were kindly offered by Pentapharm (Basel Switzerland). The Peak value of the 1st derivative of thrombin formation representing the maximum velocity of the propagation phase of thrombin generation was analysed. To establish normal range and to control the performance of the assay TDT was done on PPP from 50 healthy individuals. TDT was also performed on samples from patients treated with VKA ($n = 50$) and patients with endogenous thrombin potential (ETP) higher than 2500 nM × min, tested with Calibrated Automated Thrombogram ($n = 50$). Values of the tested samples are expressed as percentage versus a normal control sample (Calibrator) supplied from Pentapharm.

Results: The intra- and inter-assay variability was 2% et 6% respectively. The inter-individual variability was 15%. The thrombin dynamics in healthy individuals was 106.7 ± 16.5% and the Lower and Upper Normal Limit were 74% and 140%. TDT was not influenced by the sex and the age of healthy individuals. In VKA treated patients TDT was correlated with the INR ($r = 0.5$, $P = 0.006$). No corrélation was found between TDT and the values of Endogenous Thrombin Protential.

Conclusion: The TDT, using aPTT-like reagents is a user friendly global coagulation assay not requiring any particular pre-analytical conditions. TDT showed a very satisfactory performance and low inter/intra- assay variability and is not influenced by the age and the sex of the studied individuals. The inter-individual variability was 15% being similar to that of other global coagulation assays and is sensitive to detect hypocoagulablity induced by VKA treatment. TDT measures a different parameter of thrombin generation than the ETP of thrombogram.

PB2.54 – Coagulation Factor XI – I

PB 2.54-1

Ongoing risk of thrombosis with factor XI concentrate: 5 years experience in two centres

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Background: Factor XI (FXI) deficiency is an autosomally inherited bleeding disorder of variable clinical phenotype. Treatment options in the peri-operative period include expectant management, anti-fibrinolytics and plasma derived (pd) FXI concentrates. Two pd FXI concentrates are currently available in the United Kingdom. Initial use of both concentrates was complicated by reports of thrombosis. These episodes typically occurred in elderly patients, especially those with vascular risk factors. Since these episodes, changes have been made to the manufacturing process of both concentrates, but the relevance to thrombotic risk is unknown.
Aims: To retrospectively review the indications for FXI concentrate usage, efficacy and thrombotic events over a five-year period at two haemophilia treatment centres.
Methods: The clinical and laboratory data for all factor XI concentrates issued between November 2006 – November 2011 at both centres were reviewed, using paper and electronic records.
Results: Twenty-nine (17 male/12 female) patients with FXI deficiency were treated over the study period. The median age was 59 years (14–92). Twenty-one patients had severe FXI deficiency (FXI:C ≤ 15 IU/dL) and eight mild FXI deficiency (FXI:C > 15 IU/dL). The median baseline FXI:C was 9 IU/dL (< 1–51). A total of 93 doses of FXI concentrates were issued (31 Hemoleven (LFB) and 62 FXI (BPL)) over 64 treatment episodes. The indications for FXI infusions were to cover surgery in 87.5% (56/64), trauma/bleeding in 7.8% (5/64), non-surgical delivery in 3.1% (2/66) and as a test dose prior to surgery in 1.6% (1/64). The median number of infusions per treatment episode was 1 (1–10). The median dose of FXI concentrate used was 17 U/kg (6–42). The median peak FXI:C obtained was 61 IU/dL (26–172). Pharmacological thromboprophylaxis was used during 17.2% (11/64) of treatment episodes. There were six episodes of clinically significant bleeding with four requiring infusion of a FXI concentrate, two additional medical review, one a blood transfusion and one oral iron replacement therapy. The lowest recorded peak FXI:C in this group was 44 IU/dL. Three of these patients (3/6) were receiving pharmacological thromboprophylaxis. Two thrombotic episodes (2/64) were documented in the study period. Both occurred in patients using Hemoleven to cover surgery. The first (multiple pulmonary emboli) occurred in a patient with traditional vascular risk factors following complex orthopaedic surgery (peak FXI:C 50 IU/dL) not covered by pharmacological thromboprophylaxis. The second (transient ischaemic attack) occurred in a patient with no known cardiovascular risk factors following major urological surgery (peak FXI:C 70 IU/dL). No immune or infective complications were reported.
Conclusions: Our retrospective review demonstrates the efficacy and safety issues of using FXI concentrates across a wide range of indications. There were six episodes of clinically significant bleeding all of which were controlled with a single additional dose of FXI concentrate or standard intervention. Two thrombotic episodes occurred following major surgery, despite cautious FXI use. We suggest thorough assessment of vascular risk factors in patients requiring FXI concentrate to cover surgical procedures. Consideration of pharmacological thromboprophylaxis remains essential.

PB 2.54-2

Identification of two novel mutations in sequential nucleotides of the factor XI gene in a Dutch Caucasian family with inherited factor XI deficiency

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Background: Factor XI deficiency is an autosomal recessive disorder and is predominantly present in Ashkenazi Jews. To achieve sufficient haemostasis the haemostatic level of factor XI needs to be between 15% and 20%. However, the correlation between factor XI level and bleeding tendency is weak and inconsistent. Factor XI gene contains 15 exons and 14 introns located on the long arm of chromosome 4 (4q35) and more than 240 mutations have been reported.
Aim: We performed an analysis in order to determine the molecular background of factor XI deficiency in a Dutch Caucasian family.
Methods: Factor XI activity levels were determined by an one-stage clotting assay (Siemens, Marburg, Germany), which was standardised using a home-made normal plasma pool. The reference interval, based on a locally performed reference range, was 65–150 U/dL. The assay was performed using a Sysmex CA-7000 (Siemens, Marburg, Germany). Direct sequencing analysis of all 15 exons and flanking introns of factor XI gene was performed to detect mutations.
Results: Sequencing analysis showed in all three subjects with factor XI deficiency the presence of two novel heterozygous mutations in sequential nucleotides in exon 3 of the factor XI gene: a silent mutation (NM_000128.3(FXI):c.177C>T) that preserves amino acid Phenylalanine at position 59 and a potential missense mutation (NM_000128.3(FXI):c.178A>C). These mutations were not found in another sister and two daughters with normal levels of factor XI. NM_000128.3(FXI):c.178A>C may lead to a conversion of hydrophilic and median sized Threonine at position 60 to hydrophilic large Proline. Multiple alignment analysis of the human factor XI gene sequence with sequences of other vertebrates was performed using OMA browser. The alignment showed that Threonine is highly conserved among other species. To evaluate the possible effect of Threonine to Proline substitution on the molecular structure of factor XI, we used a computer-based model (2f83) in Swiss PDB Viewer.
Threonine 60 interacts with Phenylalanine 30, Serine 96, and Valine 77 through strong hydrogen bonds. After substitution, only an interaction remains with Valine 77. Furthermore, due to its biochemical properties, Proline might result in steric hindrance with Phenylalanine 59. To prove that the novel mutations are not polymorphisms, we also performed sequence analysis in 100 unrelated healthy volunteers. No healthy volunteer carried these mutations. Additionally, these mutations may inactivate exon splicing enhancers (ESEs), leading to failure of splicing and exon skipping or inclusion of intron segments in mRNA. To test the possibility that these mutations cause a splicing defect, we used the motif-scoring matrices in a web-based program called ESEfinder. The two novel mutations did not result in an additional or abolishment of ESE.
Conclusions: It is likely that the presence of these two novel heterozygous mutations may modify the factor XI function and are associated with factor XI deficiency in a Dutch Caucasian family.

PB 2.54-3

Comparison of thromboelastographic parameters before and after fresh frozen plasma treatment in patients with factor XI deficiency and with ex-vivo samples spiked with FXI concentrate

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Background: The options for perioperative management of patients with factor XI deficiency include Solvent Detergent Fresh Frozen Plasma (SD-FFP) or factor XI concentrates. Although FXI:C levels are often monitored peri-operatively, it has been previously shown that there is poor correlation between FXI:C levels and bleeding tendency in these patients. For this reason, we used the technique of thromboelastography as an investigative tool to assess treatment response in patients with factor XI deficiency. Previous studies have demonstrated prolonged Clot Formation Times (CFT) and alpha angles on thromboelastography in these patients.

Aims: To determine the various thromboelastographic parameters before and after SD-FFP treatment in patients with factor XI deficiency undergoing surgery and after ex-vivo samples were spiked with factor XI concentrate.

Methods: Five patients with partial FXI deficiency (baseline FXI:C levels 34–47 IU/dL) and one patient with severe FXI deficiency (FXI:C level < 1 IU/dL) were treated with SD-FFP prior to surgery (dose range 13.3–24.3 mL/kg). FXI:C levels and ROTEM® thromboelastography parameters (triggered with a tissue factor concentration of 0.12 pm) were analysed prior to and after treatment with SD-FFP. In addition, in one patient with partial FXI deficiency, baseline whole blood samples were spiked ex-vivo with different amounts of FXI concentrate (equivalent to 10, 20, 30 and 40 units/kg) and similar measurements were performed.

Results: Following administration of SD-FFP to the six patients, the FXI:C levels increased by a median of 20 IU/dL (range 14–27 IU/dL). Following the ex-vivo spiking of the whole blood with factor XI concentrate, the FXI:C levels increased to 76 IU/dL (with 10 units/kg), 102 IU/dL (with 20 units/kg), 118 IU/dL (with 30 units/kg) and 143 IU/dL (with 40 units/kg).

The CFT values shortened by a median of 164s (range 45–179 s) and the alpha angle increased by a median of 21° (range 7–27°) with SD-FFP. Interestingly, although CFT values decreased to 131s and 102s and the alpha angle increased to 65° and 70° respectively with 10 and 20 units/kg of the concentrate, no further improvement were noticeable with the higher doses beyond that achieved with 20 units/kg although there was an increase in factor XI levels. This indicates a possible ceiling effect of FXI concentrate dosage on these parameters. Importantly, for clinical purposes, it was noticeable that a lower CFT (92s vs. 102s) and higher alpha angle (72° vs. 70°) were achieved with the SD-FFP *in-vivo* compared to the FXI concentrate.

Summary: Improvement in Clot Formation Time and alpha angle parameters on thromboelastography are seen following the treatment of FXI deficient patients with SD-FFP and in one patient with partial FXI deficiency following ex-vivo spiking experiments with FXI concentrate up to a dose of 20 units/kg. SD-FFP is at least as effective as factor XI concentrate in correcting thromboelastographic abnormalities in factor XI patients undergoing surgery.

PB 2.54-4

Factor XI in the relation to fetal loss: the frequency of the risk alleles

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Background: Pregnancy is a prothrombotic state and a pathological exaggeration of this hypercoagulability has been increasingly linked to pregnancy loss and placenta-mediated complications. Pregnancy loss is a very significant public health issue, associated with maternal morbidity and mortality and psychological trauma. Thrombophilia (sometimes hypercoagulability or a prothrombotic state) is an abnormality of blood coagulation that increases the risk of thrombosis. The correlation between a high level of F XI and the risk of the occurrence of venous thrombosis influenced also by genetic factors was confirmed by Leiden Thrombophilia Study and MEGA study. High levels of factor XI are associated with thrombotic diseases, and severe factor XI deficiency shows protective effects against deep vein thrombosis (DVT) and ischemic stroke

Aims: In our study we focused on coagulation factor XI, which plays the main role in the coagulation cascade. We compared the level of coagulation factor XI between patients with fetal loss vs. control subjects with no history of fetal loss and thrombosis. Subsequently evaluate the frequency of the risk alleles in the relation to fetal loss.

Methods: The study was approved by the Ethical committee, Jessenius Faculty of Medicine, Comenius University – No. EK950/2011. Informed consent was obtained from each participant.

Factor XI was determining using a coagulometer (Sysmex, CA 1500, Japan). Two single-nucleotide polymorphisms (SNPs) of factor 11 gene and one SNP of CYP4V2 gene were evaluated. DNA was extracted from peripheral blood leukocytes. Isolation of genomic DNA from the whole blood was performed with SiMaxTM Genomic DNA Extraction kit (SBS Genetech Co., Ltd., China) according to the manufacturer′s instructions. The gene polymorphisms were identified with the use of restriction fragment length polymorphism and high resolution melt analysis (in the check on process of one of tag SNP), with in-house design of individual polymerase chain reaction.

Results: We examined 55 patients with fetal loss (the average age of 31.9 ± 4.1 years) and 31 healthy controls (the average age of 32.74 ± 8.94 years). A total number of patients with one to four spontaneous abortions were: 22 (40%), 24 (44%), 6 (11%) and 3 (5%), respectively.

We found significantly higher level of factor XI ($P < 0.04$) in patients with fetal loss vs. control subjects. The occurrence of two SNPs (rs2289252, rs2036914) of the factor 11 gene and one SNP (rs13146272) of CYP4V2 gene were not significantly different between patients with fetal loss and control subjects.

Summary/Conclusion: Increased levels of factor XI may be a potential risk factor for fetal loss. High levels of factor XI diagnosed at the women with fetal loss are not probably dedicated to the presence of risk alleles (rs2289252, rs2036914, rs13146272).

Acknowledgements: The study was supported by grants VEGA 1/0016/12, VEGA 1/0029/11, APVV-0222-11, UK/381/2012 by projects 'Centre of Excellence for Perinatological Research' co-financed from EU sources (ITMS 262201200016 and 26220120036) and CEPYVET, co-financed from EU sources (ITMS 26220120053).

PB 2.54-5

A newly diagnosed with congenital factor XI deficiency in a mild hemophilia A patient by gene analysis

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Introduction: Congenital factor XI deficiency is a rare autosomal recessive bleeding disorder that is highly prevalent in Ashkenazi Jews but rare in other ethnic groups. In Japan, only 28 cases were registered in the national survey for blood coagulation disorders carried out in 2011. Patients with factor XI deficiency show a widely varied bleeding tendency after trauma and surgery. Hemophilia A is a congenital X-linked bleeding disorder caused by various mutations in the factor VIII gene. Patients with severe hemophilia experience joint and muscle bleeding, whereas bleeding symptoms in patients with mild hemophilia are usually caused only as a result of surgery or major injury. In the present study, we investigated the genes responsible for both factor VIII and XI deficiency and newly diagnosed factor XI deficiency in a patient with mild hemophilia A.

Methods: The study was approved by the Ethics Committee of Tokyo Medical University, and the patient gave his written informed consent. Genomic DNA was extracted from leukocytes. The coding regions and exon/intron boundaries in both the factor VIII and XI genes were amplified by polymerase chain reaction (PCR). The PCR products were purified and direct sequenced with an Applied Biosystems 3730 DNA Analyzer.

Patient and Results: The patient, a Japanese man in his early 70s, presented to a local hospital with nasal and gingival bleeding after tooth extractions; he was previously diagnosed as having mild hemophilia A in his 40s. He received on-demand treatment with factor VIII concentrate at that hospital. He was then admitted to our hospital for cataract surgery. His activated partial thromboplastin time was very prolonged at 125.9 s as compared with usual mild hemophiliacs. His prothrombin time was normal. Factor VIII and XI levels were 20% and 1.5%, respectively. Levels of other coagulation factors acting in the intrinsic pathway, factors IX, XII, and von Willebrand factor, were within normal range. Factor VIII and IX inhibitor, lupus anticoagulant, and anti-CLbeta2GPI and anticardiolipin antibodies were not detected. We diagnosed combined factor VIII and XI deficiency after considering these results. In the genetic analysis, we identified a novel missense mutation, Ile2032Thr, in exon 20 of the factor VIII gene. A homozygous missense mutation, Phe221Ser, in exon 7 was also identified in the factor XI gene by direct sequencing. Interestingly, this factor XI mutation was not detected in Ashkenazi Jews; however, it was reported previously in two unrelated Japanese cases.

Discussion: A recombinant Phe221Ser mutant protein expression study reported that the mutant in the factor XI gene would cause a secretion defect (*Jpn J Thromb Hemost. 2005;16:304–311*). Although difficult to diagnose only from the patient's mild bleeding symptoms, his diagnosis was confirmed by gene analysis

Conclusion: We identified both a novel lle2032Thr mutation located in the A3 domain of the factor VIII protein and a previously reported homozygous Phe221Ser mutation located in the apple 3 domain of the factor XI protein as a mutation causing hemophilia A and factor XI deficiency.

PB 2.54-6

Pharmacodynamic tests for factor XI participation in haemostasis

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Background: Patients with factor XI deficiency show an increased risk of bleeding up till 50% factor XI antigen level. Below 50% factor XI antigen bleeding risk is increased but does not correlate to antigen levels.

Correction of clotting time in the APTT is already practically achieved at low concentrations of 10% factor XI (IC50 = 5%)

This shows a clear discrepancy between APTT testing and clinical effects. A functional method to evaluate individual risks and responses to treatment below 50% factor XI is lacking

Aims: To help to solve a diagnostic need.

To develop a functional method that shows a clear dose response between 0% and 50% antigen levels of factor XI mimicking the patient information as a potential pharmacodynamic method to include individual aspects and monitor factor XI supplementation.

Methods: We prepared mixtures of normal and factor XI depleted plasma and evaluated the response on read outs of various global methods, compared to the APTT. The methods evaluated were: thrombin generation (TGA-RCH ® of Technoclone, with 5 pM tissue factor); a newly devised chromogenic test for Xa generation, started with micronized silica, made independent of thrombin by addition of an excess of hirudin; thromboelastography (Rotem ® with tissue factor or contact activation) with and without induced lysis by adding t-PA.

Results: For all methods we studied the dose response between 0% and 100% factor XI.

We identified two methods with a higher dose response range.

These methods were the newly devised factor Xa generation test and thromboelastography with low tissue factor (1:250 diluted Extem ®) and with added t-PA.

The first method revealed that the peak of factor Xa generation was clearly dose-dependently decreased and delayed in the range of antigen concentrations of factor XI below 50%. The IC 50 was found to be 10% for peak height.

The thromboelastography method showed that the lysis rate of thrombi was dose-dependently increased over the whole range from 100% to 0% factor XI. This effect may add to occurrence of less persistent haemostatic plugs.

Conclusion: We have identified two methods showing sensitivity to low factor XI in a physiological relevant range of 0–50%, thus correlating with clinical data. They represent phenotypes other than clotting times (as in APTT), notably factor Xa generation and clot lysability. These global functional methods are candidates for pharmacodynamic methods which may express effects of individual factors and might help to explain bleeding risks in patients and be useful for monitoring need/effect of supplementation in deficient patients.

PB2.55 – Coagulation Factor VIII – IV

PB 2.55-1

Factor VIII level correlates with hemolysis and may contribute to the hypercoagulability of children with sickle cell disease

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Background: Factor VIII (FVIII:C) level is often increased in sickle cell disease (SCD) which is considered as a hypercoagulable state. This state is evidenced by increased markers of coagulation activation and increased thrombin generation (TG). It is influenced by the rate of hemolysis. However, the contribution of factor VIII level to this hypercoagulability is still unclear.

Aims: (i) to assess the relationship between FVIII level, von Willebrand factor antigen level (vWF Ag), markers of hemolysis and markers of inflammation (ii) to assess the contribution of elevated FVIII levels in TG and DDimers level in SCD children at steady-state.

Methods: FVIII level, vWF Ag, TG and DDimers were measured in 50 SCD children at steady-state aged between 2 and 18 years (median: 9). TG was triggered in platelets-poor plasma using 1 pM tissue factor and 4 μM phospholipids with and without thrombomodulin. The amount of thrombomodulin added was that expected to produce a 50% reduction of total thrombin generated. Only the endogenous thrombin potential (ETP), the peak height and the percentage reduction of ETP with addition of thrombomodulin (RETP) were analyzed for TG. FVIII level (%) was measured using a one stage clotting assay. DDimers and vWF Ag levels were measured using immunoturbidimetric assays. FVIII, vWF Ag and DDimers assays were performed using the STA-evo coagulation automate (Stago, Asnières-sur-Seine, France). Total hemoglobin (THb), reticulocyte count (Retic) and lactate deshydrogenase (LDH) level were also measured as markers of hemolysis. Highly sensitive C-reactive protein (hsCRP) was measured as marker of inflammation. Correlations between variables were assessed using the Spearman's test. $P < 0.05$ was considered significant.

Results: Median (IQR) values of FVIII, vWF Ag and hsCRP levels were respectively: 258.5% (194.8–307.8), 170% (127–198) and 3.14 mg/L (1.34–6.98).

FVIII level strongly correlated with vWF Ag ($r = 0.77$; $P < 0.001$), LDH ($r = 0.50$; $P = 0.001$) and reticulocytes count expressed as percentage ($r = 0.62$; $P < 0.001$). More modest but significant correlation was also observed with THb ($r = -0.32$; $P = 0.023$), absolute reticulocytes count ($r = 0.38$; $P = 0.009$), DDimers level ($r = 0.34$; $P = 0.030$), ETP ($r = 0.37$; $P = 0.009$), peak height ($r = 0.35$; $P = 0.015$) and RETP ($r = -0.39$; $P = 0.006$) of TG. No significant correlation was found between FVIII level and hsCRP ($r = 0.20$; $P = 0.214$).

Conclusion: As expected, elevation of FVIII level in SCD is associated to parallel increase of vWF level. However, the results of this study suggest that elevation of FVIII level seems to be related to hemolysis rather than chronic inflammation. Significant correlations with DDimers level and TG indicate a probable contribution of elevated FVIII level to the hypercoagulability observed in SCD children. The negative correlation observed with RETP in TG suggests that elevated FVIII level may also contribute to increased resistance to activated protein C after addition of thrombomodulin.

PB 2.55-2

Molecular changes in LRP1 and FVIII genes in patients with venous thromboembolism and high FVIII levels

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Background: Venous thromboembolism (VTE) is a multifactorial disease, and increased levels of factor VIII (FVIII) has been demonstrated as risk factor for VTE. The low-density lipoprotein receptor-related protein 1 (LRP1) is a multifunctional receptor which plays an important role in endocytosis and catabolism of its ligands, including the FVIII.

Aims: The aim of the study was investigate if after a long-term follow-up, patients with high levels of FVIII after anticoagulant treatment. Moreover, we investigated in these patients, the presence of molecular changes in specific exons of LRP1 and FVIII genes which encode regions of interaction between these two proteins.

Design and Methods: Our initial cohort consisted of 300 adult patients with a first episode of acute VTE occurring between January 1990 and September 2004. In 2004, in a first assessment, FVIII levels were evaluated in all these patients and in 300 matched controls. In 2011, all patients from this initial cohort who originally presented FVIII levels above the 90th percentile ($n = 100$) were recruited for a second assessment of FVIII activity (FVIII:C). In total, 68 patients agreed to participate. Control subjects were again selected according to age, gender, ABO blood group. FVIII levels were measured by a one-stage clotting assay. The regions of LRP1 and FVIII genes of VTE patients were analyzed by automated capillary sequencing.

Results: Median age of the 68 patients (19 male and 49 female) was 47 years, and median of the first DVT episode was 10 years. In the first measure of FVIII, in our initial cohort, the median of the first DVT episode was 3 years. The control group consisted of 74 subjects (28 male and 46 female) with a median age of 45 years.

FVIII levels was significantly higher in patients when compared to controls [158.0 IU/dL, (82.0–216.0) vs. 126.1 IU/dL, (83.4–187.1); $P < 0.001$]. Those patients also showed higher plasma FVIII when compared to controls [235.8 IU/dL, (200.3–510.0) vs. 127.2 IU/dL, (80.1–211.0); $P < 0.001$] when previously analyzed. FVIII levels were significantly lower at the moment when compared to the initial assessment ($P < 0.001$).

We evaluated the exons 14–20 and junctional intronic regions of LRP gene, and the exons 10, 11, 16, 26 and junctional intronic regions of FVIII gene. We identified five molecular changes in the LRP gene, including two silent alterations not previously described, C2767T and G3376A (Ser970Ser), two intronic alterations in the intron 16: G3137 + 81A (previously described), C3137 + 54T and one in the intron 18 (T3380 + 114C) both not previously described. None molecular change was found in the regions of FVIII gene.

Conclusions: We demonstrated a persistent increase of FVIII levels in a subset of patients after 10 years of the first DVT, but in a much lower magnitude than that observed in the first 3 years of the disease. Despite we have found five molecular changes in the LRP gene oh VTE patients, further evaluations in a healthy group with normal FVIII levels are necessary to determine the role of these molecular changes on the modulation of FVIII levels and/or VTE risk.

PB 2.55-3

Effect of unstructured polypeptide insertions on the recombinant expression of human factor VIII

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Background: Fusion of unstructured polypeptides of defined amino acid composition and low immunogenic potential, known as XTEN technology, has proven to be an effective means of extending *in vivo* half-life of biotherapeutics. We are employing the XTEN technology to improve the half-life of human coagulation factor VIII (hFVIII). One or more XTENs at varying lengths were inserted to inter-domain sites, intra-domain sites, and/or C terminus of hFVIII. The insertion sites were identified based on FVIII structure analyses excluding genetic mutations reported in hemophilia A patients.

Aim: The aim of this study was to determine the effect of XTEN insertion(s) on the expression of recombinant hFVIII.

Methods: An expression vector encoding human B-domain-deleted FVIII (BDD-FVIII) was constructed using pcDNA4.1, and the XTEN modifications were generated following optimized molecular cloning procedures. The XTENs used in this study were of three different lengths (composed of 42, 144 or 288 amino acids) and of two types with slightly different amino acid compositions – AE XTEN (containing A, E, G, P, S and T) and AG XTEN (containing A, G, P, S and T). The FVIII-XTEN fusion constructs were transiently expressed in HEK293F cells using polyethyleneimine as a transfection agent. Five days post transfection the conditioned cell culture media were analyzed for FVIII activity using a chromogenic assay (Coatest); for FVIII variants with single XTEN insertion, the conditioned media were also analyzed for FVIII antigen level by ELISA.

Results: The impact of XTEN insertion on FVIII expression was found to be insertion site dependent. FVIII-XTEN remained active after single XTEN insertions at many sites, predominantly in the A domains, while insertions at some sites abolished expression. We iden-

tified three insertion sites, B domain (N745), a3 domain (Q1656) and C terminus, where a single XTEN resulted in consistently higher levels of antigen and activity than non-modified BDD-FVIII, though this could be due to either higher expression or greater stability of the protein in the cell culture media due to the XTEN insertion. Increasing the number of insertions (up to six) decreased the observed activity in a progressive fashion. In the case of multiple insertions, an XTEN at a3 domain (Q1656) partially rescued the suppression of expression resulting from XTEN insertion at another site such as those in A1, A2 and A3 domains. In general AG XTENs resulted in slightly higher observed activity than AE XTENs.

Conclusions: Human FVIII retained activity following the insertion(s) of one or more XTENs when insertion points are appropriately selected, even when one or more insertions were at intra-domain sites of FVIII. Single XTEN insertions at three specific sites enhanced the FVIII activity level. These results indicate that active FVIII-XTEN fusion proteins can be generated, providing multiple candidates for assessment of half-life extension in *in vivo* studies.

PB 2.55-4

Pharmacokinetic results from a Phase I/III study of a novel recombinant single-chain factor VIII (rVIII-SingleChain) compared to octocog alfa in severe haemophilia A patients

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Background: rVIII-SingleChain (CSL627, CSL Behring, Marburg) is a homogeneous, more stable recombinant FVIII containing a covalent bond linking the light and heavy chains. It has increased affinity to von Willebrand factor (VWF) resulting in faster and more efficient binding compared to other rFVIII molecules. VWF, the natural carrier protein of FVIII, increases the half-life of all FVIII. After activation, the resultant FVIIIa is identical to that derived from activation of a two-chain FVIII.

Aims: This multinational (Austria, Germany, Italy, USA) clinical trial is designed to assess the pharmacokinetics (PK), efficacy, tolerability and safety of rVIII-SingleChain. We report on the PK of rVIII-SingleChain compared with octocog alfa (Baxter, Westlake Village, CA)

Methods: Up to 30 PTPs ≥ 18 year-old with severe hemophilia A (FVIII:C < 1%) with > 150 exposure days to factor VIII were eligible for enrollment. Following an infusion of 50 IU/kg octocog alfa, PK was measured over 72 h, and after a 4 day washout, PK was repeated after an infusion of rVIII-SingleChain. Subjects were then assigned to treatment with rVIII-SingleChain either as prophylaxis or on-demand treatment for a minimum of 6 months and 50 EDs. Prophylaxis subjects were treated with rVIII-SingleChain at 20–40 IU/kg every second day or 20–50 IU/kg two to three times per week at the investigator's discretion. Patients were assessed monthly: reviewing bleeding events, response to rVIII-SingleChain, adverse drug reactions including the incidence of inhibitors, laboratory safety and virus safety parameters.

The pharmacokinetics of rVIII-SingleChain and octocog alfa were assessed on the basis of FVIII activity measured using both the one-stage and chromogenic substrate assay. The following PK parameters were calculated for baseline-corrected FVIII activity using a non-compartmental model analysis with WinNonlin Phoenix (Version 6.3): The area under the plasma activity-time curve from time zero to the last quantifiable concentration (AUC_{last}); the area under the plasma activity-time curve from time zero to infinity (AUC_{inf}); the observed maximum plasma activity after drug administration (C_{max}); incremental recovery (IU/mL/IU/kg) defined as FVIII activity (IU/mL) obtained 30 min following infusion; clearance (CL), and terminal elimination half-life ($t_{½}$).

Results: Currently 25 patients (median age 32 years, range: 19–60 years) have completed PK studies with octocog alfa and rVIII-SingleChain and the study continues to recruit subjects for PK and treatment. Individual plasma and median activity-time profiles for rVIII-SingleChain and octocog alfa were compared and show favorable kinetics for incremental recovery, AUC_{last} and half-life. Differences between FVIII levels measured with one-stage and chromogenic substrate assay were noted.

We will present detailed PK for rVIII-SingleChain and comparator, safety and efficacy data.

Summary/Conclusions: rVIII-SingleChain has excellent PK, and satisfactory safety and efficacy to advance to late-stage development. PK parameters indicate advantages over two-chain rFVIII.

PB 2.55-5

Influence of age on recombinant factor VIII pharmacokinetics: results from clinical pharmacology studies of turoctocog alfa

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Background: Five open-label, multi-centre clinical studies (three phase 1 and two phase 3 trials) conducted in 18 countries worldwide (Brazil, Croatia, Germany, Israel, Italy, Japan, Lithuania, Macedonia, Malaysia, Poland, Russia, Serbia, Spain, Switzerland, Taiwan, Turkey, the UK and the US) have explored the pharmacokinetics (PK), safety and efficacy of turoctocog alfa, a human recombinant coagulation factor VIII (rFVIII) developed for the prevention and treatment of bleeding in patients with haemophilia A.

Aims: To investigate the influence of age on the PK characteristics of turoctocog alfa.

Methods: PK characteristics of turoctocog alfa were assessed in previously-treated paediatric (< 12 years of age) and adolescent or adult (≥ 12 years of age) patients with severe haemophilia A (FVIII activity level ≤ 1%) in a non-bleeding state and with no history of FVIII inhibitors. PK parameters were assessed following a wash-out period (≥ 3 days in paediatric patients; ≥ 4 days for adolescent/adult patients) and a subsequent single intravenous 50 IU/kg dose of turoctocog alfa. All patients were previously treated with ≥ 50 exposure days (paediatric patients) and ≥ 150 exposure days (adolescent/adult patients) to any FVIII product. Blood was sampled at regular intervals throughout a 48-hour period post-dose. PK parameters (incremental recovery of FVIII, AUC, and terminal half-life [$t_{½}$]) were based on FVIII activity, measured using a one-stage clot assay and a two-stage chromogenic substrate assay. Plots of mean PK endpoints by age or bodyweight across trials were used for a descriptive evaluation of dependency.

Results: Data from a total of 61 patients (aged 1' '54 years, including 28 patients < 12 years of age) were included, totalling 83 assessments. PK parameterswere comparable between young children (aged 0' '< 6 years) and older children (aged 6' ' ≤ 12 years). Overall, AUC and $t_{½}$ tended to increase with increasing age, with lower AUC and shorter $t_{½}$ seen in children (aged 6' '≤ 12 years) compared with adults (aged ≥ 18 years), whereas there was no clear relationship between age and incremental recovery of FVIII. Similar apparent relations between age and pharmacokinetic parameters (AUC and $t_{½}$) were also observed within the adult population alone (> 18 years). However, it cannot be excluded that factors other than age also may have influenced the differences observed.

Summary/Conclusions: Our results suggest that there may be differences in the PK characteristics of turoctocog alfa according to age. The observed apparent positive relation between age and PK parame-

ters for turoctocog alfa is in line with previous findings for commercially available FVIII products.

PB 2.55-6

N-Glycosylation of rVIII-SingleChain, a novel recombinant single-chain factor VIII

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rVIII-SingleChain represents a novel type of factor VIII (FVIII) product, a homogeneous recombinant single-chain FVIII, that is currently under clinical development. Commercially available FVIII products so far are two-chain molecules consisting of light and heavy chains. The pharmacokinetic properties as well as activity and immunogenicity of a protein may be affected by its post-translational modifications. Especially N-glycosylation of a protein has an impact on size, hydrophobicity and charge. It is known that the N-glycosylation pattern of recombinant proteins strongly depends on the cell type used for expression. Production cells should therefore also be selected thoroughly in view of the resulting N-glycosylation. In the present study the N-glycosylation pattern of rVIII-SingleChain was investigated and was also compared to different commercially available rFVIII products.

rVIII-SingleChain was expressed in CHO cells and purified by chromatographic methods. BDD rFVIII and full-length rFVIII were obtained from commercial sources. The investigation of the respective carbohydrate structures was performed after release of N-glycans by PNGase F and subsequent fluorescence labeling. The labeled N-glycans were analyzed by anion exchange chromatography at high pH (HPAEC) and ESI-MS.

rVIII-SingleChain manufactured in CHO cells is characterized mainly by complex type, core fucosylated N-glycan structures that are highly sialylated. As expected also high mannose type N-glycan structures were detectable. A similar pattern was observed for commercially available recombinant rFVIII products, a BDD rFVIII and a full-length rFVIII product, both expressed in CHO cells. The N-glycan structures of human plasma-derived FVIII expressed in the liver were also considered for comparison purposes. Plasma-derived FVIII contains sialylated bi-antennary complex type N-glycans as main structure besides some high mannose type oligosaccharides (T. Hironaka et al., JBC (1992), 267(12): 8012–8020). Based on the experimental and clinical experience with the commercially available rFVIII molecules it can be assumed that the known CHO cell-type specific N-glycosylation patterns of rFVIII are suitable models when choosing a cell line for expression of a new biocompatible and safe rFVIII product.

PB2.56 – Natural Anticoagulants – II

PB 2.56-1

Gas6 plasma levels in elderly patients with acute venous thromboembolism (VTE) predict major bleeding under anticoagulation and mortality but not recurrent VTE

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Background: Growth arrest-specific gene 6 (Gas6) product is a vitamin K-dependent protein which plays a role in thrombus stabilization as *Gas6* deficient mice are protected against venous and arterial thrombosis. Patients with venous thromboembolism (VTE) display elevated Gas6 plasma levels as compared to healthy volunteers.

Aims: We aimed to assess whether elevated Gas6 levels are associated with VTE recurrence, major bleeding, and all-cause mortality.

Methods: In a multicenter Swiss cohort, we prospectively enrolled consecutive patients aged ≥ 65 years with acute, symptomatic VTE between September 2009 and March 2012. Follow-up was until october 2012. Gas6 level was measured in plasma at baseline by ELISA. We defined elevated Gas6 levels as results above the median, low Gas6 levels being those below the median. Baseline characteristics of patients were compared with Gas6 levels below and above the median. We estimated the cumulative incidence of a first VTE recurrence, major bleeding under anticoagulation, or death event using the Kaplan-Meier technique and compared incidence rates among patients with elevated and low Gas6 levels by logrank tests. Associations between Gas6 levels and the time to the first event were assessed by Cox-regression. Adjustment was done for age, gender, and known risk factors for VTE recurrence, bleeding, and mortality.

Results: Of the 853 patients studied, 435 (51%) patients had elevated Gas6 levels. The median follow-up time was 18 months (interquartile range 9.5–24.2). Patients with elevated Gas6 were older (76 vs. 74 years) and were more likely to have immobilization, active cancer, post-thrombotic syndrome, history of major bleeding, chronic liver disease, anemia, high risk of fall, and a lower arterial oxygen saturation than patients with low Gas6.

The cumulative incidence of recurrent VTE did not differ between patients with elevated and low Gas6 levels (5.3 vs. 4.8 events per 100 patient-years; $P = 0.71$). However, patients with elevated Gas6 had a significantly higher cumulative incidence of major bleeding (10.5 vs. 5.9 events per 100 patient-years; $P = 0.03$) and overall mortality (18.6 vs. 5.7 events per 100 patient-years; $P \leq 0.001$) than patients with low Gas6. After adjustment, Gas6 levels were significantly associated with major bleeding (hazard ratio [HR] 2.13, 95% confidence interval [CI] 1.07–4.26) and overall mortality (HR 6.55, 95% CI 4.01–10.70) but not with recurrent VTE (HR 1.50, 95% CI 0.62–3.60).

Conclusions: Our study demonstrates that patients with elevated Gas6 levels are older and sicker than those with low Gas6 levels. Elevated Gas6 levels at baseline are significantly associated with future major bleeding under anticoagulation and overall mortality but not with VTE recurrence.

PB 2.56-2

Influence of the 3K3A-activated protein C variant on the *in vitro* fibrinolytic activity of tPA

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Background: Tissue plasminogen activator (tPA) is used to treat ischemic stroke, but its use is limited by a narrow safety window and by increased bleeding risk. Activated protein C (APC) is neuroprotective in preclinical ischemic stroke models. Remarkably, APC, in spite of having intrinsic anticoagulant activity, reduces tPA-induced bleeding and neurotoxicities primarily due to its ability to initiate cell signaling that provides anti-inflammatory and anti-apoptotic activities and stabilizes vascular endothelial barriers, thereby reducing leakage.

Aims: The APC variant 3K3A-APC in which the three lysines 191–193 are replaced by three alanines is neuroprotective in stroke models and the subject of current efforts for translation to a novel biologic for ischemic stroke therapy when combined with tPA. Because APTT and fibrinolytic *in vitro* assay data have implications for bleeding risks and for achieving reperfusion, we used these assays to study 3K3A-APC and tPA combinations.

Methods: APTT assays were performed in the presence of recombinant tPA and human plasma-derived APC or recombinant 3K3A-APC. Fibrinolytic turbidometric assays employed plasma clots containing tPA, APC and/or 3K3A-APC. Clot lysis time was defined as the time for 50% clot lysis.
Results: The anticoagulant potency of APC and of the 3K3A-APC variant in the presence or absence of 3.0, 1.0, and 0.5 μg/mL of tPA was determined from the prolongation of the APTT. Data showed that in the absence of tPA, the mutations in the 3K3A-APC variant caused loss of approximately 90% of anticoagulant activity compared to APC. There was no significant effect of tPA on the anticoagulant activity of 3K3A-APC, although at the highest concentration of tPA (3.0 μg/mL) in presence of 90 and 140 nM, several additional seconds of APTT prolongation was seen. When human normal pooled plasma that contained 20 nM APC or 3K3A-APC and tPA at varying concentrations was clotted by addition of thrombin and then clot lysis was monitored over several hours, the clot lysis time was dose-dependently shortened by increasing tPA. The presence of 20 nM of either normal or variant APC had no statistically significant effect on the ability of tPA to induce clot lysis compared to buffer controls. When the fibrinolytic activity of 0.25 μg/mL tPA was assayed in the presence of varying concentrations of each APC species, there was no statistically significant effect for either APC species when the APC level was ≤ 50 nM. However, plasma-derived APC exhibited profibrinolytic effects when present at ≥ 70 nM. But the 3K3A-APC mutant with its greatly reduced anticoagulant activity did not influence the fibrinolytic activity of tPA even when present at levels up to 140 nM.
Conclusions: Based on these results, when 3K3A-APC is present at very high levels in plasma, there appears to be no deleterious effects of tPA on the APTT or of 3K3A-APC on the fibrinolytic activity of tPA. Thus, if one considers tPA and 3K3A-APC combinations for ischemic stroke, this study failed to generate concerns about any undesirable effects of one agent on the other agent's activities that are reflected in APTT and fibrinolytic assays.

PB 2.56-3
Differences in isoforms of antithrombin in infants and adults

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Background: Antithrombin (AT), a key anticoagulation protein, has been shown to exist in various isoforms in adult plasma: native AT (NAT), cleaved AT, pre-latent AT (pre-LAT) and latent AT (LAT). The NAT isoform is present in two glycoforms, α-AT and β-AT, where a single glycosylation site difference leads to a significant difference in the affinity of these isoforms for heparin. The AT isoforms present in infants and children are yet to be characterized and are likely to play an important role in the interaction of AT with heparin. Knowing that 15% of patients at tertiary paediatric hospitals are administered some form of heparin therapy, research focusing on age-specific differences AT isoforms may have vast clinical implications.
Aims: To investigate the differences in AT isoforms in healthy infants compared to adults.
Methods: Citrated, platelet-poor plasma samples from infants (28 days–1 year old) and adults were collected and stored at −80°. All participants were healthy individuals, without previous thromboembolic events, family history of bleeding or thrombosis, and not subjected to any form of anticoagulant therapy. This protocol was approved by the Royal Children's Hospital, Melbourne, Ethics Committee (# 20031).
AT was purified from plasma using an AKTA FPLC system and a 1 mL HiTrap NHS-activated column (GE Healthcare) which was coupled with 6 mg of polyclonal sheep anti-human AT-specific antibody (Affinity Biologicals). The elution profiles were compared between the pooled sample from infants and adults.
Results: The AT elution peak obtained for the adult samples was made up of two peaks with a retention time of 27.65 and 28.07 min in comparison to the single AT elution peak for infants with a retention time of 28.07 min.
Summary/Conclusions: These results indicate that there are differences in the AT isoforms between infants and adults. The profiles indicate that the AT isoforms present in infants may be more similar to the first peak observed in the adult samples which elute at the same times. This needs to be confirmed in a larger number of samples. The specific differences in AT between the samples will be further characterised using mass spectrometry to retrieve sequence information, and to establish the precise molecular weight of the different AT isoforms. Furthermore, the specific heparin binding of the different AT isoforms has never been determined.
The final results regarding age-specific differences in the ratio of these isoforms in age-specific plasma samples is important when considering the clinical implications that these variations may have on heparin therapy.

PB 2.56-4
Effect of functional alpha2macroglobulin and antithrombin concentration on thrombin generation and decay in liver cirrhosis patients

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Background: α2Macroglobulin (α2M) has been reported to increase two- to four-fold in liver cirrhosis patients, whereas the level of antithrombin and other serpins (AT) as well as of procoagulant factors decreases proportionally to the severity of the disease. α2M and AT are responsible for the decay of thrombin. The increase of one inhibitor and the decrease of others may result in a net change in the inhibitory capacity of the plasma. A net decrease of the over-all inhibitory capacity might counterbalance the decrease of procoagulant factors.
Aims: To relate the over-all decay constant of thrombin as well as the isolated α2M dependent decay constant to the plasma levels of α2M and AT, both in a set of normal controls as well as in a series of patients with liver cirrhosis.
Methods: AT concentrations were determined by titration with purified α-thrombin. α2M was determined by saturation with excess α-thrombin and removal of excess free thrombin with AT-heparin. Thrombin generation was measured with the whole blood (WB) CAT technique. The pseudo first order decay constant of thrombin was obtained from the descending slope of the TG curves; the pseudo-first order constant of α2M-dependent decay from the formation velocity of the α2M-thrombin complex. Inhibitor concentrations were correlated to decay parameters by Pearson's correlation analysis. Differences in decay parameters and inhibitor concentrations between the patient and control group were analysed with the Mann-Whitney test.
Results: The α2M and AT assays performed well, with intra-assay CVs of 5.1% and 3.1% and inter-assay CV's of 11.9% and 7.8%. Mean α2M and AT concentrations in the group of healthy subjects were 3.6 ± 0.9 and 2.1 ± 0.3 μM, respectively, which is comparable to values reported in the literature. α2M levels in liver cirrhosis plasma samples were significantly increased (5.0 ± 1.0 μM; $P = 0.002$) compared to the control group, and AT levels were found to be significantly decreased (1.6 ± 0.7 μM; $P = 0.037$). The over-all thrombin decay constant in healthy controls was 0.375 ± 0.030/min (CV 8%) and the α2M dependent one 0.028 ± 0.013/min (CV 46%). In the cirrhosis group these figures were 0.285 ± 0.083 and 0.036 ± 0.013/min.
In the ensemble of the samples total thrombin decay and thrombin decay by serpins is significantly correlated with the AT level ($P = 0.005$ and $P = 0.001$, respectively). The α2M level is significantly

associated with the α2M dependent decay parameter only ($P = 0.002$). The overall parameter of thrombin decay in a TG curve was on the mean 24% lower in cirrhosis patients than in controls ($P = 0.006$), showing that an increase in α2M cannot completely compensate for the loss of AT in liver cirrhosis patients.

Conclusion: Inter-individual variation in thrombin inhibitor concentrations causes variation in thrombin decay (CV 8%) and thereby contributes to variation in net thrombin generation (CV 16%). In liver cirrhosis AT decreases and α2M increase but the latter increase cannot compensate for the former decrease. This makes that over-all TG is less affected than could be expected from the decrease of procoagulant factors.

PB 2.56-5

Antithrombin assays can vary in sensitivity to homozygous antithrombin Budapest III, a defect that causes heparin resistance

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Background: Antithrombin (AT) is an important inhibitor of thrombin and FXa, and deficiency results in increased tendency to venous thrombosis. AT deficiency may be due to lack of production (type I) or to production of functionally defective AT (type II), the defect may affect the reactive site (type RS), heparin binding site (type HBS) or have more than one effect (type PE). Subjects with homozygous HBS defects may have an inadequate laboratory response to heparin therapy. Low molecular weight heparin (LMWH) may be measured using anti-FXa reagents that may or may not include added AT. Antithrombin level is routinely assayed by measuring AT inhibition of thrombin or FXa in the presence of heparin, by means of chromogenic substrates.

Aims: We present data on two patients homozygous for the type II AT deficiency, AT Budapest III. Both patients showed poor laboratory response to LMWH when tested with a chromogenic anti-Xa assay that does not include added AT (AFXaA). We report discrepancies between commercial AT assays.

Methods: Blood from two subjects (A and B) was taken into 0.109M sodium citrate approximately 4–6 h post injection of LMWH, and plasma anti-FXa activity was measured by Coamatic AFXaA on Sysmex analyser. Antithrombin activity was measured on ACL TOP analyser (Instrumentation Laboratory, IL) by four chromogenic assays: Berichrom (Siemens) bovine thrombin-based assay modified by using 30s inhibition time; Innovance human FXa-based assay (Siemens); Liquid Antithrombin assay using bovine FXa (IL); and Coamatic assay utilising bovine FXa (Quadratech). Antithrombin antigen was measured by Liatest ATIII assay (Stago) modified for ACL TOP analyser. Two dimensional electrophoresis (2d CIE) of AT was performed in the presence and absence of heparin using antibodies from Dako, and genetic analysis was carried out by direct sequencing of *SERPINC1*.

Results: Both patients had anti-FXa levels of < 5 IU/dL 4–6 h post injection of maximal therapeutic dose LMWH, and this level did not increase when mixing patient plasma with an equal volume of normal plasma. AT activity (Berichrom) and AT antigen levels were reduced in both patients, (activity/antigen IU/dL) levels were 38/50 in patient A and 45/60 in patient B. In both subjects 2d CIE of AT indicated defective heparin binding, and both were found to be homozygous for AT Budapest III. Chromogenic AT level also varied by FXa-based assay, patient A had Liquid Antithrombin level of 41 IU/dL and Innovance AT level of 17 IU/dL, whereas patient B had Liquid Antithrombin level of 52 IU/dL and Innovance AT level of 14 IU/dL. In one sample from patient A, Berichrom AT was 31 IU/dL, and Coamatic AT was 30 IU/dL.

Summary/Conclusions: The inadequate anti-FXa response 4–6 h after injection of therapeutic dose LMWH was not corrected by addition of AT (pooled normal plasma), which may indicate increased clearance of plasma LMWH. AT activity levels varied by assay type, and the assay utilising human FXa showed significantly lower AT activity than the other assays, the lower level might better explain the poor anti-FXa response to LMWH in these two patients with AT Budapest III.

PB 2.56-6

Anticoagulant effect of two new estrogens (R- and S-Fenetame)

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Introduction: Estrogenic therapies increase the risk of thrombosis. Continuing with our program of design and synthesis of new estrogen compounds with antithrombotic activity, we had designed and synthesized N-[(3-Hydroxy-1,3,5(10)-estratriene-17β-il)]-(R)-(+)-β-methylphenethyl-amine (R-fenetame) and N-[(3-Hydroxy-1,3,5(10)-estratriene-17β-il)]-(S)-(+)-β-methylphenethyl-amine (S-fenetame).

Aim: Characterize by X-ray spectroscopy R- and S-fenetame and determine the whole blood clotting time.

Material and Methods: The estrogens derivatives were designed and synthesized at the Institute of Chemistry, UNAM. The compounds were dissolved in DMSO and administered subcutaneously to male mice, 29–33 g of weight, strain CD1 which remained at controlled temperatures 21–24 °C, food and water *ad libitum*, light-dark cycles of 12–12 h. After 24 h of the administration, we measured the whole blood clotting time with 25 μL of a whole blood sample, obtained of the dorsal vein of the mice. The blood was made alternately flow by gravity between two marks in a capillary tube (angles ± 60° with respect to the horizontal plane). We record the time until the blood is clot and stop to flow in the capillary tube. The protocol was approved by the institutional scientific and ethic committees.

Results: We obtained the the X-ray molecular structure of (i) R-fenetame and (ii) S-fenetame, in a solid state. The blood clotting time was measured by (sec, mean and SE). Results show the difference respect to the control group (in percent). Both estrogen enantiomers treated mice have to increase de blood clotting time. S-fenetame and R-fenetame, to dose of 1, 2 year 4 mg/kg: increased 16, 43 and 20% and 61, 184* and 27%, respectively. *$P \leq 0.05$ vs. vehicle. ANOVA, with Dunnett. Prism 5.0

Conclusions: The results show that both enantiomers increased the blood clotting time in a dose dependent manner. R-fenetame showed a two times stronger increase the blood clotting time compared with S-fenetame.

PB2.57 – Fibrinogen/Fibrin – III

PB 2.57-1

Genotype and phenotype of a large series of patients with congenital dysfibrinogenemia

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Background: Congenital dysfibrinogenemias (CD) are characterized by biosynthesis of a structurally abnormal fibrinogen molecule that exhibits reduced functional properties compared to level of fibrinogen antigen. To date a large number of mutations have been identified in patients with CD, although few are predictive of the clinical phenotype. In order to better understand the clinical complications in relation to the genotypes, we report a survey of 65 CD diagnosed in our laboratory.
Methods: Biological and clinical characteristics were recorded at reception of samples using a standardized case report. After isolation of genomic DNA, exons and intron-exon junctions of the fibrinogen genes were amplified by polymerase chain reaction (PCR) and sequenced.
Results: Between 2005 and 2012 we diagnosed 65 CD. Samples were sent to our laboratory principally to perform a familial screening (n = 23, 36%), before surgery (n = 14, 21%), following thrombosis (n = 8, 12%) and for investigation of bleeding (n = 7, 11%). The median age at time of DNA analysis was 36 years (range 1–76 years). The median fibrinogen activity (Clauss method) was 0.66 g/L (range 0.1–2.3) and the median antigen fibrinogen was 3.5 g/L (range 1.4–5.3). Mutations were identified in the coding sequences of fibrinogen genes in all 54/54 (100%) probands. Heterozygous missense mutations in residues Arg35(Arg19) of FGA in exon 2, Arg301(Arg275) of FGG exon 8 and surrounding residues accounted for almost 75% of CD. Twenty-three patients (43%) had mild bleeding episodes, easy bruising, epistaxis and menorrhagia being the most frequent. Thirteen (20%) and 3 (5%) patients experienced at least one venous or arterial thrombosis, respectively. A total of 30 pregnancies were reported, including 6 (20%) miscarriages. Deliveries were not associated with bleeding complications.
Conclusions: Mutations in exon 2 of FGA and in exon 8 of FGG are the most frequent causes of CD. At the time of diagnosis half of patients experienced mild bleeding or thrombotic events. CD is associated with miscarriage. An ongoing trial is evaluating the long term follow-up of these patients in order to better define the true incidence of bleeding complications and/or thrombotic events during the course of disease.

PB 2.57-2

Proteases in human pancreatic juice degrade both liquid and carrier-bound fibrin sealants *in vitro*

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Background: Fibrin sealants are used in a variety of surgical procedures mainly to control bleeding and to reinforce suture lines. Furthermore, these products are frequently applied to enhance tissue sealing for purposes other than induction of hemostasis in procedures including liver, lung, and pancreatic surgery. We have previously shown that fibrin sealants are unstable in the presence of human bile, and this observation may explain the lack of efficacy of fibrin sealants to avoid bile leak-related complications following liver surgery. Fibrin sealants have been used in pancreatic surgery for almost 35 years with the main aim of preventing fistula formation as a consequence of leakage of pancreatic juice. However, the clinical efficacy of this approach is still unclear.
Aims: Here we investigated the stability of commercially available liquid and carrier-bound fibrin sealants in the presence of pancreatic fluid using *in vitro* experimentation.
Methods: Fibrin clots were generated *in vitro* from two commercially available liquid fibrin sealants and exposed to saline or human pancreatic fluid. Degradation of clots was assessed by weighing clots or by examination of release of fibrin degradation products. Also, pancreatic fluid was exposed to the carrier-bound fibrin sealant Tachosil, and stability of the sealant was assessed qualitatively and quantitatively by measuring fibrin- and collagen degradation products.
Results: Clots generated from liquid fibrin sealants degrade rapidly in pancreatic fluid, but not in normal saline, as evidenced by a rapid reduction in clot weight and release of fibrin degradation products. Exposure of Tachosil to pancreatic fluid results in rapid degradation of both the fibrin and collagen part of the sealant, as evidenced by release of fibrin- and collagen degradation products and a visual inspection of sealant integrity. Protease inhibitor cocktails or individual serine protease inhibitors reduce breakdown of both liquid sealants and Tachosil, and a collagenase inhibitor reduces breakdown of Tachosil.
Conclusions: Proteases present in pancreatic juice effectively degrade both liquid and carrier-bound fibrin sealants *in vitro*. These results may imply that the use of these products in pancreatic surgery with the aim to prevent fistula formation as a result of leakage of pancreatic fluid may serve limited purpose.

PB 2.57-3

The same mutations in patients with congenital dysfibrinogenemia leading to different clinical manifestations

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Background: Congenital dysfibrinogenemia is known as a disorder with various manifestations. Fifty percent of affected people are asymptomatic, while 25% of affected ones suffer bleeding and the other 25% have thrombotic risk.
Aims: This study is to investigate the different clinical manifestations appeared in two unrelated dysfibrinogenemia families with the same mutations. Proband A has haemorrhagic manifestations, while proband B undergoes thrombosis performance.
Methods: PCR amplification and direct sequencing technology were used to identify the mutations in these patients. The antigen level (Fg:Ag) and activity level (Fg:C) of fibrinogen patients were detected by immunoturbidimetric assay and clauss assays, respectively. Routine thromboelastogram (TEG) test was performed to evaluate the comprehensive coagulation status of patients and functional fibrinogen TEG test was carried out to evaluation the level of functional fibrinogen in patients. Fibrinogen clottability, fibrin polymerization and fibrinolysis measurement were applied to evaluate the functional fibrinogen in patients. Aα, Bβ and γ chains of fibrinogen were detected by Western blot.
Results: The value of Fg:C in these two probands was 0.35 and 0.6 g/L, and the ratio of Fg:C and Fg:Ag was lower than 0.5 in both of them, which can diagnose them as dysfibrinogenemia. We identified the same compound heterozygous: AαArg289Gln, which is a novel mutation, and γArg275Cys in these two patients, as well as their family members. The molecular weight of Aα, Bβ and γ chains of fibrinogen in these two probands were the same as that in normal pooled plasma shown by western blot results. The CI value in TEG test was −8.1 in proband A, showing hypocoagulated status in her body, while that in proband B was within the normal reference range, which may due to antithrombotic drugs she had taken. The MA value in functional fibrinogen TEG test totally depends on the levle of functional fibrinogen. It was 2.8 in proband A and 3.2 in proband B, which was lower than the low limit of normal reference range (10~25), suggesting

the reduced level of functional fibrinogen in both patients, which was also consistent with their diagnosis as dysfibrinogenemia. The fibrinogen clottability rate was 18.15% and 47.26%, respectively, in proband A and proband B, compared to that in normal control. The fibrinogen polymerization curve of proband A was almost on the baseline, suggesting bleeding manifestation in this patient. The takeoff time in the curve of proband two was earlier than that in normal control, indicating its thrombosis risk, although its peak value was relatively lower. The fibrinolysis rate of proband B was slower than that of proband A, and the dissolving rate was 38% in proband A and 20% in proband B, further indicating thrombosis risk in proband B.

Conclusion: Our results were consistent with clinical manifestations of the patients, which can further explain the different manifestations in these patients. We have excluded all the other polymorphisms related to thrombosis. So, we propose a hypothesis that it may be the different assembly of proteins that leads to the different clinical manifestations in these two patients.

PB 2.57-4

Novel fibrinogen gamma-chain mutation p.Asp342Asn (fibrinogen Pisa) associated with hepatic fibrinogen storage disease and hypofibrinogenaemia

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Background: Type I fibrinogen deficiencies (hypofibrinogenemia and afibrinogenemia) are rare congenital disorders characterized by low or unmeasurable plasma fibrinogen antigen levels. Their genetic bases are represented by mutations within the three fibrinogen genes, FGA, FGB, and FGG, coding for Aalpha, Bbeta, and gamma fibrinogen chains. So far, about 130 genetic defects have been reported to cause a- or hypofibrinogenemia, but only four mutations, all located in the fibrinogen gamma-chain gene, were shown to cause hepatic endoplasmic reticulum storage disease (ERSD).

Aims: To find the genetic basis of hypofibrinogenemia and to investigate the possibility of hepatic fibrinogen storage disease in a 4-year-old female with elevated serum aminotransferases.

Methods: Plasma functional and antigen fibrinogen levels were measured by an assay based on fibrin polymerisation time and by an enzyme-linked immunosorbent assay, respectively. Mutational screening was performed by direct sequencing of PCR products covering the coding sequence of FGA, FGB, and FGG genes. Liver histology was evaluated by light microscopy and immunocytochemistry.

Results: The proband had concordantly reduced functional and immunologic fibrinogen levels (136 and 117 mg/dL, respectively), distinctive of hypofibrinogenemia. Molecular screening revealed the presence of a novel heterozygous transition (c.1024G>A) in exon 8 of FGG, leading to the p.Asp342Asn missense mutation (p.Asp316Asn, according to the mature protein numbering). This non-conservative amino acid substitution involves a highly conserved residue located in the C-terminal globular D-domain of the gamma chain. The same mutation was found also in the proband's mother and grandfather, who also had similarly reduced plasma fibrinogen levels but no sign of liver disease. Histological analysis of hematoxylin-eosin stained sections of proband liver biopsy samples revealed hepatocyte cytoplasmic microvescicular steatosis and the presence of small globular and needle-like inclusions. These inclusions were negative with PAS-D and iron histochemical reactions, not refracting with polarized light and not auto fluorescent. All cytoplasmic inclusions of hepatocytes were strongly immunoreactive with anti-fibrinogen antibody.

Conclusions: This work reports the identification of the fifth mutation in the FGG gene leading to hypofibrinogenemia and liver ERSD. As described in other families, our data confirm that the link between the FGG mutation and ERSD is not as strong as that with hypofibrinogenemia.

PB 2.57-5

Molecular characterization of the fibrinogen molecule in seven unrelated patients from Cordoba, Argentina

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Background: Inherited disorders of fibrinogen (Fg) are classified according to a complete lack of Fg in plasma (afibrinogenaemia), a partial deficiency (hypofibrinogenaemia) or evidence of an abnormal circulating molecule (dysfibrinogenaemia). In addition, a mix of both qualitative and quantitative conditions (hypodysfibrinogenaemia) has been reported.

Aim: In the present report we describe the molecular characterization of the Fg molecule from unrelated patients which show abnormal laboratory results consistent with the presence of alterations in the Fg.

Materials and Methods: Blood samples from seven patients without hemorrhagic or thrombotic history were analyzed by PCR using genomic DNA under standard conditions. Primers were designed from the sequence for each fribrinogen genes (GenBank accession numbers M64982, M64983 and M10014). Activated partial thromboplastin time (APTT), prothrombin time (PT), thrombin time (TT) and reptilase time (RT) were assayed on a semi-automatic coagulometer. Plasma functional Fg (Fg:C) was performed by Clauss method and Fg antigen (Fg:Ag) by Mancini test using specific polyclonal antibodies.

Results: All the samples analyzed showed a prolonged TT and RT compared to normal values (42 ± 15 vs. 12 ± 2 s and 39 ± 23 vs. 18 ± 3 s respectively). According to the ratio Fg: C/Fg: Ag four patients were included in the group of dysfibrinogenaemias (0.39 ± 0.22) and three in the hypofibrinogenaemias (0.83 ± 0.13) compared with normal values (> 0.75). In three patients with low levels of Fg:C we found the same mutation in the gamma chain (Phe281Leu). In the remaining patients the gamma chain showed a described mutation (Arg275Hys) and in other cases new mutations were found. Alpha and beta chain mutation were absent in all the patients analyzed.

Discussion: New and known mutations in the gamma chain of Fg were found in our cohort of patients from Cordoba, Argentina and the most important finding was associated with the presence of Phe281Leu mutation associated with low levels of Fg.

PB 2.57-6

Stream with high shear rate decreases tensile strength of plasma clots

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Coagulation problems are very important for many diseases and their treatment. In natural conditions clotting mostly takes place into moving blood, but there is very little information in publications about influence of a stream on properties of the clot and thrombus.

The aim of our work was to study if the stream conditions can influence on tensile strength of plasma clots.

The samples of blood were obtained from 30 volunteers. Centrifugation was used to get plasma. Clotting took place into the cell of the special stretching measure equipment, where plasma had been flowing before stop as the result of coagulation. After that cylindrical plasma clot (diameter 6 mm) was slowly stretched before rupture and critical force was registered. The clots formed in unmoving plasma were used

as 'the control'. Two regimes of plasma stream were examined: (i) average shear rate was 45.4 + 15.8 (1/s); (ii) average shear rate was 55.1 + 23.0 (1/s). These speeds are natural for venous blood stream. In control group the rupture force equaled 177 + 53 mN. In group A it was less on 13 + 10 mN (authenticity coefficient 1.3), and in group B it was less on 22 + 9 mN (authenticity coefficient 2.44).
Thus the clots formed in flowing plasma were shown to have less tensile strength than ones formed in unmoving substrate.

PB2.58 – Other Coagulation Factors – II

PB 2.58-1

Two novel mutations in the gamma carboxylase gene causing VKCFD1

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Background: Post-translational conversion of glutamic acid (Glu) in gamma-glutamyl carboxyglutamic acid (Gla) is an essential step in biosynthesis of biologically active vitamin K-dependent coagulation factors including factor II, VII, IX, X, Protein C, and Protein S, as well as active calcium binding proteins matrix Gla protein (MGP) and osteocalcin (bone Gla protein, BGP). This conversion is catalyzed by the microsomal enzyme gamma-glutamyl carboxylase (GGCX) which requires vitamin K hydroquinone as a cofactor. The process generates equimolar amounts of vitamin K 2,3-epoxide, which in turn is reduced by vitamin K 2,3-epoxide reductase complex subunit 1 (VKORC1) to vitamin K quinone and further to vitamin K hydroquinone in the vitamin K cycle. Combined deficiency of these factors (VKCFD) is caused by defects in either GGCX or VKORC1 and represents a very rare autosomal recessive disorder.
Methods: Direct sequencing of VKORC1 and GGCX genes was applied for mutation detection in a 13 year old girl originating from Germany. Vitamin K dependant coagulation factor activity was significantly diminished. Furthermore, the girl was presented with atrial septal defect, supra valvular pulmonary stenosis, cochlear hearing loss, hypertelorism, short-neck, flat nasal bridge, and underdeveloped ear helix. Treatment with 20 μg vitamin K/day results in mostly normal VKD coagulation factor activity. This study was approved by the local ethics committee and informed consent was obtained prior to genetic analysis.
Results: In our patient, two compound heterozygous mutations in GGCX gene were detected (p.Ser284Pro; p.Trp315*). Thus, VKCFD1 can be diagnosed as cause of combined vitamin K dependant coagulation factor deficiency.
Conclusions: Due to its type, the nonsense mutation p.Trp315* must be considered causative for the observed phenotype. The missense mutation Ser284Pro to date has also not been reported, the sequence around Ser284 is highly conserved and therefore functional relevance is likely. Indeed, this region forms part of the interface of the fourth transmembrane helix and the second ER-lumenal loop. As the resulting proline due to its structure is known as a 'helix-breaker', the observed mutation might result in a disturbed topology of this enzyme. The reported defects in heart, bone, and cartilage development most probably are result of missing gamma-carboxalation of MGP, BGP, and periostin. Treatment with high doses of vitamin K normalises function of vitamin K dependent coagulation factors.

PB 2.58-2

Real-life use of high and standard initial doses of activated recombinant Factor VII (rFVIIa) in patients with haemophilia A and B with inhibitors – data from the UKHCDO/NHD registry

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Background: Activated recombinant FactorVII (rFVIIa) is an established treatment for bleeding episodes in haemophilia patients with inhibitors. In the United Kingdom, rFVIIa is recommended at standard (3 × 90 μg/kg) or high (1 × 270 μg/kg) doses, the latter providing more rapid but equally effective control of bleeding. When licensing the higher dose,EMA requested post-marketing surveillance for thrombosis in patients receiving high-dose regimens.
Aims: A post-marketing surveillance study was conducted by the UK National Haemophilia Database to assess the use and safety of low and high initial rFVIIa doses.
Methods: The UK National Haemophilia Database (NHD) collected treatment and safety data prospectively on patients treated with rFVIIa from UK centres and submitted this quarterly to Novo Nordisk for transmission to EMA between 1/1/08 and 30/6/11. Safety data included adverse drug reactions. Dosage was decided by the managing clinician. The initial rFVIIa dose was categorised as low (≤ 90 μg/kg), intermediate (90–180 μg/kg) or high (180–270 or ≥ 270 μg/kg). A single-dose regimen was defined as one dose per >26 h. The study was conducted in accordance with the Declaration of Helsinki and was terminated when the EMA were satisfied.
Results: In total, 139 patients received rFVIIa for 1356 treatment episodes. Sixty-seven patients with haemophilia A or B were treated for 1057 (78%) episodes (median 7.0 episodes/patient, range 1–124). Ninety-nine percent (1048) of episodes occurred in inhibitor patients. Most (1009 episodes) were treatments, on demand, for non-surgical bleeding. A high initial rFVIIa dose (≥ 180 μg/kg) was used for 406/1057 (38%) of episodes, while 51% of episodes were treated with lower rFVIIa doses (≤ 90 μg/kg, 16%; 90–180 μg/kg, 35%; incomplete data, 11%). Higher initial doses tended to be used in haemophilia patients compared with other bleeding disorders (median 146.3 vs. 39.1 μg/kg; mean 178.4 vs. 60.0 μg/kg). Forty-seven (70%) patients and 539 (51%) episodes were treated with a single dose of rFVIIa, representing the most frequently-used regimen. In patients with any bleeding disorder, a single rFVIIa dose was ≥ 180 μg/kg suggesting a reduced need for repeat dosing when higher-dose regimens are used. The duration of treatment (median 2.5 h, range 2.5–1616.5) and number of required doses (median 1; range 1–88) were also lower when initial doses ≥ 180 μg/kg, were used compared with lower doses (90–180 μg/kg: median duration of treatment 5.1 h [range 2.5–2498.7], median number of required doses 2 [range 1–131]). No adverse drug reactions, including thromboembolic events, disseminated intravascular coagulation or anti-rFVIIa antibody formation, were reported in any patient.
Conclusions: Real-life data for the use of rFVIIa in patients with haemophilia with inhibitors shows that a single dose is the most frequently used regimen, especially with higher initial doses, and that the use of higher initial dosing is associated with reduced treatment duration. No thromboses or adverse drug reactions were reported for any rFVIIa treatment or dosage. These data suggest that a high rFVIIa dose is effective for treating bleeding episodes, decreases the number of doses and duration of treatment without compromising safety.

PB 2.58-4

Recovery of factor XIII and thromboelastometry during pregnancy in a case of severe factor XIII deficiency

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Background: Severe factor XIII (FXIII) deficiency is a rare congenital disorder associated with bleeding and spontaneous abortion, unless replacement therapy is initiated early in pregnancy. Based on case reports the recommendation is to maintain a FXIII level above 10% from 5 weeks of gestation, but the ideal dose to secure pregnancy remains to be elucidated. *In vitro* studies using global coagulation assays such as thromboelastometry show significant alteration of coagulation kinetics in FXIII-spiked whole blood. Thromboelastometry may provide additional useful information on clot formation and stability in FXIII-deficiency, however, published data are sparse.

Aims: In a case of severe FXIII deficiency, we report the usefulness of thromboelastometry in addtion to FXIII recovery measurements in the successful management of pregnancy and delivery.

Methods and Results: A 31-year old woman weighing 95 kg with a typical history of recurrent miscarriages was referred to our department for coagulation analyses. She was diagnosed with severe FXIII subunit A deficiency with a FXIII antigen level of < 1% (HemosIL™). At the time of diagnosis she was pregnant gestation week 8 with discrete vaginal bleeding and replacement therapy with plasma derived FXIII (Fibrogammin®) was started immediately. Bleeding ceased, and there were no further complications. Initially, the patient visited the outpatient clinic every week with repeated evaluation of recovery by measurement of FXIII antigen level. The suggested starting dose of 250 ie FXIII weekly had to be increased to 500 ie before an acceptable trough level close to 10% was achieved. Subsequently recovery was performed every second week. From gestation week 27 the dose was further increased to 750 i.e. weekly. Due to the increasing FXIII demands and a continuous propensity for epistaxis, whole blood thromboelastometry (ROTEM®) was performed before and after *in vivo* FXIII replacement in parallel with FXIII recovery in week 33. Standard assays in addition to a low tissue factor assay was applied and normal whole blood clot formation and stability was observed following *in vivo* replacement in concordance with the FXIII recovery. Hence, the dosing was considered safe and maintained unchanged. Before delivery by sectio gestation week 40 the patient received 1500 i.e. of FXIII, and there were no excess bleeding.

Conclusion: The required dose of FXIII replacement to maintain FXIII levels above 10% was higher than reported in most cases, probably due to the patients increased bodyweight. As expected dose requirements increased during the pregnancy. These observations underscore the necessity of individualized coagulation monitoring during the entire pregnancy. Furthermore, evaluation by thromboelastometry aided dose adjustment and is a useful supplement to FXIII antigen measurements.

PB 2.58-5

Health-related quality of life in hemophilia patients with inhibitors receiving prophylaxis with anti-inhibitor coagulant complex (AICC): results from AICC prophylaxis study

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Background: Hemophilia patients with inhibitors face significant challenges in controlling bleeds and are at high risk for arthropathy which may impact health-related quality of life (HRQoL).

Aims: This analysis aimed to determine whether prophylactic treatment with FEIBA (AICC) for hemophilia patients with inhibitors improved HRQoL compared to on-demand treatment as well as the impact of annual bleed rates (ABRs) on HRQoL.

Methods: A one-year prospective, randomized, open-label and parallel clinical study was conducted to compare the efficacy, safety, and HRQoL of prophylactic vs. on-demand treatment in hemophilia A or B patients with high-titer inhibitors and ≥ 12 bleeds a year. All patients gave informed consent and the study was conducted according to the Declaration of Helsinki and its amendments. Patients were randomized into two study arms: prophylaxis 85 ± 15 U/kg every other day vs. on-demand over a course of 12 months. HRQoL was assessed using the EQ-5D (age ≥ 14 years), hemophilia-specific QoL instruments: Haem-A-QoL for adults (age ≥ 16 years) and Haemo-QoL for pediatrics (age < 16 years), and Visual Analogue Scale (VAS) for Pain (age ≥ 12 years). HRQoL was collected at baseline, 6-month and termination for each arm. HRQoL changes from baseline to termination were compared between the two arms using the Student t-test and were interpreted as being clinically meaningful if they exceeded their respective minimally important difference (MID) threshold. Regression for repeated measure using data at baseline, 6-month and termination was conducted to assess the impact of ABRs on physical HRQoL.

Results: Thirty-six subjects (mean age = 27.4 years) met the study criteria, of these, 17 received prophylaxis and 19 received on-demand treatment. After 12 months of follow up, patients on prophylaxis reported a mean improvement of 0.075 on EQ-5D index score and 15.7 on EQ-5D VAS, both larger than the established MIDs for these measures (0.07 and 7, respectively). Little change in EQ-5D scores was seen in the on-demand arm. The EQ-5D health profile showed that a larger percentage of patients reported improved function compared to the on-demand patients, particularly in mobility (16.7% vs. 6.3%), self-care (25.0% vs. 6.3%), and usual activities (25.0% vs. 12.5%). With regard to Haem-A-QoL, prophylaxis, not on-demand, patients reported improvement in physical HRQoL (21.9) and overall HRQoL (9.5) larger than estimated MID thresholds (11.4 and 7.7, respectively). For Haemo-QoL, analysis was limited due to the small number of pediatric patients who completed the questionnaire. In addition, prophylaxis patients showed a larger reduction in general pain as compared to on-demand patients (23.2 vs. 5). While no statistically significant differences in HRQoL were found between the treatment arms, this study was not statistically powered to detect the difference in these parameters. When controlling for age and time, ABR was found to have significant negative impact on hemophilia-related physical HRQoL ($P < 0.05$). Patients experienced higher ABRs had significantly worse physical HRQoL than those with lower ABRs.

Conclusions: Patients on prophylaxis showed greater improvement in HRQoL than those who received on-demand treatment. In addition, reduced ABRs led to better physical HRQoL among hemophilia patients with inhibitors.

PB 2.58-6

Plasma levels of factor VIIa-antithrombin complex in normal pregnancy and in patients with pre-eclampsia

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Background: Increasing tissue factor (TF)-dependent activity has been reported to occur both during normal pregnancy and in patients who develop pregnancy complications (e.g. pre-eclampsia (PE)). During normal pregnancy there is a progressive increase in TF dependent activity as the pregnancy advances making it difficult to differentiate the advance of normal pregnancy from the development of pregnancy complications like PE. Simple diagnostic tests are needed that may give early warning of developing complications so that earlier treatment can be initiated. At present no sensitive routine tests for TF activity is available, probably as a consequence of the low concentrations of TF in the circulation. In normal subjects, TF activity is considered to be < 10 pM, much lower than the sensitivity of any present available routine assay. One approach that could be used is to use an assay that can indirectly reflect increasing TF activity. One candidate for this may to measure the plasma levels of factor VII-antithrombin complex (FVIIa-AT). FVIIa-AT has been shown to reflect the degree of intravascular exposure and production of TF and may thus have some utility in monitoring pregnancy.
Aim: A case-control study to evaluate plasma levels of FVIIa-AT and FVIIa during normal pregnancy and in patients with PE.
Methods: One hundred and five pregnant women referred to the Section of Maternal Foetal Medicine of University Hospital of Padua, were entered into the study. These included $n = 30$ women in the first (T1), $n = 30$ in the second (T2), $n = 30$ in the third (T3) trimester of pregnancy and 15 women with PE. As controls samples were collected from 30 normal non-pregnant women. Samples were only collected after obtaining informed consents from the patients.
FVIIa-AT complex levels were determined in plasma using a specific ELISA and FVIIa measured using a clot based assay based on activation by truncated TF (both from Diagnostica Stago, Asnieres, France).
Results: Plasma FVIIa-AT complex levels (mean ± standard deviation, SD) were significantly higher in pregnant (119 ± 24pM) than in healthy (103 ± 18pM, $P = 0.001$) women. FVIIa-AT increased with advancing pregnancy but the increase did not reach statistical significance. No significant difference in FVIIa-AT levels between women during T3 and with PE was observed. FVIIa levels were stable through out pregnancy but were found to be lower in PE (this decrease did not reach statistical significance). Women with PE had significantly higher FVIIa-AT/FVIIa ratio than women during T3 and this change was statistically significant (2.01 ± 0.44 vs. 1.50 ± 0.29, $P = 0.001$).
Conclusion: Increased levels of FVIIa-AT were found in normal pregnancy reflecting the expected increase in TF activity. Using either the FVIIa-AT or FVIIa assay alone, could not be used to discriminate patients with PE from advancing normal pregnancy. This study did show that the FVIIa-AT/FVIIa ratio may have clinical utility for detection of PE. Further studies are needed to confirm our results and to see if the increase in FVIIa-AT/FVIIa ratio has clinical utility in predicting the occurrence of PE. Studies are also needed to see if this method can detect developing PE at an earlier stage than present methods.

PB2.59 – Regulation of Coagulation and Fibrinolysis – II

PB 2.59-1

Acute atherothrombotic stroke is not associated with systematic enhancement of thrombin generation

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Background: Thrombosis and atherosclerosis are major contributors to the development of ischemic atherothrombotic stroke. However thrombosis is more than a local reaction leading to the obstruction of the atherosclerotic vessel or the embolization of the vessels above the atherosclerotic lesion. We hypothesised that arterial thrombosis triggered by plaque rupture is related to a generalized hypercoagulable state. In the present study we explored the evolution of thrombin generation in acute ischemic stroke patients.
Methods: We enrolled 21 patients with ischemic stroke. Eligibility criteria were: age > 18 years, first-ever in life stroke of atherosclerotic origin, symptom onset < 3 h before admission. *Exclusion criteria*: non atherothrombotic stroke (i.e. lacunar, cardioembolic,cryptogenic), liver or renal insufficiency, active cancer and recent chemotherapy, rheumatoid disease, VKA, UFH or LMWH treatment during the last 15 days, contraindications to antiplatelet treatment. Platelet Poor Plasma (PPP) samples were tested with Calibrated Automated Thrombogram using PPP-5pM TF reagent. Samples were obtained at the following time-points: T1: on admission (after the confirmation of the diagnosis of acute stroke); T2: 12 h and T3: 24 h after initiation of aspirin; T4: at discharge from the stroke unit at 10 days after treatment initiation. Patients received prophylaxis LMWH after T3. The control group consited of 21 age and sex-matched healthy individuals. The endogenous thrombin potential (ETP), the Peak and the mean rate index (MRI) of TG were analysed.
Results: At inclusion (T1), thrombin generation was not significantly different between acute stroke patients and the control group. At time points T2, T3 and T4 thrombin generation was not significantly different as compared either to T1 or to the control group. s well as was similar between controls and patients at time-points 1, 2 and 3. At time point 4 a significant increase of ETP was observed, compared to the control group and to the previous time points. A significant increase was observed for the Peak of thrombine at time point 1 and 4. TtPeak was slightly shorter at time-point 1. The mean rate index (MRI) of the propagation phase of thrombin generation was significantly lower in patients (at each studied time point) compared to the control group.
Conclusion: The present study demonstrates that patients with atherothrombotic stroke do not present biological signs of activation of blood coagulation either at the acute phase or during the hospitalization period. These data support the concept of the preponderance of platelet activation in the pathogenesis of acute atherothrombotic stroke.

PB 2.59-2

A novel methodology for modulation of fibrin clot lysis: the role of fibrinogen-targeted artificial binding proteins

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Background: Cardiovascular disease (CVD) remains the main cause of morbidity and mortality in the developed world. Fibrin clot structure and fibrinolysis can determine predisposition to CVD and manipulating the prothrombotic environment can reduce the risk of vascular events. However, current treatment strategies are only partially effective at reducing the thrombotic milieu, and frequently associated with adverse events secondary to bleeding. Therefore, identification of new therapeutic targets is necessary to reduce thrombosis potential in high risk subjects.

Aims: The aim of the research was to utilise an artificial binding protein (adhiron) phage display library to screen fibrinogen for binding peptides that could interfere with clot lysis, in particular interaction with plasminogen inhibitor (PI).

Methods: A phage display library consisting of two loops of nine amino acids, constrained in a protein scaffold was used to screen against fibrinogen (library size: 10^{10} adhirons). After multiple rounds of panning, high affinity binding adhirons were released by the addition of excess PI. To determine if the adhirons were acting at functionally relevant sites, fibrinogen-binding adhirons were tested in turbidimetric assays using plasma and purified systems. Time from full clot formation to 50% lysis was taken as clot lysis time. In a plasma system, adhiron/fibrinogen molar ratio was kept at 5:1, whereas this ratio was reduced to 2:1 in the purified system.

Results: Numerous adhirons were seen to bind fibrinogen and therefore only those with the highest binding affinities were tested, including a total of eight adhirons. In a plasma system, time to 50% clot lysis was 840 s, with four adhirons increasing this by a mean of 894 s (range 144–1872). One adhiron abolished clot lysis, another adhiron reduced clot lysis by 48 s whereas two had no effect. We then tested adhirons that prolonged, reduced and had no effect on plasma clot lysis in a purified system, both in the presence and absence of PI.

In a purified system and in the absence of PI, adhiron A2 prolonged clot lysis from (mean ± SD) 648 ± 34 to 996 ± 85 s ($P < 0.05$), whereas adhirons G2 and G4 had no significant effect at 708 ± 51 and 672 ± 34 s, respectively. Clot lysis time in the presence of PI was 1332 ± 85 s, which was further increased to 2016 ± 68in the presence of adhiron A2, whereas G4 had the opposite effect with clot lysis time reduced to 1152 ± 68 s ($P < 0.05$). G2 had no significant effect on PI-mediated prolongation of clot lysis (1356 ± 51 s).

Summary: The use of a phage display system allows rapid screening of proteins with numerous random peptides for the investigation of coagulation protein interactions. In this instance the interactions between fibrinogen and PI were analysed. We discovered two adhirons that bind to fibrinogen at functionally active sites and were capable of enhancing and reducing the effects of PI respectively. These adhirons could be the focus of novel therapeutic agents for thrombotic disorders and may even be beneficial in those with bleeding tendencies.

PB 2.59-3

Bradykinin enhances sympathetic nerve-induced cardiac tPA release possibly by transactivation of the β2-adrenergic receptor

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Background: Both infusion of the endogenous vasodilator bradykinin (BK) and stimulation of cardiac sympathetic nerves (SS), induce cardiac tPA release. Electrophysiological studies on transfected frog oocytes have shown that BK-receptor 2 (BK_2R) and β2-adrenergic receptors (β_2-AR) form heterodimers which may allow cross-talk between the receptors, and transactivation of β_2-AR by BK (KK Haack et al, 2010). However, clinical consequences of this receptor interaction have so far not been shown.

Aims: In the present study in pigs, we investigated *in vivo* the interplay between BK and SS stimulation on cardiac tPA release, and furthermore if ß-blocker had an effect on this potential interaction. Finally, we examined interaction between BK_2R and β_2-AR in left ventricular myocardium (LV).

Methods: Six pigs were subjected to 4 min of electrical SS_1. Thirty min later, 9 min of coronary BK_1 infusion was given, and the last 4 min with simultaneous SS_2 (BK_1/SS_2). Subsequently, β-blocker (propranolol) was given and the stimulations were repeated (BK_2 and BK_2/SS_3). Blood was collected frequently and simultaneously from a shunt in the adjacent coronary vein and from the femoral artery, and cardiac tPA release was determined. Mean of three samples in each stimulation period was used to estimate total tPA release. Co-immunoprecipitation studies in lysates from isolated LV biopsies were performed using antibodies against β_2-AR and BK_2R.

Results: SS_1 induced a 6.3 ± 2.2-fold increase in tPA release compared to baseline, whereas BK_1 induced a 13.2 ± 4.8-fold transient increase in tPA release with return to baseline (at 3 min) despite continuing BK infusion, probably due to desensitization. At this point of BK-desensitization, SS_2 combined with continued BK infusion induced 13.5 ± 4.7-fold increase in cardiac tPA release, an increase of 2.3 ± 0.3-fold as compared to SS_1. Following β-blockade, BK_2 and BK_2/SS_3-induced tPA release was not significantly different from baseline release. BK_2R co-precipitated with β_2-AR demonstrating interaction between BK_2R and $ß_2$-AR in LV.

Summary/Conclusions: The present study demonstrates physical interaction between BK_2R and $ß_2$-AR in pig LV myocardium. Despite desensitized BK_2R *in vivo*, BK still enhanced SS-induced cardiac tPA release indicating BK-transactivation of β_2-AR. β-blocker seemed to inhibit both BK and combined BK/SS-induced tPA releases. Importantly, this is the first *in vivo* study showing interplay between SS and BK stimulation through BK-transactivation of $ß_2$-AR, i.e. heterodimerized with BK_2R, thus leading to enhanced SS-induced cardiac tPA release.

PB 2.59-4

Processed vs. pooled plasma enhances thrombin generation and reduces tissue factor pathway inhibitor

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Background: Standardized solvent/detergent treated pooled human plasma (Octaplas®, Octapharma) is indicated for the management of bleeding patients requiring replacement of multiple plasma coagulation factors, for patients with coagulation deficiencies due to hepatic disease, for patients undergoing cardiac surgery or liver transplantation, and for the treatment of thrombotic thrombocytopenic purpura (plasma infusion or plasma exchange). Severe protein S deficiency is a contraindication for administration of plasma, the reason being historical coincidence of thromboembolic complications. However, the mechanism behind this contraindication remains uncertain, as the levels of natural anticoagulants in Octaplas® are reported to represent normal references.

Aim: Our aim was to study thrombin generation capacity and the content of tissue factor pathway inhibitor (TFPI) in various plasma samples.

Methods: As plasma samples Octaplas®, standard human plasma (SHP, Siemens) for clinical chemistry grade and pooled unprocessed

plasma (PP) (both fresh and freezed) were used. Thrombin generation was measured with calibrated automated thrombogram (CAT, Thermo Labsystems) in the absence of corn trypsin inhibitor and free and total TFPI antigen with specific ELISA (Asserachrom® Free TFPI, and Total TFPI, Stago). The lipid content regarding total cholesterol, triglycerides (TG), low (LDL) and high density lipoproteins (HDL) and protein S free antigen and protein C activity were measured with routine techniques and APTT with coagulometer (Amelung KC4, Sigma) using Actin FSL reagent (Siemens).

Results: APTT baseline results were similar, 35, 30 and 30 s in Octaplas®, SHP and PP, respectively. In CAT, however, all variables indicated enhanced thrombin generation in Octaplas®; time to peak (ttpeak) was 4 s and peak 280 nM, in SHP ttpeak was 7 s and peak 225 nM, while in PP ttpeak was 8 s and peak only 155 nM (65% of that with Octaplas®). Total TFPI concentration was compatible with the CAT data. In PP total TFPI was highest, 53.7 ng/mL and free TFPI was 12.5% of the total. In Octaplas® (two batches, blood group O and A) total TFPI was 60% less, being 33.4 and 36.7 ng/mL, respectively, but free TFPI was 25% of the total. In SHP total TFPI was 45.7 ng/mL free TFPI representing 25%. The lipid content was the lowest in Octaplas® (TG 0.65; LDL < 0.1; HDL 0.49 mM) in comparison to SHP (TG 1.02; LDL 2.0; HDL 0.97 mM) and PP (TG 0.86; LDL 2.5; HDL 1.55 mM). Protein S free antigen and protein C activity were 81% and 103% in Octaplas®, 90% and 98% in SHP and 95% and 125% in PP, respectively.

Conclusions: Octaplas® demonstrated most thrombin generation and least TFPI antigen concentration in comparison with SHP and pooled plasma. This finding may bear implications in patients with deficiencies of natural anticoagulants, and suggest that Octaplas® units are effective already at smaller quantities.

PB 2.59-5

Resveratrol and its dimers down-regulate protein S mRNA expression in HepG2 cells

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Background: Protein S is an anticoagulant factor that acts as a cofactor of activated protein C and its plasma level is decreased in high-estrogen conditions such as pregnancy and oral contraceptive use. Protein S deficiency is known to be genetic as well as environmental risk factor for venous thromboembolism. Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene), a phytoestrogen in red wine and various plants, has been reported to possess many health benefit effects including anti-atherosclerotic, anti-cancer and anti-aging effects. We previously demonstrated that resveratrol down-regulates protein S expression in HepG2 cells independently of estrogen receptor (Hiroto Y. et al. Thromb Res 2011;127:e1-e7).

Aims: This study aimed to determine whether the newly synthesized resveratrol dimers, having no affinity with estrogen receptor, affect protein S expression in HepG2 cells.

Methods: The resveratrol dimers, UHA4002 and UHA4003, were prepared by heat-treatment of resveratrol in alkaline solution, followed by purification with reverse-phase HPLC and a LH-20 gel-column. The structure of the dimers was determined by the physical data of nuclear magnetic resonance and mass spectrometry. HepG2 cells were incubated with resveratrol and resveratrol dimers, and then the mRNA expressions of protein S, protein C and b chain of C4b-binding protein (C4BP-b) were analyzed by reverse transcription-polymerase chain reaction using a Thermal Cycler Dice Real Time System (Takara).

Results: One hundred mM resveratrol down-regulated protein S mRNA expression in HepG2 cells to about 30% of control (0.1% DMSO); however, protein C and C4BP-b mRNA expressions were not affected, as previously reported. At a concentration of 10 mM, both UHA4002 and UHA4003 down-regulated protein S mRNA expression to 40–50% of control; while resveratrol showed no effect. Although C4BP-b mRNA expression was not affected by resveratrol dimers, protein C mRNA expression was inhibited by 10 mM UHA4003.

Conclusion: Both resveratrol dimers down-regulated protein S mRNA expression in HepG2 cells at a concentration of less than one-tenth of resveratrol, furthermore, UHA4003 suppressed protein C mRNA expression. These two dimers conserve the two hydroxyls at carbon-3 and -5 of resveratrol, which may be prerequisite for the protein S mRNA suppression by resveratrol and its dimers.

PB 2.59-6

Twelve weeks of daily exercise reduces thrombin generation in young, overweight males

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Background: Physical activity has several acute effects on blood coagulation. Notably, enhancement of coagulation can be detected immediately after exercise. Much less is known about the effects of long-term exercise programs on coagulation variables, some of which are risk markers for cardiovascular disease (CVD). Obesity is an important risk factor for CVD, and treatment of obesity is challenging and the results often disappointing. Therefore, individuals who are overweight but not yet obese constitute a subpopulation that undoubtedly has a potential of benefit regarding primary CVD prevention.

Aims: We hypothesized that 12 weeks of strictly controlled exercise with-out diet modification would induce changes in thrombin generation in plasma from moderately overweight subjects. The present study aimed to quantify these changes and investigate whether the effects of exercise on thrombin generation were dose-dependent.

Methods: A total of 61 overweight (BMI: 25–30, percentage body fat: ≥ 25%) and sedentary (VO_2 max < 45 mL/min/kg) male subjects between 20 and 40 years were randomized to one of three groups. One group was instructed to perform aerobic exercise corresponding to 600 kcal/day (~60 min/day, HIGH), another to perform aerobic exercise corresponding to 300 kcal/day (~30 min/day, MOD) and the last to maintain their habitual lifestyle (CON). Three times per week, the subjects in the HIGH and MOD groups exercised at an intensity above 70% of VO_2 max. The subjects were monitored throughout the program and instructed not to change their dietary habits. Fasting venous blood samples were collected before and after the intervention. Thrombin generation was assessed by the automated calibrated thrombogram method.

Results: The 12-week intervention was completed by 53 individuals, who showed excellent training compliance (MOD: 98.9%; HIGH: 96.1%). There were no significant differences in the measured variables between the three groups at baseline. The exercise-induced reduction in body weight (MOD: −3.6 kg, $P < 0.01$; HIGH: −2.7 kg, $P < 0.01$) and increases in aerobic capacity were significant in the HIGH and MOD, but not the CON group. The endogenous thrombin potential (ETP) was significantly reduced in the HIGH (2164 nM*min vs. 2001 nM*min, $P = 0.038$, $n = 18$) and MOD (2224 nM*min vs. 2044 nM*min, $P < 0.01$, $n = 18$), but not in the CON (2144 nM*min vs. 2179 nM*min, $P = 0.24$, $n = 17$) group. The changes in ETP were not significantly different between the HIGH and MOD group (−163 vs. −180 nM*min; $P = 0.85$). However, when the CON group was compared with the HIGH and MOD group combined, the change in ETP differed significantly (33 vs. −174 nM*min, $P = 0.02$).

Conclusions: We found that young, overweight men following an intensive exercise program for 12 weeks had a significant reduction of around 8% in ETP. The reduced coagulability is of clinical interest,

since it could reflect a reduced risk for CVD. Notably, this effect was not dependent on dose of exercise, since the reductions in ETP in the HIGH and MOD group were similar, but different from the CON group. Further studies are needed to elucidate whether the observed effects can be ascribed to exercise alone or the induced weight loss.

PB2.60 – Cancer and Thrombosis – V

PB 2.60-1

High resolution transcriptomic analysis of trousseau syndrome

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Background: As first described by Armand Trousseau in 1865, there is a strong association between cancer and venous thromboembolic disease. This association has been validated in several studies demonstrating high incidence of malignancy in individuals who present unprovoked venous thromboembolism. Although this association has been validated by recent studies, its underlying molecular mechanisms remain elusive.

Aims: In this study we have examined the association between cancer and venous thromboembolism with the objective to determine whether idiopathic thromboembolism, cancer, cancer plus thromboembolism cases and healthy controls have a specific molecular signature characterizing each of these groups.

Methods: One hundred and twenty blood samples (30 idiopathic venous thromboembolism, 30 epithelial cancer, 30 epithelial cancer plus venous thromboembolism and 30 healthy controls) were recruited in this study. Peripheral blood samples were collected in PAXgene tubes. Total RNA was isolated by PAXgene Blood RNA Kit according to manufacturer's instructions. RNA quantity was assessed spectrophotometrically (NanoDrop). RNA integrity was checked on Agilent Bioanalyzer using RNA 6000 Nano Kit. cRNA samples were prepared using Affymetrix GeneChip 3′ IVT Kit. RNA samples were hybridized to Affymetrix GeneChip HGU-133 Plus 2.0 arrays. CEL files were analysed by dCHIP software. Differentially expressed gene lists were obtained for each comparison after the quality control, preprocessing, variance filtering steps. Functional annotation cluster and pathway analyses were conducted on DAVID bioinformatics resources.

Results: We have determined differentially expressed gene sets for each pairwise comparison using rigorous bioinformatic analyses. The number of genes in these sets was 278 for Cancer vs. Control, 98 for Cancer vs. Idiopathic Venous Thrombosis, 217 for Cancer plus Thrombosis vs. Control, 122 for Idiopathic Venous Thrombosis vs. Cancer plus Thrombosis, 331 for Idiopathic Venous Thrombosis vs. Control, 147 genes for Cancer vs. Cancer plus Thrombosis groups. Hierarchical cluster analysis showed that these gene sets were able to distinguish four sample groups from each other. Pathway and gene ontology analyses demonstrated that the genes in differentially expressed gene sets mostly belong to cancer, spliceosome, Jak-Stat signaling pathway and Leukocyte migration pathways.

Conclusions: In this exploratory study, we have identified specific gene expression profiles that are able to distinguish the four sample groups from each other. The ongoing confirmatory experiments along with gene ontology and pathway analyses of these gene expression profiles will shed light on the pathology of Trousseau syndrome.

PB 2.60-2

Estrogens downregulate TFPI expression in breast cancer cells

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Background: Estrogens influence the pathology and development of hormone-sensitive cancers such as breast, ovarian and endometrial cancers. Tissue factor pathway inhibitor (TFPI) is a Kunitz-type serine protease inhibitor of the extrinsic coagulation pathway and has recently been associated with breast cancer cell development. Moreover, low levels of TFPI have been detected in the plasma of healthy post-menopausal women receiving hormone replacement therapy (HRT), indicating a possible link between estrogen and TFPI.

Aims: To investigate how estrogen and estrogen analogues may regulate TFPI expression.

Methods: Estrogen receptor alpha (ERα) expressing cells (MCF-7) and ERα negative cells (MDA-MB-231) were treated with estrogen and different estrogen analogues and the relative expression of TFPI mRNA and protein was measured using qRT-PCR and ELISA, respectively. Transient ERα overexpression in MDA-MB-231 and downregulation in MCF-7 cells was induced by cDNA and siRNA transfections, respectively, and was followed by stimulation with estrogens. Luciferase reporter gene constructs were used to evaluate whether the effects of estrogens were mediated through the TFPI promoter.

Results: The relative expression of TFPI mRNA and protein was significantly downregulated by more than 50% after 6 h incubation following treatment with 17β- estradiol (E2) and another potent estrogen agonist, 17α-ethynylestradiol (EE2), in ERα expressing MCF-7 cells, but not in the ER negative MDA-MB-231 cells. Moreover, TFPI mRNA was unaffected by raloxifene, a selective estrogen receptor modulator (SERM). The E2 mediated downregulation of TFPI mRNA was abolished by fulvestrant, an estrogen receptor antagonist. Furthermore, the downregulation of TFPI was not dependent on newly synthesised proteins suggesting that the estrogen mediated downregulation of TFPI may be through the ERα receptor. Overexpression of ERα in MDA-MB-231 cells induced E2 mediated downregulation of TFPI mRNA. Results from on-going ERα knockdown studies in MCF-7 cells and TFPI promoter activity assays will also be presented.

Conclusions: Estrogens significantly downregulate TFPI in ERα expressing breast cancer cells and our results indicate that their effect on TFPI promoter may be primary. These findings may help explain the aggressiveness of hormone-sensitive breast cancers.

PB 2.60-3

Internalized Protein C inhibitor (PCI) reverses impaired histone modification in Jurkat T-cell lymphoma cells

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Background: Acetylation and methylation of Lys-residues in the tails of histones has a crucial role in regulation of chromatin structure and gene expression. In cancer epigenetic modification of histones is altered. A significant loss of monoacetylation of Lys16 and trimethylation of Lys20 of histone H4 has been shown in lymphoma cells (Fraga et al., 2005). So far little is known about mechanisms leading to impaired histone modification in cancer cells. A nuclear isoform of cathepsin L removes the N-terminal tail of histone H3 during differentiation of mouse embryonic stem cells (Duncan et al., 2008). This H3 cleavage by cathepsin L removes binding sites for histone modifying

enzymes: for methylation of H4Lys20, the methyltransferase Suv4-20 h has to bind to H3Lys9 (Gonzalo, 2005).
PCI is a secreted serine protease inhibitor. Low PCI expression is associated with malignant phenotype (Naggara et al., 2008). PCI inhibits cathepsin L and decreases tumor cell migration (Fortenberry et al., 2010). PCI is internalized by leukocytes and translocates to the nucleus (Baumgärtner et al., 2007).
Aims: We wanted to determine if internalized PCI inhibits the processing of histone H3 by cathepsin L in cancer cells, and thereby increases the level of trimethylated Lys20 of histone H4.
Methods: Subcellular fractionation, Western blotting, acid extraction of isolated nuclei, separation of histones by HPLC, separation of non-acetylated H4 from monoacetylated H4 by capillary electrophoresis, and estimation of the degree of methylation of these two H4 forms by mass spectrometry.
Results: PCI prevented the cleavage of H3 by cathepsin L *in vitro*, as judged from Western blots. Analysis of subcellular fractions of blood leukocytes in Western blots revealed localization of PCI antigen in the nuclei of normal lymphocytes. To assess whether PCI prevents H3 cleavage *in vivo*, Jurkat lymphoma cells, which contain very little endogenous PCI, were incubated for 2 h with 300 nM recombinant mouse PCI (mPCI) and subcellular fractions were prepared. Fractions were analyzed by Western blotting with anti-mouse PCI, allowing differentiating between endogenous PCI and internalized mPCI. mPCI was found in different nuclear fractions. Cathepsin L colocalized with mPCI in the nuclease- digested fraction and in the nuclease-resistant fraction. In these fractions an additional high-molecular weight form of cathepsin L, probably corresponding to a covalent complex of PCI with cathepsin L, was seen. In cells incubated with mPCI no cathepsin L cleavage product of H3 was detected, and histone H4Lys20 trimethylation was increased to 18.8% (nonacetylated form) compared to 7.3% in control cells. Monoacetylated H4Lsy20 showed 5.7% trimethylation in mPCI-treated cells and 2.1% in control cells.
Summary: We hypothesize that internalized PCI inhibits cathepsin L thus preventing cleavage of histone H3 and rescuing trimethylation of histone H4 at Lys20 in Jurkat lymphoma cells.

PB 2.60-4
The factor XII-driven intrinsic coagulation cascade contributes to prostate cancer-associated pulmonary embolism

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Background and Aim: Deep venous thrombosis and subsequent pulmonary embolisms are frequent and life-threatening complication in prostate cancer (PC) patients. Mechanisms of PC-driven thrombosis are poorly understood and safe strategies to interfere with cancer-driven procoagulant activity remain a major medical challenge. We report a pathway by that PC-cell released particles (prostasomes) induce thrombosis *in vivo*.
Methods and Results: Clotting analyses revealed that PC-cell derived prostasomes are highly procoagulant in human and murine plasma. Prostasome-driven procoagulant activity was defective in plasma lacking the intrinsic pathway protease factor XII or its substrate factor XI. Factor XII inhibitors interfered with prostasome-driven procoagulant activity. To analyze the *in vivo* relevance of prostasomes for venous thrombosis, we challenged genetically modified mice in a model of pulmonary embolisms. Intravenous infusion of cell and PC patient purified prostasomes triggered lethal pulmonary embolism in wild-type animals. In contrast, ablation of factors XII or XI, or combined deficiency in factors XII and XI largely protected mice from prostasome-induced lethal pulmonary embolism.
Conclusion: The data identify the factor XII-driven intrinsic coagulation pathway triggered by prostasomes as a new player in PC-associated pulmonary embolisms. Inhibition of this pathway offers a novel and safe therapeutic strategy to interfere with thrombosis in PC patients.

PB 2.60-5
Analysis of the expression of RNase in blood cells involved in homeostasis in the vascular system

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Background: Extracellular RNA may be released from cells in cases of injury, vascular disease, or malignancy in the vasculature. It has been recognized as a novel procoagulatory and permeability-increasing factor *in vivo* and *in vitro* and it is counteracted by pancreatic-type ribonuclease (RNase 1) in endothelial cells (PNAS 2007;104:6388–93, Thromb Haemost 2011;105:345–55). Based on the identification of RNase 1 in plasma and serum, it is proposed that the enzyme is expressed by vascular cells to regulate extracellular RNA. Conversely, RNase inhibitor (RI) may be expressed in a variety of cells to protect RNA against degradation by certain RNases.
Aims: Although a gene expression profile analysis of vascular vs. lymphatic endothelium revealed strong expression of RNase 1 in the vascular endothelium, no detailed characterization of the expression and functions of RNase in the vasculature has been carried out so far. We aimed to investigate the expression and function of RNases in cells which come in contact with blood, such as blood cells and malignant cells.
Methods: We investigated the expression and activity of RNase 1 and RI using RT-PCR, western blot analysis, immunocytochemical staining and RNase activity tests in various leukemia cell lines, a multiple myeloma cell line, peripheral blood mononuclear cells, platelets, and red blood cells. We compared them with the human umbilical vein endothelial cell line EAhy926.
Results: Analyzing cell lysates and supernatants, the expression of RNase 1 and RI was determined variously at the mRNA and protein levels in all blood cells. RI was ubiquitously expressed in the blood cells. RNase activity in supernatants derived from EAhy926 cells was higher than in supernatants derived from the other cells. When incubated for different times in serum-depleted culture medium, RNase activity in supernatants derived from EAhy926 cells increased progressively, but it decreased gradually in supernatants from the other cells.
Summary/Conclusions: The RNA/RNase 1 in blood cells and malignant cells may be a novel contributor to the regulation and maintenance of vascular homeostasis.

PB 2.60-6
Inhibition of TFPI increases the sensitivity of thrombin generation assay to procoagulant microvesicles

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Introduction: Patients with cancer have a 7- to 10-fold increased risk of developing venous thromboembolism (VTE). Circulating microvesicles (MVs) could be a predictive biomarker for VTE in cancer. Thrombin generation assay (TGA) is a useful technique to detect procoagulant activity of MVs. However, TGA suffers from a lack of sensitivity due to the presence of Tissue Factor Pathway Inhibitor (TFPI) in plasma.
Aims: To improve the sensitivity of TGA to tissue factor (TF) by limiting the interference of TFPI.

Methods: Serial dilutions of MDA-MB231 cells were incubated for 45 min at 37 °C to generate MVs. Samples were then centrifuged and supernatants which contain MVs were used for TGA. Normal pooled plasma was incubated with inhibitor of TFPI or was diluted twice to decrease plasma level of TFPI. Lagtime was used as a surrogate marker of TGA to detect procoagulant activity of MVs.
Results: (i) Inhibition of TFPI decreased twice the cell concentration needed for a significant reduction of lagtime and decreased 2.4-fold the intra-assay variability. (ii) Plasma dilution had no impact on the TGA sensitivity when TGA was triggered by MVs derived from MDA-MB-231.
Conclusions: Thrombin generation is a very sensitive method to study the procoagulant activity of TF-MVs. The sensitivity can be increased by inhibition of TFPI with specific monoclonal antibody against its Kunitz Domain I. A two times plasma dilution is an interesting cheaper alternative to study the procoagulant activity of MV by TGA with a good sensitivity, especially when low plasma quantities are available.

PB2.61 – Cancer and Thrombosis – VI

PB 2.61-1

Poor predictive value of the pulmonary embolism severity index in hospitalized cancer patients with acute pulmonary embolism

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Background: Active cancer increases the risk of pulmonary embolism (PE) and the development of a thromboembolic event is associated with a worse prognosis. Specific risk stratification tools are needed to optimize the management of oncological patients with PE. The PE Severity Index (PESI) and the Simplified PESI (sPESI) accurately classified the general PE population in low or high risk of mortality; however the prevalence of cancer patients did not exceed 25%.
Aims: The aim of this analysis was to evaluate the performance of PESI and sPESI in oncological patients with PE.
Methods: Consecutive patients admitted to the tertiary hospital of Varese (Italy) with an objectively diagnosed PE between January 2005 and December 2009 were included. Information on clinical presentation, diagnostic work-up, risk factors, treatment and mortality rate at 1- and 3-month follow-up was collected. This sub-analysis examines the 150 oncological patients extracted from the entire population.
Results: Mean age was 69.6 (± SD 11.6) years and 84 (56%) patients were male. Most common primary sites of cancer were lung (25.2%), colon-rectal (13.3%) and bladder (8.4%). Active cancer was present in 121 patients, of whom 47 had a metastatic disease.
Overall mortality rate was 20.7% (95%CI, 15.0–27.8%) at 1-month and 38.7% (95%CI, 31.2–46.7%) at 3-month. The high risk category was overestimated, since the PESI classified 96.7% of patients and the sPESI 100% of patients at high risk of mortality. As a result, the PESI showed a high sensitivity but a very low specificity among cancer patients (100% and 4.2% respectively at 1-month follow-up, 98.2% and 4.3% respectively at 3-month follow-up). The accuracy of these two prognostic rules was lower than in the general PE population (AUC for PESI 0.66, 95%CI 0.57–0.75, and AUC for sPESI 0.69, 95%CI 0.59–0.79, at 1-month, P for comparison = 0.401). These results were confirmed at the 3-month follow-up.
Conclusions: The results of this analysis suggest that PESI and sPESI lose their discriminatory ability when applied to a pure oncological population. The creation of a new clinical prediction rule is advisable in order to redefine the risk of death in cancer PE patients.

PB 2.61-2

A retrospective review of myeloma patients over a 6 year period to investigate the prevalence of venous thromboembolism (VTE) and the risk factors associated with it

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Background: Multiple myeloma is a malignant disorder characterized by clonal proliferation of plasma cells. VTE is one of the leading causes of death in cancer patients. Patients with haematological malignancies have the highest risk, adjusted for age and sex. Myeloma therapies such as thalidomide and lenalidomide (IMiDs) further increase the risk. There are few dedicated studies in the literature looking at the prevalence of VTE in myeloma patients. Almost all published data is extrapolated from treatment studies where they look at the incidence of thrombotic side effects in highly selected populations. Myeloma is a disease of the elderly and in this population, co-morbidities are inherent. Therefore, looking at the rate of VTE in randomised controlled trials designed to evaluate treatment efficacy may not be generalisable. Many assessment scores have been suggested to estimate the risk of VTE in the cancer setting such as the Khorana score (*Khorana et al Blood 2008*) but these have mainly been validated in solid organ tumours.
Aims: To estimate the prevalence of VTE in a 'real world' myeloma population, to assess the risk factors associated with VTE and to investigate the validity of the Khorana score to assess the risk of VTE in myeloma patients.
Methods: The medical charts of patients with a diagnosis of myeloma attending a tertiary referral centre in Ireland between Jan 1st 2007 and Dec 31st 2012 were reviewed. The pre-treatment Khorana score was then calculated.
Results: Two hundred and seventeen patients were identified with an average age at diagnosis of 65 ± 12.26 years (Range 30–89).The male to female ratio was 5:3. Myeloma subtypes included IgG 53%, IgA 24%, Light Chain 17%, Non secretory 4%, IgD 2% and IgM < 1%. The ISS stage was known for 77–28% (49/168) were stage I, 49% (82/168) were stage II and 23% (39/168) were stage III. Sixty-six percent (143) received 1–2 lines of therapy, 32% (69) received 3–4 and 2% (5) received > 4. Sixty-nine percent (149) received IMiDs and 98%(146) of these were administered with either high dose steroids or chemotherapy. Seven percent had a second malignancy. Khorana scores were follows: 1–59%, 2–36%, 3–4% and 4–1%. Overall, there were 9675 months of follow-up with an average of 44.58 ± 37 months (Range 1–231 months) per patient. Twelve percent (27) had an episode of VTE (7% PE, 5% DVT). Non-parametric tests were performed to see if there was an association between use of IMiDs, co-existing malignancy, no. of treatment courses or Khorana score and the occurrence of VTE in this population but none were significant.
Conclusions: The prevalence of VTE in this population was similar to previously published reports. Myeloma patients have many risk factors for VTE but in this population, no one was predictive. The Khorana score was not helpful in assessing risk in this population. This is likely due to myeloma patients being under represented in cancer-associated thrombosis studies. As myeloma therapies and outcomes are rapidly changing, a dedicated prospective study is *r*.

PB 2.61-3

Survival in renal cell carcinoma patients with venous tumor thrombus: a retrospective case–control study

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Background: Renal cell carcinoma (RCC) with tumor thrombus (TT) remains a challenging topic in urological oncology. Approximately 4–10% of RCC patients have tumor invasion of the venous system, with extension into the renal vein (RV) and the inferior vena cava (IVC) in 65% and 35%, respectively. Despite advances in immunotherapy and targeted therapy, the standard of care for RCC with tumor thrombus is surgical resection, including radical nephrectomy and tumor thrombectomy. While it has been well established that tumor thrombus is associated with a worse prognosis, controversy remains as to the clinical importance of the localization of tumor thrombus. Some studies have shown survival differences based on the level of tumor thrombus (e.g. RV vs. IVC involvement) whereas others did not.
Aims: Our objective was to evaluate the impact of tumor thrombus and tumor thrombus level on survival in RCC patients treated with radical nephrectomy and tumor thrombectomy.
Methods: A case-control study of patients with RCC treated at our hospital from January 1st, 2005 to July 1st, 2012 was undertaken. Imaging studies and pathology reports of all included patients were reviewed to identify TT and its location. TT was defined as the presence of vascular invasion on the pathology report or an intra-luminal filling defect in the renal, hepatic veins, or IVC (infra and supradiaphragmatic) directly extending from a renal mass detected on computed tomography or magnetic resonance imaging. The primary endpoint was overall survival during the follow-up period. Hazard ratios (HR) for death and corresponding 95% confidence intervals (CI) were obtained.
Results: From a cohort of 927 RCC patients, a total of 193 stage 3–4 patients that underwent surgery were included: 66.8% were males, median age was 62.0 years (range 22–88) and 48.2% were stage 4. Tumor thrombus was present in 108 cases (12%). TT was limited to the RV in 70.4% (76/108), infradiaphragmatic IVC in 13.0% (14/108), within the hepatic vein in 1.9% (2/108), and supradiaphragmatic IVC in 13.0% (14/108). The median survival time was 47.0 months (95% CI: 31.7–52.3) in the tumor thrombus group and 65.0 months (95% CI: 42.2–87.8) in the control group. Compared with controls, all patients with tumor thrombus had worsened overall survival [HR = 1.8; 95% CI: 1.1–2.9; $P = 0.02$]. Patients with RV involvement only did not have worsened overall survival compared to those with infradiaphragmatic IVC [HR = 2.0; 95% CI: 0.9–4.3; $P = 0.07$] and supradiaphgramatic IVC [HR = 1.6; 95% CI: 0.8–3.3; $P = 0.2$]. Further, no significant difference in survival was noted between patients with infra and supradiaphragmatic IVC involvement [HR = 1.3; 95% CI: 0.5–3.4; $P = 0.5$].
Conclusions: Patients with RCC are at high risk of tumor thrombus (12%). Patients with RCC and tumor thrombus undergoing radical nephrectomy and tumor thrombectomy have a reduced survival compared to surgical RCC patients without tumor thrombus. Localization of tumor thrombus level was not associated with worse overall survival. New therapies are needed to complement surgical intervention to improve survival among RCC patients with tumor thrombus.

PB 2.61-4

The association of folate-related gene polymorphisms with colorectal cancer risk in Koreans

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Background: Folate is important for cell division and homeostasis because it has the essential role in the synthesis of S-adenosyl-methionine, which is the methyl donor required for all methylation reactions in cells. In addition to the physiologic function of cell homeostasis, it has been investigated that folate may has important role in the carcinogenesis, especially in colorectal cancer (CRC) development.
Aims: We performed this study to investigate relationship between folate-related gene polymorphisms and CRC prevalence.
Methods: In the present study, we analyzed seven polymorphisms of *MTHFR* (677C>T and 1298A>C), *TS* (*TSER* 2R/3R and 1494 0/6 bp), and *RFC-1* (−43T>C, 80G>A, and 696C>T), in 514 healthy controls and 477 patients with CRC. The genotyping of was performed using polymerase chain reaction or polymerase chain reaction-restriction fragment length polymorphism assays.
Results: *MTHFR* 1298CC was associated with CRC in age > 62 years, female, rectal cancer, and ≥ 5 cm cancer groups. *TSER* 2R2R was connected to CRC prevalence of normotensive and non-lymph node invasion groups. *RFC-1* −43CC increased CRC risk in nondiabetic, ≥ 5 cm cancer, and lymph node invasion groups. In gene-environmental interaction analysis, *MTHFR* 1298CC with hypertension and *MTHFR* 1298CC with diabetes mellitus showed synergistic risk effects.
Summary/Conclusion: Our study suggests that the pivotal roles of folate-related gene polymorphisms on CRC development.

PB 2.61-5

Chemotherapy as a risk factor of DIC and thrombophilia in ovarian cancer patients

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Background: Patients with gynecologic cancer represent group of the highest risk of thrombotic and hemorrhage complications, especially during chemotherapy. The chemotherapy are significant contributed the risk of VTE and hemorrhage complications in cancer patients, especial with widespread malignant process. Chemotherapy stipulates for endothelium damage, direct platelet activation and reduction of fibrinolytic activity.
Purpose: To determine necessary range of laboratory tests for a high-grade estimation of haemostasis state in ovarian cancer patients undergoing chemotherapy.
Materials and Methods: One hundred and sixteen patients with ovarian cancer undergoing chemotherapy were divides at random on two groups: I group 56 has received biosimilar LMWH Hemapaxan 0.4 mL (4000 IU) before each chemotherapy curse and II group 60 has not received any anticoagulant prophylaxis during chemotherapy.
Laboratory tests: Platelet aggregation tests with different stimulators: Adrenaline, Ristocetin and ADP in various concentrations, platelet factor 4 (PF4). DIC and thrombophilia marker tests: D-dimer, TAT complexes, F1 + 2 prothrombin fragments. Fibrinolytic activity tests: determine PAI level, Protein C and S levels.
Results: Before operation thrombofilia and DIC was detected in 65% cancer patients, subcompensated forms was in 35%.
In postoperative period in 96% cancer patient were detected thrombofilia and DIC, and about third of patients has thrombocytopatia and coagulopatia. The rate of the subcompensated forms of DIC was 57%, decompensated 21%.

We have detected the sign of thrombofilia and DIC in more than 90% patient during chemotherapy. The rate of the subcompensated forms of DIC was 30%, decompensated 23%. It was observed damage of fibrinolytic activity due to iatrogenic effects of chemotherapy: reduction in proteins C and S levels, increase PAI concentration, platelets hyperaggregation in ristotetin presence.
In I group normalization of lab test results was detected during 2–3 days after chemotherapy course in comparison with II group normalisation was in 5–7 days in 22% and in 7–12 day in 58%, in 20% was not registered spontaneous normalisation.
Conclusion: Due to endothelium protection activity LMWH in ovarian cancer patients during chemotherapy significantly reduce intensity of thrombofilia and DIC. 85–90% patients with cancer of female genitals required permanent preventive anticoagulant prophylaxis.

PB 2.61-6
Prophylaxis for venous thromboembolism in patients treated for acute lymphoblastic leukemia – a systematic review

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Background: Venous thromboembolism (VTE) occurs frequently in patients with acute lymphoblastic leukemia (ALL). Reported incidences vary between 2% and 36%. Occurrence is often associated with treatment components, particularly asparaginase. The efficacy and optimal approach of VTE prevention during ALL treatment are unclear.
Aims: To investigate the efficacy and safety of systemic thromboprophylaxis, using either blood-derived products, i.e. fresh frozen plasma, cryoprecipitate or antithrombin concentrate, or anticoagulant agents, i.e. (low-molecular-weight) heparin, fondaparinux or oral anticoagulants (vitamin K antagonists), on VTE incidence in pediatric and adult patients treated for primary ALL with asparaginase therapy. Also, the impact of thromboprophylaxis on overall survival and treatment outcomes of ALL were investigated.
Methods: We systematically searched The Cochrane Central Register of Controlled Trials (CENTRAL, *The Cochrane Library*, Issue 7 2012), MEDLINE (January 1966 to August 2012; accessed via Pubmed) and EMBASE (January 1980 to August 2012; accessed via OVID). We handsearched conference proceedings and checked references of included studies. Randomized controlled trials (RCTs) that assessed the efficacy and safety of systemic thromboprophylaxis in patients treated for primary ALL with asparaginase therapy were eligible. Interventions included any dose of fresh frozen plasma, cryoprecipitate, antithrombin concentrate, (low-molecular-weight) heparin, fondaparinux or oral anticoagulants, in comparison with no intervention or placebo, or a comparison of two different interventions. Three authors independently assessed eligible articles for inclusion in the review and systematically extracted the data from selected articles. If necessary, study authors were contacted for more information. Discrepancies were resolved by discussion or with the opinion of a fourth author. Risk of bias, quality of evidence, potential heterogeneity and reporting biases were explored, a sensitivity analysis applied if applicable.
Results: Of 304 identified citations, 44 articles were selected for full-text evaluation. Cross-referencing of articles yielded another 20 articles. Finally, one RCT enrolling 109 patients fulfilled our inclusion criteria and was analyzed for our review. This study assessed a randomization between antithrombin concentrate infusions (once weekly for 4 weeks) and no intervention in children treated for primary ALL. Outcomes were symptomatic and asymptomatic thrombosis (by radiographic screening following completion of the induction phase), and bleeding events. seven of 25 analyzed children with antithrombin had thrombosis (28.0%) vs. 22 of 60 patients without antithrombin (36.7%; OR 0.67; 95% CI 0.3–2.3, $P = 0.43$). One major bleeding event (1.7%) occurred in the non-antithrombin arm vs. no major but two minor bleeds in the antithrombin arm. Impact of thromboprophylaxis on survival or ALL treatment outcomes was not assessed.
Conclusions: Only one RCT in children with ALL was identified. In this study, no statistically significant effect of antithrombin infusions was seen on the outcomes of interest, but the sample size was small with a skewed randomization ratio, and may have missed a clinically important effect. No RCTs were found addressing other blood-derived products or anticoagulants. Therefore, the efficacy and safety of thromboprophylaxis to prevent VTE during ALL treatment remain unclear. The use of thromboprophylaxis during ALL treatment, in particular during asparaginase therapy, needs to be assessed in randomized controlled trials.

PB2.62 – Antiphospholipid – III

PB 2.62-1
Myocardial ischaemia and coronary atherosclerosis in patients with antiphospholipid syndrome

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Background: In recent years it became apparent that mortality in patients with generalized autoimmune diseases like antiphospholipid syndrome (APS) and/or systemic lupus erythematosus (SLE) is mainly due to severe cardiovascular complications. Conventional risk factors for coronary artery disease do not explain this increased risk. Suggested non-coventional factors include, among others, the presence of antiphospholipid antibodies (aPL). Their elevated levels have been associated with arterial and microvascular thrombosis.
Aims: To evaluate possible influence of aPL on myocardial ischaemia assessed by single-photon emission computerized tomography (SPECT) and coronary atherosclerosis assessed by multidetector computerized tomography (MDCT) in APS patients.
Methods: SPECT (Tc 99 m sestamibi) and MDCT–based coronary calcium scoring were performed in consecutive APS patients (12 females, four males, aged 28–62, mean 47.3) without clinical signs of heart disease. Anticardiolipin (aCL) and anti-beta2 glycoprotein I ($a\beta_2$-GPI) antibodies (IgG and IgM class) were measured by ELISA and lupus anticoagulant (LA) according to ISTH guidelines.
Results: SPECT revealed myocardial perfusion defects in 12 (75%) patients: in 3 (19%) exercise-induced, and in 9 (56%) persistent. MDCT revealed coronary calcifications in 5 (31%) patients. The number of plaques ranged from 1 to 11 (median 2), volume 3 – 201.7 mm^3 (median 7), calcium scores 1.3–202.6 (median 5.7). In patients with perfusion defects or coronary calcifications ($n = 12$), as compared to patients without these pathologies ($n = 4$) the levels of IgG aCL and/or IgG $a\beta_2$-GPI antibodies were significantly higher and LA present more often (IgG aCL: 63.25 ± 23.1 vs. 19.22 ± 8.8, $P = 0.05$; IgG $a\beta_2$-GPI: 44.05 ± 21.3 vs. 11.25 ± 5.3, $P = 0.05$; LA: 10/12 (83%) vs. 2/4 (50%), respectively). No such association was seen for IgM-class antibodies.
Conclusions: In many symptom-free, relatively young APS patients SPECT shows myocardial perfusion defects and, in one third of them, coronary calcifications. These well-known cardiovascular risk markers, were associated with the presence of LA and higher levels of aCL and $a\beta_2$-GPI antibodies. In such patients advanced search for cardiovascular risk factors and proper preventive measures are warranted.

PB 2.62-2

Confirmation of the initial antiphospholipid antibody positivity depends on antiphospholipid antibody profile

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Background: The revised classification criteria for the antiphospholipid syndrome (APS) state that antiphospholipid (aPL) antibodies [Lupus Anticoagulant (LAC) and/or anticardiolipin (aCL) and/or anti β2-glycoprotein I (aβ2GPI) antibodies] should be detected on two or more occasions at least 12 weeks apart. Consequently, classification of patients' risk and adequacy of treatment may be deferred by 3 months.
Aims: In order to early classify patients risk, we evaluated whether initial aPL positive profiles were predictive of aPL persistence in patients testing positive for aPL.
Methods: Consecutive patients, referred to our Center, initially testing positive to one or more of the tests exploring the presence of aPL were evaluated again after 3 months. Tests were performed according to the latest guidelines. LAC, aCL and aβ2GPI antibodies were considered positive when their value was above the 99th percentile of 40 healthy subjects. Positive aPL subjects were classified as triple positive (LAC+, aCL+, aβ2GPI+, same isotype), double positive (LAC-, aCL+, aβ2GPI+, same isotype) and single positive (LAC or aCL or aβ2GPI antibodies as the sole positive test).
Results: During a 4-year period, of the 1520 initially screened individuals, 225 were found positive to one or more tests for aPL. Of these 161 were available for confirmation after 3 months. Patients were classified as triple positive (n = 54: LAC+, aCL+, aβ2GPI+, same isotype), double positive (n = 50: LAC-, aCL+, aβ2GPI+, same isotype) and single positive (n = 57: LAC or aCL or aβ2GPI antibodies as the sole positive test). Among subjects with triple positivity at initial testing, 98% (53 out of 54) had their aPL profile confirmed after 12 weeks. Double and single positivity groups had data confirmed in 84% (42 of 50) and 40% (23 of 57) of the individuals, respectively.
Conclusions: Our results show that patients initially testing positive for all three aPL tests (high-risk subjects with triple positive aPL profiles) remain positive after 12 weeks. An anticipation of diagnosis may be beneficial for an early decision on the type of antithrombotic treatment.

PB 2.62-3

Establishment of standardized international units for IgG anti-β2glycoprotein antibody measurement

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Background: Current classification criteria for antiphospholipid syndrome (APS) calls for 'standardized' positive lupus anticoagulant, IgG/IgM anticardiolipin (aCL) and/or IgG/IgM anti-β2glycoprotein (aβ2GPI) antibodies in the presence of classic symptoms for identification of the disease. Despite numerous efforts aimed at standardization of antiphospholipid testing there still exists considerable inconsistency.
Aims: We sought to prepare a reference preparation (RP) and to establish international consensus units (IU) for the measurement for IgG aβ2GPI antibodies.
Methods: Whole IgG fractions were affinity-purified (AP) from a blend of sera from two primary APS patients (PAPS) with high IgG aβ2GPI levels using a Protein G Sepharose column followed by an NHS-activated Sepharose column coupled to β2GPI. Purity of β2GPI AP fractions was confirmed using SDS-PAGE; fractions with high activity were pooled, concentrated and sterile-filtered. The protein concentration of the AP material was determined using spectrophotometric measurement at 280 nm and Bradford protein assays and assigned a value based on the definition that 1 IU/mL equates to 1 μg/mL of AP aβ2GPI. A reference preparation (RP) serum blended from the two original PAPS patients was assigned an IU value using original AP material as a calibrator. The RP and 30 samples were sent to six commercial companies for testing in their respective kits (eight total) according to an approved protocol to enable evaluation of linearity, unit equivalency and commutability. Companies (and kits) included INOVA Diagnostics (QUANTA Lite ® β2GPI IgG ELISA, QUANTA Flash ® β2GPI IgG chemiluminescent assay), Bio-Rad Laboratories (BioPlex ® 2200 APLS IgG Kit, Anti-β2Glycoprotein I IgG EIA Test Kit) TheraTest (EL-β2GPI IgG kit), Corgenix (IgG Anti-β2Glycoprotein I Test Kit), Phadia (EliA β2Glycoprotein I IgG) and Instrumentation Laboratory (HemosIL® AcuStar anti-β2Glycoprotein-I IgG).
Results: The pooled AP material had a protein concentration of 103.1 μg/mL (OD280 nm) and 108.8 μg/mL (Bradford) and was assigned a value of 100 IgG aβ2GPI IU/mL. RP had a value of 270 IgG aβ2GPI IU/mL. The linearity (R^2) of the RP curve for the various assays ranged from 0.9600 to 0.9983 excluding the Bio-Rad EIA test kit, which had a value of 0.8994. When a 4-PL curve was used for that assay the correlation was improved. The value of the RP in the various arbitrary kit units ranged from 115 to 9993.1 units. Commutability samples fit very well within 95% prediction intervals and had excellent correlation when comparing assays.
Conclusion: The RP demonstrates excellent linearity in the various aβ2GPI IgG assays and can be considered adequate to be used as a calibrant/reference material. Available assays will be able to use this RP material effectively since there is very good correlation of results of commutability samples among the various assays, including ELISA formats and some new assays with wider analytical measuring ranges that use different arbitrary units of measurement for calibration. New international consensus units as well as an adequate RP are now available to be used for the measurement of IgG aβ2GPI antibodies. These studies contribute significantly to the much-needed standardization of aβ2GPI immunoassays.

PB 2.62-4

An open-label prospective pilot mechanistic study of fluvastatin in persistently antiphospholipid antibody-positive patients

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Background: Antiphospholipid antibodies (aPL) induce a proinflammatory and pro-thrombotic state by upregulating the production of various cytokines, chemokines, and tissue factor (TF). Fluvastatin reduces TF expression and the thrombogenic effects of aPL as shown *in vitro* and in mice studies.
Aims: The purpose of this prospective pilot study was to examine the effects of fluvastatin on pro-inflammatory and pro-thrombotic biomarkers in persistently aPL-positive subjects.
Methods: Persistently aPL-positive patients (as per APS classification criteria) received fluvastatin 40 mg daily for at least 3 months. At 3 months, patients were instructed to stop fluvastatin and they were followed for another 3 months. Serum samples were collected at base-

line and monthly thereafter for 6 months. Exclusion criteria included pregnancy, statin use, prednisone > 10 mg/day, and immunosuppressive use (except hydroxychloroquine (HCQ). Interferon (IFN)-α, Interleukin(IL)1β, IL6, IL8, inducible protein (IP)10, tumor necrosis factor (TNF)-α, vascular endothelial growth factor (VEGF), and soluble CD40 ligand (sCD40L) levels were determined by a multiplex assay (Millipore, Billerica,MA) in the sera of patients and controls. Plasma samples were used to detect sTF using a chromogenic assay. aCL (IgG, IgM and IgA), aβ_2GPI (IgG, IgM and IgA), soluble intercellular cell adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, and E-selectin (E-sel) were evaluated by ELISA. We analyzed the monthly change in biomarker levels for the entire cohort (Spearman test) and also for four subgroups (Kruskal–Wallis).

Results: Of 41 patients recruited, 24 completed the study (mean age: 44.6 ± 13.6 year; female: 70%; Primary APS:8, SLE/APS:7, Asymptomatic aPL:5; SLE/aPL:4; HCQ: 61%; and anticoagulation: 43%). The withdrawal reasons were: 15 were lost at follow-up and two experienced muscle crumps with no evidence of elevated CPK or liver enzymes. The treatment with fluvastatin resulted in decreased biomarker levels and the proportion of patients with decreased levels ranged from 25% to 89% of the 24 aPL positive patients that completed the study. The mean maximum biomarker reduction was significant for IL6, IL1β, VEGF, IFNα, IP-10, sCD40L, TNFα and sTF when compared to baseline values. Fluvastatin did not significantly affect levels of IL-8, sICAM-1, sVCAM1 and sE-sel. The mean maximum reduction of biomarkers was achieved between 30 and 54 days of treatment for all biomarkers. aCL and anti-β2GPI titers remained unchanged. After discontinuing treatment with fluvastatin, many patients that had fluvastatin-induced reduction of biomarker levels had subsequent increases in these levels. The proportion of patients that had a rebound elevation in biomarker levels ranged from 14% to 90% for the various biomarkers. The mean maximum biomarker increase after stopping fluvastatin treatment was statistically significant for IL-1β, VEGF, TNFα, IP-10, sCD40L, and sTF.

Conclusion: Our pilot study demonstrating that fluvastatin can reversibly reduce the pro-inflammatory and prothrombotic biomarkers in persistently aPL-positive patients with or without SLE provides the basis for future larger randomized-controlled trials to examine the effects of the statins on the aPL-induced biomarkers as well as on the aPL-related clinical manifestations.

PB 2.62-5

Establishment of standardized international units for IgM anti-β2glycoprotein antibody measurement

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Background: Current classification criteria for antiphospholipid syndrome (APS) calls for positive 'standardized' antiphospholipid tests including IgG/IgM anti-$\beta_2$glycoprotein (aβ_2GPI) antibodies in the presence of classic symptoms for identification of the disease. Despite numerous efforts aimed at standardization there still exists considerable inconsistency in antiphospholipid testing. We sought to prepare a reference preparation (RP-M) and to establish international consensus units (IU) for the measurement for IgM aβ_2GPI antibodies.

Methods: Whole IgM fractions were affinity-purified (AP) from sera from APS patients with high IgM aβ_2GPI levels using a 2-mercatopyridine-coupled Sepharose column followed by an NHS-activated Sepharose column coupled to β_2GPI. Purity of β_2GPI AP fractions was confirmed using SDS-PAGE; fractions with high activity were pooled, concentrated and sterile-filtered. The protein concentration of the AP material was determined using Bradford protein assays and assigned a value based on the definition that 1 IU/mL equates to 1 μg/mL of AP aβ_2GPI. A reference preparation (RP-M) serum from an APS patient with high titers of IgM aβ_2GPI activity was assigned an IU value using original AP material as a calibrator. The RP-M and 30 samples were sent to several commercial companies for testing in their respective kits according to an approved protocol to enable evaluation of linearity, unit equivalency and commutability. Companies (and kits) included INOVA Diagnostics (QUANTA Lite ® β_2GPI IgM ELISA, QUANTA Flash ® β_2GPI IgM chemiluminescent assay), Bio-Rad Laboratories (Anti-β_2Glycoprotein I IgM EIA Test Kit) Corgenix (IgM Anti-β2 Glycoprotein I Test Kit), and Phadia (EliA ß2-Glycoprotein I IgM).

Results: The pooled AP material had a protein concentration of 15.125 μg/mL and was assigned a value of 15 IgM aβ_2GPI IU/mL. The RP-M had a value of 220.3 IgM aβ_2GPI IU/mL. The linearity (R^2) of the RP curve for the various assays ranged from 0.9649 to 0.9983. The value of the RP-M in the various arbitrary kit units ranged from 72.5 to 1143.4 units. Commutability samples fit very well within 95% prediction intervals and had excellent correlation when comparing assays.

Conclusion: The RP-M demonstrates excellent linearity in the various aβ_2GPI IgM assays and can be considered adequate to be used as a calibrant/reference material. The results of commutability studies suggest that this material can be used on a wide array of assays including ELISA formats and some newer assay formats with wider analytical measuring ranges that use different arbitrary units of measurement for calibration. New international consensus units as well as an adequate RP-M are now available to be used for the measurement of IgM aβ_2GPI antibodies. These studies contribute significantly to the much-needed standardization of aβ_2GPI immunoassays.

PB 2.62-6

Laboratory diagnosis of the antiphospholipid syndrome: evaluation of two new automated chemiluminescent assays for anticardiolipin and anti-β2glycoprotein I detection

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Background: The antiphospholipid syndrome (APS) identifies a condition of vascular occlusion increased risk and/or pregnancy complications. APS diagnosis requires the combination of at least one clinical (vascular occlusion and/or pregnancy complications) and one laboratory criterion; this has to be confirmed 12 weeks apart.

The Anticardiolipin (aCL), anti-$\beta_2$glycoprotein I (aβ_2GPI) and Lupus Anticoagulant (LAC) tests are the laboratory criteria for diagnosis of APS. Standardization of aCL and/or aβ_2GPI assays still remain difficult, automation could be helpful to improve inter- and intra-laboratory variability.

Aim: Aim of our study is the evaluation of two new fully automated chemiluminescent systems (ACL AcuStar and Zenit RA) for the detection of aCL (IgG – IgM) and aβ_2GPI (IgG – IgM).

Methods: Ninety retrospective patient samples (serum or platelets-poor plasma; LAC pos:15/86 and LAC neg: 71/86), referred to our Center for laboratory diagnosis of APS, have been tested for aCL (IgG – IgM) and aβ_2GPI (IgG – IgM) on both analyzers.

Results: Positive samples according to manufacturer's cut-off indications: 16/90 aCL IgG, 11/90 aCL IgM, 15/90 aβ_2GPI IgG and 8/90 aβ_2GPI IgM with ACL AcuStar; 13/90 aCL IgG, 12/90 aCL IgM, 14/90 aβ_2GPI IgG and 13/90 aβ_2GPI IgM with Zenith RA, respectively.

Analytical agreement between the two methods were 92.2% for aCL IgG and 96.7% for aCL IgM; 96.7% for aβ_2GPI IgG and 92.2% for aβ_2GPI IgM.

Eleven samples were positive for all of three tests including LAC with both chemiluminescent systems evaluated.
Conclusion: In comparison to classic ELISA testings, these two fully automated walk-away analyzers with random-access capabilities are easy to use and significantly reduce the hands-on time.
We have observed good analytical agreement for both aCL and aβ_2GPI assays. It is important that each laboratory will establish its own reference intervals.
Despite last Lupus guidelines published in 2009, we have noticed that only few request for diagnosis of APS were correct, including all three panels together (aCL, aβ_2GPI and LAC), and aβ_2GPI was less frequently requested.

PB2.63 – Arterial Vascular Disorders – III

PB 2.63-1

Circulating beta antithrombin glycoform increases during the acute ischemic cerebrovascular event

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Despite the control of thrombin is crucial in the development of atherothrombosis, antithrombin, the main endogenous inhibitor of thrombin, has been classically considered to play a role only in venous thrombosis. Additionally, available methods to study antithrombin do not discriminate between the two main glycoforms present in plasma. Thus, no study has evaluated the levels of β-antithrombin in thrombotic disorders, despite this glycoform lacking a glycan at N135 residue, has higher heparin affinity and shows predominant vascular localization. These features support that β-antithrombin might function as the major inhibitor of thrombin *in vivo* even being the less abundant form in plasma (10%). Firstly, we have developed a simple, cheap, and fast method able to specifically distinguish β-antithrombin by using only few microliters of plasma. The method quantifies the anticoagulant activity of β-antithrombin by activating the molecule with heparin under a high salt concentration that does not allow a-antithrombin activation. This method was validated with a and β glycoforms of antithrombin purified from plasma of healthy subjects, as well as with recombinant antithrombin glycoforms. We used this novel method and the classical one that determines the whole antithrombin anticoagulant activity (α + β) in plasma of patients with ischemic cerebrovascular disease during the acute event and 1 year later. We recruited 117 consecutive and unrelated patients (mean age 73 years, 52% males). The total levels of antithrombin did not significantly differ from that of a cohort of 97 healthy control subjects (97.6 ± 12.7% vs. 99.9 ± 6.4%, respectively $P = 0.105$) and did not significantly change 1 year after the event on available samples (100 ± 13.1%). However, it is important to point out that five cases had significant deficiency of antithrombin (values < 70%) during the acute event. In two cases, a consumption of antithrombin can be suggested since values increased after 1 year and/or values of other anticoagulants (protein C or S) were also reduced during the acute event. In contrast, in the other three cases, a congenital deficiency of antithrombin could be suspected based on antithrombin levels 1 year after the event and/or in relatives. Interestingly, sequencing of the *SERPINC1* gene revealed a heterozygous missense mutation responsible for the Budapest III variant (L99F) in the patient with the strongest deficiency at the moment of the stroke (32%): a 50 year-old male who suffered a lacunar stroke but had no previous thromboembolic events. In contrast, plasma β-antithrombin of patients during the acute event was significantly higher than in the control cohort (105.0 ± 17.7% vs. 97.1 ± 12.2%, respectively; $P < 0.001$). Moreover, in available samples, we observed a significant decrease of β-antithrombin over 1 year time ($P = 0.04$) and 1 year after the thrombotic event, β-antithrombin levels reached values observed in controls. These results might be explained by the release of β-antithrombin from the vascular compartment to the circulation during the acute event.
Conclusions: These data sustain a role of antithrombin in stroke and shows new knowledge on β-antithrombin, one potentially relevant glycoform that might be studied in different thromboembolic diseases using the method that specifically detect this glycoform.

PB 2.63-2

Toll like receptor (TLR)-4 modulates the effects of hyperglycemia and hyperinsulinemia on tissue factor procoagulant activity in blood

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Background: Tissue factor (TF) is the principal initiator of blood coagulation. We have shown: (i) TF levels are elevated in type 1 and 2 diabetes mellitus (DM) patients; (ii) in healthy subjects and T2DM patients hyperglycemia (HG), hyperinsulinemia (HI) and combined HG + HI (24 h infusion clamps) increased whole blood TF-procoagulant activity (TF-PCA); (iii). However, in T1DM patients, neither HG or HI, nor HG + HI increased TF-PCA. Aims: To understand the mechanisms for these differences, we studied the effects of HG and HI *in-vitro* in whole blood from healthy individuals under unstimulated and lipopolysaccharide (LPS)-stimulated conditions. T2DM patients have enhanced platelet-monocyte activation and elevated plasma LPS, a ligand for TLR-4. We postulated that TLR-4 modulates TF-PCA.
Methods: Glucose (200 mg/dL, HG) and insulin (1, 10 or 100 nM, HI) were added either alone or in combination (HG + HI), in the presence or absence of 1 mg/mL LPS to blood from six healthy subjects and incubated for 4 h at 37 °C. TF-PCA (2 and 4 h) was measured in whole blood lysates by a two-stage clotting assay and TLR-4 (4 h) by ELISA.
Results: Basal plasma glucose and insulin were 75 ± 4 mg/dL and 99 ± 24 pM ($n = 6$, mean±SE), respectively. At 2 h HG increased TF-PCA from 22 ± 5 to 41 ± 7 U/mL ($P = 0.006$). Insulin increased TF-PCA: insulin 10 nM (to 33 ± 8, $P = 0.033$) and 100 nM (to 44 ± 12, $P = 0.04$). HG + HI (insulin 1, 10 or 100 nM) increased TF-PCA to 42 ± 6, 50 ± 9 and 63 ± 12 nM (all $P < 0.05$ over basal), respectively. LPS markedly increased TF-PCA at 2 h (22 ± 5 U/mL to 826 ± 93, $P < 0.001$). HG induced a small increase over LPS. However, insulin inhibited LPS-induced TF-PCA at 10 nM (from 826 ± 93 to 587 ± 119, $P = 0.037$) and 100 nM insulin. In presence of LPS, HG + HI (1 nM) markedly increased TF-PCA over that with LPS alone (from 826 ± 93 to 2369 ± 234 U/mL, $P = 0.003$). TLR-4. HG alone increased TLR-4 (3.6 ± 1–18 ± 6, $P = 0.04$) with an uptrend with HI. HG + HI (100 nM) increased TLR-4 to 15.6 ± 3 ($P = 0.02$). LPS increased TLR-4 levels at 4 h by 11-fold (4.4 ± 0.8–49.3 ± 5 ng/mL). Added HG did not cause further increase; however, HI inhibited LPS-induced TLR-4 (1 nM HI: 14.6 ± 4.8 vs. 41.7 ± 9.7, $P = 0.006$). With HG + HI, TLR-4 levels were comparable to those with LPS alone; thus, HG overrides insulin inhibition. TLR-4 levels strongly correlated with TF-PCA ($r = 0.71$, $P \leq 0.0001$). Whole blood TLR-4 in T2DM subjects (0.7 ± 0.1 ng/mL, $n = 6$) was lower than in T1DM (3.2 ± 0.9 ng/mL, $n = 8$) and healthy subjects (3.6 ± 1.1 ng/mL, $n = 4$).
Conclusions: (i) In non-diabetic subjects HG, HI and HG + HI increased whole blood TF-PCA. Insulin inhibited LPS-induced increase in TF-PCA. Thus, insulin effect is influenced by the cellular activation state and HG overrides the inhibitory effect. (ii) TLR-4 plays a major role in modulating TF-PCA levels. (iii) Differences in cellular activation state and TLR-4 levels may be the basis for the differences in TF-PCA responses in healthy subjects, T1DM and T2DM in our infusion studies.

PB 2.63-3

Hemostatic factors as predictors of recurrent vascular events up to 12 years after ischemic stroke: the Sahlgrenska Academy Study on ischemic stroke outcome

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Background: The hemostatic factors tissue plasminogen activator (t-PA), von Willebrand factor (VWF) and fibrinogen are known from prospective studies to predict arterial thrombotic events. Previously we reported an association between convalescent plasma levels of the Thrombin Activatable Fibrinolysis Inhibitor (TAFI) activation peptide (AP) with the composite outcome recurrent vascular events and/or death within 2 years after stroke in the Sahlgrenska Academy Study on Ischemic Stroke (SAHLSIS). Here, we examined the associations between plasma levels of t-PA antigen, VWF, fibrinogen, and TAFI and the long-term risk of recurrent vascular events in SAHLSIS Outcome.
Methods: SAHLSIS Outcome comprises 600 consecutively recruited ischemic stroke patients aged 18–69 years that have been prospectively followed for up to 12 years. Blood was drawn under strictly standardized conditions both in the acute (day 1–10, median 4) and the convalescent phase, 3 months after index stroke. Plasma levels of fibrinogen were measured by an automated clot-rate assay, and the levels of t-PA antigen, VWF, intact TAFI and TAFI-AP by ELISAs. Vascular deaths, recurrent stroke, and coronary events were registered through national registers and medical records. Hazard ratios (HR) for associations between plasma levels and recurrent vascular events were calculated using Cox Regression models.
Results: The mean follow-up time was 8.5 (SD 1.6) years. No patient was lost to follow-up. Seventy-five vascular deaths, 119 recurrent strokes, and 53 coronary events were registered. In univariate analyses acute and 3-month levels of t-PA antigen, VWF and fibrinogen, but not TAFI or TAFI-AP, showed significant associations with vascular death. Similar results were obtained when analysing all events combined (n = 184). No significant associations were observed with recurrent stroke. In multivariate analyses, adjusting for vascular risk factors and hsCRP, the association for t-PA and vascular death was retained; HR per 1 SD increase in t-PA antigen 1.40 (95% CI 1.05–1.88, P = 0.02).
Conclusion: In young and middle-aged ischemic stroke sufferers, the convalescent plasma level of t-PA was an independent predictor of long-term risk of vascular death. Levels of t-PA, VWF and fibrinogen were associated with an increased risk of the combined outcome of fatal and non-fatal vascular events, but these associations were not independent of vascular risk factors in our study.

PB 2.63-4

Does the immature platelet function (IPF) in chest pain patients presenting to the emergency department aid in the diagnosis of acute coronory syndrome?

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Background: Patients presenting to the emergency department (ED) with chest pain and suspected acute coronary syndrome (ACS) are assessed by history, physical examination, electrocardiogram and serum markers of myocardial necrosis. However, early and accurate identification of patients with ACS vs. non-cardiac chest pain remains problematic. Previous studies reported that the mean platelet volume (MPV) is elevated in ACS and that elevated MPV may be predictive of recurrent myocardial infarction. Based on the hypothesis that platelet turnover is increased in ACS, resulting in elevated levels of younger, larger and potentially more active platelets, MPV may be an indirect measure of the immature platelet fraction (IPF). Indeed, studies suggest that the IPF is increased in ACS. We therefore hypothesized that the IPF may assist in the diagnosis of ACS in chest pain patients in the ED.
Aims: To determine if the measurement of IPF assists in the diagnosis of ACS in patients presenting to the ED with chest pain.
Methods: In this single-center study, adult patients (≥ 18 years of age) presenting to the ED with chest pain and/or suspected ACS were considered for enrollment. Exclusion criteria included trauma, pregnancy, elevated serum creatinine, anemia, thrombocytopenia and evidence of bleeding. In addition to routine ED admission laboratory tests, an EDTA-anticoagulated blood sample was drawn. Within 24 h of collection, blood samples were analyzed in a Sysmex XE-2100 hematology analyzer for platelet count, MPV and the percentage and concentration of immature platelets (IPF and IP concentration, respectively). IRB-approved written informed consent was obtained for the use of data from patients meeting enrollment criteria. Patients were followed in the hospital to determine their final diagnosis. Results were stratified into ACS or non-ACS (non-cardiac, non-thrombotic) patient groups.
Results: A total of 280 patients were enrolled in the study. Eleven subjects were excluded due to measurement error, consent withdrawal, inconclusive diagnosis or exclusions as per the study protocol. Two patients were diagnosed as non-cardiac, thrombotic (i.e., not classified as either ACS or non-ACS) and were excluded from analyses. Data from 226 ACS-negative (non-cardiac, non-thrombotic) and 41 ACS-positive patients were analyzed. Platelet counts and MPV were not statistically different between ACS-negative and ACS-positive patient groups. The IPF was 6.0 ± 1.3% and 5.1 ± 0.4% and the IP concentration was 10.0 ± 0.3 and 11.7 ± 1.3 × 10^3/μL for non-ACS and ACS, respectively, with no statistically significant differences between ACS-negative and ACS-positive groups for either measurement.
Summary/Conclusions: In patients presenting to the ED with chest pain, no differences in IPF, IP concentration or MPV were observed in non-ACS vs. ACS patients (n = 267). This study suggests that the IPF, the IP concentration and MPV do not assist in the diagnosis of ACS in chest pain patients presenting to the ED.

PB 2.63-5

Fractal dimension (Df): a novel biomarker to assess change in clot structure in cerebrovascular disease following therapeutic intervention

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Background: Stroke is the second largest cause of death Worldwide.[1] Abnormal haemostasis plays an important role in the pathophysiology of cerebrovascular disease (CVD) due to the development of an abnormally dense clot structure[2,3]. Standard techniques assessing clot structure have limited clinical use. Fractal Dimension (D*f*) (of the incipient clot) is a promising novel biomarker [4] that can quantify clot structure.
Aims: This study aims to explore the role of D*f* in quantifying changes in clot structure and evaluating the efficacy of therapeutic intervention in stroke and Transient Ischaemic Attack (TIA).
Methods: A prospective observational study of 50 patients with CVD (stroke/TIA) with a control group of 50 healthy volunteers. This study was given a favourable ethical opinion by the South West Wales Research Ethics Committee. Only patients or legal representatives

who gave written informed consent or assent respectively were enrolled onto the study. Rheological testing (D*f* analysis) was performed in addition to standard coagulation tests. Further assessment took place at 24 and 72 h to assess the effects of therapeutic intervention on clot structure.

Results: Our previous analysis has confirmed that D*f* in normal subjects is 1.74 (SD 0.04). On admission in CVD subjects mean D*f* was 1.78 (SD 0.05). After 24 and 72 h the mean D*f* was 1.78 (SD 0.05) and 1.74 (SD 0.05) respectively. Although there was no change in D*f* at 24 h, at 72 h it was significantly lower than the mean at presentation ($P < 0.05$; one way ANOVA). These findings also show that D*f* is significantly higher in patients with CVD than in healthy subjects at presentation but not at 72 h.

Conclusion: In this study D*f* accurately quantified the effects of CVD and therapeutic intervention on clot structure. D*f* was significantly elevated in the acute phase and returned to normal at 72 h.

PB 2.63-6

MMP-10: a new biomarker in peripheral artery disease?

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Background: Peripheral arterial disease (PAD) is associated with a poor prognosis in terms of cardiovascular morbidity and mortality. Matrix metalloproteases (MMPs) contribute extensively to vascular remodeling by degrading extracellular matrix (ECM) components. Circulating levels of MMP-10 are associated with different inflammatory markers, increased carotid intima media thickness, and the presence of carotid plaques. Moreover, MMP-10 expression is detected in endothelial cells (ECs) and macrophages within human atherosclerotic plaques in association with C-reactive protein (CRP), suggesting a role for MMP-10 in cardiovascular diseases.

Aims and Methods: To analyze the role of this protease in PAD, MMP-10 was measured in 139 patients (71 ± 11 years), and sex and age matched healthy controls ($n = 148$, 64 ± 8 years). Moreover, the role of MMP-10 *in vitro* was determined in endothelial cells from WT and MMP-10 deficient mice (*Mmp10*−/−).

Results: Subjects with PAD were older, and presented higher frequency of diabetes and hypertension ($P < 0.05$). The levels of fibrinogen (493 ± 135 vs. 293 ± 72 mg/dL; $P < 0.01$), hs-CRP (1.7 ± 2.9 vs. 0.5 ± 1.3 mg/L; $P < 0.01$) and MMP-10 (987 ± 520 vs. 729 ± 320 pg/mL; $P < 0.01$) were increased in PAD patients compared to controls while TIMP-1 was reduced (205 ± 88 vs. 410 ± 61 g/mL; $P < 0.01$). We then divided the population in patients presenting intermittent claudication (IC, $n = 76$), and critical leg ischemia (CLI, $n = 63$) based on Rutherford's score. CLI subjects were older, more diabetic and hypertensive compared to IC patients ($P < 0.05$), and presented increased levels of fibrinogen (552 ± 158 vs. 444 ± 89 mg/dL; $P < 0.01$), hs-CRP (3.1 ± 3.8 vs. 0.6 ± 1.0 mg/L; $P < 0.01$) and MMP-10 (1080 ± 467 vs. 905 ± 549 pg/mL; $P < 0.01$). *In vitro Mmp10*−/− endothelial cells presented reduced migration, proliferation and tube formation capacity compared to WT ($P < 0.05$).

Conclusions: Our results suggest that MMP-10 may be a biomarker in PAD and that it could be involved in the pathological changes underlying this disease.

PB2.64 – Diagnosis of VTE – III

PB 2.64-1

Are standardized algorithms used in clinical practice in seven different European countries to aid in the diagnostic work-up for suspected venous thromboembolism?

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On behalf of the working group in post-analytical external quality assessment (WG-PEQAS) of the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM).

Background: It has been shown that the need for radiologic imaging can be reduced by 30–40% if standardized algorithms consisting of clinical decision rules (CDRs) and D-dimer tests are used in the diagnostic work-up for patients with suspected venous thromboembolism (VTE).

Aims: The aim of this study was to explore if algorithms combining CDRs and D-dimer testing are used as recommended in different European countries.

Methods: Physicians working within emergency and internal medicine departments were invited, through the national medical associations, to answer a web based questionnaire. The physicians were presented with two case histories with questions related to diagnosis and exclusion of suspected pulmonary embolism (Patient A, high pretest probability) and deep venous thrombosis (Patient B, low pretest probability). The physicians were asked to estimate the probabilities of VTE before and after D-dimer testing, to answer how they would use and interpret D-dimer and their use of radiologic imaging. They were also asked if they regularly used clinical decision rules and type of rule.

Results: Altogether, 548 physicians from seven different European countries responded, after exclusion of 78 physicians who did not regularly diagnose VTE. For patient A, most physicians (63%) suggested a high pretest probability or likely diagnosis of PE. Still, 11.1% stating high probability would request D-dimer alone and 68.8% both D-dimer and radiologic imaging. Only 19% requested radiologic imaging alone. If the D-dimer result was negative in this patient, 16.6% suggesting a high probability even would regard PE as excluded. For patient B, the majority (82.7%) of the physicians suggested a low pretest probability or unlikely DVT diagnosis. D-dimer alone was requested by 65.6% of physicians, while 13% of clinicians would request *both* D-dimer and radiologic imaging and 10.8% only radiologic imaging. CDRs were always or often used by 31.9% of the physicians. The pretest probability estimates in percent varied substantially for both patients A and B, regardless of the use of CDRs. If CDRs were used often and/or always, the probability that the physician would do the correct action was higher for several questions compared to if CDRs were seldom or never used, but the probability estimates for VTE were not different between these groups.

Conclusions: Exclusion of VTE by negative D-dimer results might underdiagnose VTE in high probability patients and puts the patients into significant risk. Applying radiologic imaging without or in parallel with D-dimer testing in low probability VTE cases imply extra burden on the health care system, and could be avoided if standardized algorithms were followed. Results from this study suggest that standard-

ized algorithms are not systematically followed by a substantial amount of physicians.

PB 2.64-2

Association between treatment regimen and quality of life assed by EQ-5D-3L and pain interference in adults with haemophilia with and without inhibitors in the global HERO study

Tricarico A

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Background: Treatment options for congenital haemophilia A (HA) and B (HB) with or without inhibitors include on-demand and prophylactic factor replacement. Little is known about the relationship between treatment regimen and quality of life (QoL) in people with haemophilia (PWH) with and without inhibitors.

Aims: To examine the association between treatment regimen and QoL in adult PWH with and without inhibitors from the Haemophilia Experiences, Results and Opportunities (HERO) Study.

Methods: A *post-hoc* analysis of data from PWH ≥ 18 years from 10 countries (AR/DZ/CN/CA/DE/FR/IT/ES/GB/US)) participating in the HERO Study. PWH completed a standard EQ-5D-3L[TRADEMARK] assessment. EQ-5D index was calculated based upon the US method (Shaw et al. 2005).

Results: In total, 675 PWH completed qualitative and quantitative questions on QoL and well-being. Most PWH reported HA (498), 86 reported HB and 91 reported HA/HB with inhibitors. Half (n = 45) of those reporting inhibitors were from the US. PWH with inhibitors were younger (median/maximum age 34/69 years) than those without inhibitors (36/86 years). At a global level, treatment was somewhat evenly split between on-demand (289, 43%) and prophylaxis (207, 31%); fewer reported on-demand plus situation prophylaxis (146, 22%). This was similar for PWH with inhibitors (on-demand 43 [47%] including 20 US, prophylaxis 29 [32%] including 22 US, on-demand with situational prophylaxis 7 [8%]). Most adult PWH (598, 89%) and PWH with inhibitors (86, 95%) reported pain had interfered with their daily life in the past 4 weeks; of those, 301 (50%) reported constant pain (38 [44%] with inhibitors). Mean EQ-5D index for PWH with inhibitors was 0.707; it was 0.745 for HA and 0.741 for HB; median EQ-5D index was the same for HA, HB and PWH with inhibitors (0.778). Mean EQ-5D for PWH on prophylaxis was 0.707, 0.712 for PWH on-demand and 0.731 for PWH treated on-demand with situational prophylaxis. Almost half of PWH reported no issues with mobility (HA: 41%, HB: 38%, inhibitors: 44%). Most PWH reported no issues with usual activity (HA: 81%, HB: 79%, inhibitors: 74%). For PWH with inhibitors, the percentage reporting no issues with usual activity was similar across treatment regimens (on-demand: 72%, prophylaxis: 79%, on-demand with situational prophylaxis, 71%). Approximately half of PWH reported no issues with self-care (HA: 56%, HB, 49%, inhibitors: 43%) or anxiety/depression (HA: 54%, HB: 53%, Inhibitors: 54%).. Few PWH denied any pain/discomfort (HA: 26%, HB: 28%, inhibitors: 19%). Self-care is the only index that shows a significant difference between the types of haemophilia. Statistical comparison for mobility and usual activity was not possible due to the small number of PWH who responded to each question.

Conclusions: Globally, adult PWH with inhibitors in HERO had a lower mean EQ-5D index and more frequently reported problems in individual EQ-5D domains than those without inhibitors. While differences in age and treatment regimen across participating countries limit descriptive analysis; multivariate analyses may yield further insights. The underlying reasons for treatment regimens (e.g. pain vs. bleed control, desired activity participation, employment) likely also impact quality of life, and need to be factored into future studies.

Performance of fibrin monomer in the evaluation of patients with suspected pulmonary embolism

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Background: During thrombin generation, fibrinopeptide A is released from the fibrinogen molecule, leading to the formation of fibrin monomer and cross-linked fibrin. Simultaneous triggering of the fibrinolytic system results in plasmin clipping of cross-linked fibrin with D-dimer release. Fibrin monomer, therefore, is an early indicator of thrombin generation. Studies evaluating this molecule for the exclusion of venous thromboembolism have had inconsistent results; however, the high specificity of fibrin monomer for venous thrombosis has been suggested as a potential advantage over D-dimer in this setting.

Aim: We sought to evaluate the performance of fibrin monomer (STA LIATEST®FM) in a large cohort of patients with suspected pulmonary embolism (PE).

Methods: Seven hundred and ninety-four available frozen samples from a prospective cohort study of 808 consecutive patients with suspected PE were tested for both fibrin monomer and D-Dimer (STA LIATEST®DDi), according to the manufacturer's instructions. All patients underwent pre-test probability assessment using the Wells model. Patients were characterized as PE positive or negative according to the results of objective testing and 3 month follow-up. Suspected venous thromboembolism was adjudicated centrally. The study protocol was approved by the institutional review board of each participating center and is consistent with the principles of the Declaration of Helsinki.

Results: One hundred and two patients (12.8%) were diagnosed with PE. Five hundred sixty-five had low pre-test probability, while 172 and 57 were classified as having a moderate and high pre-test probability, respectively. Concordance between D-dimer and fibrin monomer results was moderate to low (Pearson r – 0.41). The normal range for fibrin monomer is < 8.0 μg/mL. Using this cutpoint, the assay had an overall sensitivity of only 42.2% (95% CI, 32.6–52.3%) and a specificity of 88.7% (95% CI, 86.1–90.9%), with a corresponding negative predictive value (NPV) of 91.2% (95% CI, 88.8–93.2%) and positive predictive value (PPV) of 35.5% (95% CI, 27.2–44.8%). Decreasing the cutpoint to 5.0 μg/mL resulted in a slight improvement in sensitivity (66.7%; 95% CI, 57.2–75.6%) with resultant loss in specificity (66.6%; 95% CI, 63.0–70.1%). The NPV remained too low for this test to be used to confidently exclude PE (93.1%; 95% CI, 90.4–95.1%), even in patients with a low pre-test probability (95.9%; 95% CI, 93.3–97.6%). In comparison, using a cutpoint of 0.5 μg FEU/mL, the D-dimer assay had an overall sensitivity, specificity, and NPV of 98.0%, 42.5%, and 99.3%, respectively. Although increasing the fibrin monomer cutpoint to 50 and 100 μg/mL resulted in specificities of over 90%, even in patients with a high pre-test probability, the prevalence of disease was not sufficiently high (35.1%) to result in a PPV sufficient to confidently rule-in PE (62.5% [95% CI, 25.9–89.8%] and 66.7% [95% CI, 24.1–94.0%], respectively). In order for a positive result to generate a PPV of over 90%, the population prevalence of PE would need to be at least 70%.

Conclusions: The fibrin monomer is not sufficiently sensitive to be used to exclude PE, even in patients with a low pre-test probability. Although this assay has a higher specificity than D-dimer, it is insufficient to be used to rule-in PE.

PB 2.64-3

PB 2.64-4

Assessment of thrombus age with contrast-enhanced MR-Venography

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Background: Current treatment of DVT is limited to anticoagulation and compression stockings. However, at least 20% of patients continues to have leg complaints even after 1 year of (extended) treatment. Contrast-enhanced MR-Venography allows for high detail imaging of the deep vein system. This enables us to visualize detailed thrombus characteristics. Being able to assess thrombus age may contribute to early identification of patients at risk for suboptimal treatment outcomes and safer patientmanagement.

Aims: To assess the age of thrombus with CE-MR-Venography and compare the results with the clinical estimate of the age of the DVT based on duration of complaints.

Methods: Forty-one patients (27 male and 14 female), age 17–74 years (mean 49, SD 17), underwent MR-Venography to evaluate the presence and extend of their DVT. Thrombus age was assessed at the level of the common femoral vein (35 patients). For those patients in whom the thrombus did not extend into the common femoral vein, the most proximal extension was evaluated (six patients). Thrombus age was scored, blinded to the clinical duration of complaints, as either acute, subacute or chronic. Thrombus was identified as acute if the thrombosed vein was dilated, showed a thin enhancing wall and a homogenous signal intensity. Thrombus was identified as subacute if the thrombosed vein was still dilated, with a thicker enhancing wall and signs of recanalization within the thrombus (visual as areas of lower and higher intensity within the thrombus). Thrombus was identified as chronic if the thrombosed vein was no longer evidently dilated, only partially filled with thrombus and no evident signs of residual vein wall enhancement were present. Additionally, signs of trabeculations or fibrotic strands were identified as post-thrombotic. Results of image assessment were subsequently compared to the clinical information with regard to duration of complaints.

Results: In all 41 patients the radiologist was able to distinguish the thrombus age into three categories (acute, sub acute, chronic), based on MR characteristics. Thrombus was identified as acute in patients with a mean duration of complaints of 6.5 days (range 2–13 days). Thrombus was identified as sub acute in patients with a mean duration of complaints of 13.4 days (8–18 days,). Chronic thrombus was identified in patients with a mean duration of complaints of 22.2 days (15–32 days).

Summary/Conclusions: Contrast-enhanced MR-Venography may enable differentiation between acute, sub acute and chronic thrombi. This could potentially impact treatment options in both the acute and chronic phase.

We might be able to predict suboptimal treatment outcomes, and consider more aggressive treatment options, such as catheter directed thrombolysis.

Keywords: DVT, thrombus, age, MR-venography.

PB 2.64-5

Values of pretest probability tests for diagnosis of acute symptomatic proximal DVT in Thai patients

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Background: Clinical diagnosis of deep venous thrombosis is difficult because clinical signs and symptoms lack sensitivity and specificity. Some authors have developed pretest probability tests for diagnosis of acute DVT based on history and physical examination before confirmation by objective testing such as duplex ultrasonography or venography. These scores have not been evaluated for the accuracy of diagnosis of acute symptomatic proximal DVT in Thai patients.

Aims: To compare the accuracy of clinical predictor scores in diagnosis of acute symptomatic proximal deep vein thrombosis of lower extremity and to identify risk factors of acute proximal deep vein thrombosis of lower extremity in Thai patients'

Methods: Patients who had symptoms suspected acute deep vein thrombosis (DVT) were examined by duplex ultrasonography for detecting proximal deep vein thrombosis at common femoral veins and popliteal veins. Clinical predicting scores of acute deep vein thrombosis including Wells, Kahn, and St. Andre′ and Ambulatory constant scores were recorded. Sensitivity and specificity of all clinical scores were calculated by comparing with the result of duplex ultrasonography. We also identified the clinical predicting factors associated the diagnosis of acute symptomatic proximal deep vein thrombosis by duplex ultrasonography by univariate and multivariate analysis. In addition, we developed the new clinical predictor score (Siriraj DVT score) by using the predicting factors which had statistical significant in multivariate analysis.

Results: Five hundred patients who had symptoms suspected deep vein thrombosis were included in this study. Proximal deep vein thrombosis was confirmed by duplex ultrasonography in 133 patients (26.6%). The Ambulatory constant score was a best predictor score in diagnosis of deep vein thrombosis following by the Wells and St. Andre′ and Kahn scores respectively. According to the Ambulatory constant score, the area under the ROC curve was 0.646 (95% confidence interval [95%CI]: 0.59–0.69), for the Wells score 0.644 (95%CI: 0.59–0.69), 0.575 (95%CI: 0.52–0.63) for the St. Andre′score, and 0.540 (95%CI: 0.48–0.59) for the Kahn score. We found that history of cancer, confinement to bed, unilateral lower limb pain, local warmth, whole limb enlargement, calf enlargement more than 3 cm compared to the other sites, and previous history of venous thromboembolism were statistically significant in univariate analysis. In multivariates analysis, confinement to bed, unilateral lower limb pain, calf enlargement > 3 cm compared to the other sites and previous history of venous thromboembolism were the predicting factor which had significant statistic. We also developed the new predicting score (Siriraj DVT score) by these four parameters and the area under the ROC curve was 0.717 (95%CI: 0.67–0.77).

Conclusion: Ambulatory constant score had highest accuracy in diagnosis of acute symptomatic proximal DVT in Thai patients. Confinement to bed, unilateral lower limb pain, calf enlargement > 3 cm compared to the other sites and previous history of venous thromboembolism were the important clinical predicting factors for diagnosis of acute symptomatic proximal deep venous thrombosis in Thai patients.

PB 2.64-6

Using model-based clustering to identify patterns of INR trajectories following warfarin initiation

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Background: Achieving stable warfarin therapy as defined by the International Normalized Ratio (INR) being within the therapeutic range is often challenging due to intra- and inter-individual variability regarding patient response to warfarin. Characterizing patterns of INR trajectories following initiation of warfarin may help identify patients early on with stable vs. unstable INR patterns, and in turn help guide management decisions in patients with venous thromboembolism.

Aim: We aimed to identify distinct patterns of INR trajectories following initiation of warfarin therapy by applying an approach to model-based clustering of longitudinal data recently developed by the authors.

Methods: We analyzed retrospective data collected from 99 patients presenting to the anticoagulation clinic at a single tertiary-care center, who were also enrolled in a previous study. We determined INR values at the time of warfarin initiation and up to 6 months thereafter. Our approach to model-based clustering of longitudinal data treats a subject's observed trajectory as arising from a finite mixture of probabilistic models for curves, such that each component identifies a distinct pattern of time evolution (described by a mean curve and variance-covariance matrix). Our algorithm estimates from a sample of observed curves both the number of distinct patterns and the parameters describing each pattern. The algorithm also produces estimates, for each individual, the probabilities of a patient belonging to each pattern.
Results: In all, we included 99 patients with an average number of INR values per patient of 13 (Standard Deviation (SD) 4) following warfarin initiation and a mean follow-up of 189 days (SD 31). We identified two distinct patterns of INR trajectories. The most frequent pattern included 80% ($n = 79$) of the population and describes patients with favorable INR control. They achieved target INR values (between 2.0 and 3.0) early after warfarin initiation and maintained stable INR control during follow-up. The mean INR was 2.3 (SD 0.8). The less frequent pattern describes 20% of the population ($n = 20$) with less favorable INR control. In this group, INR instability was observed early during initiation of warfarin therapy with target INR values difficult to achieve and maintain. The mean INR was 2.8 (SD 1.4).
Conclusion: Our study shows that using model-based clustering can identify stable and unstable patterns of INR trajectories in patients initiating warfarin therapy. Future work is needed to verify our method in a larger population, and to determine patient characteristics predictive of INR patterns. Ultimately, predicting INR instability may help identify patients who may benefit from alternate anticoagulants.

PB2.65 – Diagnosis of VTE – IV

PB 2.65-1

Clinical impact of findings supporting an alternative diagnosis on computed tomography pulmonary angiography in patients with suspected pulmonary embolism

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Background: Computed tomography pulmonary angiography (CTPA) is commonly used as the first-line imaging test in the diagnostic work-up of patients with suspected pulmonary embolism (PE). Other CTPA findings may provide an alternative explanation for the signs and symptoms in this group of patients; however, the clinical impact of these findings is not clear.
Aims: To assess the clinical impact of findings supporting alternative findings on CTPA ordered in the diagnostic work-up for PE, in terms of diagnostic or therapeutic consequences. Besides, we investigated whether findings supporting alternative diagnoses were already established or highly suspected before CT-scanning.
Methods: In 203 consecutive patients with suspected PE, the clinical implication of abnormalities on CTPA was prospectively evaluated. Alternative diagnoses were defined on clinical grounds prior to performing CTPA; afterwards, all findings were systematically registered. Diagnostic and therapeutic consequences were assessed using *a priori* defined criteria.
Results: A total of 39 (19%) patients were diagnosed with PE and 61 (30%) had no abnormality on CTPA. Before CTPA, alternative diagnoses were suspected in 97 (48%) patients. Findings supporting an alternative diagnosis were detected in 88 (43%) patients. In 28 patients this was a new finding, in 18 patients, a conclusive alternative diagnosis for the complaints was made based on the outcome of the CTPA. Overall, findings supporting alternative diagnoses had therapeutic consequences in 10 (4.9%) patients. Incidental findings (nodules/lymph nodes) requiring diagnostic procedures were present in 17 (8.4%) patients, of which one (0.5%) had a therapeutic consequence.
Conclusion: In patients undergoing CTPA for suspected PE, findings supporting alternative diagnoses were found in almost half of the patients. However, in only few patients this had therapeutic consequences. Hence, CTPA should principally be used to find or exclude PE in high probability patients but not to establish an alternative diagnosis.

PB 2.65-2

Inter-observer agreement for Wells DVT score and empiric unstructured estimate of pretest probability

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Background: Evaluation for potential DVT is challenging due to inadequacy of clinical exam to identify clot and lack of 24 h duplex Doppler ultrasonography in some settings. The Wells DVT score has achieved less attention by clinicians and researchers than the corresponding score for PE. Increased use of an approach to identify of low probability patients (< 10%) could facilitate increased use of D-dimer testing with improved diagnostic efficiency. Currently most patients in US EDs are evaluated primarily with ultrasonography as the primary and only diagnostic tool.
Objectives: To determine interobserver agreement among two clinicians for components of the Wells DVT score, as well as agreement for empiric clinician judgment.
Methods: We conducted a prospective, observational single site study including patients evaluated in the emergency department for possible lower extremity DVT. IRB approval was provided for human subjects research. We excluded patients with: known DVT on warfarin with INR > 2.0 or other non-aspirin anticoagulation, DVT diagnosed within last 1 month regardless of INR, or known results of duplex Doppler. Two clinicians (attending, resident or mid-level) independently evaluated each patient prior to test results and completed a web-based data collection instrument including components of the Wells Score and overall clinician empiric estimation of DVT probability (< 10%, 10–19%, ≥ 20%). Structured medical record review determined combined VTE outcome (any acute DVT or PE over 45d). Kappa statistic assessed inter-observer agreement.
Results: One hundred and thirty-six patients were enrolled; mean age was 54 years, 64% were female. The median time to obtaining a duplex Doppler was 3.4 h. Acute DVT was diagnosed in 18% (95% CI 12–26%) acute PE without DVT in 2% (5–6%). Forty-four percent of patients were pretest probability < 10% by clinician unstructured estimate and 49% were DVT unlikely by Wells. Kappa values (and agreement%) were: previous DVT 0.90 (98%), active cancer 0.85 (98%), leg immobility 0.75 (97%), bedridden or surgery 0.56 (92%), calf swelling 0.58 (81%), entire leg swelling 0.42 (77%), unilateral pitting edema 0.36 (72%), alternative diagnosis at least as likely 0.32 (70%), deep venous system tenderness 0.23 (66%), superficial collateral veins 0.15 (86%). Kappa for binary empiric unstructured gestalt (< 10% vs. ≥ 10%) was 0.42 with 71% agreement. Prevalence of acute DVT/PE in the empiric < 10% group was 8.3%. Acute DVT/PE in the Wells ≤ 1 'unlikely' group was 12.5%.
Conclusion: Highest precision exists for the patient history components of the Wells DVT score rather than physical exam components. Almost half of the patients were non-high probability, suggesting possible increased opportunity for a combined pretest probability and D-dimer testing strategy.

PB 2.65-3

Accuracy of diagnosing incidental pulmonary embolism on routinely performed contrast-enhanced CT-imaging in patients with malignancy

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Introduction: The routine use of modern CT scanners has led to an increased detection of incidental PE. The accuracy of diagnosing PE on normal CT scans that were not performed for diagnosis of clinically suspected PE is unknown.

Methods: Consecutive patients with active malignancy in whom incidental PE was diagnosed were included. All CT-images were reassessed in a blinded fashion by an experienced thoracic radiologist, to determine the location of the emboli, the obstruction index by the method of Qanadli and the level of confidence in diagnosis. A sample of 19 cancer patients, who had undergone staging CT-imaging, in whom no incidental PE was reported, were included to ensure blindness and control of the expert reader.

Results: A total of 62 patients with incidental PE were included. A central pulmonary embolism was diagnosed in 32.8%, and a segmental localization in 67.2%. All patients received anticoagulant treatment upon diagnosis. In only one patient (1.6%; 95% CI: 0.04–8.7%) expert reading refuted the diagnosis of PE. The Kappa level of agreement between initial reading and expert reading for the diagnosis of PE was 0.97. The median obstruction index was 17.5% (IQR: 10–30%), which was lower compared to a reference sample of 113 patients with symptomatic PE diagnosed on CTPA ($P = 0.008$).

Conclusion: This study is the first to assess the accuracy of diagnosing PE on CT-scans that were conducted for other reasons than symptomatic PE. It demonstrates an excellent agreement between initial reading and expert reading for the diagnosis of incidental PE.

PB 2.65-4

Fibrin monomer (FM) might be used as a marker for venous thromboembolism in pregnancy

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Background: Pre-test probability of VTE combined with D-dimer measurement can be used to decide on exclusion of venous thromboembolism (VTE) or further diagnostic work-up (radiologic imaging) in non-pregnant patients when VTE is suspected. Although we have suggested a model for interpretation of D-dimer based on the within-subject variability of D-dimer in pregnancy, the usefulness of D-dimer measurement in pregnancy and post-partum period, when VTE is suspected, is complex. There is a gradual increase in D-dimer concentration during pregnancy, not returning to normal levels until about 6 weeks post-partum. Studies have suggested that fibrin monomer (FM) can be used as a biomarker for VTE. But to be used in pregnancy, FM concentration should first be shown to be stable throughout the whole pregnancy in healthy women.

Aims: The aim of this study was to compare FM concentration in healthy pregnant and non-pregnant women to evaluate if FM concentration is as stable in healthy pregnant as in healthy non-pregnant women.

Methods: Blood samples were obtained every 4 weeks in 20 healthy non-pregnant women and every 4 weeks during pregnancy in 20 healthy pregnant women. D-dimer (STA Liatest D-dimer, Stago) and FM (STA Liatest FM, Stago) were analysed on all samples. Informed consent was obtained and the study was approved by the Regional Committee of Medical Ethics (REK) of Western Norway.

Results: The median FM in non-pregnant women was 4.75 μg/mL (97.5th percentile 8.2 μg/mL) and in pregnancy 6.2 μg/mL (97.5th percentile 15.4 μg/mL), after exclusion of two pregnant women who had elevated concentrations of both FM and D-dimer in several samples (one of the women was Factor V Leiden homozygous). The median fibrin monomer concentration were slightly lower in the 1st trimester (5.7 μg/mL) compared to 6.2 and 6.8 in the 2nd and 3rd trimester, but there was a large overlap of concentrations in the three trimesters, and there was no statistical significant increasing trend from the 1st to the 3rd trimester (linear regression, $P = 0.55$). The analytical variation (CVa) based on duplicate measurements was 11% and 14% in samples from pregnant and non-pregnant, respectively.

Summary/Conclusion: The concentration of FM seems to be relatively constant during pregnancy, as opposed to D-dimer. The suitability of FM to be used in the exclusion of VTE in pregnant women should be assessed in clinical studies.

PB 2.65-5

Age adjusted D-dimer for exclusion of venous thromboembolism: results from a hospital emergency department

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Background: A clinical decision rule, the Wells score, can be used to exclude venous thromboembolism (VTE), when used in conjunction with a normal D-dimer test. The instrument/reagent combination CS2100i Sysmex coagulation analyzer along with the INNOVANCE® D-dimer is used for the conventional 500 μg/L FEU D-dimer cut-off. Higher cut-off values may provide advantage for low Wells score. An age-adjusted cut-off of the D-dimer (patient's age x 10) may increase the number of elderly patients in whom VTE can be safely excluded.

Methods: D-dimer data was collected in a hospital laboratory prospectively and sequentially over a period of 38 days. Four hundred and fifty-eight consecutive patients were included of whom 266 were > 50 years (58%). No patients were excluded. The patients in the dataset were all from the emergency department. We retrospectively assessed the presence or absence of VTE and the Wells Score using chart reviews. This included review of the hospital charts on all emergency department visits and with additional follow-up information on those patients admitted to hospital, but not on those patients discharged from the emergency department. The proportion of patients in whom VTE could be excluded, with low Wells score, combined with age-adjusted D-dimer test was calculated and compared with the conventional D-dimer cut-off of 500 μg/L and a higher cut-off of 650 μg/L.

Results: When combined with a low Wells score (0–2) the negative predictive value for negative D-dimer results is excellent (100%) for conventional, higher and age adjusted cut-offs. For patients under the age of 50, raising the conventional D-dimer cut-off to 650 μg/L yields a higher specificity than the 500 μg/L cut-off. Lastly, for patients over the age of 50, the age-adjusted D-dimer cut-off yields a modest improvement in specificity for a low Wells score and a doubling of the specificity (20% to 41%) for moderate probability Wells score, compared to using the conventional D-dimer cut-off, with no change in sensitivity.

Conclusion: Using age-adjusted cut-off yields a higher specificity for both the low and moderate (2–6) Wells scores in emergency department patients.

PB 2.65-6

Patient delay in the diagnosis of acute pulmonary embolism does not lead to higher thromboembolic burden or worse right ventricular function

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Background and Aims: It has earlier been demonstrated that the time between symptom onset and objective diagnosis of pulmonary embolism (PE) – does not influence outcome on re-thrombosis and mortality. It is unknown whether the patient's delay – defined as time between symptom onset and presentation at the hospital – is of influence on the thromboembolic burden and right ventricular function. We sought to evaluate this by measuring Qanadli-score and RV/LV-ratio in PE patients with and without patient's delay.

Materials and Methods: *Post-hoc* analysis of an observational prospective outcome study in 113 consecutive CT proven PE patients. In all patients Qanadli-score and RV/LV ratio were scored and duration from symptom onset until the clinical presentation was requested. Also mortality and hospital readmission in a 6-weeks follow-up period were collected.

Results: Twenty patients with and 93 patients without delay, defined as more than 7 days after symptom onset, with identical baseline characteristics and co-morbidities, were included. In a linear analysis, Qanadli-scores were not correlated to delay with a R^2 of 0.021 ($P = 0.130$). RV/LV-ratio had a $R^2 < 0.001$ ($P = 0.991$). Likewise, longer delay was not associated to 6-week mortality (Odds ratio: 0.65; 95%CI 0.08–5.57) or hospital readmission (Odds ratio: 0.75; 95%CI 0.15–3.65).

Conclusion: In our patient cohort, patient's delay was not associated with higher thrombus load or right ventricular dysfunction. This could provide a possible explanation for the lack of association between delay in diagnosing PE and clinical outcome as found in earlier studies.

PB2.66 – Hormones, Pregnancy, Women's Issues – II

PB 2.66-1

Oral contraceptive formulations with estetrol as an estrogen, in combination with levonorgestrel or drospirenone, show minor effects on haemostasis

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Introduction: Current contraceptive preparations contain either ethinyl estradiol or the natural 17β-estradiol. The natural fetal estrogen, estetrol, has recently been added to the estrogen portfolio and was successfully evaluated for contraconception and vaginal bleeding patterns with either levonorgestrel or drospirenone as progestogen.

Aims: To determine the effects on haemostasis variables (in relation to estrogenicity markers) of combinations of estetrol (5, 10, 20 mg) with levonorgestrel (LNG:150 μg) and estretrol (5 and 10 mg) with drospirenone (DRSP: 3 mg) in comparison with Yaz® (20 μg ethinyl estradiol with 3 mg DRSP). All combinations were administered according to a 24–4 day regimen.

Methods: A randomised, open-label, comparative, parallel study in 111 assigned women of which 109 were actually treated with six different hormone combinations was performed with analysis of variables at the pre-treatment sample, after one cycle at day 22–23, after three cycles at day 22–23 and post treatment after 1 month. Yaz® effects were taken as reference; the third cycle data were taken for comparisons.

Results: We selected to compare the results for 10 mg estetrol with either LNG or DRSP with the results of Yaz®.

The change from baseline in the third cycle was respectively + 15% and −23% for SHBG and + 19% and −9% for angiotensinogen relatively compared to Yaz® (set at 100%) ($P < 0.001$). This indicates a low estrogenicity with DRSP and shows the estrogen-antagonistic effect of LNG.

Comparisons for important haemostatic variables shows a reduction in APC-resistance of 1% and 10% for DRSP and LNG respectively while Yaz® shows an increase of 179% ($P < 0.001$). Free TFPI shows a reduction of 17% for DRSP and an increase of 7% for LNG while Yaz® shows a decrease of 45% ($P < 0.001$). Antithrombin shows increases of 2% and 1% for DRSP and LNG respectively, while Yaz® shows a decrease of 5% (n.s.). Protein S activity showed increases of 3% and 17% for DRSP and LNG and a decrease of 27% with Yaz® ($P < 0.001$).

Most remarkable were decreases in F 1 + 2 of 3% and 10% for DRSP and LNG respectively while Yaz® showed 63% increase ($P = 0.001$), and D-dimer which showed decreases of 26% and 31% for DRSP and LNG respectively while Yaz® showed 31% increase ($P = 0.008$).

It is concluded that impact of estetrol containing preparations on markers of haemostatic activation (F1 + 2 and D-dimer) suggests a decrease in coagulation activation. This is supported by changes in the coagulation inhibitors antithrombin, protein S and TFPI showing no or less strong decreases compared to Yaz®. The APC resistance test is slightly changed in a favourable direction unlike for Yaz® where it is strongly increased.

It is suggested that the biomarker analysis of the effects of estetrol containing oral contraceptive formulations on haemostasis indicate a low increase of risk or even protection against venous thrombosis.

PB 2.66-2

Circulating nucleosomes and plasma free DNA are increased in abnormal pregnancies

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Background: Recent data have analyzed the fundamental coupling between coagulation and innate immunity, indicating the central role of neutrophil-derived circulating nucleosomes on the promotion of coagulation and thrombus growth. Indeed, nucleosomes, as DNA/histones molecular complexes, a component of the neutrophil extracellular traps, target the proteolytic action of neutrophil serine proteases on Tissue Factor Pathway Inhibitor and factor XII, thus worsening coagulation *(Massberg S et al. 2010)*.

Pregnancy is an acquired hypercoagulability state. The placental function is central to the fetal development. Thrombotic impairments of the placental vessels may participate to placental insufficiency and may favor the related clinical complications, the so-called ischemic placental diseases (IPDs).

Aim: These data prompted us to perform an exploratory prospective study. Its aim was to compare plasma nucleosomes and cell free DNA (fDNA) concentrations between non-pregnant women, pregnant women with a normal pregnancy and pregnant women with an obvious clinical complication. Pregnancy complications, defined according to international guidelines, were diagnosed by reference practitioners and included: gravidic hypertension, IPDs (pre-eclampsia, intrauterine fetal growth restriction with evidence of placental insufficiency), venous thromboembolism, gestational diabetes, acute infections, cholestasis of pregnancy, abnormally implanted placenta, threats of premature labour and premature rupture of membrane. The study was

approved by the University Hospital of Nîmes Institutional Review Boards and Ethics Committee. This clinical investigation was performed according to the Helsinki declaration of 1975 as revised in 1996 (ClinicalTrials.Gov ID: NCT01736826). Informed consent was obtained from all patients.

Methods: Quantification of circulating nucleosomes and DNA were respectively determined by a commercially available ELISA kit and Q-PCR. Age, white blood cell (WBC) and neutrophils counts were documented for each woman. Blood samples from pregnant women were characterized by gestational age and pregnancy-related pathology, if any.

Results: We included 124 patients from October 2011 to May 2012: 35 women with a normal pregnancy, 73 women with an abnormal pregnancy and 16 non-pregnant women.

We found increased plasma concentrations of nucleosomes ($P = 0.02$) and fDNA ($P < 0.0001$) in pregnant women, concentrations being the highest in abnormal pregnancies.

Nucleosomes ($P = 0.002$) and fDNA ($P < 0.0001$) concentrations differed depending on the type and severity of pregnancy-related complications: very high rates in the ischemic placental disease group.

Nucleosomes:WBC, fDNA:WBC, nucleosomes:neutrophils and fDNA:neutrophils ratios were significantly higher in pregnant women with a symptomatic complication.

Conclusion: This preliminary study shows high nucleosomes and fDNA concentrations during pregnancy, which culminate in severe pregnancy complications. These data encourage the systematic evaluation of the pathogenic and aggravating role of circulating total nucleosomes and fDNA in ischemic placental diseases.

PB 2.66-3
Could polycythemia vera be a vascular 'risk factor' in woman?

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Background: The life expectancy of polycythemia vera (PV) patients is strongly affected by thrombotic events. Investigation of risk factors of thrombotic events separately in women should be prominently important, since changes in their lifetime conditions such as pregnancy or climaterium, and commonly prescribed oral contraceptives or oral hormone replacement therapies (HRT) could have an additional effect on the relatively frequent occurrence of vascular complications in these patients.

Aims: To evaluate the impact of major cardiovascular risk factors (hypertension, cigarette smoking, diabetes mellitus, obesity and hyperlipidemia) on thrombotic events in woman diagnosed with polycythemia vera.

Methods: Women with or without of thrombotic events were compared by a series of variables such as age, presence of JAK2 V617F mutation, measured platelet, red blood cell, haemoglobin and leukocyte counts at diagnosis, cardiovascular risk factors, and thrombotic events before and after diagnosis. Mann-Whitney tests were performed to explore overall effects of these variables. Multivariate binary logistic regression was also run to estimate the probability of thrombotic events.

Study participants: Fifty- eight women with median age 62 years; (range: 24–80 years) diagnosed with polycythemia vera between 1999 and 2011 at our Department, were enrolled to the study. Patients who used oral contraceptives or HRT were excluded from the study.

Results: JAK2 V617F-positivity was proven in 41 patients. Twenty-one thrombotic events were recorded in the prior history of PV patients, (before the clinical diagnosis of PV): myocardial infarction in five cases (8.6%), ischemic stroke or transient ischemic attack in nine cases (15.5%), venous thrombotic events in seven cases (12.1%). During the follow up period, six new thrombotic events were recorded: ischemic stroke or transient ischemic attack in five cases (8.6%), venous thrombotic event in one case (1.7%). Thirty-seven patients (63.8%) had high blood pressure. Eleven patients (19%) had hyperlipidaemia, nine patients (15.5%) had diabetes mellitus, and seven patients (12.1%) were cigarette smoker. Thirty-six women (62.1%) had normal weight (BMI 18.5–25), 22 (37.9%) woman were obes (BMI > 25).

The univariate analysis of the individual cardiovascular risk factors, the presence of diabetes mellitus ($P = 0.576$) or obesity ($P = 0.664$) did not show association with an increased risk of thrombosis in our study. However high blood preassure ($P = 0.053$), hyperlipidaemia ($P = 0.042$), and cigarette smoking ($P = 0.003$) are all associated with a significantly increased risk of thrombotic events. The association of cigarette smoking could be also confirmed using the multivariate binary logistic regression analysis ($P = 0.046$).

Summary/Conclusions: Based on our findings we concluded that polycythemia vera female patients, with cardiovascular risk factors (especially high blood pressure, hyperlipidemia, smoking) may have a higher risk for thrombotic events. In the future, in a prospective analysis we plan enlarge our study group for comparing the risk status of female PV patients who use or do not use oral contraceptives or HRT.

PB 2.66-4
Carbetocin increases thrombin generation after cesarean section

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Background: Uterine atony is the first cause of haemorrhages at delivery. To prevent post partum major bleeding many strategies are adopted and uterotonic prophylactic drugs are commonly used after caesarean section. Thrombin generation, as expression of the balance between hyper- and hypo-coagulability, could give information about haemostatic assessment and modifications.

Aims: Aim of the present pilot study is to evaluate if women treated with carbetocin and oxytocin, two different uterotonic agents, show differences in thrombin generation test (TGT) after caesarean section (CS).

Methods: Twenty-eight women undergoing cesarean section, without previous bleeding or thrombotic events, were matched for age, weight, parity, race and for type of intervention (planned or emergency). Fourteen women were treated with a standard 2-h oxytocin IV infusion (10 IU) and 14 with carbetocin 100 μg IV. Blood samples for TG study and blood cell count were collected before delivery (T0), 1 h (T1) and 24 h (T2) after drug infusion. Blood samples were immediate centrifuged and poor platelet plasma (PPP) stored at −80°C. Thrombin generation was performed with a commercial assay (TGA Ceveron α, Technoclone). TG measured lag time, peak, velocity, endogenous thrombin potential (ETP). The activation of coagulation was obtained by adding small amounts of tissue factor and phospholipids. ETP results were expressed as nM-Thrombin/min.

Results: No differences were observed in TG profile between the two groups at T0, confirming homogeneity between the two groups. At T1 a significant increase in ETP was observed in the carbetocin group (ETP mean ± DS = 3810.8 ± 661.95) compared with oxytocin treated patients (ETP mean ± DS = 3588.7 ± 711.36) with $P < 0.05$. T2 showed the persistent ETP increase in carbetocin group, even if it didn't reach the statistical significance. Also other parameters like peak and velocity were significantly increased in the carbetocin compared with oxytocin group, both at T1 and T2. No differences in bleeding were observed in the two groups.

Conclusion: Our pilot study show a major ETP increase in patients treated with carbetocin, probably reflecting a much important thrombin activation associated with uterotonic action and longer half-life, in comparison with oxytocin. TGT showed to be sensitive in describing uterotonic agent effects on the haemostatic system.

PB 2.66-5

Changes in haemostatic parameters during the menstrual cycle and drospirenone-containing oral contraceptive use

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Background: Oral contraceptives (OC) increase the risk of venous thromboembolism. The risk is dependent on the OC formulation and can be explained by increased thrombin generation and acquired resistance to activated protein C (APC). OC containing a new progestogen-drospirenone (DRSP-OC) associated with an increase of thrombosis risk that is similar to that during the use of third generation OC and two times higher as compared to second generation OC. There is limited information available on the effect of DRSP-OC on thrombin generation, APC resistance and other coagulation parameters.
Aim: to investigate changes in the coagulation system during the menstrual cycle and DRSP-OC use.
Methods: In a study population consisting of 14 healthy women between 21 and 33 years of age we investigated the effect of menstrual cycle and subsequent use of DRSP-OC on thrombin generation, APC resistance, the function of the tissue factor pathway inhibitor (TFPI) system and on the plasma levels of prothrombin, antithrombin, factor (F) V, FX, FVIII, protein C, total and free protein S, and full length and free TFPI.
Results: During DRSP-OC use we observed a significant increase in APC resistance (128%), thrombin generation at low (66%) and high tissue factor concentrations (40%), plasma concentrations of prothrombin (20%), FX (28%), FVIII (17%) and protein C (32%). DRSP-OC use impaired the function of the TFPI system and decreased plasma levels of antithrombin (−7%), FV (−21%), total protein S (−23%), free protein S (−21%), full length TFPI (−44%) and free TFPI (−49%). All studied parameters remained unchanged during menstrual cycle.
The changes in total and free protein S caused by DRSP-OC use correlated with $r = 0.89$ and slope 0.97 (95%CI: 0.66–1.28), indicating that the decrease of functionally active free protein S is likely due to the reduction of the levels of total protein S and not to a shift in the distribution between free and bound fractions of protein S. In contrast, although both total and free TFPI decreased during DRSP-OC use, no correlation between changes in these two forms of TFPI was detected, suggesting that DRSP-OC may influence TFPI activity at various regulatory levels, such as synthesis, cleavage, binding to lipoproteins or clearance.
Conclusions: DRSP-OC induce multiple changes in coagulation, which lead to an increase of thrombin generation, particularly at low tissue factor concentrations and which more than doubles APC resistance. The changes in anticoagulant pathways such as protein C- and TFPI-systems are more pronounced than the changes in the coagulation pathways.

PB 2.66-6

Venous thromboembolism (VTE) prophylaxis in hospitalized obstetric patients in Ireland: a multicentre cross-sectional study

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Background: VTE complicates 1–2/1000 pregnancies, and the risk increases with age, mode of delivery, and presence of co-morbidities. It continues to be one of the leading causes of maternal death. Prophylaxis with low molecular weight heparin (LMWH) is safe. Despite having a population of only 4.5 million, Ireland has 20 maternity hospitals. Given this large number, it is difficult to standardize practice with international best practice.
Aims: To assess the prevalence of VTE risk in pregnant women in the hospital setting and to determine the proportion of at-risk patients who receive effective prophylaxis.
Methods: The study period was September 2011 to November 2012. All patients admitted to the participating hospitals on the day of investigation were assessed for risk of VTE on the basis of hospital chart review. Risk was assessed in accordance with the 2009 Royal College of Obstetricians and Gynaecologist Guidelines. Patients undergoing procedures or on the labour ward at the time of review were excluded. Ethical approval was obtained from the ethics committees governing all centers.
Results: Five hundred and forty pregnancies were reviewed across 16 centers. The average age of was 31 ± 5.65 years (Range 16–47) with 21.87% (117/535) aged over 35. Twenty-two percent (118/535) had a parity of three or more. The average weight was 71.51 ± 14.482 kg (Range 42–134 kg). Data on BMI was available for 77–34% were overweight and 21% were obese. One percent (6/420) had a BMI > 40. Thirty-one percent (168/540) were antenatal and 69% (372/540) were postnatal.
Sixty-three percent (105) of antenatal patients were low risk (< 2 risk factors), 35% (59) were intermediate risk (two or more risk factors, prophylaxis should be considered) and 2% (4) were high risk. All the high risk patients were on prophylaxis at an appropriate dose. Four percent (6) of the low risk patients were on prophylaxis unnecessarily. Only 7% (4/59) of the intermediate risk patients were on prophylaxis (3/4 were on too low a dose).
Among postnatal patients, 41% (153) were low risk (< 2 risk factors), 58% (217) were intermediate risk (two or more risk factors, require prophylaxis) and < 1% (2) were high risk. Eighty percent (296) were appropriately risk stratified and put on LMWH if necessary. Fifty-nine percent (219) of patients should have been on LMWH but only 42% (157) were (92% Tinzaparin and 8% Enoxoparin). This included eight patients who were on LMWH unnecessarily. Thirty-eight percent (59/157) were on too low a dose.
Conclusion: VTE prophylaxis is an important issue in obstetrics given its prominent role in maternal morbidity and mortality and the increasing prevalence of risk factors such as obesity and increasing maternal age. It is clear that while there is good awareness of the risk in the postnatal period, there is less emphasis on risk assessment in antenatal patients where prophylaxis is rarely used. Those on prophylaxis are also likely to be on too low a dose. Given the number of maternity hospitals inIreland, there is a role for a national guideline to standardize care for all pregnant women.

PB2.67 – Inflammation: Basic – II

PB 2.67-1

Factor VII-activating protease in concert with DNase removes nucleosomes from necrotic cells

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Background: Circulating cell-free DNA e.g. nucleosomes can be a potent immune activator, which is released into the circulation during inflammation. Nucleosome levels correlate with disease severity and mortality in patients with sepsis. Little is known about the mechanism on how DNA in the form of nucleosomes is released from dead cells. It has been shown that plasma can remove nucleosomes from late apoptotic cells and the plasma serine protease Factor VII-activating protease (FSAP) is responsible for this release. FSAP circulates as an inactive single-chain protein, which is activated upon contact with either apoptotic or necrotic cells. In contrast to late apoptotic cells

however, purified FSAP is not able to remove nucleosomes from necrotic cells.
Aim: We hypothesized that upon necrosis, endogenous DNase I activity may be required to facilitate efficient nucleosome release by FSAP and aimed to investigate the role of these serum proteins in this process.
Methods: Necrotic jurkat cells were incubated with serum or serum components and nucleosome release was monitored by measuring residual DNA content by flow cytometry. In addition nucleosomes were measured in the supernatant of these samples by means of a nucleosome specific ELISA. Finally, the extent of chromatin fragmentation of these samples was determined on agarose gel.
Results: Both serum and a combination of purified FSAP and DNase I are equally efficient to release nucleosomes from necrotic cells. However, purified FSAP or DNase I alone is not able to convey nucleosome release. Furthermore, both serum in which FSAP is blocked by a neutralizing antibody and FSAP-deficient serum are unable to induce nucleosome release from necrotic cells. Finally we show that in contrast to FSAP or DNase I alone, a combination of both FSAP and DNase I can induce chromatin fragmentation.
Conclusion: FSAP and DNase cooperate in the release of nucleosomes from necrotic cells. This synergistic activity might play a crucial role in the release of pro-inflammatory nucleosomes upon inflammation or tissue damage.

PB 2.67-2
SMTP-7, a novel small-molecule thrombolytic with an anti-inflammatory potential, improves primate thrombotic stroke with reduced hemorrhage risk

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Background: SMTP-7, a small molecule that promotes plasminogen activation through relaxation of plasminogen conformation, has excellent therapeutic activities in cerebral infarction in several rodent models, possibly due to its thrombolytic and anti-inflammatory, crebroprotective potentials.
Objective: Detailed evaluation of SMTP-7 efficacy in a primate stroke model is anticipated in view of novel, safe drug development, especially in terms of reduced hemorrhage risk.
Methods: A monkey photochemical-induced thrombotic middle cerebral artery (MCA) occlusion model, as well as several other rodent models, was utilized to evaluate therapeutic potentials of SMTP-7.
Results: In the monkey model, in which thrombotic MCA occlusion was followed by recanalization/reocclusion, Compared with saline control, SMTP-7 (10 mg/kg, iv infusion) increased post-infusion MCA recanalization rate (32.5-fold, $P = 0.043$) and ameliorated post 24-h neurological deficit (by 29%, $P = 0.02$), cerebral infarct (by 48%, $P = 0.025$), and even cerebral hemorrhage (by 51%, $P = 0.013$). In normal monkeys, SMTP-7 did not affect general physiological and hemostatic variables including coagulation and platelet parameters. Investigations on rodent models of transient and permanent focal cerebral ischemia as well as endothelial-injured arterial thrombosis and bleeding tests, suggested an importance of the drug's crebroprotective properties as well as its regulated profibrinolytic action that served as a physiological on-demand system coping with thrombotic events.
Conclusions: SMTP-7 is effective in treating thrombotic MCA occlusion stroke in monkeys, ameliorating neurological deficits, infarct size, and even hemorrhagic transformation, which may be attributable to both regulated thrombolytic promotion and neuroprotective properties as demonstrated in several other animal models.

PB 2.67-3
Oral anti-thrombin abrogates microvessel permeability during experimental ischemia

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Background: Non-valvular atrial fibrillation (AF) increases the risk and severity of thromboembolic stroke, however prevention of thrombus formation with an anti-thrombotic agent increases intracerebral hemorrhagic risk. The RE-LY trial demonstrated significant reductions in thromboembolic stroke and intracerebral hemorrhage with the direct thrombin inhibitor dabigatran compared to warfarin. Thrombin is generated acutely in the wall and abluminal space of cerebral microvessels during focal ischemia at a time when the (blood-brain) permeability barrier is compromised, and thrombin is itself a putative mediator of increased microvessel permeability.
Aims: We hypothesized that dabigatran could decrease the risk of intracerebral hemorrhage by direct inhibition of thrombin-induced increases in microvascular permeability. These studies focus on the cellular components of the microvessel.
Methods: Based on *in vivo* work in the non-human primate focal ischemia model, we developed high-quality *in vitro* systems mimicking endothelial cell-matrix-astrocyte interactions of cerebral microvessels. Primary murine brain endothelial cells (mBEC) without or with astrocytes were exposed to purified murine thrombin (0–10 U/mL) in serum-free media for 1 h prior to measuring permeability to a molecular weight range of FITC-labeled dextrans. Co-culture with astrocytes significantly decreases endothelial permeability. Cell viability was confirmed by a propidium iodide uptake assay. Coincident expressions of endothelial cell integrin and the tight junction protein claudin-5 following thrombin exposure were measured by flow cytometry. In separate studies, experimental ischemia (oxygen-glucose deprivation, OGD) was induced by placing cells in low-glucose media in an airtight container purged with N_2 and incubating 18 h at 37°C.
Results: Thrombin increased mBEC permeability to 4 kDa FITC dextran in a concentration-dependent manner (P_{app} for control: $2.98 \times 10^{-6} \pm 0.87 \times 10^{-6}$ cm/s, for 10 U/mL thrombin $4.37 \times 10^{-6} \pm 0.39 \times 10^{-6}$ cm/s, $n = 6$, $2P = 0.0109$), without endothelial cell demise ($\leq$ 20 U/mL thrombin). Pre-treatment of mBEC with dabigatran (24 h, 500 nM) completely abrogated the effect of 10 U/mL thrombin on permeability under conditions of normoxia. The results were similar when endothelial cells and primary astrocytes were grown in apposition, and subject to thrombin ± dabigatran exposure. Thrombin-induced permeability was not due to decreased expression of beta 1 integrins or claudin-5. OGD also increased permeability of mBEC, and this effect was also abrogated by treatment with dabigatran.
Summary/conclusions: Dabigatran blocks thrombin-induced brain endothelial cell (*in vitro* microvessel) permeability. These findings indicate that thrombin increases mBEC permeability by direct modification of other tight junction protein(s) than claudin-5 or via a transcellular mechanism. They imply that the development of thrombin during an embolic ischemic stroke (in the setting of AF) contributes to increased microvessel permeability. Ongoing studies further detail the mechanisms by which thrombin increases cerebral microvascular permeability and hemorrhagic risk.

PB 2.67-4
Connective tissue growth factor (CTGF/CCN2) is over-expressed via TGF beta pathway in synoviocytes upon cell-to-cell interaction with platelets

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Background: CTGF/CCN2 is an important molecule for extracellular matrix development and remodeling. It has potent angiogenic prop-

erties and it is present in fibrotic tissue and on the surface of atherosclerotic plaques. In inflammatory conditions such as rheumatoid arthritis (RA), CTGF is increased in plasma and synovial fluid, and it might contribute to increase inflammation, angiogenesis and synovial fibrosis. Interestingly, CTGF is also stored in the α granules of platelets and it is released upon activation. Platelets have been shown to infiltrate into the synovial fluid during RA and contribute to the inflammation of the joint. Thus, we hypothesize that CTGF is involved in (i) worsening the formation of atherosclerotic plaque via platelet activation and granule-content release with subsequent platelet adhesion and in (ii) the perpetuation of synoviocyte hyperplasia.

Aims: (i) To evaluate platelet adhesion in the presence of CTGF/CCN2 (ii) To evaluate the potential role of platelet/synoviocyte interaction for the expression of CTGF/CCN2 and (iii) to delineate potential signaling pathways in platelets and synoviocytes leading to CTGF/CCN2 expression.

Methods: We performed *in vitro* studies on platelets and platelets/fibroblast-like synoviocytes co-cultures, analyzing CTGF/CCN2 effects by immuno-fluorescence, adhesion assay, flow-cytometry and Western blot analyses.

Results: Platelets adhere and spread on recombinant (r) CTGF-coated slides in a concentration dependent manner similar to platelet adhesion on fibrinogen. The platelet spreading area on CTGF surface (1 μg/mL) was significantly increased (8-fold) in comparison to control (BSA). Furthermore, platelets pre-treated with rCTGF (50 nM) increased ERK phosphorylation upon ADP stimulation. In contrast, pretreatment with rCTGF did not cause platelet aggregation or secretion by itself. Platelet/synoviocyte co-cultures treated with rCTGF showed an increase in cell-cell aggregates formation, assessed by flow cytometry and immunofluorescence. CTGF/CCN2 was also increased when synoviocytes were co-cultured with resting platelets in a time dependent manner assessed by Western blot analyses. In order to understand the role of synoviocytes in CTGF/CCN2 production, we analyzed the SMAD proteins, which are part of the TGFβ pathway. Activation of the TGFβ-receptor will result in phosphorylation of SMAD2, formation of a SMAD2/3 complex and subsequent CTGF/CCN2 expression. SMAD2 phosphorylation was increased in synoviocytes after co-culture with resting platelets, suggesting that synoviocytes increased CTGF/CCN2 expression.

Conclusions: Platelets adhere and spread into CTGF/CCN2 surfaces, suggesting a possible atherogenic effect for CTGF/CCN2 *in vivo*. Furthermore, CTGF/CCN2 exposure could augment platelet ability to adhere to synoviocyte. Overall, these data indicate that CTGF/CCN2 may contribute to increase and/or sustain joint inflammation, suggesting a possible target for future therapeutic strategies in RA

PB 2.67-5

Thrombin modulates the expression of a set of proinflammatory genes including tissue factor in human monocytes

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Background: Besides its role in blood coagulation, thrombin is known to act as a cell signaling molecule via activation of protease-activated receptors (PAR), thereby connecting this coagulation protease to specific cellular/inflammatory responses. Previous studies demonstrated that thrombin is capable of modulating chemotaxis of monocytes and also inducing expression of monocyte chemotactic protein (MCP-1), plasminogen activator inhibitor type 2 (PAI-2), matrix metalloproteinase-9 (MMP-9) or interleukin (IL) eight in monocytes. However, no study has analyzed the gene expression changes of thrombin-stimulated monocytes in a systematic way using microarray experiments.

Aim: The aim of this work was to evaluate the gene expression profile of thrombin-stimulated monocytes by microarray technology.

Methods: Peripheral blood mononuclear cells (PBMC) were isolated from human blood by density gradient centrifugation using Ficoll-Paque PLUS and affinity adsorption using anti-CD14 monoclonal antibody-coated microbeads combined with a magnetic cell separation system. Monocytes were stimulated with 10 U/mL human alpha-thrombin at 37 °C for 5 h, and after washing cells, total RNA was isolated using the RNeasy mini Kit (Qiagen). Labeled cRNAs were hybridized to the Whole Human Genome Oligo Microarray Kit 4x44K v2), including cRNA fragmentation, hybridization (Agilent Technologies, protocol V5.7). Slides were scanned on the Agilent Micro Array Scanner G2565CA. DAVID database (http://david.abcc.ncifcrf.gov) was used to dynamically assign gene expression data to the Gene Ontology (GO) biological-process categories.

Results: Eighty-three genes were selected which showed differential expression changes; 68 genes were up-regulated whereas 15 genes were down-regulated. Near 30% of the upregulated genes were related to the inflammatory response. Thus, genes encoding several cytokines and chemokines, associated with the recruitment and activation of leukocytes, were differentially expressed in monocytes in response to thrombin. Moreover, the gene for tissue factor was also up-regulated. Real-time reverse transcriptase polymerase chain reaction (RT-PCR) was used to confirm the microarray results of some of these genes. Moreover, higher expression of tissue factor in monocytes exposed to thrombin was also demonstrated by immunofluorescence and by shortening of clotting time.

Conclusion: Our results provide new evidences to the thrombin-mediated link between coagulation and inflammation through changes in gene expression in monocytes.

PB 2.67-6

The impact of glycation on aspirin-induced acetylation of human serum albumin: an *in vitro* study

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Background: Aspirin (ASA) acetylation is a post-translational modification that occurs through the transfer of its acetyl residue to the amino groups of proteins located at lysine and N-terminal residues. This modification plays a key role in medicine, since it improves the level of inflammation and partially prevents the risk of cardiovascular ischemic events. However, the biological effects of ASA on platelet function as well as its clinical benefit are lower in diabetic patients, a phenomenon described as 'aspirin resistance'. The effect of ASA was demonstrated in several human plasma proteins including haemoglobin, fibrinogen and serum albumin (HSA). HSA is the main transporter of drugs, hormones, fatty acids and metabolites, plays an important role in the regulation of osmotic pressure and is considered as an aspirin esterase. Considering the widespread use of aspirin, investigation of the acetylation and glycation of albumin is of interest.

Aim: The purpose of this study was to identify the albumin acetylated residues after incubation of HSA with an increasing ASA concentration by immunoblotting and mass spectrometry; and to determine whether pre-incubation of HSA with glucose inhibits ASA-acetylation.

Methods: HSA was incubated with an increasing ASA concentration (0, 0.02, 0.2, 2 and 20 mM) and the acetylation and glycation levels were measured by immunoblotting and high-resolution tandem mass

spectrometry (MS). In a second analysis, HSA was first incubated with glucose (10 mM) followed by ASA (20 mM). The acetylation and glycation levels were again measured.

Results: Incubation of HSA with ASA resulted in a dose-dependent increase of acetylated sites, among which only one was found as endogenous in native HSA, while six and 32 sites were identify at very low and very high ASA concentration, respectively. A significant decrease in the acetylation level was observed by Western blot when HSA was previously incubated with glucose. This result was confirmed by MS. Four sites were found to be common targets for both acetylation and glycation modifications.

Summary/Conclusions: We evidenced here a dose-dependent increase of the number of acetylation sites on HSA. Glycation had a major impact in decreasing the aspirin-induced acetylation process on HSA. The preferential amino acid sites for both post-translational modifications were identified as well. The influence of glycation on HSA acetylation could impair its biological functions, potentially leading to the worsening of the typical diabetes complications. This proof-of-concept study may pave the way to a better understanding of the acetylation – glycation interactions at the protein level in diabetic patients.

Keywords: aspirin, acetylation, human serum albumin, glycation.

PB2.68 – Inflammation: Clinical – I

PB 2.68-1

Rhinovirus infection induces procoagulant changes in parallel with eosinophilic airway inflammation

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Background: Asthma exacerbations are frequently triggered by rhinovirus infections. Asthma itself is associated with activated coagulation and increased risk of venous thromboembolism[1] and also respiratory viruses may activate hemostasis. *Vice versa*, a prothrombotic state in the lung can also induce or aggravate pulmonary inflammation.

Aim: To determine whether rhinovirus infection and asthmatic airway inflammation act on the local and systemic hemostatic balance in patients *in vivo*.

Methods: In a two-groups parallel study design 28 volunteers (14 patients with mild asthma (seven females, 19–26 years) and 14 healthy controls (13 females, 19–31 years)) were experimentalliy infected with low-dose rhinovirus serotype 16 (RV16). Patients with mild asthma were stable after discontinuation of their asthma medication 2 weeks prior to RV16 inoculation. Venous plasma and bronchoalveolar lavage fluid (BAL fluid) were obtained 1 day before and 6 days after rhinovirus challange to evaluate several key markers of coagulation activation in plasma and BAL fluid, as well as the coagulant features of microparticles in BAL fluid. Thrombin-antithrombin complexes (TATc), von Willebrand factor (vWF), Plasmin-antiplasmin complexes (PAP), Plasminogen activator inhibitor type-1 (PAI-1), and eosinophil cationic protein (ECP) in plasma and BAL fluid were measured by immunoassay. Endogenous thrombin potential (ETP) was analysed using the Calibrated Automated Thrombogram® and tissue factor bearing microparticles, measured by fibrin generation test (FGT). Eosinophils were counted on cytospin preparations. Comparisons and correlations were performed by non-parametric testing.

Results: In plasma, RV16 challange resulted in increased PAI-1 levels in patients with asthma after viral infection (26.5 ng/mL in patients with astma vs. 10.0 ng/mL in healthy controls, $P = 0.01$) and decreased PAP levels (318 vs. 534 ng/mL resp., $P = 0.04$). Changes in PAI-1 levels were significantly elevated in asthma than in control subjects (3.0 vs. −3.5 ng/L respectively, $P = 0.024$), while changes in TATc, D-dimer, vWF and ETP did not differ between both groups. In BAL fluid, the FGT shortened after viral infection in asthma (t = −1 day: 689s vs. t = 6 days: 516 s; $P = 0.011$), but not in healthy controls (t = −1 day: 695s vs. t = 6 days: 672 s; $P = 0.79$). The changes in TATc and PAP did not differ between both groups and vWF, D-dimer and PAI-1 were below the detection limit.

Both FGT and TATc in BAL fluid correlated (Spearman) with eosinophil counts and ECP ($r = -0.583$ and -0.682 resp. for FGT and $r = 0.535$ and 0.619 resp. for TATc, all $P < 0.01$)

Conclusion: Experimental rhinovirus infection induces procoagulant changes in patients with asthma systemically through PAI-1 and in the airways by TF-bearing microparticles. This did not lead to changes in global assays of hemostasis, probably due to the relatively mild infection in patients with mild to intermittent asthma. In the airways, microparticle-associated procoagulant changes are associated with eosinophilic inflammation, suggesting that virus infection and eosinophilic inflammation both act on the hemostatic (dis)balance during asthma exacerbations.

Reference:

1. J.D. de Boer et al., Blood 2012;119(14):3236–44

PB 2.68-2

Role of platelets and MMP2 in osteoarthritis joint inflammation: effect of hyaluronic acid

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Background: Osteoarthritis (OA) is the most common form of arthritis and, although mainly a degenerative disorder, it is now demonstrated that inflammation takes part in the early development and in the progression of this chronic degenerative joint disease.

OA is characterized by cartilage breakdown, consequent to extracellular matrix (ECM) degradation caused in particular by an increased local formation of matrix metalloproteinases (MMPs), a family of zinc-dependent endoproteinases.

Platelets are involved in different inflammatory conditions, including rheumatoid arthritis (RA), but their role in OA has not been investigated yet.

Platelets contain and release several MMPs, including MMP-2, and they enhance MMPs expression by other cell types. We have recently shown that MMP-2 contributes to joint degeneration in group B Streptococcus-induced arthritis (Puliti M. et al., Arthritis Rheum. 2012 Apr;64(4):1089–97).

Viscosupplementation by the intra-articular administration of hyaluronic acid (HA), a non-sulphated glycosaminoglycan physiologically present in synovial fluid, is a widely used treatment for OA.

Aims: Aims of our study were to evaluate the role of platelets in joint inflammation in OA patients and to assess the effect of HA on platelets and on their interaction with synoviocytes (FLS).

Methods: We collected synovial fluid (SF) from 38 patients with knee-OA (T0). Twenty-seven out of these 38 patients were re-analyzed after 5 weekly HA intra-articular administrations (T1). The following parameters were measured in SF: total cell count, platelet count and platelet-leukocyte aggregates (by flow-cytometry) and MMP-2 levels (by zymography).

Moreover, FLS isolated from SF of OA patients were co-cultured with platelets in the presence or not of HA, and MMP-2 release was measured by zymography. Platelet P-selectin was measured as an index of platelet activation.

Results: HA treatment significantly reduced total cell count (T0: 943.9 ± 144.1 vs. T1:573.1 ± 62.97 $P < 0.05$), platelet count (T0: 32.83 ± 22.31 vs. T1: 3.70 ± 1.06 $P < 0.05$), platelet-leukocyte aggregates (T0: 7.55 ± 2.31 vs. T1:2.19 ± 0.67 $P < 0.05$) and MMP-2 levels (T0: 468.1 ± 72.69 ng/mL vs. T1: 428.2 ± 68.89 ng/mL $P < 0.05$) in SF.

In vitro, platelets increased MMP-2 release by FLS (FLS: 45.27 ± 11.80 ng/mL vs. FLS + Plts: 64.76 ± 15.50 ng/mL $P < 0.05$). Platelet activation, evaluated as P-selectin expression, increased significantly after 24 h of co-culture (Plts: 8.75 ± 2.15 ng/mL vs. Plts + FLS: 20.70 ± 3.19 ng/mL $P < 0.05$). HA decreased significantly the release of MMP-2 by FLS-platelet co-cultures (FLS + Plts: 64.76 ± 15.50 ng/mL vs. FLS + Plts + HA: 20.84 ± 4.78 ng/mL $P < 0.05$).

Conclusions: Platelets are involved in OA and may participate in cartilage degradation by facilitating the expression and release of MMP-2 by synoviocytes. Platelet P-selectin contributes to this activation. Treatment with HA reduces platelet activation in synovial fluid of OA patients and reduces the release of MMP-2 by platelet-synoviocyte co-cultures.

PB 2.68-3

Defining platelet function in polytrauma patients upon admission to the emergency department

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Background: Haemostatic function is impaired by multisystem injury and impacts survival. Recent description of Trauma Induced Coagulopathy (TIC) has found significant relationships between the onset and degree of coagulopathy and injury severity in trauma patients.

Aims: To characterize the relationships between platelet function and injury severity with clinical outcomes in response to trauma.

Methods: We conducted a prospective observational trial of clot formation in trauma patients presenting to a U.S. level I trauma center. Blood samples and clinical data were collected upon arrival to the emergency department and platelet function was determined using a comprehensive panel of tests including viscoelastic clot strength and platelet mapping, platelet-associated thrombin generation, platelet-induced clot contraction and effect on clot structure, platelet aggregation, flow cytometry, prothrombin time (PT), activated partial thromboplastin time (aPTT) and fibrinogen. Exploratory hierarchical clustering analysis of patient admission data resulted in clustering using the Injury Severity Score (ISS); mild/moderate (1–15), severe (14–24) and profound (=25). ISS groups were then used to group patients and compare compare to healthy volunteers.

Results: The ISS score was available on 94 trauma patients including 36% with mild/moderate injury; 26% with severe injury and 38% with profound injury. The presence of traumatic brain injury (TBI) was significantly greater in the profound injury group (42%) compared to the other groups ($P = 0.019$). When compared to healthy patients, the mild/moderate, severe, and profound ISS groups, respectively had relatively lower fibrinogen concentrations (median [range] 247.0 [215.75, 324.00], 226.5 [176.50, 342.00], 175.7 [114.50, 221.50] mg/dL, respectively, [$P < 0.001$]). Severe and profound injury had severe base deficits, respectively (median [range] −5.3 [−7.13, −1.15]; −4.7 [−10.45, −2.13] mEq/L). Profound injury patients had normal TEG maximum amplitude (mean [SD] 57.5 [9.3] mm), reduced collagen impedance aggregation (median [range] 12.3 [7.75, 15.00] Ω), and prolonged PT (17.5 [15.03, 21.48] s).

Summary/conclusion: All ISS groups showed signs of TIC, but only patients with severe and profound ISS classifications had significant tissue hypo-perfusion, prolonged PT and relatively reduced fibrinogen levels, suggesting a disseminated intravascular coagulopathy pattern. As there were more TBI patients in the profound ISS group, this may suggest an association between TBI and altered haemostasis. The ISS may be a useful tool to predict the degree of coagulopathy in polytrauma patients, but further prospective trials are needed.

PB 2.68-4

Changes of platelets status in pts with inflammatory cardiac pathology

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Background: Viruses are able to induce myocarditis and lead to subsequent inflammatory dilated cardiomyopathy (DCM) development. Cardiac rhythm and conduction disturbances can be direct consequence inflammation in myocardium. Coxsackie-adenovirus receptor (CAR) a protein of tight junctions generally present at the cardiac intercalated disks recently was shown on platelets in the sites of intracellular communications.

The aim of our study was to evaluate platelets property in patients (pts) with DCM/myocarditits compared to pts with conduction delays with structurally normal heart.

Methods: Platelet spontaneous and ADP-induced aggregation (studied by simultaneous analysis of the mean aggregate size changes kinetics and light transmission (LTA) using an aggregation analyzer BIOLA (Russia) as well as CAR expression was investigated in 13 pts with DCM/myocarditis and in 19 pts with different grade atrioventricular and left bundle branch block, which were diagnosed by routine clinical investigation. Diagnosis of DCM/myocarditis was confirmed according endomyocardial biopsy (EMB) data. Ten healthy subjects (HS) were included as a control. Platelets of pts of both study groups and also HS were analyzed using immunofluorescent method on CAR persistence by microscopic evaluation and also by flow cytometry. In eight pts the persistence of connexin 45 was assessed.

Results: In whole blood of nine from 13 DCM/myocarditits pts were found circulating microaggregates. These group of pts demonstrated high level of platelet activation which was induced by release reaction. The level of 1.0 μM ADP and 5.0 μM ADP induced aggregation was 43 ± 21% ($N < 25\%$) and 52 ± 24% ($N < 68\%$) respectively. Spontaneous aggregation was 3.7 ± 1.8% at the first 5 min, but to the 9–14 min its level grew up to 37 ± 17.3% similar to ADP induced aggregation ($P = 0.2$) as a result of platelet release reaction that was never observed in HS. Microaggregates persistence and high level of platelet aggregation can possible reflect microvascular changes due to inflammatory infiltration revealed by endomyocardial biopsy data in all these pts. The level of aggregation of the rest four pts was in normal ranges that can be explained by aspirin uptake according clinical indications. Similar induced aggregation was demonstrated in nine pts from group with conduction delays (1.0uM ADP was 40 ± 19.1% and 5.0uM ADP was 51 ± 17.6%, $P = 0.1$). Spontaneous aggregation was 1.76 ± 0.72 r.u.(normal range < 1.4 r.u) without release reaction. Complex of clinical investigation confirmed structurally normal heart in these pts. The estimation of platelets with CAR expression was much higher in DCM/myocarditis pts (14 ± 9.9% vs 3.5 ± 1.9% in pts with conduction delays and HS). CAR persisted predominantly at the sites of intracellular communication in the aggregated and was co-localized with connexin 45 which was firstly detected at human platelets. The level of CAR expression was higher in DCM/myocarditis pts with clinical and EMB data confirmed the sigh of active inflammation.

Summary/Conclusions: Platelet activation can reflect microvascular changes in DCM/myocarditis pts. The high level of CAR expression, its appearing in the sites of intracellular connection and co-localization with connexin 45 may indicate the role of these two receptor in platelets changes during inflammation.

PB 2.68-5

Protein chip array and thrombotic biomarker profiling of plasma samples undergoing bypass surgery

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Introduction: In cardiovascular surgical procedures, such as coronary artery bypass grafting (CABG) and valve repair/replacement, patients are exposed to large doses of heparin and protamine sulfate. There is a high prevalence of anti-heparin/platelet factor-4 antibodies in these patients. Although a fraction of these patients develop HIT syndrome, the role of circulating anti-heparin/platelet factor-4 antibodies is not clear. Such thrombogenic mediators as microparticle and tissue factor may be up regulated in these patients. The relevance of thrombotic mediators and inflammatory processes remains to be further explored in these patients. Recently the protein chip array approach using SELDI/TOF mass spectrometry methods have been employed to identify unique biomarkers in various diseases. The purpose of this study was to determine the protein chip array profile and various mediators of thrombotic activation in patients who have undergone coronary artery bypass surgery.
Materials and Methods: Plasma samples from 79 cardiovascular patients were collected prior to surgery and one and 2 weeks afterwards. Protein chip array profiling was carried out on a SELDI/TOF mass spectrometric method (PCS4000, BioRad; Richmond, CA) employing a gold chip array in the molecular weight range of 3000–150,000. The intensity of unique peaks was also calculated in terms of relative intensities. Cellular microparticles were measured using a functional method (Hyphen Biomedical; France) and tissue factor antigen levels were measured using an ELISA method (Hyphen). The anti-heparin/platelet factor-4 antibodies were also measured using a commercially available ELISA method (GTI; Wisconsin).
Results: Of the 79 patients, 20 showed a unique peak around 11–12 kDa in the pre-op samples, which was absent from normal controls. Seventy-seven of the patients showed this unique biomarker peak at 1 week, whereas only 48 patients exhibited the peak at 2 weeks after surgery. The relative intensity of the 11.6 kDa biomarker was much higher at 1 week (6-fold) and was decreased at 2 weeks (3-fold). In addition to this unique peak, additional biomarker peaks were noted at 15.1 and 15.8 kDa. In comparison to the normal controls, the microparticle levels were higher in the baseline sample (10.1 ± 3.2 nM) and increased to 19.3 ± 6.1 and 24.5 ± 8.1 nM. Similarly, the tissue factor levels were increased at the 2 weeks time period. The anti-heparin/platelet factor-4 titer rose 28% from the baseline at week 1 and 33% at week 2.
Conclusions: The results on the biomarker profile are consistent to one of our earlier findings, where the presence of a unique biomarker in the range of 11–12 kDa has been reported in patients with high prevalence of anti-heparin/platelet factor-4 antibodies. The increased level of microparticles and tissue factor at post-surgical periods of 1 and 2 weeks suggests endogenous activation of thrombogenic mechanisms, which appears proportional to the up regulation of the anti-heparin/platelet factor-4 antibodies. Thus, this data indicates that the non-functional anti-heparin/platelet factor-4 antibodies may result in the activation of cellular processes leading to thrombogenesis. Further characterization of the unique biomarkers identified in these patients may be useful in the understanding of the pathogenesis of thrombogenesis in cardiovascular surgery patients.

PB 2.68-6

Effect of long-term intake of serotonin reuptake inhibitors on neutrophil function in humans

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Background: Blood serotonin is stored in the dense granules of platelets and released upon activation. Treatment with serotonin reuptake inhibitors (SRIs) depletes platelet serotonin levels and thus reduces serum serotonin. This reduction results in reduced leukocyte rolling and adhesion in mice.
Aims: Based on findings in mice we asked, whether long-term intake of SRIs impairs neutrophil function in humans.
Methods: We compared 29 patients treated for major depression with SRIs to seven depressive patients not treated with SRIs. A control group consisted of 17 non-depressive study subjects. Severity of depression was documented by Beck Depression Inventory II. The frequency of respiratory tract or urogenital infections, infective gastroenteritis and pharyngitis was assessed by a questionnaire. Blood cell count and CRP measurement was conducted. The concentration of serum serotonin was determined by ELISA. Expression of neutrophil surface antigens involved in neutrophil recruitment was investigated by flow cytometry, as was the production of reactive oxygen species (ROS) and intracellular calcium flux upon activation with Phorbol 12-myristate 13-acetate (PMA) and Leukotriene B4.
Results: Serum serotonin levels in patients treated with SRIs were reduced by 75% ($P < 0.02$). No difference in the frequency of infective disorders was seen. The production of ROS upon stimulation with PMA was significantly reduced in patients treated with SRIs whereas intracellular calcium flux was increased. Flow cytometry showed a trend towards higher expression levels of MAC-1 in patients treated with SRIs (mean fluorescence intensity 916 ± 253) compared to patients with no treatment (MFI 491 ± 119) and healthy study subjects (MFI 519 ± 119, Kruskal–Wallis-$P = 0.08$).
Summary: Long-term intake of SRIs was not associated with a change in the frequency of infections as assessed by a questionnaire. Flow cytometry however showed differences in calcium flux, ROS production, and surface expression of some markers, warranting further research on the influence of SRIs on immune functions.

PB2.69 – Inherited Risk Factors Venous Thrombosis: Basic – II

PB 2.69-1

Haplotypes of the endothelial protein C receptor gene and circulating protein C levels

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Background: The protein C pathway plays a crucial role in the regulation of blood coagulation, by controlling the generation of thrombin. Protein C circulates in plasma as an inactive zymogen and is activated on the vascular endothelial cell membrane by the thrombin-thrombomodulin complex, a process further enhanced when protein C binds to its membrane receptor, the endothelial-cell protein C receptor. The information about the genetic factors that determine the variation in plasma protein C (PC) levels is limited. It has been reported that, apart from the PC gene itself, the endothelial PC receptor gene (*PROCR*) and the *FOXA2* gene explain part of the variation.
Aims: To analyze the association between two of the *PROCR* haplotypes, H1 and H3, and PC plasma levels.

Methods: We determined the levels of PC and soluble (s) EPCR and analyzed the *EPCR* haplotypes H1 and H3 in 467 healthy individuals, in 358 patients with a history of venous thromboembolism (VTE) and in 225 young patients with myocardial infarction (MI). Informed consent was obtained from all subjects of the study and it was approved by the medical ethics committee of our Institution.
Results: Among controls, PC level was higher in the 81 carriers of the H3 haplotype (115 ± 22%) than in the 386 non-carriers (102 ± 18%) ($P < 0.001$), but similar in the 133 carriers of the H1 haplotype (105 ± 19%) and in the 339 non-carriers (104 ± 19%), and there was a significant correlation between PC and sEPCR levels ($r = 0.312$, $P < 0.001$). Among VTE patients, PC levels were also higher in the 64 carriers (113 ± 25%) than in the 294 H3 non-carriers (102 ± 23%; $P < 0.001$), but similar in the 114 carriers of the H1 haplotype (105 ± 23%) than in the 244 non-carriers (101 ± 24%), and there was again a significant correlation between PC and sEPCR levels ($r = 0.307$; $P < 0.001$). Finally, among MI patients the 26 carriers of the H3 haplotype had PC levels (117 ± 18%) higher than the 199 non-carriers (104 ± 18%; $P = 0.001$), with a significant correlation between PC and sEPCR levels ($r = 0.276$, $P < 0.001$).
Conclusion: These results suggest that the *PROCR* H3 haplotype is, in part, responsible of the variations in plasma PC levels. We do not know the mechanism by which the H3 haplotype influences plasma PC levels. As this haplotype is associated with elevated sEPCR levels, it has been suggested that PC may bind to sEPCR and become more stable. However, this is unlikely because the concentration of sEPCR in plasma is much lower than that of PC. We hypothesize that carriers of the H3 haplotype have less membrane-bound EPCR, either because H3 may induce a lower expression of the *EPCR* gene and/or because H3 is associated with increased shedding, or both. As part of the circulating PC is bound to the membrane-bound EPCR, less membrane-bound EPCR in carriers of the H3 haplotype will led to less PC bound to the membrane and higher free, circulating PC. These data may help to further increase the information on the physiological mechanisms involved in the regulation of PC.

PB 2.69-2

Association of haplotypes (H) 1 and 3 of the endothelial protein C receptor gene (PROCR) with venous thromboembolism

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Background: Protein C is a vitamin K-dependent glycoprotein that circulates in plasma as a precursor of a serine protease and is activated on the endothelial cell surface by thrombin bound to its receptor, thrombomodulin, localized on that surface. This activation is further enhanced when protein C binds to its membrane receptor, the endothelial-cell protein C receptor (EPCR). Activated protein C (APC) regulates blood coagulation by selectively inactivating factors Va y VIIIa by limited proteolysis. Several studies have reported that plasma levels of APC and soluble forms of EPCR (sEPCR) are determined by haplotypes H1 and H3 of the EPCR gene (*PROCR*), respectively. However, there are conflicting results as to the associations of *PROCR* H1 and H3 with the risk of venous thromboembolism (VTE).
Aim: To analyzed these associations in a case-control study.
Methods: We studied 676 VTE patients and 505 sex- and age-matched healthy subjects (controls). The g.4678G>C (which targets the *EPCR* H1) and the g.4600A>G polymorphism (which targets the *EPCR* H3) were genotyped by sequencing the corresponding amplified region. We also measured APC levels by a homemade ELISA and sEPCR levels with a commercial ELISA (Asserachrom sEPCR, Stago) in 461 VTE patients and 505 controls in whom adequate plasma samples was available. Informed consent was obtained from all subjects of the study and it was approved by the medical ethics committee of our Institution.
Results: The frequency of the *PROCR* H3 was similar in patients and controls ($P = 0.99$). However, the frequency of the H3H3 genotype (4600GG) was higher in patients than in controls (OR 12.7, CI95% 0.7–220.0, $P = 0.024$): eight VTE patients and none of the controls carried the 4600GG genotype. The level of sEPCR was higher in carriers than in non-carriers of the 4600G allele, both in patients and in controls ($P < 0.001$). In contrast, the level of APC was lower in the eight patients carrying the 4600GG genotype than in the 379 carrying the 4600AA genotype ($P = 0.005$). A significant difference was observed between cases and controls for the frequency of the *PROCR* H1 (OR 0.7, 0.5–0.9, $P = 0.002$). Among controls, the level of APC was higher in carriers than in non-carriers of the *PROCR* H1 ($P = 0.006$).
Conclusion: These data confirm previous result indicating that the *PROCR* H1 protects against VTE, possibly via increased APC levels. They also confirm previous reports showing that the *PROCR* H3 is not associated with the risk of VTE. However, although the number of cases is small, carriers of the 4600GG (H3H3) genotype showed an increased risk of VTE in our population (Valencia, Spain), via increased sEPCR levels and decreased APC levels, suggesting that the 4600GG genotype is associated with lower membrane-EPCR expression and lower APC generation.

PB 2.69-3

Sixteen different mutations in the SERPINC1 gene with antithrombin deficiency causing venous thrombosis

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Introductions: Antithrombin is the primary inhibitor of thrombin and other activated serine proteases in the blood coagulation system. Inherited impaired function or deficiency of this molecule significantly increases the risk of thrombosis. We previously identified 10 different mutations in the *SERPINC1* gene, which encodes antithrombin, from 10 unrelated Japanese antithrombin deficient patients with thrombotic disease. In the present study, we analyzed the *SERPINC1* gene in six newly identified patients with low levels of antithrombin activity using direct sequencing and a multiplex ligation-dependent probe amplification (MLPA) assay and performed diagnosis of the carrier detection of their family members.
Patient and Methods: The patients were unrelated Japanese adults, five of the six patients had developed deep vein thrombosis. Antithrombin activity levels were determined by amidolytic heparin cofactor assay with chromogenic substrate (S-2238) for anti-thrombin activity. Antithrombin antigen levels were determined by latex photometric immunology assay. The *SERPINC1* gene of the patients and family members was analyzed to define the diagnosis with their informed consent in our protocol, which was approved by the ethical committee of Tokyo Medical University. Genomic DNA was extracted from leukocytes. Purified PCR products from genomic DNA were sequenced using a BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems). We performed a MLPA assay using SALSA P227 SerpinC 1 (MRC-Holland).
Results: Levels of both antithrombin activity and antigen in five of the six new patients were reduced to approximately half the mean normal value, and these patients were diagnosed as having type 1 deficiency. Because the remaining patient's antithrombin activity was 37% and antigen level was 95%, the patient was given the diagnosis of type 2 deficiency, a qualitative deficiency in which nearly normal antigen levels are found in association with low antithrombin. Five different mutations were identified in type 1 deficiency in the heterozygous state, two novel deletions (g.2578_2581delTTCT and g.6414delG) and three previously reported point mutations (c. 5311C>T, p. Arg161*; c. 6420C>T, p. Arg229*; and c. 7450T>C, p. Leu302Pro). In the remain-

ing patients, a previously reported point mutation (c. 5272T>C, p. Ser148Pro) in the homozygous state was identified. This missense mutation was detected in the heparin-binding site of antithrombin protein.

Discussion: Both the novel 4-bp deletion in exon 2 and the 1-bp deletion in exon 4 resulted in a frame-shift followed by a premature termination codon occurring at amino acid 113 and 282, respectively. Therefore, two frame-shift mutations caused a quantitative deficiency. The patient with a missense (p.Ser148Pro) mutation in the homozygous state was diagnosed with type 2, heparin-binding site defects type, deficiency. The mutation in the heterozygous state have been reported to associated with thrombosis (*Blood* 81, 1993). However, vein thrombosis was not observed in our patient.

Conclusion: Taken together with our previous studies, a total of 16 different mutations, comprising nine missense mutations, five nonsense mutations, and two small deletions, were analyzed in *SERPINC1* of 16 unrelated Japanese patients. Of these, 13 patients were classified as type 1 deficiency and three patients were classified as type 2.

PB 2.69-4

Novel causative and neutral mutations in a patient with protein C deficiency

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It is often difficult to make a diagnosis of congenital protein C (PC) deficiency based on the results of hemostatic laboratory tests. Analysis of the *PROC* gene is helpful for accurate diagnosis, and a considerable number of mutations have been reported. However, it cannot be concluded that the gene mutation detected in affected patients, particularly missense mutation, is responsible for PC deficiency because the *PROC* gene has many single nucleotide polymorphisms (SNPs). In this study, we found three novel mutations in a patient with PC deficiency, and identified one causative and two neutral mutations.

A 20-year-old woman was referred to our hospital because of deep vein thrombosis. Her PC antigen and activity were 55.0% and 46.8%, respectively, suggesting type I PC deficiency. Her *PROC* gene had three missense mutations: E61Q, E375K, and V407A. To determine which mutation(s) played a causative role in PC deficiency, we established stable HEK293 cell transformants expressing wild-type or mutant PCs. Western blot analysis detected the E61Q and E375K mutant proteins in culture supernatants at the same level as wild-type PC, and their PC activities were comparable to those of wild-type PC. In contrast, the V407A protein was not detected in the supernatant or cell lysates, suggesting that V407A mutation impairs the intracellular synthesis of PC. Our study provides evidence that V407A is a causative mutation for type I PC deficiency and that E61Q and E375K are neutral mutations. Genetic information that allows differentiation between causative and non-causative mutations would contribute to accurate diagnosis of congenital PC deficiency.

PB 2.69-5

Vascular tone regulating genes polymorphism can modulate the risk of early-onset venous thromboembolism in individuals with inherited thrombophilia

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Background: Venous thromboembolism (VT) is one of the most actual multifactorial diseases in the world. Genetic predisposition plays a significant role in pathogenesis of VT. Mutations in the factor II (FII G20210A) and factor V (G1691A, FV Leiden) genes are the most frequent inherited risk factors for VT and could be detected in about 9% and 20% of VT cases, respectively, in the population of North-Western Russia. Endothelial dysfunction is an important mechanism underlying thrombosis, and it frequently occurs as a result of imbalance between vasoconstriction and -dilatation processes. Variations in the genes coding for components of the renin-angiotensin system (RAS) and endothelial NO-synthase (eNOS) can lead to changes in their structure and/or functional activity and modulate the risk of VT.

Aims: To investigate the role of angiotensinogen (AGT), angiotensin II receptor type 1 (AGTR1), angiotensin-converting enzyme (ACE) and eNOS genes polymorphism in the development of VT at young age in individuals with inherited thrombophilia.

Methods: Retrospective study involved 181 patients with early-onset VT (mean group age 34.0 $\pm$ 8.6 years) and 156 sex- and age-matched healthy controls (HC). All individuals originated from the North-Western region of Russia and gave informed consent for participation in the study. Variations in the FII (G20210A), FV (G1691A, Leiden), ACE (Ins/Del), AGT (T704C, Met235Thr), AGTR1 (A1166C) and eNOS (T−786C) genes were discriminated by PCR-RFLP method. The differences in genotype distributions between groups were estimated by Fisher's exact test.

Results: The distributions of alleles and genotypes of the vascular tone regulating genes in patients without known inherited risk factors, as well as in those having FII G20210A mutation were not significantly different from HC. At the same time, the positive association between the FV G1691A and eNOS −786CC genotypes was observed in the VT group (OR = 3.2; 95% CI: 1.3–7.5; $P = 0.01$). Homozygosity for the eNOS −786C allele was more frequently seen among carriers of FV Leiden mutation than in patients with normal FII and FV genotypes (29.7% vs. 13.1%, respectively, $P = 0.024$). The 'unfavorable' variants of the RAS genes were also over-represented in patients with FV Leiden. In particular, the simultaneous presence of the ACE Del/Del and AGT 704CC genotypes was almost 5-times more frequently seen in these individuals than in patients with normal FV and FII genotypes (15.2% vs. 3.1%, respectively, $P = 0.018$) Interestingly, neither the eNOS −786CC variant nor the 'ACE Del/Del-AGT 704CC' combination was detected in VT patients having FII G20210A mutation and normal FV genotype.

Conclusions: We suggest that polymorphism of the vascular tone regulating genes can affect the imbalance between vasoconstriction and vasodilatation processes and play a provocative role in the development of early-onset VT among patients with FV Leiden variant.

PB 2.69-6

Mutation analysis of ATIII in a small cohort of Greek patients with VTE

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Background: Antithrombin III (ATIII) belongs to Serpins (Serine Protease Inhibitors) and is the main inhibitor of thrombin. ATIII normal levels range between 80% and 120%. Low ATIII levels are an important cause of Venous Thromboembolism (VTE). ATIII deficiency is inherited with autosomal dominant pattern. Various mutations have been referred, that cause quantitative (type I) or qualitative defects (type II)

Aims: In this study we present preliminary results of mutation detection in ATIII gene in patients with VTE

Methods: Forty patients with VTE and reduced ATIII levels were examined. ATIII:C and ATIII:Ag mean levels were 46% (range 19–62%) and 58%, range (38–113%) respectively. Whole blood DNA was isolated (QIAGEN) and ATIII 7 gene exons were amplified either separately (PCR) or simultaneously (multiplex PCR). Amplified exons were analyzed on dHPLC (WAVE analysis). PCR products were heat denatured and then reannealed with graduate cooling in order to form

hetero-duplexes. The existing mutations are observed due to the formation of characteristic chromatograms (detection of point mutations, small deletions or insertions). Further analysis via sequencing was obtained for samples with characteristic chromatograms. Multiplex PCR was used for the detection of heterozygous exon deletions, difficult to be seen due to the existence of the normal allele.

Results: Until now 14 patients have been analysed and gave the following results:

In six patients the mutation 'Budapest III – L99', on exon 2, was identified. Five were heterozygous and 1 was homozygous. One patient carries the mutation W189 on exon 4 (transition, TGG > CGG, Trp > Arg) at the conservative position 189 in heterozygous state. One patient carries the mutation D342 on exon 5 (transition, GAT > GGT, Asp > Gly) at the conservative position 342 in heterozygous state. One patient carries the mutation R359X (CGA > TGA, Arg > STOP) on exon 6 (at position 9819) that leads to abolishment of exons 6 & 7 (thrombin binding site). One patient carries the mutation Q334X (CAG > TAG, Gln > STOP) on exon 5 (position 7711) that leads to abolishment part of exon 5, exons 6 & 7 (thrombin binding site). At the same exon, this patient carries one more synonymous point mutation in heterozygosity that causes the polymorphism GAG > GAA (codes for the amino acid Glu) that it has not been referred until now to ATIII data base. Three patients have the unknown polymorphism G > A on intron 6 and two of these patients are close family members.

One patient has a heterozygous frame shift on exon 2 that is under further investigation.

The patients that do not have any known mutation in seven exons are investigated for large exon deletions with multiplex PCR. Until now, four patients were examined and no deletion at any exon was found.

Conclusion: Mutation analysis could be one important tool in therapeutic management of patients with VTE and ATIII deficiency and that knowledge could improve care of VTE patients.

PB2.70 – Non-Inherited Risk Factors VT – III

PB 2.70-1

Socioeconomic status and the risk of venous thromboembolism – the Tromsø study

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Background: Indicators of socioeconomic status (SES) usually include number of years with formal education, occupational status, personal income, and neighbourhood environment. Some, but not all, previous studies have found an inverse association between SES and risk of venous thromboembolism (VTE). However, these studies have only studied limited components of SES, and mostly refrained from investigating the impact of behavioural risk factors, psychosocial aspects and comorbidities on the relationship between SES and VTE.

Aims: We wanted to investigate the association between the Socioeconomic Condition Index (SCI) and risk of VTE, and explore how the associations were affected by behavioural risk factors, psychosocial aspects and comorbidities.

Methods: Information about the SCI components, as well as potential confounders, was collected by self-administered questionnaires, blood samples and a physical examination in 26,473 men and women (≥ 25 years) participating in the Tromsø study in 1994–95. Information about personal income was obtained from Statistics Norway. The SCI included educational level, employment status, self-perceived health, satisfaction with number of good friends, and personal income with predefined categories for each component. The total SCI score ranged from 0 to 18, and was divided into quartiles for men and women separately. Participants were followed from date of enrolment in 1994–95 until December 31, 2010. Cox proportional hazard regression models were used to estimates hazard ratios with 95% confidence intervals (CI). All analyses were stratified by sex due to statistical interaction. The study was approved by the regional committee for research ethics, and all participants gave their informed written consent to participate.

Results: There were 602 VTE events during a median follow-up of 15.8 years (incidence rate: 1.72, 95% CI: 1.59–1.86). An inverse association between SCI and risk of VTE was found among women. Women in the highest quartile (SCI 15–18) had lower risk of VTE in age-adjusted analyses (HR 0.44, 95% CI: 0.27–0.72) (*P* for trend across quartiles of SCI: < 0.001) and in multivariable adjusted analyses (HR 0.62, 95% CI: 0.36–1.05) (*P* for trend across quartiles of SCI: 0.02) compared to those in the lowest quartile (SCI 0–8). Multivariable analyses were adjusted for behavioural factors (body mass index, smoking, physical activity, alcohol and coffee consumption), psychosocial factors (feelings of happiness and optimism) and comorbidities (cancer, arterial cardiovascular disease and diabetes). All the individual components of the index displayed an inverse relation to risk of VTE, but educational level and self-perceived health were the strongest predictors for the inverse association between SCI and VTE among women. Behavioural factors, psychosocial factors and comorbidities explained 30–40% of the association between SCI and VTE (age-adjusted β coefficient: −0.31 vs. multivariable-adjusted β: −0.21). No associations were found between SCI or its individual components and risk of VTE in men.

Conclusions: We found an inverse association between the SCI and risk of VTE in women, but not in men. Behavioural factors, psychosocial factors and comorbidities explained 30–40% of the inverse association between the SCI and risk of VTE in women.

PB 2.70-2

A study to analyze the utility of PNH screening in patients with intra-abdominal thrombosis

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Background: Paroxysmal Nocturnal Hemoglobinuria (PNH) is an acquired hematologic disorder, characterized by intravascular hemolysis, thrombosis or bone marrow failure. The clinical manifestations are due to deficiency of glycosyl phosphatidylinositol (GPI) anchored protein. Fluorescently labeled inactive toxin aerolysin (FLAER) binds to GPI anchor specifically and lack of FLAER binding to granulocytes, measured by multiparameter flow cytometry, indicates presence of PNH clone. FLAER testing is recommended for primary screening of PNH clones.

Thrombosis, often at unusual sites, intra-abdominal, cerebral or dermal may be the presenting feature of PNH. However there is a paucity of data on how many cases of PNH present primarily with thrombosis. Current guidelines recommend screening for PNH in patients presenting with thrombosis at unusual sites. We screened patients with intra-abdominal thrombosis for PNH clone by FLAER analysis.

Aim: To assess the utility of PNH screening in patients presenting with intra-abdominal thrombosis

Methods: Patients with intra-abdominal thrombosis -Budd Chiari Syndrome (BCS), Extra Hepatic Portal Vein obstruction (EHPVO), mesenteric, iliac or renal vasculature as confirmed by imaging studies and referred for thrombophilia workup to the department of Hematology, PGIMER, Chandigarh were included in the study. EDTA peripheral blood samples were analysed by multiparameter flow cytometry using FLAER + CD24 on neutrophils (gated on CD45 and CD15 side scatter) and FLAER + CD14 on monocytes (gated on CD45 and CD33 side scatter). Ten thousand events were acquired and analysed on the FACS Canto II (BD Biosciences). Patients with > 5% deficient neu-

trophils/monocytes were labelled positive for PNH clone and further tested for PNH clone in RBC by CD55+ CD59. Normal controls were included in each run.
Results: Eighty one (65 adults and 16 pediatric) cases of intra-abdominal thrombosis were tested for PNH clone. Mean age of these patients was 30 years (range = 1.5–65 years) and male: female ratio = 1.1:1. There were 34 (42%) cases of EHPVO and 27 (33%) of BCS. Remaining 20 (25%) cases included portal vein thrombosis, iliac vein thrombosis, superior mesenteric vein thrombosis and recurrent thrombosis
Out of the 81 patients tested only 1 (1.2%) patient was positive for PNH clone. This patient had BCS and positive PNH clone in both neutrophils (FLAER/CD24 negative cells = 82%) and monocytes (FLAER/CD14 negative cells = 62%). Further testing for PNH clone in RBC revealed 21% CD55 + CD59 negative cells. None of the 76 controls and remaining 80 patients revealed > 5% PNH positive cells.
Summary/Conclusion: In this study we evaluated the utility of PNH screening in patients with intra-abdominal thrombosis by flow cytometry with FLAER in neutrophils and monocytes. Large PNH clones were infrequent in the study population, seen in only 1 (1.2%) out of 81 patients tested. PNH with a primary thrombotic presentation is therefore rare in cases with intra-abdominal thrombosis. Our results would indicate that, in a resource limited setting, routine screening of all patients with intra-abdominal thrombosis for PNH may be of limited value.

PB 2.70-3
Impact of different measures of renal impairment on future risk of venous thromboembolism. The Tromsø study

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Background: Mild to moderate chronic kidney disease (CKD) is associated with increased risk of venous thromboembolism (VTE). Creatinine, the current clinical standard to detect CKD, is influenced by several factors. Thus, the most reliable equation for estimation of renal function is debatable. A recent study suggests that a combination of creatinine and cystatin C is more accurate than either marker alone for estimating glomerular filtration rate (eGFR).
Aims: The aims of this population-based cohort study were to investigate the ability of eGFR, based on serum creatinine (eGFRcrea), cystatin C (eGFRcys) and both (eGFRcrea-cys) to identify persons at risk, and to elucidate the impact of stages of impaired kidney function on risk of VTE in static and time-dependent analyses of exposure.
Methods: Cystatin C and creatinine was measured in 16,297 men and women, aged 25–84 years, who were enrolled in the fourth (1994–95) and sixth (2007–8). Survey of the Tromsø study, Creatinine, cystatin C and the combination of both markers was used to determine GFR according to the CKD-EPI equations. Incident VTE-events during follow-up were registered from the date of inclusion through the end of the study period, December, 31, 2010. Cox-regression models were used to calculate hazard ratios (HR) with 95% confidence intervals (CI) for VTE adjusted for age, sex, body mass index, diabetes and smoke. Moreover, to minimize regression dilution effects, Cox-regression was performed in subjects with two recordings of eGFR during follow-up, allowing for time-dependency of the exposure variable and important co-variates. The study was approved by the regional committee for research ethics, and all participants gave their informed written consent to participate.
Results: There were 451 incident VTE-events, of which 205 (45%) were unprovoked, during a median of 12.5 years of follow-up. eGFRcys identified 24% of the population to have mildly impaired kidney function (60–89 mL/min/1.73 m^2) and 3.2% to have moderate/severely impaired kidney function (15–59 mL/min/1.73 m^2), and the HRs for total VTE were 1.53 (95% CI 1.21–1.93) and 2.05 (95% CI 1.36–3.10), respectively, compared to those with a normal kidney function (≥ 90 mL/min/1.73 m^2). Likewise, eGFRcys-crea identified 24% with mildly impaired and 1.5% with moderate/severely impaired kidney function, with corresponding HRs for total VTE of 1.65 (95% CI 1.18–2.29) and 1.67 (95% CI 0.95–2.88), respectively. Although 26% with mildly impaired kidney function were identified, no significant associations were found between mildly impaired kidney function assessed by eGFRcrea and risk of VTE (HR 1.07 (95%CI 0.87–1.35). However, for moderate/severely vs. normal kidney function) there was an association (HR 1.83, 95%CI 1.05–3.20) between eGFRcrea and VTE. The risk estimates from the time-dependent analyses were essentially similar.
Conclusions: The present study showed that all CKD-EPI equations identified similar proportions with mild to severely impaired kidney function and that eGFRcys and eGFRcys-crea equally estimated the future risk of VTE among subjects with mildly impaired kidney function.

PB 2.70-4
Venous thromboembolism in patients undergoing shoulder surgery: findings from the Recos registry

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Background: Sparse informations are available about venous thromboembolic (VTE) complications after different types of shoulder surgery.
Aim: The primary end-point of the study was to determine the incidence of symptomatic deep vein thrombosis (DVT) and pulmonary embolism (PE) within 90 days of follow-up in patients undergoing shoulder surgery. Risk factors for VTE and thromboprophylaxis practices were also studied.
Methods: RECOS is a prospective multicenter registry of consecutive patients undergoing shoulder surgery recruited in nine hospitals inItaly. Patients were followed-up at 7, 30 and 90 days for major clinical outcomes. Cumulative rates of VTE were estimated according to the Kaplan-Meier method; a Cox regression model was used to calculate adjusted hazard ratios (HRs) and 95% confidence intervals (CIs) for some variables that were identified as potential risk factors for VTE.
Results: From June 2009 to June 2011 1366 evaluable patients (males 54.4%; mean age 55.65 ± 15.3 years) were enrolled in the registry. The shoulder surgical procedures were the following: arthroscopy (72%), arthrotomy (19.1%) total replacement (8.9%). After 90 days, the incidence rate (95% CI) of symptomatic DVT and PE was 0.7% (95CI% 0.2–11.2). Mean age of patient with VTE complications was significantly different from patients who did not displayed complications (55.6 ± 0.42 vs. 25.7 ± 0.2, $P = 0.024$). Duration of surgery > 60 min (HR: 10.99; 95CI% 1.26–95.89; $P = 0.030$) was found as independent risk factor for VTE. Pharmacological thromboprophylaxis was prescribed in 33.5% ($n = 457$) of the patients; in 95.8% of them the recommended duration of prophylaxis was > 10 days.
Conclusions: The risk of symptomatic VTE in patients undergoing shoulder surgery is not negligible. This risk and the potential need for thromboprophylaxis should be taken into account by orthopaedic surgeons.

PB 2.70-5

Thromboprophylaxis in mutliple myeloma – a tale of two cities

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Background: Multiple myeloma is associated with an increased risk of thrombosis. This is greater with immunomodulatory (iMID) drug combinations and during the first 6 months of therapy. Bortezomib may have a protective effect. First-line therapy for myeloma in the UK generally comprises a thalidomide-containing regimen. This contrasts with New Zealand, where bortezomib is commonly used upfront. In both countries, lenalidomide is customarily given third-line. British guidelines recommend thromboprophylaxis with aspirin in patients receiving an iMID-containing regimen who are at moderate risk of thrombosis. The optimal strategy for high risk patients is yet to be determined.

Aims: To compare and contrast the current practice of thromboprophylaxis in myeloma patients in two centres on opposite sides of the globe: one in the United Kingdom (Musgrove Park Hospital, Somerset) and the other in New Zealand (Christchurch, Canterbury District Health Board).

Methods: Audits were performed to assess current practice of thromboprophylaxis in all patients with myeloma under active follow-up in each centre. NZ data were collected in 2012, while UK data were collected in 2013. Additionally, retrospective data were collected on live patients who had experienced a thrombotic event since being diagnosed with myeloma. Results were compared with BCSH guidelines for management of thromboprophylaxis in myeloma, which recommend that all patients for an iMID-containing regimen should have a thromboprophylaxis risk assessment, giving a gold standard of 100%. In parallel, national consultant surveys were performed to review thromboprophylaxis for myeloma patients in New Zealand and the South West of England.

Results: Ninety-eight patients with myeloma under active follow up were identified in the UK centre and 151 in the NZ centre. In each centre, approximately half of the patients on active therapy were receiving an iMID (50% in the UK and 52% in NZ). In the UK centre, 96% of patients on an iMID were in receipt of thromboprophylaxis. This figure was lower at 67% in the NZ centre meaning that thromoboprophylaxis was potentially not being adequately addressed for a third of patients on iMIDs.

Despite higher upfront use of thalidomide-containing regimens in the UK, the proportion of patients experiencing thrombosis was similar in the two centres (7% in the UK centre and 8% in the NZ centre). In the NZ cohort, 50% of these thrombotic events ($n = 6$) took place during induction therapy without thromboprophylaxis and only one patient was on an iMID. In the UK centre 43% of these events ($n = 3$) took place during induction with a thalidomide-containing regimen despite thromboprophylaxis.

Summary/Conclusions: Optimal thromboprophylaxis in myeloma is still a matter for debate although there is a greater consensus when using lenalidomide-containing therapy. Thromboprophylaxis according to an individual risk profile should be considered for every patient with myeloma, particularly at the start of treatment.

PB 2.70-6

Postoperative deep vein thrombosis in patients undergoing general, colorectal and orthopedic surgeries in Taiwan- an institutional experience

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Background: It has been believed for a long time that the incidence of venous thromboembolism is lower in Asian countries, as compared to that in Western countries. Such belief was challenged by recent studies of postoperative deep vein thrombosis (DVT) in postorthropedic surgeries.

Aim: We aimed to study the incidence of postoperative DVT following general, colorectal and orthopedic surgeries seen in middle part ofTaiwanduring August 2009 to December 2012.

Material and Methods: Patients 195 patients undergoing general surgeries [mean age 63.6 years (range 29–91 years), 118 males and 77 females], 128 patients receiving colorectal surgeries [66.2 (37–91), 77 males and 51 females] and 120 patients undergoing orthopedic surgeries including 57 total knee replacements (TKR), 23 total hip replacements (THR), and 21 Hip replacements (HR) and 17 fracture surgeries, two of them received TKR and THR concurrently[69.4 (20–95), 46 males and 74 females] were studied.

Methods: Compression ultrasonography (CUS, Philips HD11) examination was performed for each patient before and 5–10 days after operation. Thrombin generation (TG) analysis using Fluoroscan Ascent and microparticle (MP) activity test using ZYMUPHEN MP activity test kit were done before and after operation. Venography was also performed in some patients for comparison. Univariate and multivariate logistic regression analysis for the risk factors associated with development of postoperative DVT including sex, age, tumor staging (in colorectal cancer only), type of operation, anesthesia duration, operation duration, bed rest days, previous cerebral and cardiac disorders, previous DVT, TG endogenous thrombin potential and MP activity were done in each group of patients.

Results: *Validation of the CUS examination:* Twice CUSs were performed after operation including six DVTs and 60 normal veins, all showed identical results. Parallel examinations of CUS and venography were done in 19 patients including four DVTs and 15 normal veins, all showed identical results except that in one patient CUS showed partial thrombosis of left external iliac vein and common femoral vein, but venography revealed no evidence of thrombosis and external compression by left common iliac artery was suspected.

General surgery: Only 1 (0.5%) patient was found to have asymptomatic distal DVT among 195 patients received general abdominal surgery. Univariate and multivariate logistic regression analysis showed no associated risk factor.

Colorectal surgery: Among 128 colorectal cancer-surgical patients, 3 (2.3%) had proximal DVT before operation, 9 (7.0%) developed new DVT after operation, all were distal. Both univariate and multivariate logistic regression analysis showed that previous VTE patients has higher incidence of postoperative VTE.

Orthopedic surgery: Nine of 120 patients (7.5%) developed DVT, Five had proximal DVTs, four distal DVT; all detected on the operated leg. Venography was also done to confirm the diagnosis in four of them. Univariate risk analysis showed patients received TKR had significant higher risk. Multivariate logistic regression analysis showed no associated risk factor.

Conclusion: The incidence of postoperative DVT in our patients undergoing general, colorectal and orthopedic surgery was only about 4.3% (0.5%,7.0%,7.5% respectively) as low as recent reports from Asian countries (Singapore: Fong 2000, Nathan 2003, China: Lu 2007).

PB2.71 – Recurrent Venous Thrombosis – II

PB 2.71-1

Factors associated with early and late hypercoagulability after stopping anticoagulation for idiopathic venous thromboembolism

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Background: The recurrence rate is especially high in the 3 months after stopping anticoagulation for a first episode of idiopathic venous thromboembolism (VTE). A hypercoagulable state, as expressed by an abnormal D-dimer, in these 3 months has been shown to be a risk factor for both early and late recurrence.

Aim: To evaluate factors associated with an abnormal D-dimer within and beyond 3 months after stopping anticoagulation for a first episode of idiopathic VTE in the PROLONG II study.

Methods: in a prospective multi-center study patients underwent repeated D-dimer testing after anticoagulation suspension. D-d was measured with a qualitative method (Clearview Simplify D-dimer, Inverness Medical Professional Diagnostics, Bedford, UK) on the day of anticoagulation withdrawal and after 30 days. If D-dimer was abnormal during anticoagulation or at 1 month, patients continued or resumed VKAs, while if D-dimer was normal at 1 month after stopping anticoagulation patients did not resume treatment and repeated D-d testing every 2 months for a year.

Results: Early hypercoagulability (abnormal D-dimer in the 3 months after stopping anticoagulation) was associated with age > 65 year (odds ratio-OR: 3.6 (95% confidence interval-CI:1.9–6.7; $P = 0.0001$), the presence of comorbidities (OR: 2.7; 95% CI:1.5–5.1; $P = 0.001$) and thrombophilia (OR: 1.9; 95% CI:1.1–3.4; $P = 0.041$) when compared with a persistent normal D-dimer over a year. Late hypercoagulability (abnormal D-dimer after 3 months since anticoagulation withdrawal) was associated with the presence of comorbidities (OR: 2.8; 95% CI:1.5–5.5; $P = 0.002$) and of residual venous obstruction (RVO) (OR: 1.9; 95% CI:1.1–3.8; $P = 0.037$) when compared with a persistent normal D-dimer over a year. Late hypercoagulability was associated with male sex (OR: 2.3, 95% CI:1.1–4.7; $P = 0.029$) and age < 65 year (OR:2.2; 95% CI:1.1–4.6; $P = 0.039$) vs. early hypercoagulability.

Conclusions: Hypercoagulability after stopping anticoagulation for idiopathic VTE is influenced by age, comorbidities, thrombophilia and RVO.

PB 2.71-2

The incidence of recurrent venous thrombosis associated with different clinical risk profiles

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Background: Acquired, clinical risk factors are easily assessed after a patient suffers a first venous thrombotic event and may be useful in the prediction of recurrence.

Aim: The aim of this study was to assess whether the risk of recurrent venous thrombosis differed per clinical risk profile, i.e., in patients in whom the first venous thrombosis was surgery provoked, non-surgery provoked, or unprovoked.

Methods: Analyses were performed in the Thrombophilia, Hypercoagulability, and Environment and the risk of Venous ThromboEmbolism (THE-VTE) study. Between March 2003 and December 2008, patients with a first, objectively diagnosed episode of deep venous thrombosis or pulmonary embolism, aged 18–75 years, were included in the anticoagulation clinics inLeiden, NL andCambridge, UK. Controls were partners of patients. Individuals with active malignant disease were excluded. In total, 796 patients and 531 controls were included. Consent and ethical approval were obtained.

Patients were subsequently followed for recurrent thrombosis. Potential recurrences were classified as certain or unlikely based on the time between the first and recurrent event and the location of the recurrence. Patients with an unlikely recurrence were censored at the time of the event. The end of follow-up was defined as the date of recurrence, date of death, or end of follow-up.

The incidence rates (IR) of recurrence with 95% confidence interval (CI) were calculated overall and after stratification in risk groups (surgery provoked, non-surgery provoked, and clinically unprovoked first event).

Surgery provoked was defined as having had a surgical procedure in the 3 months prior to the first event. Non-surgery provoked as having had a plaster cast, injury, immobilization > 4 consecutive days, hospitalization, or hormone use in the 3 months prior to the first event. In patients with an unprovoked first event, none of the above risk factors were present in the 3 months prior to the first event. Complete information on these risk factors was available for 760 patients.

The 2- and 4-year cumulative incidences of recurrence were calculated in the before-mentioned risk groups separately.

Results: After a mean duration of follow-up of 5.1 years, 117 (14.7%) of the 796 patients developed a recurrence: IR = 29/1000 person years (py); 95% CI: 23.7–34.2.

The distribution in risk groups was as follows: surgery provoked first event $n = 135$ (17.8%), non-surgery provoked first event $n = 225$ (29.6%), clinically unprovoked first event $n = 400$ (52.6%). The incidence of recurrence was lowest in patients with a provoking factor (surgery: IR = 21.7/1000 py; 95% CI: 11.1–32.4, non-surgery: IR = 19.5/1000 py; 95% CI: 11.4–27.6). The incidence of recurrence was highest after a first clinically unprovoked event, IR = 37.8/1000 py; 95% CI: 29.3–46.3.

The difference in recurrence is present after 2 years of follow-up and remains also after longer duration of follow-up (4-year cumulative incidences: surgery provoked: 9.6%, 95% CI: 5.8–13.5, non-surgery provoked: 7.6, 95% CI: 4.1–11.0; unprovoked: 12.8, 95% CI: 9.5–16.0).

Summary/Conclusion: Assessment of clinical risk factors at the time of a first venous thrombosis predicts the risk of a recurrent event. The risk of recurrence is highest in patients with an unprovoked first event.

PB 2.71-3

Whole blood gene expression profiles to distinguish venous thromboembolism phenotypes

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Background: Recurrent venous thromboembolism (VTE) occurs in 10–30% of individuals after completing a course of anticoagulant therapy for an initial unprovoked VTE. Currently, there is limited understand-

ing of the biological mechanisms that predispose patients to recurrent VTE and it would be useful to have biomarkers to help predict which patients are at a higher risk for recurrent VTE. Using clinically well-defined patient groups, we explored the use of whole blood gene expression profiles to distinguish patients with recurrent VTE as well as provoked and unprovoked VTE.

Methods: Adults with one or more VTE were recruited at four sites participating in the Thrombosis Research and Prevention Network supported by the Centers for Disease Control and Prevention. Participants were allocated into three groups: (i) 'low-risk' individuals had sustained one or more provoked VTE ($n = 34$); (ii) 'moderate-risk' individuals had sustained one unprovoked VTE (with or without provoked VTE) ($n = 33$); and (iii) 'high-risk' individuals had sustained more than one unprovoked VTE (with or without provoked VTE) ($n = 40$). Patients with antiphospholipid syndrome were excluded. Individuals with no history of VTE were enrolled as healthy controls ($n = 25$). The study was approved by the review boards at each institution and informed consent was obtained from all participants. Blood for RNA was collected in PAXgene tubes; citrated plasma and serum were collected at the same time. Total RNA was isolated and hybridized to Illumina HT-12v4 Beadchips to assay whole genome expression with over 47,000 probes. Blood levels of selected biomarkers identified by gene expression were measured by ELISA. Class prediction analysis and differential expression analysis were used to compare the groups.

Results: Class prediction analysis was able to distinguish high-risk individuals from healthy controls with good receiver operating curve characteristics (AUC = 0.88). We also distinguished high-risk individuals from low-risk individuals, moderate-risk individuals from healthy controls and low-risk individuals from healthy controls with AUC's of 0.72, 0.77 and 0.72, respectively.

Differential expression analysis identified genes that were differentially expressed when comparing the high-risk group to the low-risk group or healthy controls, and the moderate-risk group to the healthy controls. Several genes relevant to coagulation, immune response and/or vascular biology, including *SELP* and *CD46,* were differentially expressed in at least two of the comparisons. This distinction in whole blood was limited to gene expression, since plasma or serum protein levels of several of these genes did not differ between the different groups.

Conclusion: Gene expression profiles were able to distinguish individuals with recurrent, unprovoked VTE from individuals with provoked VTE only and healthy controls. In contrast, this approach could not distinguish individuals with a single, unprovoked VTE (the moderate-risk group) from patients with recurrent unprovoked VTE or patients with provoked VTE, indicating heterogeneity among the moderate risk group. Prospective studies are needed to determine whether the gene expression profiles identified by this approach will indeed distinguish patients at high-risk for recurrent VTE and thus provide a guide for which patients would benefit from extended anticoagulant therapy.

PB 2.71-4

Efficacy and safety of weight-adjusted dosing of low-molecular-weight-heparin for prevention and treatment of acute VTE in obese patients: a systematic review and meta-analysis

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Background: Obese patients are often excluded from clinical trials or are not recruited in sufficient number to assess safety and efficacy of LMWH in this population. It is unclear if standard (i.e. non-adjusted) thromboprophylaxis doses of low-molecular weight heparin (LMWH) provide adequate coverage for obese patients. Similarly, weight-adjusted dosing of LMWH is recommended for the acute treatment of venous thromboembolism (VTE). However, product monographs of different LMWH manufacturers state not to exceed their recommended maximum dosage. It is not known if using the actual weight-adjusted dose of LMWH (instead of capping the dose) is safe and effective for obese patients treated for acute VTE.

Aims: To summarize the event rates of VTE (or recurrent VTE) and major bleeding episodes in obese patients receiving weight-adjusted doses of LMWH for the prevention and treatment of VTE.

Methods: A systematic literature search was performed using MEDLINE and EMBASE. The primary outcome measures were VTE and major bleeding events. Venous thromboembolism was defined as symptomatic proximal lower limbs (popliteal vein or more proximal) deep vein thrombosis or pulmonary embolism. Major bleeding was defined as per the ISTH definition. Weight-adjusted thromboprophylactic LMWH dosing was defined as the use of higher than standard recommended dosing for obese patients. Weight-adjusted therapeutic LMWH dosing was defined as a dose calculated according to patient's weight regardless of dosage cap as per the manufacturer's instructions. Rates of the primary outcomes were generated for the indications: (i) Thromboprophylaxis in medically-ill patients; (ii) Acute treatment of VTE. Pooled proportions for the different outcomes during hospitalization (prophylaxis medically-ill patients) and up to 3 months of follow up (acute treatment of VTE) were calculated.

Results: A total of eight studies (four on thromboprophylaxis in medically-ill patients; four on the acute treatment of VTE) met inclusion criteria. Two thousand two hundred and twenty patients were included in the systematic review (1200 in thromboprophylaxis studies; 1020 in treatment studies).

Medically ill hospitalized patients receiving weight-adjusted thromboprophylactic LMWH had a VTE rate of 2.1% (95% CI: 0.1–6.7%) compared to a rate of 2.9% (95% CI: 1.7–4.4%) for patients receiving standard doses of LMWH thromboprophylaxis. The rate of major bleeding in the weight-adjusted prophylaxis studies was 1.0% (95% CI: 0.02–4.6%). None of the studies included in the systematic review reported the risk of major bleeding among obese patients receiving standard doses of LMWH thromboprophylaxis.

The 3-month risk of recurrent VTE and major bleeding in obese patients receiving weight-adjusted LMWH for acute VTE was 2.7% (95% CI: 0.7–5.9%) and 0.8% (95% CI: 0.3–1.6%) respectively. No studies have reported the risk of recurrent VTE or major bleeding among obese patients receiving capped doses of LMWH.

Summary/Conclusions: Weight-adjusted doses of LMWH seem safe and effective in the prevention and treatment of VTE in obese patients. Rates of VTE, recurrent VTE and major bleeding episodes compare favorably to previously reported rates in non-obese patients. Future studies assessing the efficacy and safety of different dosing strategies among obese patients are needed.

PB 2.71-5

Residual pulmonary embolism as a predictor for recurrent thromboembolic events after a first unprovoked episode: data from the REVERSE cohort study

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Background: We sought to assess if residual pulmonary embolism after completion of 5–7 months of oral anticoagulant therapy for unprovoked pulmonary embolism is associated with an increased risk of recurrent venous thromboembolism.

Methods: Patients with a first unprovoked venous thromboembolism were enrolled after completion of 5–7 months of oral anticoagulant therapy over a 4 year period and completed a mean of 18 months

follow-up. Patients with pulmonary embolism (with or without deep venous thrombosis) had a baseline V/Q scan performed before stopping oral anticoagulant therapy. The percentage of vascular obstruction was determined for all patients. This is a Multi-centre multinational prospective cohort study done in tertiary care centres.
Main Outcome: During follow-up off oral anticoagulant therapy, all episodes of suspected recurrent venous thromboembolism were independently adjudicated with reference to baseline imaging.
Results: Annual incidence rates of recurrent venous thromboembolism were 2.8% (95%CI 0.3–5.2%) in patients with a percentage of vascular obstruction of 0% (no residual obstruction), 6.7% per year (1.3–12.0%) in patients with percentage of vascular obstruction of 1–5%, and 11.7% per year (5.3–18.0%) for patients with a percentage of vascular obstruction > 5%.
Summary/Conclusion: In our study, the presence of residual pulmonary vascular obstruction at the time of oral anticoagulant therapy withdrawal was associated with a statistically significant higher risk of subsequent recurrent venous thromboembolism. Percentage of vascular obstruction assessment might be useful to guide duration of oral anticoagulant therapy.

PB 2.71-6
Two years outcome and features of isolated distal vein thrombosis

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Background: Isolated distal deep vein thrombosis (IDDVT) is a frequent finding in symptomatic outpatients, but its natural history is still uncertain.
Aims: To assess the long term outcome of IDDVT and ascertain the impact of IDDVT characteristics on outcome.
Methods: In a prospective, single center study we enrolled 90 symptomatic outpatients (age 60.7 ± 18.3, male 48.9%) in whom a complete real-time B-mode and color Doppler ultrasonography examination revealed an IDDVT. Among the 88 treated patients, 56 received low molecular weight heparins (LMWH) for 30 days and 32 with vitamin K antagonists for 3 months. The outcomes of the present study included death, major bleeding, pulmonary embolism, proximal deep vein thrombosis (DVT), and IDDVT. Follow-up visits were at 1, 3, 12 and 24 months.
Results: The most frequent risk factors for thrombosis in the study population were: significantly reduced mobility (34.4%), obesity (25.3%), surgery (15.7%), and previous DVT (15.7%). Cancer was present in eight patients (8.9%). During the follow-up (median 24 ± 2 months), 18 patients (20%) developed an outcome, which included one non cardiovascular death (cancer), one major bleeding (during LMWH therapy), three pulmonary embolism (two among patients with cancer), four proximal DVT (one among patients with cancer), and nine IDDVT. According to Cox regression analysis, male patients (HR 4.0 CI95%: 1.3–12; $P = 0.018$) and patients with cancer (HR 6.1 CI95%: 1.4–26; $P = 0.016$) were at higher risk for reaching the end-point, whereas the anatomical characteristics and the type (provoked vs. non-provoked) of IDDVT, the type of anticoagulant therapy, age, BMI, and history of DVT were not associated with the outcome. Normal d-Dimer values at IDDVT diagnosis were associated with lower risk of recurrent venous thromboembolism (HR 0.76 CI95%: 0.67–0.87 $P = 0.023$).
Conclusions: The risk of recurrent venous thromboembolism after IDDVT may be relevant, especially in male patients or in patients with active cancer. Larger studies are needed to address this issue.

PB2.72 – Thrombophilia – III

PB 2.72-1
FIX-Padua increases fibrinolytic resistance through a TAFI-mediated mechanism

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Background: Thrombin activatable fibrinolysis inhibitor (TAFI), upon activation to TAFIa, down-regulates fibrinolysis by removing plasminogen binding sites from fibrin. As thrombin is considered the main activator, TAFI represents a link between coagulation and fibrinolysis. Therefore, hypercoagulability is often associated to a hypofibrinolytic state determined by increased TAFI activation. The substitution R338L in the gene for coagulation factor IX (FIX-Padua) has been reported in a young patient with venous thrombosis as a gain-of-function mutation that causes an 8-fold increase in the clotting activity of FIX. To date, it is not known whether FIX hyperactivity can also impact on TAFI activation.
Aims: To evaluate the influence of FIX-Padua on TAFI-mediated regulation of fibrinolysis.
Methods: Blood was collected from the proband (hemizygous for FIX-Padua), two family members (mother, heterozygous, and father, normal) and six unrelated healthy controls (all providing written informed consent), and anticoagulated with citrate plus the inhibitor of the contact system corn trypsin inhibitor (CTI). Plasma clot lysis induced by 40 ng/mL t-PA was studied by a microplate turbidimetric assay. TAFI-dependent inhibition of fibrinolysis was calculated as the ratio between lysis times in the absence and in the presence of the specific TAFIa inhibitor PTCI (PTCI-ratio). TAFI activation was assessed by an ELISA specific for TAFIa and its inactive derivative TAFIai (TAFIa/ai). Thrombin generation was evaluated by calibrated automated thrombinography (CAT). In all experiments, clotting was induced by very low concentrations of tissue factor (0.05 pM) in order to better reproduce the physiological conditions.
Results: Under our conditions, in three different experiments TAFI-dependent inhibition of fibrinolysis was low in control samples (PTCI-ratio: 1.18 ± 0.22), intermediate in the heterozygous mother (1.55 ± 0.19), and high in the proband (1.92 ± 0.15) ($P < 0.001$ by one sample t-test). Thrombin generation was markedly enhanced in the proband as compared to his mother and controls, lag time being 13, 27, and 32.3 ± 4.6 min, respectively ($P < 0.001$) and endogenous thrombin potential (ETP) being 705, 320, and 186 ± 27 min ($P < 0.001$). Accordingly, TAFIa/ai accumulation at 30 min after the start of experiments amounted to 115 ng/mL in the proband as compared to 55.7 ± 14.3 in controls ($P < 0.01$). When a higher concentration of TF (5 pM) was used to make thrombin generation independent of FIX, the differences in PTCI-ratio, thrombin generation and TAFI activation between the proband and controls became much smaller and statistically insignificant ($P > 0.05$).
Conclusions: FIX-Padua increases fibrinolytic resistance by enhancing thrombin-mediated TAFI activation. This finding, on the one hand, may provide an additional explanation for the thrombotic risk of patients carrying the mutation; on the other hand, it might represent a further haemostatic mechanism encouraging the use of FIX-Padua for the gene therapy of haemophilia B.

PB 2.72-2

Audit of requests for thrombophilia laboratory tests post introduction of clinical guidelines in a referral laboratory

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Background: Thrombophilia testing represents a significant portion of test requests in a tertiary referral coagulation laboratory but such testing has not been shown to be clinically useful in the acute or longterm management of the majority of patients with Venous Thromboembolism (VTE). Guidelines for laboratory testing for thrombophilia were introduced at the National Centre for Hereditary Coagulation Disorders (NCHCD) in August 2012 to assist clinicians in determining appropriate indications for thrombophilia testing. Following implementation, requests for thrombophilia tests were only processed if the clinical indication was in keeping with the guidelines or the specific clinical circumstances had been discussed with a Consultant Haematologist. Requests for testing received without clinical information or appearing to be outside the guidelines were put on hold in the laboratory until additional clinical information was received from the requesting clinician. The final decision regarding laboratory testing was made by the Consultant Haematologist.
Aim: The aim of this audit was to examine the changes in thrombophilia requesting patterns and test processing pathways in the coagulation laboratory following the introduction of thrombophilia test guidelines.
Method: Samples are received for thrombophilia testing (functional and molecular tests) from within St James's Hospital (in-patient and out-patient services), from other referral hospitals throughout Ireland and from general practitioners. Test numbers for the period September to December 2012 were compared with the same period in 2011. Total test numbers and a breakdown of requesting source were examined. An audit of all requests that had been put on hold was conducted and the review rate and final testing decision by the Consultant Haematologist was determined.
Results: There was a 56% reduction in thrombophilia test numbers following implementation of the thrombophilia testing guidelines. The greatest reduction in test numbers was seen in tests requested from external agencies and general practitioners (> 60%). A total of 129 requests were put on hold during this period awaiting further clinical information. The reason for holding the request was either lack of clinical information (n = 66) or the clinical information provided appeared to be outside the testing guideline (n = 63). Requests included full thrombophilia screen (n = 86), Lupus Anticoagulant testing (n = 25) and prothrombotic molecular testing (n = 18). Of the 129 requests on hold, additional information was received in the laboratory on 35/129 requests. Following consultant review, testing was approved in 20 of these requests consisting of full thrombophilia screen (n = 4), Lupus Anticoagulant screen (n = 14) and prothrombotic molecular analysis (n = 2). The most common reason for approval of testing was recurrent pregnancy loss and Lupus Anticoagulant testing (n = 9).
Conclusions: Guidelines for laboratory testing improve the appropriate selection of patients for thrombophilia testing and result in a significant reduction in test requests. There is however an increased administrative workload for laboratory and clinical staff in processing test requests.

PB 2.72-3

Novel mutations of antithrombin gene in inherited AT deficiency

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Background: Antithrombin (AT) is a natural anticoagulant agent to produce a marked inhibitory effect on thrombin and other activated blood coagulation factors (factor Xa、IXa、XIa) and is pertained to SERPIN (serine protease inhibitor) superfamily. Inherited AT deficiency is associated with a high risk of deep vein thrombosis (DVT) and pulmonary embolism (PE), owing to the mutations of antithrombin gene. It is considered that inherited AT deficiency, as autosomal dominant inheritance, is the main cause of thrombophilia in China.
Aims: The phenotype and genotype in three propositus with DVT and their family members were analysed, in order to identify the mutations in AT gene and investigate the pathophysiologic mechanism.
Methods: The blood sample were collected from three propositus and their family members. The AT activity level in plasma, using chromogenic, were detected in our laboratory. The Protein C activity and Protein S activity were also measured. Genomic DNA was extracted from blood cells. All exons in AT gene were amplified by PCR, then PCR products were directly sequenced to further identify the genotype.
Results: The AT activity in plasma were significantly decreased in propositus and their affected family members, but the Protein C activity and Protein S activity in plasma were normal. We found three kind of heterozygous mutations in AT gene in three unrelated Chinese patients with AT deficiency, 18,390–18,391 InsCT in exon 6 leading to frameshift as to nonsense mutation (433 Thr>stop), 10446 C>T in exon 3 resulting in nonsense mutation (164 Arg>Stop), and 7747T>C in exon 2 causing 78Leu>Pro missense mutation. These mutations can cause premature termination or protein structural abnormality in AT protein. According to the antithrombin mutation database (http://www1.imperial.ac.uk/departmentofmedicine/divisions/experimentalmedicine/haematology/coag/antithrombin/type1/), we confirmed the 18,390–18,391 InsCT mutations and 7747T>C in AT gene were novel mutations.
Summary: These mutations in AT geng, found in three unrelated Chinese patients with DVT, resulted in a premature termination of AT protein or dysfunctional AT protein in plasma, which were the intrinsic reason for the occurrence of DVT.

PB 2.72-4

Inherited thrombophilia and recurrent pregnancy loss

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Background: Recurrent fetal loss (RFL) is a common health problem, with three or more loses affecting 1–2% and two or more losses affecting up to 5% of women at the reproductive age.
The polymorphisms G20210A of prothrombin gene (FII G 20210A), G 1691A), of factor V gene (Factor V Leiden, FVL) and C677T of methylene tetrahydrofolate reductase gene (MTHFR C677T) are the most extensively studied thrombophilic mutations in association to recurrent miscarriage.
Aim: Our aim is to compare the prevalence of FII G20210A and FVL polymorphisms and their combinations in a series of patients with three or more consecutive, miscarriages with control group.
Methods: The study group included 90 west southern Iranian patients with three or more consecutive miscarriages with the same partner in < 20 weeks gestation. We obtained medical histories, performed physical examinations, routine laboratory tests, endocrinologic examinations, and immunologic tests for auto antibodies for patients. Exclusion criteria were: anatomic abnormalities, endocrinologic dysfunction, liver function, abnormalities, inflammatory pelvic disease and PCO. The control population consisted of 44 women of similar age to the patients, with at least one live born children and no history of pregnancy loss. Functional activity of protein C & S, activated protein C resistance (APCR), FV Leiden assay by PCR and prothrombin gene mutation were assessed. The polymorphism frequencies were recorded for each of the groups and comparisons were made using odds ratios (OR), together with their 95% confidence intervals. Chi-squared or Fisher's exact test were also used for comparisons of poly-

morphism distribution between the groups. P-value < 0.05 was considered significant.

Results: The mean age of patients (29.21 years, S.D. 5.9) did not differ from that of the controls (28.75 years, S.D. 5.2) (t-test, $P = 0.66$). Four patients (4.4%) had protein C deficiency while only 1 (2.3%) of control group had protein C deficiency. The mean functional activity of protein C was 109.74 and 102.35 s for case and control groups respectively ($P > 0.05$). Protein S deficiency was detected in 9 (10%) patients. The mean functional activity of protein S was 91.32 and 85.42 s for case and control groups respectively ($P > 0.05$).

There were three homozygous and 15 heterozygous cases of Factor V Leiden in patient group. Carrier ship for Factor V Leiden did not significantly increase the risk for recurrent miscarriage (OR 2.25, 95% CI 0.41–7.55).

There were no homozygous cases in either group. Carrier ship for G20210A (homozygous or heterozygous vs. the wild type) did not significantly increase the risk for recurrent miscarriage (OR 1.95, 95% CI 0.49–13.90)

Conclusion: Factor V Leiden and FII G20210A are associated with pregnancy loss in both the first and second trimester. We found that two common thrombophilic mutations (Factor V Leiden and Prothrombin G20210A), alone or in combination, is associated with increased risk for recurrent miscarriage.

PB 2.72-5

Thrombophilia screening in patients with ocular venous thrombosis

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Background: Ocular venous thrombosis (OVT) is a frequent cause of visual impairment. Patients with OVT are commonly treated with anticoagulants, although no recommendations concerning anticoagulant treatment exist. We here report upon treatment of and thrombophilia screening in 90 patients with OVT.

Aims: To reveal the importance of thrombophilia screening in patients with OVT and to get an overview over anticoagulant treatment in these patients.

Methods: Ninety patients with OVT (46 male, 44 female, age: 22–75 years) were examined. At time of examination none of the patients was acutely ill. Thrombophilia parameters were determined in all patients.

Results:

1 Thrombophilia screening: antiphospholipid antibodies were detected in 12, lipoprotein (a) elevation in 19, factor VIII:c elevation in 11, antithrombin deficiency in 2, factor XII deficiency in 1, hyperhomocysteinemia and Prothrombin (G20210A) polymorphism in 2, Factor V Leiden mutation in eight patients.
2 Treatment: 56 patients were initially treated with acetylsalicylic acid (ASA), two with ADP antagonists, two with low molecular weight heparin (LMWH), 11 with vitamin K antagonists. Ten patients had no treatment at all. Eleven patients had recurrent OVT under treatment with ASA, whereas three patients developed OVT under vitamin K antagonist treatment (one of them suffering from antiphospholipid syndrome). One patient developed two consecutive OVTs under treatment with ASA (100 and 300 mg/day, respectively) despite of proved efficacy of ASA.

Conclusion:

1 Common risk factors of venous thromboembolism like Factor V Leiden mutation, Prothrombin polymorphism, antithrombin deficiency and factor VIII:c elevation were relatively rare in OVT patients, while antiphospholipid antibodies and lipoprotein (a) elevation seem to play a major role. Therefore it is questionable, whether it is necessary to perform a 'complete' thrombophilia screening in OVT patients.
2 ASA was more frequently used than vitamin K antagonists. Nevertheless, recurrence rate of OVT in patients under treatment with vitamin K antagonists was lower in comparison to patients treated with ASA.

PB 2.72-6

The course of vWF multimers and ADAMTS13 in cardiac surgery

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Introduction: Several cases of postoperative TTP following cardiovascular surgery have been recorded. It is still unclear whether the use of extracorporeal circulation (Heart-Lung Machine) during surgery has an impact on vWF multimers and ADAMTS13 and thus on the occurrence of postoperative TTP.

Methods: In order to analyse the course of vWF multimers and ADAMTS13 we included 47 patients undergoing cardiac surgery at the University Hospital Frankfurt/Main. Thirty-nine patients were operated with the use of an HLM (23 coronary artery bypass grafts, 17 cardiac valve operations). Eight patients were operated without the use of an HLM (Off Pump Coronary Artery Bypass). Six blood samples were taken from each patient; before the operation, at 60 & 120 min during operation and 2, 4 and 6 days postoperatively.

Results: We found that ADAMTS13 activity significantly decreased during surgery (from 66% to 50%, $P > 0.001$ in the HLM group, from 67.5% to 48.5%, $P = 0.02$ in the OPCAB group), as well as the ADAMTS13 antibodies. Postoperatively the antibodies rose to a maximum of 14 U/L in the HLM group but stayed lower at 7.7 U/L in the OPCAB group.

The percentage of large vWF multimers increased significantly during surgery in the HLM group from 27% to 30.8% ($P = 0.04$). The amount of vWF antigen measured did not significantly change during surgery in either group.

VWF multimers were quantified using densitometric gel analysis. To test the difference between measuring points the Friedman-Test was used with a 5% level of significance. For the vWF multimers and antigen the Wilcoxon-Matched-Pairs-Test was used.

Conclusion: Our investigations showed significant changes in ADAMTS13 activity during cardiac surgery. There is also a difference in the presence of large vWF multimers when comparing extracorporeal circulation to OBCAB surgery. These findings might support the thesis that TTP may develop after cardiac surgery with extracorporeal circulation.

PB2.73 – TTP/Thrombotic Microangiopathies – I

PB 2.73-1

Hematologic outcomes at 2 years in eculizumab-treated atypical hemolytic uremic syndrome patients with long disease duration and chronic kidney disease

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Background: Atypical hemolytic uremic syndrome (aHUS) is a rare, systemic genetic disease characterized by chronic, uncontrolled complement pathway activation leading to platelet activation, thrombosis, hemolysis, and thrombotic microangiopathy. Patients with aHUS frequently present with abnormal hematologic parameters, as well as

renal and end-organ damage. Within 1 year of diagnosis, up to 65% of patients develop permanent renal damage, end-stage renal disease, or die.[2] Eculizumab, a terminal complement inhibitor, is a humanized monoclonal antibody that binds with high affinity to the human C5 complement protein, blocking the generation of proinflammatory C5a and C5b-9. Eculizumab is the first approved treatment for aHUS in pediatric and adult patients.

Aims: Two-year hematologic and renal outcomes were investigated from a phase 2 study of eculizumab treatment in aHUS patients with long disease duration, and chronic kidney disease (CKD) with prior chronic plasma exchange/plasma infusion (PE/PI).

Methods: Twenty patients age ≥ 12 years receiving chronic PE/PI on a stable regimen with no platelet count decrease > 25% during an 8-week observation period were enrolled in the study and received eculizumab for 26 weeks; 19 of 20 patients entered the long-term extension. Patients provided informed consent and the study was approved by each center's institutional review board. Data are reported at 104 weeks unless otherwise noted.

Results: Patients (median age 28 years, 60% female, 40% with prior renal transplant) had a median time from diagnosis to screening of 48 months. All patients received PE/PI prior to eculizumab treatment. Mean ± SD hemoglobin and haptoglobin levels at baseline were 107.7 ± 16.5 and 0.9 ± 0.6 g/L, respectively. Mean ± SD baseline estimated glomerular filtration rate (eGFR) was 30.8 (19.0). By data cutoff, 18 of 20 patients (90%) achieved hematologic normalization (platelet count ≥ 150′ 10^9/L and lactate dehydrogenase [LDH] ≤ upper limit of normal sustained for at least two consecutive measurements spanning ≥ 4 weeks), 19 of 20 patients (95%) sustained LDH levels below the upper limit of normal, and only one patient (5%) required PE/PI (two fresh frozen plasma infusions) while continuing eculizumab. Mean ± SD increase in hemoglobin from baseline was 13.6 ± 16.2 g/L ($P = 0.0044$). Hemoglobin increased to a normal level in six of the 12 patients with a low level at baseline. At 100 weeks, mean ± SD haptoglobin increase from baseline was 0.2 ± 0.7 g/L ($P = 0.2770$). The eGFR increase from baseline (mean, 95% CI) was 7.2 (0.76–13.6; $P < 0.05$), an increase of 42.0% (23.2–60.7; $P < 0.0001$). Earlier eculizumab intervention (shorter duration of aHUS clinical manifestation prior to treatment) was associated with greater increases in eGFR ($P = 0.001$).

Summary/Conclusions: In aHUS patients with long disease duration and CKD with prior chronic PE/PI, treatment with eculizumab led to improvements in both hematologic and renal outcomes at 2 years. There was significant reduction in hemolysis in association with ongoing improvements in renal function. These results underscore the important role that hematologists may play in the diagnosis and management of this life-threatening disease, as well as the need for early and ongoing eculizumab treatment in aHUS patients.

References:

1. Loriat C, Frémeaux-Bacchi V. *Orphanet J Rare Diseases*. 2011;6:60.
2. Caprioli J et al. *Blood*. 2006;108:1267–1279.

PB 2.73-2

A new heterozygous mutation in the metalloprotease domain of ADAMTS13 in a patient with thrombotic thrombocytopenic purpura

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Background: Hereditary thrombotic thrombocytopenic purpura (TTP) is caused by mutations in the von Willebrand factor (VWF) cleaving protease ADAMTS13. ADAMTS13 deficiency prevents processing of ultra-large (UL) VWF multimers into normal, less reactive molecules. Hence, surviving UL-VWF multimers spontaneously react with platelets thereby inducing intravascular thrombosis, severe thrombocytopenia and hemolytic anemia.

Aim: We aimed at identifying the genetic defect in the *Adamts13* gene in a patient who developed TTP during pregnancy and at characterizing the functional effect of the ADAMTS13 mutation *in vitro*.

Methods: Genomic DNA was isolated from the patient's blood cells. Twenty-nine ADAMTS13 exons with exon-intron boundaries were amplified by PCR and sequenced. The identified mutation was introduced by site-directed mutagenesis in the pcDNA6.1 huADAMTS13 expression vector. Wild type and mutant ADAMTS13 were stably expressed in HEK293T cells using blasticidin selection. Recombinant proteins were partially purified using heparin chromatography. ADAMTS13 antigen levels were determined using an in house developed ELISA. ADAMTS13 activity was measured in a FRET assay. The presence of inhibitory antibodies was tested using a commercial ELISA kit.

Results: We here report the identification of a mutation in the *Adamts13* gene in a 39-year old female who developed TTP during pregnancy. In the acute phase of the disease she was diagnosed with severe thrombocytopenia and schistocytes. ADAMTS13 activity levels were severely decreased (8%, normal values in controls are > 40%). As no inhibitory anti-ADAMTS13 antibodies were found, we looked for the genetic defect in the *Adamts*13 gene of this patient. Sequence analysis of all 29 ADAMTS13 exons with exon-intron boundaries revealed three homozygous single nucleotide polymorphisms in exons 5 (c.420 C>T), 15 (c.1716 A>G) and 19 (c.2280 C>T) and a heterozygous c.559 G>C mutation in exon 6. This missense mutation changes D187 to H (p.Asp187His) in the metalloprotease domain of ADAMTS13 and has not been described before in any other patient. However, D187 was previously shown to be functionally involved in the high affinity Ca^{2+} binding site as mutating D187 to A resulted in a 10 fold reduced catalytic activity. In line with this, at an enzyme concentration of 0.5 nM, the activity of the D187H mutant as measured in the FRETS assay was abolished while wild type ADAMTS13 exhibited normal activity.

Conclusion: In this study we show that the p.Asp187His mutation leads to an inactive ADAMTS13 protein, confirming that the patient mutation, when expressed in a homozygous state, abolishes ADAMTS13 activity. The heterozygous state of the p.Asp187His mutation in our TTP patient however does not explain her severely reduced ADAMTS13 activity levels. Expression studies mimicking the heterozygous state and analyzing the contributions of the SNPs to ADAMTS13 expression levels will help to further explain this discrepancy. Nevertheless, the presence of the p.Asp187His mutation in our TTP patient supports the physiological relevance of the role of the D187 residue in the high affinity Ca^{2+} binding site of ADAMTS13.

PB 2.73-3

Atypical hemolytic uremic syndrome patients with progressing thrombotic microangiopathy treated with ongoing eculizumab have favorable hematologic outcomes at 2 years

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Background: Atypical hemolytic uremic syndrome (aHUS) is caused by inherited and/or acquired defects of complement system regulators, resulting in chronic, uncontrolled complement activation that causes platelet activation, thrombosis, hemolysis, and thrombotic microangiopathy (TMA). Patients may present with end-organ damage despite plasma exchange/plasma infusion (PE/PI), frequently in association with anemia, thrombocytopenia, undetectable haptoglobin, and/or elevated lactate dehydrogenase (LDH).[1] Within 1 year of diagnosis, up to 65% of patients develop permanent renal damage or die.[2] Eculizumab, a terminal complement inhibitor, is a humanized monoclonal

antibody that binds with high affinity to the human C5 complement protein and is the first approved treatment for aHUS in pediatric and adult patients.

Aims: Hematologic and renal outcomes were investigated in this phase 2 study of eculizumab in aHUS patients with progressing TMA.

Methods: Patients age ≥ 12 years with clinical evidence of progressing TMA (platelet count < 150×10^9/L after ≥ 4 PE/PI sessions in the prior week and platelet count decrease > 25%) were enrolled and received treatment with eculizumab. Patients provided informed consent and the study was approved by each center's institutional review board. Data are reported at 104 weeks.

Results: Seventeen patients (median age 28 years, 71% female, 41% with prior renal transplant) had a median time from diagnosis to screening of 10 months. Hematologic parameters were abnormal at baseline: mean ± SD platelet count was $109.0 \pm 32.1 \times 10^9$/L, LDH level was 323.0 ± 138.2 U/L, hemoglobin level was 89.1 ± 14.0 g/L, and haptoglobin level was 0.5 ± 0.4 g/L. All patients presented with renal impairment (estimated glomerular filtration rate [eGFR] < 60 mL/min/1.73 m^2). At 104 weeks, mean ± SD platelet count increase vs. baseline was $93.9 \pm 55.5 \times 10^9$/L ($P = 0.001$), LDH decrease was 205 ± 107.2 U/L ($P = 0.013$), hemoglobin increase was 36.1 ± 30.5 g/L ($P = 0.008$), and haptoglobin increase was 0.9 ± 0.4 g/L ($P = 0.006$). The median (range) time to the start of platelet count normalization (first platelet count ≥ 150×10^9/L) was 7 days (range, 1–218). Only two patients (12%) required PE/PI after starting eculizumab, (one patient after 10 days of treatment, the other after 84 days). Significant renal improvements were observed in association with the hematologic effects: eGFR change from baseline (mean, 95% CI) was 35.2 (17.3–53.1; $P = 0.0005$), and earlier eculizumab intervention (shorter duration of aHUS clinical manifestation prior to treatment) was associated with greater increases in eGFR ($P < 0.01$). In addition, four of five patients (80%) receiving dialysis at baseline were able to discontinue after eculizumab initiation.

Summary/Conclusions: Ongoing treatment with eculizumab led to sustained improvement in hematologic outcomes at 2 years, including reduced hemolysis, in association with ongoing improvements in renal function. These results underscore the important role that the hematologist may play in the diagnosis of this life-threatening disease, as well as the need for early and ongoing eculizumab treatment in patients with aHUS and progressing TMA.

References:

1. Loriat C, Frémeaux-Bacchi V. *Orphanet J Rare Dis*. 2011;6:60.
2. Caprioli J, et al. *Blood*. 2006;108:1267–1279.

PB 2.73-4

A novel heterozygous missense ADAMTS13 mutation (G3368A) in a patient diagnosed with thrombotic thrombocytopenic purpura due to reduced ADAMTS13 activity and low titer of anti-ADAMTS 13 antibody

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Background: Thrombotic thrombocytopenic purpura (TTP) is characterized by microvascular thrombosis, associated with deficiency of ADAMTS13, induced by mutations in the gene and autoantibodies.

Aims: In order to explain the patient's phenotype of the ADAMTS13 deficiency, it was decided to explore the mechanistic effect of a mutation found by means of expression studies in mammalian cells.

Methods: Patient with a history of TTP episodes that began at 13 years of age. Several plasma samples were measured for ADAMTS13 activity (ac) and antigen (ag), IgG anti-ADAMTS13 antibody and VWF multimers. The wild type (WT) and the ADAMTS13 mutant (site-directed mutagenesis) construct were transiently expressed in HEK 293 cells. Culture medium and cell lysate were evaluated by ELISA kit for ADAMTS13 ac and ag levels.

Results: In the patient's plasma were detected: ag = < 0.5% (nv = 70–160%), ac = 0.6 ± 0.6% (nv = 40–130%), 26% ULVWF multimers (normal value, nv < 15%) and IgG anti-ADAMTS13 antibody = 19 ± 3 U/mL (nv < 15 U/mL). The mean ± SD of ag, ac and IgG anti-ADAMTS13 antibody from three samples of the patient was informed.

To exclude the possibility that the novel mutation (in heterozygous state) found in our patient is not a disease-related polymorphism, we screened 50 healthy donors. The '*in vitro*' expression of the ADAMTS13 mutant led to a defect of secretion (ag = 7.5, ac = 17) of protease, causing intracellular accumulation (ag = 116, ac = 83). The cotransfection of the mixtures of the mutant and the WT revealed a secretion defect of the protease (ag = 86, ac = 69). All samples were compared to WT (considered 100%).

Conclusions: Mutagenesis suggests that the novel mutation G3368A (in TPS1-8 domain, exon 25) is critical for ADAMTS13 secretion.

The patient had low antibody titers to ADAMTS13 although the levels of ac and ag always were < 1.2%.

This patient's ADAMTS13 deficiency was probably caused by a combination of the G3368A mutation (in heterozygous state) and IgG anti-ADAMTS13 antibodies, although it is unclear why the latter were present.

PB 2.73-5

Release of fibrinolytic microvesicles in atypical haemolytic-uremic syndrome

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Background: Complement deposition on platelets and endothelium results in increased release of microvesicles that may participate in the thrombotic microangiopathy (TMA) of atypical hemolytic-uremic syndrome (aHUS). However, activated endothelial cells may also release microvesicles with compensatory fibrinolytic activity. Treatment with eculizumab, an anti-C5 mAb, arrests the TMA process.

Aims: We hypothesized that eculizumab may modify the release of fibrinolytic microvesicles from endothelial cells. Thus, we monitored the release pattern of fibrinolytic microvesicles in a patient with aHUS treated with eculizumab.

Methods: Blood samples were drawn before and after each dose of eculizumab. Microvesicles were isolated from plasma by sequential centrifugation and their concentration was evaluated by microvolume protein quantitation (A280 nm). The fibrinolytic activity of microvesicles was detected by fibrin-agarose zymography and by measuring the generation of plasmin using a photometric microtitre play assay.

Results: Microvesicle concentration and fibrinolytic activity before eculizumab were similar to the control group. A higher concentration of microvesicles and a parallel increase in fibrinolytic activity was observed after each dose of eculizumab. This increase was sustained all along the treatment period (6 month). The plasminogen activator responsible for this fibrinolytic activity was identified as tPA, thus suggesting an endothelial origin.

Conclusions: Eculizumab induced the release of fibrinolytic microvesicles that may participate in the arrest of the TMA during the course of aHUS.

PB 2.73-6

Recurrent disease in patients with thrombotic thrombocytopenic purpura: data from Serbian TTP Registry

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Background: Thrombotic thrombocytopenic purpura is a severe multisystem disorder characterized by fever, thrombocytopenia, microangiopathic hemolytic anemia, neurologic symptoms, and impaired renal function. Despite greater understanding of pathophysiology and effective treatment with plasma exchange thrombotic thrombocytopenic purpura is still intriguing disease since mortality from acute episode as well as risk of recurrent disease in survivors remains substantial. Recurrent episodes occur in up to 50% of patients who survive initial episode, developing a chronic recurrent form of the disease

Aims: To analyze risk of recurrent TTP and treatment options in such cases.

Methods: Registry of patients with TTP was established in Blood Transfusion Institute of Serbia (BTIS) in 2004. From August 2004 to December 2012 consecutive patients with clinical diagnosis of TTP were followed up and treated in Belgrade at the University Clinical Centre of Serbia, Military Medical Academy and Mother and Child Health Care Institute of Serbia. Prospective/retrospective analysis of 51 consecutive TTP patients was performed. Patients' informed consents were obtained and the study was approved by the BTIS Medical Ethics Committee.

Results: Seven of 51 patients (12%) did not survive acute episode of the disease. Among survivors, 32/44 (72%) patients presented with their initial TTP episode, 7/44 (16%) patients presented on their relapse in the range of 5–11 years after their initial TTP episode, whereas 5/44 (12%) patients presented at our observation in remission lasting 1–5 years after the initial TTP episode. There were 32 female (73%) and 12 male (27%) patients. The age range for the initial TTP episode was from 20 to 68 years, median value 37 years. Idiopathic TTP was observed in 36 patients and secondary TTP in eight patients. In the course of the disease none of the patients with secondary TTP manifested recurrence, while recurrent disease was present in 15/36 (42%) patients with idiopathic TTP. Recurrent disease occurred with a spectrum of presentations ranging from a single relapse to several episodes developing with variable frequency. In our cohort six patients had relapse only once, five patients relapsed two times, and four patients had three recurrent episodes. The period between initial TTP episodes and relapses ranged from 2 months to 11 years, median value 53 months. Most recurrent episodes were treated efficiently by plasma infusion or therapeutic plasma exchange. Due to repeated relapses splenectomy was performed in six patients.

Summary/Conclusions: Relapses are common in patients with idiopathic TTP. Patients follow up is necessary not only immediately upon the episode because of the risk of delayed TTP recurrence. In one of our patients, we have seen the first relapse as late as 11 years after initial episode. In patients with frequent relapses splenectomy as therapeutic option should be considered.

PB2.74 – Thrombosis: Miscellaneous

PB 2.74-1

Successful management for pregnancy in three patients with Upshaw-Schulman syndrome

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Background: Upshaw-Schulman syndrome (USS) is caused by a deficiency of ADAMTS13 activity (AC) due to its gene mutation. When ADAMTS13:AC is deficient, unusually large von Willebrand factor (VWF) multimers are not cleaved, which induces platelet thrombi formation in the microcirculation under high shear stress. We found nine female patients who were diagnosed with USS in their pregnancy. These pregnancies often result in premature delivery or fetal loss. Detail therapeutic protocol including FFP infusion for pregnant women with USS has not been established.

Case Series: Patients G3 (c.686 + 1 G>A/p.R1123C) was diagnosed with USS at 14 years old (yo). Her first pregnancy at 21 yo resulted in spontaneous abortion at 7 weeks of gestation (WG) without regular FFP infusion. In the second pregnancy at 22 yo, prophylactic FFP infusion was started at as following dose and intervals: 5–25 WG 5 mL/kg biweekly, 26–28 WG 5 mL/kg every 10 days, 29–33 WG 5 mL/kg weekly, after 34 WG 8 mL/kg every other days. At 35 WG, she delivered a baby girl (weighting 1446 g) with intrauterine growth retardation but no anomaly by a cesarean section (CS). Just before delivery, her platelet counts were 179×10^9/L, and ADAMTS13:AC was 34%. In her third pregnancy at 24 yo, she started FFP infusion at 7 WG. In addition to the FFP infusion of the same protocol as 2nd pregnancy, she was treated with asprin (100 mg/day) between 12 and 28 WG. She delivered a healthy baby boy (2632 g) at 37 WG by CS. Her platelet counts were 223×10^9/L, and ADAMTS13:AC was 10.5% just before delivery.

Patients K4 (p.Y304C/p.G525D) was diagnosed with USS at 25 yo in her first pregnant. After diagnosis, she received regular FFP infusion (5 mL/kg) every 3 weeks until nine WG of 2nd pregnancy at 30 yo. Between 9 and 29 WG, she received 5 mL/kg of FFP infusion biweekly. In 30 WG, she delivered a baby girl (1522 g) by CS after infusion of 9 mL/kg FFP. Her platelet counts were 179×10^9/L, and ADAMTS13:AC was 34% just before delivery.

Patients LL4 (p.C438S/p.G909R) was confirmed the diagnosis of USS at 27 yo by ADAMTS13 gene analysis. She became pregnant at 30 yo. She received 7 mL/kg FFP biweekly in 9–17 WG, 7 mL/kg weekly in 18–31 WG, and 9 mL/kg weekly after 32 WG. In addition to FFP, she was treated with aspirin (100 g/day) between 9 and 34 WG. At 39 WG, she delivered a healthy baby girl (3476 g) without anomaly. Her platelet counts and ADAMTS13:AC just before delivery was 208×10^9/L and 15%, respectively.

Conclusion: Plasma VWF level increases with progressing gestation in pregnancy. To prevent platelet thrombi in USS, much more ADAMTS13 supplementation may be necessary in the later term of gestation of USS. Therefore, we shortened the interval and increased the volume of FFP infusion with progressing gestation. This protocol brought successful delivery in four pregnancies of three patients with USS. The administration of low dose aspirin might be useful in USS pregnancy in addition to FFP infusion.

PB 2.74-2

Size of pulmonary embolism in relation to persisting symptoms

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Background: Up to two third of patients experience persisting symptoms after pulmonary embolism (PE). In nearly all of them no distinct (cardiopulmonary) pathology is detected. Predicting which patients will develop persisting symptoms is difficult.
Aims: We explore whether the size of PE, as defined by amount of perfusion defects and/or level of D-dimer, is able to predict development of residual symptoms.
Methods: Consecutive patients who have participated in PE-studies, who were at least 1 year after PE and had undergone perfusion lung-scanning as part of the diagnostic test and/or in whom a D-dimer was measured at PE were eligible. They were evaluated for persisting symptoms. Perfusion defects were scored by using a validated scoring-chart. D-dimer were measured by Vidas (BioMerieux) and by Roche Modular (Roche Diagnostics)-tests. Patients were classified as having increased fatigue when they reported any increase in fatigue with or without loss of condition after PE; their daily routine had to be unchanged. Patients were classified as having decreased performance when reporting any change in daily routine due to residual symptoms following PE. All other patients were classified as having no symptoms.
Results: Of 110 patients who participated in PE-studies between January 2000 and December 2009, 85 were evaluable. In 45 patients a perfusion-scan was available, in 74 D-Dimer had been measured at PE and in 34 both tests were available.
Median age at time of PE in patients with perfusion-scan was 50.5 (range 22.6–76.5) years. Time since PE was median 4.2 (1.0–8.6) years, 44% of patients were female. Perfusion defects were median 3.00 (0.25–12.25) segments. No symptoms were reported by 16 (36%), increased fatigue by 15 (33%) and decreased performance by 14 (31%) patients.
Perfusion defects in patients without symptoms were median 1.75 (range 0.25–12.00) segments vs. 2.50 (0.50–8.25) segments in patients with increased fatigue vs. 3.88 (0.50–12.25) in patients with decreased performance, overall $P = 0.325$. When comparing asymptomatic patients with those with symptoms, number of mismatched segments was median 1.75 (range 0.25–12.00) vs. 3.25 (0.50–12.25) segments, $P = 0.307$.
D-dimers were measured from 2003 on. Median age at time of PE in the D-dimer-group was 50.3 (20.8–85.7) years, time since PE median 1.5 (1.0–5.8) years and 41% were female. No symptoms were reported by 38%, increased fatigue by 34% and decreased performance by 28% of patients. D-dimer-levels were median 3059 (581–41,870). Observed levels of D-Dimer were similar before and after change of assay. D-dimer were in patients without symptoms median 2749 (581–18259) vs. 3094 (944–41870) in patients with increased fatigue vs. 3082 (765–8859) in patients with decreased performance.
Conclusions: Median larger perfusion-defects in perfusion lung-scanning seem to be related to persistent symptoms. This relation is not strong enough to yield a useful predictor at diagnosis for development of persistent symptoms. D-dimer was not related to symptoms.

PB 2.74-3

D-dimer, factor VIII, and thrombotic burden during the acute phase of leg deep venous thrombosis and early signs and symptoms of post-thrombotic syndrome

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Background: Post-thrombotic syndrome (PTS) is the most common complication of deep vein thrombosis (DVT). Few data are available on the time course of D-dimer, FVIII and thrombotic burden in relation with the risk of post-thrombotic syndrome.
Aim: The aim of the study was to evaluate the course of D-dimer, FVIII levels, and the burden of residual thrombus during the acute phase of proximal DVT of the lower limbs and the PTS signs and symptoms after 90 days of vit K antagonist therapy.
Methods: Fifty-eight patients with a proximal DVT of the lower limbs (age 64; range: 20–88 years; male 57%) were enrolled from the day of diagnosis with a follow-up of 90 days. All subjects received LMWH for 5–7 days, overlapped and followed by oral anticoagulation for 3 months. A complete ultrasound examination of the deep vein system of the lower limbs was conducted on the day of diagnosis (D0) and 7 (D7), 30 (D30), and 90 (D90) days afterward. On the same days, blood samples were taken for measuring D-dimer (Vidas d-Dimer, BioMerieux, France, n.v. < 0.5 μg/mL) and FVIII (chromogenic assay). Villalta scale was evaluated at D30 and D90. The thrombotic burden was defined according to a new score, which considered the number of districts with thrombi and the degree of occlusion.
Results: D-dimer was 4.1 ± 3.4 μg/mL at D0 and decreased till 0.4 ± 0.5 μg/mL $P < 0.001$ at D90. D-d levels did not correlate with thrombotic burden at diagnosis and during follow-up; FVIII levels did not change during follow-up (D0: 2.5 ± 0.7 IU/mL; D90 2.6 ± 0.6 IU/mL, P = ns) and did not correlate with thrombotic burden. At D90, nine patients had a Villaalta score ≥ 5, the mean Villalta score was 2.3 IQR 0–3 and was not correlated to D-dimer or FVIIII. Neither D-dimer nor FVIII time course correlated with Villalta score, whereas the burden of residual thrombus at D30 and D90 was correlated with the Villalta scale (rho 0.4 $P < 0.01$).
Conclusions: D-dimer decreases according to a power decay during treatment for acute DVT but is not correlated with PTS symptoms and signs. FVIII levels are stable during the acute phase of DVT. The burden of residual thrombus is correlated with signs and symptoms for PTS after 90 days of oral anticoagulation.

PB 2.74-4

The effect of initiating combined antiretroviral therapy on endothelial cell activation and coagulation markers in a south african HIV-infected cohort

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Background: An increased prevalence of venous thrombo-embolism (VTE) including deep venous thrombosis (DVT) is observed in HIV-infected patients with an increased incidence vs. HIV negative individuals [1, 2]. A beneficial effect of anti-retroviral therapy (ART) on coagulation markers is described. [3, 4, 5, 6]. Clinical risk factors for VTE in HIV-infected patients are advanced age, immobilisation, presence of a central venous catheter, AIDS-defining opportunistic infections and a CD4 cell count < 500 cells/mm^3. Protein C, protein S or antithrombin deficiencies as well as antiphospholipid antibodies and elevated levels of factor VIII and von Willebrand factor (vWF) are associated with VTE in HIV [7].
Objectives: To examine the effect of initiating ART on endothelial cell activation and coagulation markers in a South African ART-naive HIV-infected cohort.
Patients/Methods: Prospective cohort study of 123 HIV-infected individuals. Markers of endothelial cell activation, coagulation and anticoagulation were measured at baseline (ART naive) and at follow-up (on ART) in HIV-infected individuals and compared to the levels in an HIV-uninfected control population. A venous ultrasound of both legs was performed to detect asymptomatic DVT.
Results: Before initiating ART significantly elevated von Willebrand factor and D-dimer levels, increased activated protein C resistance (APCR) and decreased protein S and C levels were observed. At follow-up all markers, except APCR, improved towards the normal range for controls without showing complete normalisation. In a subgroup of 56 patients no asymptomatic DVT was found.

Conclusions: Compared to the controls, abnormal levels of coagulation markers were observed in HIV-infected individuals before and after the initiation of ART. Most markers improved after starting ART, but remained significantly different from the controls, indicating a persistent disturbed haemostatic balance. In this cohort no increased prevalence of asymptomatic DVT was observed.

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PB 2.74-6

Markers of endothelial damage are associated with successful recanalization in acute stroke

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Background and Purpose: We aimed to determine the association of pretreatment levels of selected endothelial biomarkers with arterial recanalization and clinical outcome in acute ischemic stroke (AIS) patients treated with recombinant tissue plasminogen activator (rtPA).

Methods: We prospectively recruited 64 AIS patients treated with intravenous and/or intra-arterial rtPA. Blood samples were collected before thrombolysis and analyzed for von Willebrand factor (vWF), soluble thrombomodulin (sTM) and soluble endothelial protein C receptor (sEPCR). Patients were divided into three groups according to documented arterial occlusion followed or not by complete recanalization (Thrombolysis In Myocardial Infarction score of 3). Favorable clinical outcome was defined by modified rankin score of 0–2 at 90-day.

Results: Of the 64 included patients, 33 had a documented arterial occlusion and 14 (42%) of them had a complete recanalization after thrombolysis. After adjustement for confounding factors, these patients presented lower levels of sTM and sEPCR than patients with a persistent occlusion (median sTM values, 21 vs. 48 ng/mL, $P = 0.008$; median sEPCR values, 78 vs. 114 ng/mL, $P = 0.018$), but similar levels than patients without documented occlusion (median sTM values 22 ng/mL; median sEPCR values 77 ng/mL). Levels of vWF did not differ between the three groups. None of the selected biomarkers were significantly associated with favorable outcome.

Conclusions: The efficacy of thrombolytic therapy to recanalize intracranial vessels is associated with low levels of sTM and sEPCR but not with vWF levels. This suggests that thrombolysis resistance might be related to the impairement of the activated protein C pathway resulting from an increased shedding of endothelial surface receptors rather than a more global endothelial dysfunction including VWF secretion. Further larger studies are warra

PB 2.74.5

Aspirin intake AM or PM?: effect on platelet reactivity in the morning

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Background: Platelets play a crucial role in the development of acute thrombotic events and platelet activity follows a circadian rhythm, with a peak in the morning. This may contribute to the observed peak of cardiovascular disease (CVD) in the morning. Aspirin is one of the cornerstones in secondary prevention of CVD and is usually taken on awakening, although evidence regarding optimal time of intake is lacking. Due to its short half life, aspirin only inhibits platelets which are present at the time of intake while new platelets formed throughout the day will not be inhibited. These uninhibited platelets contribute to the morning peak of platelet reactivity. The proportion of uninhibited platelets which are present in the morning may be higher with aspirin intake on awakening compared with aspirin intake at bedtime. Thus, the timing of aspirin intake may influence its effect on platelet reactivity during the morning hours.

Aim: We hypothesized that aspirin intake at bedtime compared with on awakening attenuates platelet reactivity in the morning.

Methods: This study is part of the Aspirin in Reduction of Tension II trial (clinicaltrials.gov number: NCT01379079), which is a randomized crossover trial comparing the effect of aspirin intake on awakening with intake at bedtime on blood pressure and platelet reactivity. Two hundred and fifty patients using aspirin for secondary prevention of CVD randomly use both aspirin on awakening and at bedtime during two intervention periods of 3 months. Blood is drawn in the morning at fixed time points for each patient at the end of both intervention periods. Platelet reactivity is measured with the VerifyNow® Aspirin assay in 125 of the 250 patients to detect a 10% reduction of platelet reactivity in the morning with aspirin intake at bedtime.

Results So Far: Until now (19 December 2012) 235 patients have been randomized, of which 109 patients have complete measurements at both time points. We expect to complete data collection of platelet reactivity measurements in March 2013, which guarantees availability of the data to be presented at ISTH 2013.

Potential Impact: Results of this study will show whether aspirin intake at bedtime attenuates the morning peak in platelet reactivity compared with intake on awakening in patients with CVD. Simply changing the time of intake could make aspirin even more effective in preventing recurrent thrombotic events in the morning without any additional costs.

PB3.21 – Antiplatelet agents: aspirin – II

PB 3.21-1

Diabetes modulates the fibrinolytic properties of aspirin without altering the platelet inhibitory actions: a possible mechanism for aspirin treatment failure

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Background: Aspirin is an anti-thrombotic agent that has a dual mode of action by inhibiting platelet function and modulating fibrin clot lysis. This agent is used in individuals at high risk of atherothrombosis, including those with diabetes. However, recent evidence suggests reduced clinical efficacy of aspirin in diabetes by mechanisms that are not entirely clear.

Aims: We hypothesised that hyperglycaemia in diabetes interferes with aspirin action, consequently comrpomising cardiovascular protection. Therefore, the aim of this work were to investigate the effects of short and medium term hyperglycaemia on response to aspirin therapy.

Methods: To analyse the effects of medium term hyperglycaemia on response to aspirin therapy, we investigated the ex vivo effects of this agent on platelet function and clot structure/fibrinolysis in 25 individuals with type 1 diabetes not on any treatment other than insulin. Results were compared with an age and sex matched control group of 23 individuals.

In addition, we investigated the effects of short term hyperglycaemia on response to aspirin therapy by ex vivo addition of excess glucose (20 mM) to blood samples in the presence and absence of aspirin, followed by plasma separation. Platelet function, after stimulation with different agonists, was monitored by the multiplate assay and fibrinolysis was tested using turbidimetric analysis.

Results: Platelet aggregation to arachidonic acid (AA) after treatment with 0, 1 and 10 mg/L aspirin in T1DM subjects was 62.7 ± 2.8, 54.6 ± 3.0 and 31.4 ± 3.3 AU, respectively ($P < 0.05$). However, failure to respond to low aspirin concentration was noted in individuals with poorly controlled diabetes ($n = 9$, HbA1c>9%). Excess glucose abolished the platelet inhibitory effect of low aspirin concentration but had no effect on inhibition by high aspirin concentration. Similar findings were demonstrated in 23 healthy controls with low aspirin concentration having a non-significant effect on platelet inhibition in the presence of excess glucose (69.2 ± 5.0, 64.3 ± 4.8; for 0 and 1 mg/L aspirin, $P > 0.05$ and 37.6 ± 4.7 au for 10 mg/L, $P < 0.05$). Aspirin had no effect on ADP-induced platelet stimulation in the diabetes group or controls.

Fibrinolysis in individuals with diabetes showed no difference in the presence of 0, 1 and 10 mg/L aspirin (654 ± 49, 672 ± 43.8 and 657.7 ± 41.5 s, respectively; $P > 0.1$). This was not affected by adding excess glucose to blood samples. In contrast, fibrin clot lysis was enhanced by aspirin treatment in healthy controls (600 ± 63.5, 475 ± 26.6 and 489 ± 29 s, respectively; $P < 0.05$). However, facilitation of fibrinolysis by aspirin in the control group was abolished when excess glucose was added to blood samples.

Summary: Our data indicate that medium term glycemic control and high glucose concentrations affect platelet response to low aspirin concentrations following AA stimulation. Moreover, the fibrinolytic properties of aspirin are lost in diabetes, which appear to be due to both chronic and acute elevation of blood glucose levels. Future work is warranted to investigate the relationship between clinical aspirin treatment failure and medium/short term glycaemia in aspirin-treated individuals with diabetes.

PB 3.21-2

Anti-platelet effect of aspirin in medically treated patients with acute myocardial infarction: Influence of age and gender

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Background: Aspirin is recommended as a first-front anti-platelet treatment for patients who present with acute myocardial infarction (AMI). The pharmacological target of aspirin in platelets is the inhibition of thromboxane A_2 (TXA_2) synthesis. Data on platelet function testing in medically managed patients with AMI without coronary interventions are scarce in the literature, particularly those testing platelet TXA_2.

Aims: To assess the effect of aspirin on platelet TXA_2 synthesis and platelet reactivity in medically treated patients with AMI and the possible influence of age and gender.

Methods: We studied 220 consecutive medically treated patients with AMI (mean 65 ± 13 years of age, range 31–91; 164 men, 56 women). Of them, 135 patients had electrocardiographic ST-segment elevation (STEMI) and 85 did not (NSTEMI). All patients were treated with aspirin and clopidogrel at the onset of the acute event, and 40% of the STEMI patients received fibrinolytic treatment. The study was approved by the Institutional Review Board of the hospital; all patients gave informed consent. Platelet function testing was performed within 48 h of the onset of the acute event. The tests, performed as described[1], included: collagen-induced TXA_2 synthesis, ^{14}C5HT release and recruitment (proaggregatory activity of the cell-free releasates) in whole blood; light transmission aggregometry (LTA)-induced by arachidonic-acid (AA, 1 mM), collagen (1 μg/mL), ADP (20 μM), U46619 1 μM and TRAP 15 μM and the occlusion times in the PFA-100 system with collagen/epinephrine (CEPI) and collagen/ADP cartridges (CADP). TXA_2 inhibition in individual patients was considered partial if > 3.5 ng/mL (< 95% inhibition compared with that of aspirin-free volunteers)[1].

Results: Partial TXA_2 inhibition was detected in 58/220 (26%) of the patients without differences between STEMI and NSTEMI or the fibrinolytic treatment. Those patients had markedly higher TXA_2 synthesis as compared to the rest of the patients (mean ± SEM 45.1 ± 4.6 vs. 0.23 ± 0.03 ng/mL, $P = 0.000$). This was associated with increased platelet dense granule release, collagen-induced aggregation and recruitment, AA-induced aggregation, and with reduced closure time in the CEPI test (in all instances $P = 0.000$). LTA-induced by ADP, U46619 and TRAP or the CADP test were unchanged. When patients were stratified according to age (< 75 years of age and ≥ 75 years of age) the older patients had 2-fold higher platelet TXA_2 synthesis (18.0 ± 4.1 vs. 9.0 ± 2.0 ng/mL, $P = 0.026$). Comparison of TXA_2 synthesis by gender indicated that men had higher TXA_2 synthesis than women (15.4 ± 2.3 vs. 2.4 ± 1.1 ng/mL, $P = 0.002$).

Conclusions: The effectiveness of aspirin for TXA_2 suppression early at the onset of the acute event is less than optimal in a relevant proportion (26%) of the medically treated patients with AMI. The aspirin effect was lower in patients more than 75 years of age and in male patients. Since this was associated with elevations of other markers of platelet reactivity, patients with partial inhibition of TXA_2 by aspirin may be at higher risk of poor outcome or recurrence.

Grants: PI07/0463; Retics06/0026;[1] Santos MT et al. J Thromb Haemost 2008; 6:615–621.

PB 3.21-3

Low-dose acetylsalicylic acid therapy monitored with ultra high performance liquid chromatography

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Background: Assessment of compliance in patients on low-dose acetylsalicylic acid (ASA) therapy is crucial when evaluating the antiplatelet effect of ASA. Assessment of compliance is however limited to interview, pill counting, witnessed ASA intake, and measurement of thromboxane B_2 levels.
Aims: We validated a new, sensitive ultra high performance liquid chromatography assay for analysing blood levels of ASA and the metabolite salicylic acid (SA) in order to develop a reliable and sensitive method to assess compliance in patients receiving ASA.
Methods: Ten healthy volunteers not taking any drugs containing ASA were included and had blood samples taken prior to and one, six and 24 h after intake of 75 mg ASA. Besides that, 50 ASA-treated patients with stable coronary artery disease were included and blood samples were obtained one and 24 h after intake of 75 mg ASA. Whole blood was collected into chilled Lithium Heparin tubes and plasma was separated by centrifugation at 4 °C and afterwards immediately stored at −80 °C until analysed. Before analysis plasma was mixed with acetonitrile, and 2-methylbenzoic acid was added as internal standard. After filtration, ASA and SA were quantified with ultra high performance liquid chromatography. Separation was accomplished with an isocratic flow of acetonitrile in phosphate buffer pH 2.5, and a Zorbax RRHD C_{18} analyticalcolumn. Detection was achieved with wavelength photodiode array detection set at 237 nm. The study was approved by the Danish Data protection Agency and it was conducted in accordance with the Helsinki II declaration.
Results: The ASA- and SA-assay showed linearity ($r^2 > 0.999$) within the range of 0.2–200 μg/mL, with detection limits in plasma of 170 and 53 ng/mL, respectively. Limits of quantification in plasma were estimated to be 0.2 μg/mL for both ASA and SA. The intra- and inter-day imprecision for ASA and SA were 2.2–5.9%, 3.4–11.7% and 1.8–8.0%, 3.8–9.4%, respectively. Recovery was between 89% and 103% for both assays. More than 60 coadministered drugs were investigated for interference, and only one drug interfered with the ASA-assay whereas none of the coadministered drugs interfered with the SA-assay. ASA was measurable 1 h after intake of 75 mg ASA in most participants (54 of 60), and SA was measurable 1 and 6 h after ASA intake in all participants. No difference in ASA or SA concentrations was found between healthy volunteers and coronary artery disease patients (*P*-values of 0.13 and 0.27, respectively). Inter-individual variance of 31–52% was observed for both ASA and SA after low dose ASA intake.
Conclusion: We developed a new, fast and reliable ultra high performance liquid chromatography assay with a high sensitivity and selectivity, suitable for monitoring compliance in patients treated with low-dose ASA.

PB 3.21-4

Aspirin response: how to explain striking differences in serum TxB2 levels across clinical studies and what use for serum TxB2?

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Background: Quantification of serum thromboxane B_2 (TxB_2) is a highly specific assay to assess the biological response of platelets to aspirin. Data on the prognostic value of higher levels of TxB_2, in compliant patients, are conflicting while mean TxB_2 can display up to 10-fold differences in different cohorts
Aims: To identify factors responsible for differences in TxB_2 in two different cohorts of cardiovascular patients originally tested with two different Elisa assays.
Methods: TxB_2 levels in two sets of serum samples ($n = 39$ and $n = 34$) from representative subgroups of aspirin-treated patients from the ADRIE study[a] ($n = 656$) and from the BOSTON study[b] ($n = 562$) were quantified with both of the original assay (GE and RD Elisas) and also with liquid chromatography and tandem mass spectroscopy (MS) as the gold standard. The concordance between the two assays and with the MS was evaluated. Variables known to affect TxB_2 were compared between both population subgroups and further included in a multiple linear regression model. TxB_2 was log-transformed for linear regressions
Results: The median TxB_2 in the entire ADRIE and BOSTON study populations were respectively 7 ng/mL (IQR 3–12) and 0.6 ng/mL (IQR 0.2–1.4) as originally tested with a GE and RD Elisa assays respectively. This difference persisted in the selected subgroups for MS-TxB_2: medians were 6.1 ng/mL (IQR 2.4–16) and 0.6 ng/mL (IQR 0.2–1.2) in the ADRIE and BOSTON subgroups, respectively ($P < 0.0001$). Concordance between the GE and RD assays was poor raising the possibility of assay bias. Several clinical factors with a potential impact on MS-TxB_2 were unbalanced in the two cohorts (aspirin dose, diabetes, gender, statin use, hypercholesterolemia, clopidogrel use, SSRI use, CRP, BMI, all with $P < 0.05$) but some of these variables were tightly linked to the study (eg aspirin dose was 81 or 325 mg in BOSTON and 100 mg in ADRIE) precluding a proper regression model on pooled data from both subgroups. Multiple regression performed independently in each subgroups showed that between 27% and 59% (R^2) of the variance of Log (MS-TxB_2) could be explained by variables such as BMI, diabetes, statin, acute coronary syndrome at inclusion. Complementary analyses on the whole cohorts (1218 TxB_2 samples) will help derive a proper model to assess the respective contribution of the different factors to the difference in TxB_2 levels between the two cohorts.
Conclusions: Differences in serumTxB_2 levels in different cohorts of aspirin-treated cardiovascular patient are important and. The differences may not only be explained by a potential bias between Elisa assays but rather by differences in individual factors. In view of such differences and in the absence of clinical trials, relying on serum TxB_2 levels to adjust the antiplatelet regimen is discouraged.
References:
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PB 3.21-5

Aspirin efficacy is reduced following off-pump coronary bypass operation

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Background: Aspirin is widely used to preserve graft patency and prevent thromboembolic complications including graft closure following coronary artery bypass surgery. Conventional 'on-pump' bypass surgery transiently reduces the *in vitro* antiplatelet effect of aspirin, but it is unclear whether this is true for modern 'off-pump' bypass surgery performed through a minimally invasive J-hemisternotomy. If so, this may be explained by a postoperative increase in platelet turnover or thrombopoietin production.

Aims: To investigate if minimally invasive off-pump bypass surgery reduces the effect of aspirin and, if so, to identify potential explanatory mechanisms related to platelet turnover and platelet production.
Methods: We performed a prospective non-interventional single-center study including 41 aspirin-treated patients undergoing off-pump bypass surgery. Blood samples were obtained 8–10 days before surgery during aspirin 75 mg maintenance treatment and at first postoperative day (8 a.m.) following an aspirin bolus of 300 mg. The effect of aspirin was evaluated by VerifyNow® Aspirin and Multiplate® Analyzer (agonist: arachidonic acid 1.0 mM). The immature platelet fraction was used as a marker of platelet turnover. Thrombopoietin was measured by ELISA to reflect the regulation of platelet production. Serum thromboxane B_2 was measured with ELISA and used to confirm compliance with aspirin.
Results: All patients were compliant with aspirin according to serum thromboxane B_2 levels (all below 13.7 ng/mL). Postoperative platelet aggregation was significantly higher than preoperative platelet aggregation according to VerifyNow® Aspirin ($P = 0.002$), but not to Multiplate® Analyzer ($P = 0.19$). Platelet turnover was increased postoperatively as indicated by an increased immature platelet fraction ($P = 0.03$). Thrombopoietin levels were also significantly increased postoperatively ($P < 0.0001$), whereas platelet count was significantly reduced ($P < 0.0001$).
Summary/Conclusions: Off-pump bypass surgery reduces the antiplatelet effect of aspirin according to VerifyNow® Aspirin. This finding may reflect that the operation, although much less invasive than conventional bypass surgery, triggers an increased platelet turnover and an up-regulation of platelet production. Using the Multiplate® Analyzer showed no difference between pre- and postoperative platelet aggregation, which is likely explained by the considerable perioperative reduction in platelet count.

PB 3.21-6

Association between the GPIIIa gene polymorphism and the response to acetylsalicylic acid in patients with type 2 diabetes mellitus

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Background: Type 2 diabetes mellitus (DM2) is a metabolic disorder associated with cardiovascular complications and hyperactivation of platelets. Acetylsalicylic acid (ASA) is an antiplatelet agent used in the prevention of atherothrombotic events by inhibition of platelet cyclooxygenase-1, thus blocking the formation of thromboxane A_2. The effect of ASA can be determined by the plasma levels of 2,3-dinor-thromboxane B_2 (2.3-dinor-TXB_2). The β3 subunit (GPIIIa) of the platelet glycoprotein GPIIb/IIIa can present a polymorphism at position 33 which consists in a replacement of a leucine (PI^{A1}) by a proline (PI^{A2}) resulting from a single nucleotide transition in the GPIIIa gene (C^{155} à T). Previous studies reported that this polymorphism is associated with increased platelet aggregation and may contribute to ASA resistance. However, this association is still controversial.
Aim: To investigate the association between 2.3-dinor-TXB_2 plasma levels and GPIIIa gene polymorphism in patients with DM2 using ASA for primary prevention of atherothrombotic events.
Methods: Blood samples from 65 patients with DM2 were collected in two distinct moments, the first immediately prior to initiation of treatment with ASA and the second, at the fifteenth day of treatment with 100 mg of this medication daily. Patients with DM2 were selected in the Santa Casa Hospital, Belo Horizonte, Brazil. These samples were analyzed to determine plasma levels of 2.3-dinor-TXB_2. Furthermore, the GPIIb/IIIa (PI^A) gene polymorphism was studied using a polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) technique. Statistical analysis was performed using Pearson Chi-Square test and was considered statistically significant when $P < 0.05$.
Results: During treatment with ASA, 29 patients (44.6%) had a higher or equal to 75% reduction in the 2.3-dinor-TXB_2 levels. The PI^{A2} allele of GPIIIa gene was found in 25.9% of the participants (23.5% heterozygous and 2.5% homozygous). It was observed that the PI^{A2} allele of GPIIIa gene was present in higher frequency in patients who had a reduction of 2.3-dinor-TXB_2 higher or equal to 75% ($P = 0.032$).
Conclusions: These results suggest that the presence of PI^{A2} allele in GPIIIa gene may be associated with a better response to ASA intake in patients with DM2.
Supported by CNPq, CAPES and FAPEMIG – BRAZIL.

PB3.22 – Platelets: Point-of-Care Tests

PB 3.22-1

A 96 well plate-based whole blood assay assessing multiple platelet activation pathways appears promising in the evaluation of antiplatelet therapy

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Background: Platelet function testing to assess the efficacy of antiplatelet drugs is increasingly being used. A variety of assays have been developed; however few offer the advantage of assessing multiple platelet activation pathways simultaneously. Recently, adaptations based on 96 well plate formats have attracted attention as they require significantly smaller volumes of blood and can provide extensive concentration-response ranges to multiple platelet agonists. We have developed a 96-well plate-based assay carried out in whole blood, where flow cytometry is used to assess the decrease in single platelets as a measure of platelet aggregate formation.
Aims: To investigate the sensitivity of the 96-well plate-based whole blood assay to platelet inhibition by aspirin and cangrelor, a P2Y12 receptor inhibitor.
Methods: Platelet function was assessed in whole blood obtained from healthy volunteers, using 96 well plates coated with 4 μL of the following agonists: arachidonic acid (AA, 0.03–1 mM), ADP (0.3–30 μM), collagen (0.1–10 μg/mL) and TRAP (0.1–10 μM), or vehicle. Whole blood was incubated with aspirin 10–100 μM, cangrelor 1–1000 nM or vehicle. Forty-six microlitre of whole blood was added to each well and the plate was shaken for 5 min at 1000 rpm at 37 °C; a fixative solution (Platelet Solutions, Nottingham) was applied to stop platelet aggregation and stabilize samples for up to 9 days and allow analysis in a central laboratory where samples were labeled with FITC-conjugated CD42a and assessed by flow cytometry. Aggregation was calculated as (Platelet count in vehicle-treated sample – Platelet count in agonist-stimulated sample)/Platelet count in vehicle-treated sample × 100.
Results: In 20 healthy volunteers, aggregation assessed in duplicate was robust and reproducible (CV < 10%). As expected, cangrelor induced a profound inhibition of ADP-induced aggregation. Aspirin dose-dependently inhibited platelet responses to AA; however, a significant drop in single platelet count was seen at the highest AA concentration (1 mM) indicating full aggregation. Addition of cangrelor 1 μM to these samples prevented the drop in platelet count, suggesting that ADP is responsible for the aggregation seen with AA 1 mM in the presence of aspirin. One possible explanation for the appearance of ADP could be some degree of red cells lysis caused by high AA concentration as previously reported in the literature. Collagen- and TRAP-induced aggregation were also impaired to a different extent by *in vitro* treatment with either antiplatelet agent, and resulted in a right shift of dose-response curves.

Conclusions: The present optimization study has shown that a 96-well plate-based whole blood aggregation assay could be used to assess platelet function and the effect of commonly used antiplatelet agents. However, AA at concentrations > 0.5 mM should be used with caution in whole blood to assess the effect of COX inhibition as the latter could be underestimated potentially due to the effect of ADP. Further work to explain this observation and evaluate the utility of the assay to detect platelet inhibition in patients requiring antiplatelet therapy is warranted.

PB 3.22-2

A simple colorimetric assay provides a novel platelet activation endpoint and distinguishes between responses to different physiological agonists

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Background: Our laboratory has demonstrated that a relatively simple dye reduction assay is comparable to the complex ^{14}C-serotonin release assay (SRA) in detecting Fc-receptor mediated platelet activation by antibodies diagnostic for heparin-induced thrombocytopenia (HIT) (Platelets 23:69,2012). The aim of these experiments was to investigate this novel activation assay using other platelet agonists. Previous literature has suggested that immune stimulation of platelets results in a higher degree of activation than other physiological agonists (Platelets 10:319,1999) and that strong platelet activation leads to apoptosis (Blood Rev 26:51,2012).

Methods: This study evaluated platelet responses to hemostatic agonists, a pro-apoptotic stimulus and platelet-activating antibodies, using the colorimetric assay in comparison to traditional platelet activation endpoints. Platelet activation by thrombin and/or collagen was compared to immune-mediated stimulation with HIT antibody plus 0.1 U/mL heparin or a commercial monoclonal antibody to CD9, and to the apoptotic agent, ABT737. The non-physiologic calcium ionophore, A23187, was included as a control. Washed donor platelets were incubated with agonists or antibodies on a rotary shaker (600 rpm) for 15–30 min prior to assessment of activation. Platelet activation endpoints included dense granule release, monitored using platelets pre-incubated with radio-labeled serotonin, platelet aggregation detected spectrophotometrically, and depletion of mitochondrial membrane potential assessed by flow cytometry using fluorescent dye (JC-1). For colorimetric assay, platelets were incubated 60 min with CellTiter 96 Aqueous One Reagent (Promega, Madison WI) at 37 °C and dye reduction measured as optical density at 490 nm (OD). With this dye, moderate platelet activation results in an increased OD, while a highly activated state results in lack of ability of platelets to metabolize the dye to its dark product, reflected by a low OD reading.

Results: Both hemostatic and immune activators caused maximal platelet aggregation and serotonin release. However, antibody-mediated platelet activation resulted in an extreme loss of metabolic potential matched only by A23187, while platelets activated by high doses of thrombin and/or collagen retained significant metabolic capacity. The colorimetric assay paralleled the loss of mitochondrial membrane potential evaluated by JC-1 uptake dye. Surprisingly both thrombin-activated and HIT antibody-activated platelets showed loss of mitochondrial membrane potential, yet only the immune-activated platelets lost dye reduction capacity. Stimulation of platelet apoptosis by ABT737 did not result in platelet aggregation, and caused increased metabolic activity as detected by increased dye reduction. In contrast platelet-activating antibodies and A23187 caused maximal aggregation and a rapid loss of metabolic activity.

Conclusion: The results of this study demonstrate that while many features indicative of apoptosis are also characteristic of highly activated platelets, the metabolic pathway to these endpoints differs. The colorimetric assay is rapid and relatively inexpensive and allows a range of doses of multiple agonists to be evaluated simultaneously in a microtiter format. The precise mechanism that is reflected in the colorimetric response will require further study. In the interim, this method may be used empirically to provide a finer distinction in the level of platelet activation compared to current methods.

PB 3.22-3

Development of a novel high throughput 96 well plate-based whole blood assay for investigation of platelet function in healthy volunteers and patients with clinically diagnosed bleeding disorders

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Background: The current gold standard in platelet function testing, light transmission aggregometry (LTA), is time- and labor-intensive, and uses platelet-rich plasma (PRP) which makes it sub-optimal for high throughput testing. We have previously investigated the utility of a 96-well plate aggregation assay (*Optimul*) in assessing platelet function with promising results (presented at the SSC2012-HDC04). However, this method still relied on PRP preparation. In order to reduce blood manipulation prior to platelet function testing and to study multiple platelet activation pathways simultaneously, we have developed a 96-well plate-based assay carried out in whole blood, where aggregation is measured as a decrease in the number of single platelets – a method we previously described (Fox et al. *Platelets* 2004; 15:85–93).

Aims: To investigate whether a 96-well plate-based whole blood assay can be used to assess platelet function.

Methods: Healthy volunteers (n = 20) and participants with clinically diagnosed excessive bleeding (n = 15) were recruited to the Genotyping and Phenotyping of Platelets study (GAPP, ISRCTN 77951167) from October 2012 to January 2013. This study was approved by the National Research Ethics Service Committee and all participants gave written informed consent. Platelet function testing was carried out by LTA, used as a reference method, and in whole blood using 96 well plates coated with 4 μL of the following agonists: arachidonic acid (0.03–1 mM), ADP (0.3–30 μM), collagen (0.1–10 μg/mL) and TRAP (0.1–10 μM), or vehicle. After addition of 46 μL of whole blood, the plate was shaken for 5 min at 1000 rpm at 37 °C; a fixative solution (Platelet Solutions, Nottingham) was applied to stop platelet aggregation and allow analysis in a central laboratory. Fixed whole blood samples were labeled with FITC-conjugated CD42a and assessed by flow cytometry. Aggregation was calculated as (Platelet count in vehicle-treated sample – Platelet count in agonist-stimulated sample)/Platelet count in vehicle-treated sample x 100.

Results: Normal ranges of platelet aggregation response on the 96-well plate were determined based on the results obtained from 20 healthy volunteers. Intra-individual variability was minimal (CV < 10%). Dose-response curves were readily assessable for all agonists tested. In patients with bleeding symptoms both assays agreed on diagnosis in 80% of cases (κ = 0.587, P = 0.013). However, while 7 (47%) participants had a platelet defect according to LTA, the 96 well plate assay found a defect in 4 (27%). Discrepancies were seen in mild Gi-type defects and possible partial COX defects which were diagnosed due to kinetic information available with LTA, and were missed with the whole blood endpoint assay.

Summary/Conclusions: Evaluation of the 96 well plate-based whole blood aggregation assay suggests it might be useful for investigation of platelet function, especially in individuals where blood sample volume is limited, such as children or those requiring multiple other blood tests. Additional developments of the assay to look at the expression of platelet activation markers in addition to single platelet counting are underway. Further investigation to determine the clinical utility of the assay in detecting acquired and inherited defects in platelet function is warranted.

PB 3.22-4

Point-of-care platelet testing accurately displays platelet dysfunction in chronic renal insufficiency, but fails to predict bleeding after kidney biopsy

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Background: The risk of major complications in kidney biopsies is low with only minor bleeding complications in 24% of the cases. Low platelet count, low hematocrit, and high uremia are found to contribute to this bleeding risk in renal failure patients. Ivy bleeding time (IBT) is under debate whether it adequately predicts this bleeding risk.
Aims: We assessed if Multiplate (MEA) and/or PFA-100 (PFA) could predict bleeding complications after kidney biopsy in renal patients more accurately than the IBT. Low platelet count and low hematocrit in MEA and PFA testing, however, produce false positive results. Therefore, a full range of reference intervals was calculated by measuring healthy volunteer blood under *in vitro* lowered conditions.
Methods: Venous blood was drawn from healthy volunteers, diluted by replacing platelet rich plasma with platelet free plasma and hereafter resuspended to obtain platelet counts of around 20, 50, 100, and 150×10^9/L (PLTlow). In order to lower hematocrit (HTlow) to around 0.25, 0.30, 0.35, 0.40, and 0.45 L/L, red blood cells were replaced by platelet free plasma and resuspended. Platelet function was assessed with MEA (agonists: TRAP, ADP, COL, and ASPI) in hirudin anticoagulated blood and PFA-100 (cartridges: COL/ADP and COL/EPI) in citrate anti-coagulated blood according to the manufacturers protocols. Using Graphpad Prism V5.0a non-linear 95% prediction bands were calculated as reference intervals for the full range of results.
Renal patients for kidney biopsy were analyzed pre-biopsy with Ivy bleeding time, PFA-100, Multiplate, complete blood count, renal function parameters, and post-procedure hemoglobin. Bland-Altman plots were calculated for IBT vs. PFA tests.
Results: Using 20 healthy volunteers per condition (MEA/PLTlow, MEA/HTlow, PFA/PLTlow, PFA/HTlow) 95% prediction bands could be calculated. MEA showed lower area under the curve (AUC) for lower platelet counts, while AUC remained equal for lower hematocrit for all agonists. In PFA both lower platelet counts and lower hematocrit gradually prolonged the closure time for both cartridges.
In total 142 patients were included. With PFA 5 (3.5%) and 6 (4.2%) patients showed prolonged results for COL/EPI and COL/ADP respectively, all others were normal in comparison to healthy volunteers. For MEA low AUC was seen in 13 (9.2%), 4 (2.8%), 33 (23.2%) and 11 (7.7%) patients for TRAP, ADP, COL, and ASPI, respectively. IBT prolongation over 4 min was seen in 7 (4.9%) patients.
Bland-Altman analysis showed for IBT vs. COL/ADP a sloping trend (bias: 38 s; SD 52) and for IBT vs. COL/EPI a widening trend (bias: 11 s; SD 58).
Median hemoglobin drop after kidney biopsy was 0.4 mM (IQR: 0.2–0.6). No transfusions were needed in any of the patients. After correcting for uremia and/or serum creatinine levels hemoglobin drop after biopsy could not be predicted with IBT, MEA, or PFA.
Conclusion: Using diluted healthy volunteer blood non-linear reference intervals were calculated for low platelet counts and hematocrit levels for Multiplate and PFA-100 tests. Although a portion of renal patients show lower Multiplate and/or prolonged PFA-100 response, none of these tests together with Ivy bleeding time could predict a significant hemoglobin drop after kidney biopsy.

PB 3.22-5

Platelet aggregation in whole blood by multiplate system is a rapid and suitable assay to evaluate platelet function in stored blood for transfusion therapy

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Background: Multiplate analyzer represents a quantitative, easy-to-use, point-of-care device with minimal technical variables. This method has been standardized for analysis of whole blood samples collected in Na-citrate or Hirudin as anticoagulants, but not for blood samples anticoagulated with citrate-phosphate-dextrose (CPD) as in blood bags from healthy donors.
Aims: The present study evaluates for the first time the applicability of Multiplate device to assess platelet aggregation in whole blood samples derived from donation bags. The influence of the ABO blood group, age, and two-day storage (at 4 °C) on the platelet aggregation is also analyzed.
Methods: Whole blood samples (300 μL) were obtained from 259 donation bags collected in CPD (242 Male/17 Female). As a comparator blood samples in Na-citrate 0.105M (9 vol.: 1 vol.) were obtained from 40 healthy control subjects (20M/20F). Platelet aggregation was assessed by Multiplate (Roche) after stimulation with ADP (6.4 μM), collagen (COLL, 3.2 μg/mL), thrombin-receptor-activating-peptide (TRAP-6, 32 μM), and arachidonic acid (AA, 0.5 mM). Pre-assay expression of platelet surface CD62P was assessed by cytofluorimetric analysis (FACS Canto, Becton Dickinson).
Results: CPD samples showed lower ($P < 0.05$) aggregation values compared to the Na-citrate samples in response to the different agonists. Specifically, in CPD samples, platelet aggregation induced by ADP and TRAP-6 was higher ($P < 0.05$) than that induced by COL or AA. Furthermore, platelet aggregation induced by ADP and COLL was significantly higher ($P < 0.01$) in the O phenotype compared to both the A and B phenotypes. No significant correlations were found between the aggregometry results and the CD62P expression or the donor age. Platelet aggregation evaluated in a subgroup of 20 blood bags stored for 2 days at 4 °C showed a significant ($P < 0.05$) reduction in platelet aggregation capacity induced by ADP, COLL and TRAP-6 as compared to fresh bags.
Summary/Conclusions: The aggregation response of CPD blood samples by Multiplate analysis, although significantly lower compared to Na-citrate samples, is retained and can be quantified after stimulation by all different agonists. An influence of the ABO phenotype on aggregation can also be detected. Therefore, the Multiplate assay can be a valid tool to monitor platelet function in blood stored for transfusion therapy.

PB 3.22-6

Optimizing whole blood impedance aggregometry in severe thrombocytopenia

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Background: Information on platelet function in thrombocytopenic patients could be of high value in order to predict the risk of bleeding episodes. However, evaluation of platelet function by impedance aggregometry is currently not possible in patients with thrombocytopenia.
Aim: This study aimed to investigate a novel approach for evaluating platelet aggregation in severe thrombocytopenia, using whole blood impedance aggregometry seeking to adjust for the platelet count and optimize the amount of applied agonist.

Methods: Following informed consent, citrated whole blood was obtained from 12 healthy volunteers, seven cancer patient with chemotherapy induced thrombocytopenia, and 12 patients with primary immune thrombocytopenia (ITP). Thrombocytopenia was induced in the healthy whole blood employing a previously validated method. Platelet aggregation was evaluated by whole blood impedance aggregometry (Multiplate®), and expressed as area under the curve (AUC).
Results: Following titration experiments a final concentration of collagen 8 μg/mL was applied as agonist providing the strongest aggregation response in thrombocytopenia. Platelet counts in the range of 10–39 × 10^9/L was obtained in whole blood from healthy volunteers. In this range, linear regression analysis displayed a strong positive correlation between platelet count and platelet aggregation ($R^2 = 0.84$) in whole blood from healthy volunteers. The prediction equation of normal platelet aggregation in thrombocytopenia was: $y = 19.9x-145$ (y = platelet aggregation (AUC), x = platelet count). The platelet aggregation response was then indexed relatively to the prediction estimate of normal platelet aggregation. The obtained platelet aggregation index was 106% (64–130) [median (interquartile range)] in thrombocytopenia modelled from healthy whole blood, 10% (7–13) in cancer patients and 170% (158–188) in ITP patients. The difference in aggregation index observed between ITP and cancer patients was highly significant ($P = 0.002$; Mann–Whitney U-test). Aggregation in ITP patients was significantly increased ($P < 0.001$) whereas aggregation in cancer patients was significantly reduced ($P < 0.001$) when compared to the platelets from healthy volunteers.
Conclusion: The findings suggest that whole blood impedance aggregometry can be optimized to detect differences in platelet function even at very low platelet counts. This may provide valuable information for prediction of bleeding risk in thrombocytopenic patients.

PB3.23 – Platelet Inhibition

PB 3.23-1
Prednisolone inhibits platelet function through targeting of the RhoA/ROCK signalling pathway

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Background: The synthetic glucocorticoid prednisolone is widely used as an anti-inflammatory, and immunosuppressive drug. Platelets are pivotal in regulating haemostasis, but can precipitate atherothrombosis associated to cardiovascular diseases. Previously, we have shown that prednisolone inhibits platelet aggregation and adhesion under conditions of flow, although the mechanisms have remained unclear.
Aims: In the present study examined the mechanisms responsible for the inhibitory effects of prednisolone, with a particular emphasis on RhoA/RhoA kinase signalling.
Methods: Washed Platelets (WP) treated with prednisolone prior to stimulation with thrombin. Platelet function was examined using light-transmission aggregometry, signalling mechanisms were studied using Western blotting and Rho A activity was detailed using a pull-down assay.
Results: Prednisolone caused concentration-dependent inhibition of thrombin-induced platelet aggregation. Threshold inhibitory responses were observed at 0.01 μM, while maximal effects were observed with 10 μM where thrombin (0.02 U/mL) induced aggregation was reduced from 56 ± 8% to 12 ± 7% ($P < 0.05$). The inhibition of aggregation by prednisolone was reversible, suggesting that this was a non-toxic effect. Thrombin caused 58 ± 8% aggregation but after 5 min incubation with pred this was reduced to 23 ± 11% ($P < 0.05$), but had returned to 61 ± 8% after 15 min. In order to understand the molecular mechanism underpinning these inhibitory effects we examined RhoA/ROCK pathway. Stimulation of platelets with thrombin led to the RhoA/RhoA kinase (ROCK) dependent phosphorylation of myosin light chain (MLC). Pretreatment of platelets with pred caused a concentration-dependent inhibition of phosphorylation of MLC-ser19. The inhibition was transient lasting for up to 5 min before returning to the level of phosphorylation found with thrombin alone. Consistent this observation, prednisolone also prevented the inhibitory phosphorylation of myosin light chain phosphatase (MLCP) at two key residues thr696 and thr853 in its MYPT1 subunit, suggesting that pred inhabits RhoA/ROCK signalling in platelets. In all cases the effects of pred were inhibited by the glucocorticoid receptor antagonist, RU486.
Conclusions: Prednisolone inhibits platelet activation by targeting RhoA/ROCK signalling events following thrombin stimulation. Modulation of the RhoA activity represents on of the no-genomic effects of this synthetic glucocorticoid on platelets.

PB 3.23-2
Natriuretic peptides and platelets: evidence of a localised cGMP synthesis

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Background: Cyclic guanosine-3′,5′-monophoshate (cGMP) is the common second messenger for the cardiovascular effects of nitric oxide (NO) and natriuretic peptides (NPs), which activate soluble and particulate guanylyl cyclases (sGC and pGC), respectively. pGC activity is carried by two membrane receptors for NPs, NPR-A and NPR-B. A third receptor, NPR-C lacks the cyclase domain and acts as a clearance receptor although it has been shown to have some signalling activity. In platelets the role of NO in regulating cGMP concentration and the consequent inhibitory effects is well established. On the contrary, contrasting data have been reported in the past supporting a role for NPs in the regulation of platelet function.
Aims: To investigate the effects of atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP), C-type natriuretic peptide (CNP) and the NPR-C agonist $cANF^{4-23}$ on platelet function.
Methods: We used platelet aggregation and dense granule secretion to evaluate the effects of NPs on platelet responses involved in haemostasis. PAR-1 peptide, collagen and ADP were used as agonists. Phosphorylation of the cyclic nucleotide-dependent protein kinase A (PKA) and G (PKG) substrate vasodilator-stimulated phosphoprotein (VASP) was studied as a readout of the kinase activation. Experiments were performed in the absence or presence of the non-selective cGMP and cAMP phosphodiesterase (PDE) inhibitor, 3-isobutyl-1-methylanxthine (IBMX).
Results: None of the NPs used in this work, even at high micromolar concentration, affected platelet aggregation or dense granule secretion in response to PAR-1 peptide, collagen and ADP either on their own or in the presence of a low concentration of IBMX. On the other hand, VASP phosphorylation at Serine 239 (mainly PKG substrate), was increased by concomitant treatment with NPs or $cANF^{4-23}$ and IBMX compared to IBMX on its own, thus indicating a small localised PKG activation. On the contrary, platelet aggregation, dense granule secretion and VASP phosphorylation at Serine 157, mainly dependent on PKA, measured under the same conditions, were not affected.
Summary/Conclusions: We conclude that treatment with NPs in platelets is able to trigger PKG activation, but that the magnitude of activation *per se* is not sufficient to exert functional inhibition of platelet involvement in haemostasis. However we cannot exclude that it would affect other platelet responses. The increase in VASP phosphorylation obtained following treatment with $cANF^{4-23}$ and IBMX suggests that the effects on PKG activation may be dependent on the NP clearance receptor (NPR-C) expressed on platelet membranes, through a not yet identified mechanism.

PB 3.23-3

Decreased phosphorylation of the linker for activated T-cells (LAT) is associated with impaired collagen activation of neonatal platelets

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Background: Collagen is an important early activator of platelets under physiological conditions. Cross-linking of glycoprotein (GP) VI by collagen leads to the Fyn/Lyn-mediated phosphorylation of an ITAM motif on the Fcγ receptor, and sequential phosphorylation of downstream targets, including the transmembrane protein LAT. These serve as the platform for the formation of a membrane raft-based signalosome that includes phospholipase Cγ2 (PLCγ2), the activation of which mediates intracellular calcium mobilization and full platelet activation. Platelets from newborn infants demonstrate impaired function, although platelet number and ultrastructure do not differ from those of older individuals. Umbilical cord blood platelets show impaired *in vitro* responses to collagen stimulation, including decreased PLCγ2 activation, calcium mobilization, granule secretion and aggregation.
Aim: To determine whether deficient collagen activation in neonatal platelets results from decreased GPVI-coupled signaling.
Methods: Cord blood was collected by umbilical venipuncture from uncomplicated term deliveries (gestational age ≥ 37 weeks, $n = 10$), and blood was drawn by venipuncture from healthy adult volunteers ($n = 10$) into citrate/citric acid/dextrose anticoagulant. Washed platelet suspensions were prepared, lysed and separated by SDS-PAGE before immunoblotting with commercial monoclonal antibodies to GPVI and GPIbα. Resting and collagen-stimulated (2 μg/mL and 10 μg/mL) cord blood and adult control platelets were lysed and immunoblotted with polyclonal anti-LAT and anti-phospho-LAT. Membrane rafts were separated from platelet lysates by sucrose density-gradient centrifugation; fractions from the gradient were separated by SDS-PAGE, followed by immunoblotting with anti-LAT antibody. Immunoblot band intensities were quantitated by scanning densitometry.
Results: The maximum aggregation of cord blood platelets was significantly less than the maximum aggregation of adult control platelets in response to collagen 10 μg/mL (31 ± 6% vs. 63 ± 8%, $P \leq 0.01$), and 2 μg/mL (9 ± 4% vs. 46 ± 12%, $P \leq 0.01$). GPVI immunoblots of whole platelet lysates did not show significant differences in mean band intensities in neonatal platelets compared to adult platelets (1944 ± 145 vs. 1961 ± 480 intensity units, n.s.). The distribution of LAT in the low-density membrane (lipid raft) fractions of the sucrose gradient was the same in adult and cord blood platelets. This distribution was not altered by collagen-stimulation. Comparison of the mean band intensities of LAT in immunoblots of platelet lysates showed no difference between cord blood and adult platelets (1218 ± 290 vs. 1428 ± 114 intensity units, n.s.). Following stimulation with 10 μg/mL collagen there was significantly less phospho-LAT in cord blood platelets than in the adult control platelets (522 ± 285 vs. 1173 ± 283 intensity units, $P < 0.05$); the ratio of phospho-LAT to total LAT was 0.82 in collagen-stimulated adult platelets, and 0.42 in collagen-stimulated cord blood platelets.
Summary/Conclusion: Comparing cord blood and adult control platelets, no difference was detected in in the level of GPVI, or in the quantity or distribution of LAT in membrane rafts. However, collagen-stimulated phosphorylation of LAT was significantly decreased in cord blood platelets, and was associated with decreased downstream platelet activation. Examination of events upstream of LAT in the GPVI signaling pathway, including Fcγ receptor ITAM phosphorylation and activation of Syk may identify the basis for the impaired collagen response in neonatal platelets.

PB 3.23-4

Tangeretin regulates platelet function through dual inhibition of phosphoinositide 3-kinase and cyclic nucleotide signalling

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***Background*:** The relationship between diet and risk factors for cardiovascular diseases has long been appreciated. Indeed, several epidemiological studies have suggested that the regular intake of dietary flavonoids reduce the risk for cardiovascular diseases. But the mechanisms of action of these dietary flavonoids in reducing cardiovascular risks are poorly understood. Tangeretin, a flavonoid abundant in the peels of citrus fruits has been demonstrated to have several beneficial effects in human health.
***Aims*:** This study was performed to determine whether tangeretin is able to modulate the function of platelets and to characterise mechanisms of its actions.
***Methods*:** The effects of tangeretin at a range of concentrations on the function of platelets were tested through measurement of platelet aggregation, fibrinogen binding, calcium mobilisation and granule secretion upon stimulation with agonists. Tangeretin was also tested for its ability to modulate human plasma clot retraction and haemostasis in mice using tail bleeding assay. The effect of tangeretin on *in vitro* thrombus formation was analysed under arterial flow conditions perfusing human whole blood over collagen-coated Vena8 biochips. To explore the mechanism of action of tangeretin on platelet function, the phosphorylation of proteins involved in GPVI, PI3K and cyclic nucleotide mediated signalling pathways was analysed in the presence and absence of tangeretin.
***Results*:** Tangeretin inhibited agonist-induced human platelet activation in a concentration-dependent manner. It inhibited integrin αIIbβ3 mediated inside-out signalling, intracellular calcium mobilisation and granule secretion. Inclusion of tangeretin reduced the plasma clot retraction indicating reduced integrin αIIbβ3-mediated outside-in signalling. Tangeretin also inhibited human platelet adhesion and subsequent thrombus formation on collagen-coated surfaces under arterial flow conditions *in vitro* and reduced haemostasis in mice. The characterisation of potential mechanisms by which tangeretin inhibits platelet function revealed dual inhibitory targets in platelets. Tangeretin was found to inhibit phosphoinositide 3-kinase (PI3K) mediated signalling and increase the phosphorylation of VASP at S239, consistent with elevated cyclic nucleotide signalling.
Conclusions: This study provides support for the ability of dietary flavonoids, particularly tangeretin, to modulate platelet signalling and function, which may impact on the risk of thrombotic disease.

PB 3.23-5

Targeting of type 1 protein kinase A to lipid rafts is required for competent platelet inhibition by the cAMP-signaling pathway

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Background: Cyclic adenosine monophosphate (cAMP)-dependent signalling modulates platelet function at sites of vascular injury. The foremost effector of cAMP signalling in platelets is protein kinase A (PKA), which acts by phosphorylating numerous platelet proteins. In platelets two PKA isoforms are present, type I (PKAI) and type II (PKAII). In other cell types, PKA isoforms are specifically targeted to distinct subcellular compartments by a family of proteins called A-Kinase Anchoring proteins or AKAPs allowing them to mediate non-redundant biological effects.
Aim: To determine the role of AKAPs in platelet inhibition by cAMP signalling.

Methods and results: Under basal conditions we observed a pool of PKAI, but not PKAII, was specifically localised into platelet lipid rafts. To establish the mechanism that facilitated localisation of PKAI we used an established cell-permeable peptide inhibitors of PKAI/A-kinase anchoring protein (AKAP) interactions called RI anchoring disruptor (RIAD-Arg_{11}). Inhibition of PKAI-AKAP interactions displaced PKAI from lipid rafts with resultant loss of PKA activity in these microdomains. Using Far-Western blotting and PKA pull-down assay followed by mass-spectrometry we established that the cytoskeletal protein moesin acted as an AKAP that bound PKAI and localised the pool of PKAI to platelet lipid rafts.

To clarify the importance of the localization of PKAI to lipid rafts we examined platelet activity. PGI_2 inhibited platelet aggregation by vWF and platelet accrual on immobilised vWF under flow. Disruption of PKAI-AKAP interactions with RIAD-Arg_{11} reduced platelet sensitivity to the inhibitory actions of PGI_2. The phosphorylation of GPIbb, a known PKA substrate and a raft protein, is though to contribute to platelet inhibition of vWF-mediated responses by PGI_2. Treatment of platelets with PGI_2 resulted in the phosphorylation of GPIbb in the lipid raft fraction. Isolation of PKAI from its potential AKAPs by RIAD-Arg_{11}, resulted in a significant decrease in raft localised phosphoGPIbβ, demonstrating that this phosphorylation is a PKAI-dependent event.

Conclusion: We show that selective localisation of PKAI into lipid rafts is required for competent platelet inhibition by cAMP. Furthermore, we identify the lipid raft-residing GPIbb as the first PKAI-specific substrate in platelets and moesin as the first platelet AKAP.

PB 3.23-6

Characterization of the effects of losartan on human platelets

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Background: Losartan, a selective, competitive inhibitor of the angiotensin II type I receptor, is routinely used to treat hypertension. It has been reported to have antithrombotic effects in animal models dependent on NO/prostacyclin and direct antiplatelet effects were described *in vitro*: competitive inhibition of U46619 binding to the thromboxane A2 receptor (TPR) (Guerra-Cuesta JI et al. *J Hypertens 1999*) and interaction with GPVI (Grothusen C et al. *Arterioscler Thromb Vasc Biol*, 2007; Ono K et al. *J Med Chem* 2010).

Aims: This study was aimed to better characterize *in vitro* the effect of losartan on platelets and to elucidate its mechanism of action.

Methods: Experiments were performed on PRP and washed platelets from healthy donors who had not taken medication for at least for 10 days. Samples were preincubated with increasing concentrations of Losartan (0–50 μg/mL) before measuring platelet aggregation and P-selectin exposure. Platelet activation was triggered by arachidonic acid (AA, 1.5 mM), APD (10 μM), thrombin-receptor activating peptide (TRAP 20 μM), the thromboxane A2 analogue U46619 (1 μM), type I collagen (Horm, 1 μg/mL), convulxin (Cvx, 0.5 nM), or the anti-FcgRIIA antibody IV.3 cross-linked by anti-mouse IgG Fab'2. Platelet adhesion to immobilized collagen was analysed using whole anticoagulated blood in flow conditions (1500/s). GPVI binding to collagen was measured using recombinant soluble dimeric GPVI (GPVI-Fc). GPVI dimerisation/clustering was analysed by flow cytometry using the specific antibody 9E18 *(Loyau S et al. Arterioscler Thromb Vasc Biol 2012)*.

Results: Losartan up to 50 μg/mL had no effect on AA, TRAP or Cvx-induced platelet aggregation and P-selectin exposure. Losartan (50 μg/mL) inhibited the second wave of aggregation in response to ADP and dose-dependently inhibited U69919- and collagen-induced platelet aggregation. However, the losartan IC50 was six times higher for U46619 than for collagen-induced platelet aggregation (18 vs. 3 μg/mL) suggesting that the inhibition of collagen-induced platelet activation was partly independent of the effect of losartan on TPR. This was confirmed by the observation that losartan still inhibited the collagen-induced residual platelet aggregation of indomethacin-treated platelets. When blood preincubated with losartan (10 μg/mL) was perfused over collagen, less platelet aggregates were formed as compared to control conditions. Losartan up to 100 μg/mL slightly reduced the binding of the anti-GPVI monoclonal antibody 9O12 to GPVI-Fc and had no effect on the high affinity binding of GPVI-Fc to collagen suggesting that losartan does not occupy the collagen-binding site on GPVI. However, interestingly, losartan (10 μg/mL) inhibited the binding of the 9E18 antibody to platelets incubated with collagen suggesting that losartan blocks collagen-induced GPVI clustering.

Conclusion: Losartan inhibits collagen-induced platelet adhesion, activation and aggregation. This effect is, at least partly, independent of TPR inhibition, and does not rely on the inhibition of the protein tyrosine kinase pathway since it does not impact FcgRIIA activation. Our data suggest that the inhibition, by losartan, of collagen-induced GPVI clustering could reduce platelet reactivity and contribute to its antithrombotic effect. The relevance of this effect in losartan-treated patients is under investigation.

PB3.24 – Platelet Proteomics

PB 3.24-1

Time-resolved phosphorylation patterns of human platelets upon treatment with Iloprost reveal novel insights into platelet inhibition

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One of the most important physiological platelet inhibitors is endothelium-derived prostacyclin which stimulates the platelet cAMP/PKA signaling cascade and inhibits virtually all platelet activating key mechanisms. Using quantitative mass spectrometry, we analyzed time resolved phosphorylation patterns in human platelets after treatment with Iloprost, a stable prostacyclin analogue, for 0, 10, 30 and 60 s to characterize key mediators of platelet inhibition and activation in three independent biological replicates.

Starting from only 100 μg of protein per condition, in total we quantified more than 2700 different phosphorylated peptides of which 360 peptides show a significant (two-fold) up/down regulation upon stimulation.

For the first time, our data provide time-resolved insights into phosphorylation-dependant processes during platelet inhibition. Some of the signaling proteins detected in this study represent so far unknown key players and signaling nodes of platelet activation and inhibition – novel candidates for monitoring functional defects or impaired responsiveness of human platelets to anti-platelet treatment, imposing an increased risk for severe cardiovascular or other adverse secondary effects.

In conjunction with our recently published first comprehensive and quantitative protein composition of human platelets [1], our novel quantitative data on platelet phosphorylation provide a rich source for further functional research and represent another step towards a deeper understanding of the mechanisms contributing to platelet physiology as well as pathophysiology.

Reference:

1. Burkhart JM, Vaudel M, Gambaryan S, Radau S, Walter U, Martens L, Geiger J, Sickmann A, Zahedi RP., The first comprehensive and quantitative analysis of human platelet protein composition

allows the comparative analysis of structural and functional pathways. *Blood* 2012; 120(15):e73–82.

PB 3.24-2

Proteomic comparison of platelets with high and low mean platelet volume

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Background: It has been supposed that platelet size is a determinant of platelet age; however, there is a lack of data to support this concept.
Aim: This study applies highly sensitive quantitative proteomics to compare the protein constitution of platelet subpopulations with high and low mean platelet volume (MPV).
Methods: Platelets were separated from whole blood ($n = 3$) by density gradient centrifugation using a percoll density gradient. Residual leukocytes were removed after labeling with magnetic CD45 beads through a magnetic column (Milteny Biotec). Platelets were washed and separated by MPV using differential centrifugation to obtain a large and a small platelet fraction. MPV was determined using a Sysmex™ cell counter. Platelets were analyzed by differential in gel electrophoresis (DIGE) and label free LC-ESI-MS/MS.
Results: Mean platelet volumes were 11.2 fL (10.6–12.2) in the large and 9.0 fL (8.2–10.0) in the small platelet fraction. The comparison of 1187 proteins revealed no significant differences in platelet proteins using the LC-ESI-MS/MS approach, whereas 76 out of 1265 protein spots displayed significantly altered abundances in the DIGE-experiment. Identification of 41 spots by MALDI-TOF-MS revealed that large cells contained more fragments of highly abundant cytoskeletal proteins such as talin, kindlin-3, and filamin.
Conclusion: The relative abundances of platelet proteins appear similar in platelet subpopulations with high and low MPV. Protein differences detected by DIGE indicate fragmentation of cytoskeletal proteins in platelets with high MPV.

PB 3.24-3

A proteomics study of the neonatal platelet plasma membrane

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Background: Neonatal platelets are hyporeactive *in vitro* to a variety of agonists. The nature and degree of this impairment is diverse, depending on the type of agonist. Poor response to thrombin and epinephrine is due to decreased numbers of respective receptors, while hyposensibility to TXA_2 is not associated with decreased receptor numbers, but a result of impaired signal transduction.
Nevertheless, primary hemostasis is not affected, as newborns are not prone to easy bruising. Additionally, we observed that adult and neonatal platelets support thrombin generation to the same extent. Both observations seem discrepant with *in-vitro* hypoaggregability of neonatal platelets.
Aims: We wanted to adopt proteomics to get an integrated picture of neonatal platelet peculiarities and focused on the plasma membrane, because it contains the majority of targets for platelet activation and inhibition.
Methods: Platelets of adult blood and umbilical cord blood were isolated, washed and lysed in their resting state. Enrichment of plasma membranes was performed by partitioning in an aqueous two-phase polyethylene glycol/dextran system followed by two-fold extraction with sodium carbonate. Proteins were subjected to a bottom-up proteomics workflow including tryptic digestion. Semiqantitative comparison was achieved by stable isotope dimethyl labeling on the peptide level. Differentially labeled pools containing five adult or neonatal samples respectively were combined and subtracted to SCX prefractionation prior to analysis with high resolution LC-MSMS. The experiment was repeated three times with pools containing different samples. Analysis of semiquantitative data was done using Thermo Proteome Discoverer 1.1.0. Gene ontology classification and enrichment analysis was performed using the database for annotation, visualization and integrated discovery (DAVID) v6.7.
Results: Semiquantitative comparison with adult samples revealed 321 differentially regulated features in neonatal platelets. The most consistently upregulated proteins are all known to be involved in platelet signalling, such as GPIbα, GP9, PECAM1, CD36, and integrin alpha 6. Notable downregulated proteins were $G_{i\alpha}$, integrin-linked kinase, and pro-platelet basic protein. Gene ontology classificaton and enrichment analysis confirmed enrichment of proteins integral or intrinsic to plasma membrane in all samples. Functional annotation clustering revealed significant enrichment of proteins involved in cell signaling and cell adhesion in the neonatal samples.
Conclusion: Higher levels of GPIbα in the neonatal samples are in agreement with our previous findings using Western blot analysis and argue for increased binding to high molecular von Willebrand factor present in neonatal blood. Additionally, higher levels of several integrins speak for increased cell-cell interaction and support of platelet adhesion reflected in the results of functional annotation clustering. Lower levels of $G_{i\alpha}$ are of particular interest, because several inhibitory signaling pathways could be affected. These impairments might not be observable with *in-vitro* platelet function testing using strong activators, but could potentially explain some controversial clinical findings.

PB 3.24-4

Towards a better characterization of the platelet secretory granule proteome

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Background: Platelets are anucleated cell fragments involved in haemostasis. They contain four types of secretory granules, which are different in term of morphology, number and content: alpha granules, lysosomes, dense granules and T granules. Granules are a cornerstone in the platelet activation and aggregation processes as well as in atherosclerosis progression and its thrombotic complications. Subcellular fractionation techniques allow enriching organelles of interest and are a promising strategy to dissect the proteome of platelet granules.
Aims: The present project aims to further characterize the protein composition of the platelet secretory granules.
Methods: Platelet granules were enriched by sucrose gradient and analyzed by tandem mass spectrometry in gas-phase fractionation mode. The presence of selected proteins was verified in the granule-enriched sucrose fraction by western blot and their localization confirmed by confocal microscopy.
Results: We identified more than 800 proteins, among which the vast majority is involved in pathways ranging from platelet granule biogenesis to secretion. A pathway analysis comparing the proteomes of the granule-enriched fractions with the whole platelets showed enrichment in ERK signaling and MHC I related pathways. Western blot confirmed the presence of Syk and Lyn, which are members of the ERK pathway. In addition, MHC I was shown to co-localize with the alpha granule marker von Willebrand factor by confocal microscopy.
Conclusion: To our knowledge, this work constitutes the largest characterization of the platelet secretory granule proteome. It identified proteins never shown in platelet secretory granules so far. Among them, ERK and MHC I related pathways have been localized in platelet granule-enriched fractions and alpha granules, suggesting a role in the plate-

let granule machinery biogenesis and cargo secretion. This work constitutes a new insight in the understanding of the platelet secretion.

PB 3.24-5

A 2D-DIGE-based proteomic analysis reveals differences in the platelet releasate depending on the platelet stimulus

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1 Undesired platelet activation and formation of arterial thrombi are implicated in many diseases, such as myocardial infarction and stroke. Once activated, platelets release a high number of proteins and other biomolecules, which is known as releasate. There are clear indications that proteins secreted by platelets are found in the atherosclerotic plaque contributing to its pathogenesis [Coppinger JA et al. Blood. 2004;103:2096–2104]. In recent years there have been several groups focusing on the study of the releasate, analyzing the effect of different agonists and antiplatelet agents such as aspirin [Coppinger JA et al. Blood. 2007;109:4786–4792].

2 Our main objective was to test the hypothesis that the platelet releasate might vary depending on the platelet stimulus.

3 We activated platelets with collagen or thrombin and compared the proteome of the releasate. Platelets were isolated from healthy volunteers and activated *in-vitro* to obtain the releasate. Proteome analysis was based on two-dimensional in-gel electrophoresis (2D-DIGE) and mass spectrometry (MALDI-MS/MS and LC-MS/MS). Validations were by 1D- and 2D-western blotting.

4 We detected 1742 spots per gel, 131 of which appeared differentially regulated between both conditions. Spots were filtered based on two parameters: fold change ≥ 2 and $P < 0.05$. So far we successfully analyzed 72 differentially regulated spots by mass spectrometry. We detected two main arrays composed of fibrinogen (12 spots) overexpressed in the releasate of collagen-activated platelets, which was somehow expected due to the ability of thrombin to transform fibrinogen into fibrin. We identified another 25 unique proteins (13 overexpressed in collagen, 11 in thrombin and one in both conditions). The identified proteins were secreted in a similar proportion by both classical and non-classical secretory pathways (58% and 42%, respectively), and some of them had not been reported previously in platelet releasate or microparticle proteome studies. Those include nucleosome assembly protein 1-like 1 (NP1L1), Myosin regulatory light chain 12B (ML12B), and cardiotrophin-like cytokine factor 1 (CLCF1). The latter is a cytokine with B-cell stimulating capability. 1D- and 2D-western blotting validation experiments demonstrated it is secreted in higher amounts by thrombin-activated platelets. On the other hand, the adhesive glycoprotein thrombospondin-1, which mediates cell-to-cell and cell-to-matrix interactions, was found to be up-regulated in the collagen-induced releasate.

5 We demonstrated the platelet releasate varies depending on the specific stimulus. This could have pathological implications given that some platelet-related diseases involve a primary role of some particular receptors and platelet secreted proteins. For example, in a recent study we showed a higher activation state of GPVI signaling in platelets from patients with acute myocardial infarction [Parguiña AF et al. Arterioscler Thromb Vasc Biol. 2011;31:2957–64]. Our results may also have pharmacological implications, assuming the platelet releasate might be modulated with anti-platelet drugs.

PB 3.24-6

What do platelets release?

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Background: Platelet activation and subsequent release of α-granule content plays an important role in healthy hemostasis, but also in many cardiovascular pathologies. Although some of these factors are routinely assayed, there is an eminent need to estimate more accurately the identity, and the protein concentrations within the releasate. In addition, it was suggested that the platelet releasate varies with different activation routes. Currently, such data is only based on single protein assays, but would benefit from a more systems-wide method.

Aim: It is our aim to develop methodology to establish an unambiguous quantitative map, including identity and quantity (ng/mL), of the platelet releasate under various stimulatory conditions.

Methods: To establish and quantify the platelet releasate we isolated platelets from healthy individuals ($n = 3$) and split each sample into two halves. One half was left to rest, while the other was fully stimulated using collagen (CRP) and thrombin (TRAP). After a standard proteomics sample preparation, each half was labeled with a different stable isotope tag (dimethyl labeling) and the samples were again combined. These mixes were analyzed with an optimized workflow incorporating 2-dimensional chromatography and LC-MS/MS analysis on an Orbitrap Velos operated with a decision tree algorithm to optimally choose ETD or HCD fragmentation for each peptide. The stable isotopes aided in the elucidation of the released proteins as these were found significantly reduced in the activated sample ($P < 0.05$). The normalized spectral index was used to establish the copy number and subsequently the released concentrations of each protein in ng/mL.

Results: Thus far, qualitative, mass spectrometry-based investigations into the platelet releasate have not exploited the power of quantitative proteomics and have yielded ambiguous results due to insufficient depth and the identification of many false positives due to uncontrolled platelet lysis. We monitored almost 4500 platelet proteins for their release characteristics and estimated copy number. We observed that full stimulation leads to the consistent ($n = 3/3$) and significant release of only 124 proteins ($P < 0.05$). At this depth, the released proteins span a concentration range of at least five orders of magnitude, as confirmed by ELISA. These released proteins contained all known factors at high concentrations (> 100 ng/mL, e.g. Thrombospondin, von Willebrand factor and Platelet factor 4). Interestingly, in the lower concentration range (< 1 ng/mL) of the releasate many novel factors were identified. With this methodology we are now ready to take on the next challenge, i.e. monitor differential release upon different platelet activation routes.

Summary/Conclusion: We have generated a quantitative map of the platelet releasat, which contained all known important factors, many novel proteins. Compared to previous work, the releasate turned out to be much smaller than anticipated.

PB3.25 – Platelet Disorders: Loss-of-Function

PB 3.25-1

Variability in clinical and biological manifestations of anti-GPVI autoantibodies: about four new patients

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Background: Chronic immune thrombocytopenia (ITP) is an autoimmune disorder manifested by immune-mediated platelet destruction and decrease in platelet production. There are only rare reports of ITP patients with antibodies against GPVI-FcRγ, the major platelet receptor for collagen. Due to scarce observations, many questions are still pending: (i) do these antibodies mediate clearance of GPVI-FcRγ by shedding or internalization, (ii) are they able to activate or inhibit platelets, (iii) and are they associated or not with bleeding?
Aims: Our goal was to collect as many data on patients with anti-GPVI antibodies to conduct a study that would offer a real understanding of the natural history of this rare platelet disorder.
Methods: Light transmission aggregometry (LTA) using standard agonists; Surface expression of platelet glycoproteins analysis by flow cytometry; Plasma anti-GPVI antibodies detection by ELISA; Platelet content in GPVI and FcRγ by immunoblot analysis; Sequencing of the *GP6* gene.
Results: Four patients with identified anti-GPVI antibody were explored. All cases (numbered P1–P4) are females. At diagnosis, their age was 46, 35, 25 and 16 years; their platelet counts were of 30–40, 60, 150 and 360 G/L respectively. Bleeding manifestations were variable, from none (P1) to mild (ecchymosis, epistaxis and/or gingival bleeding). The platelet defect started during pregnancy (P2) but was not associated to an identified event in other cases. Platelet-associated immunoglobulins were detected in P1 and P4 who were positive in MAIPA for anti-GPIIbIIIa. In P1-P3, LTA revealed no or profoundly reduced collagen- and convulxin-induced platelet aggregation and normal responses to other agonists, leading to the suspicion of a GPVI deficiency which was confirmed by flow cytometry; in P4 the GPVI deficiency was identified by flow cytometry and confirmed by LTA. Cross-tests (patient's PPP mixed with control platelets) evidenced either an inhibitory effect on collagen and convulxin- induced platelet aggregation (P1), or an activating effect (P2) or no effect (P3 and P4). Platelet GPVI content was very low as indicated by immunoblot analysis and the expression of the FcRγ chain was variably reduced: as GPVI (P2) or less than GPVI (P1, P3 and P4). The GPVI cytoplasmic fragment was undetectable in all cases. An anti-GPVI immunoglobulin was evidenced in the plasma of the four patients. In P2, purified IgGs bound to GPVI and induced platelet activation. Finally, sequencing of the GP6 gene (P1, P3, P4) ruled out a genetic disorder and identified common polymorphisms.
Conclusion: The comparative study of these four new cases of acquired immune GPVI deficiency and confrontation with literature confirm the variability of their clinical manifestations (thrombocytopenia and bleeding), which are most often mild, leading to underestimate the occurrence of GPVI deficiency. The apparent absence of GPVI shedding suggests that antibody-triggered GPVI internalization and degradation could be a current process. More systematic research of anti-GPVI antibodies using a standard protocol would help to identify whether the different mechanisms of action and manifestations could be linked to specific target areas on GPVI.

PB 3.25-2

Platelet dense granule but not alpha granule secretion defect in cystic fibrosis patients

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Background: Cystic fibrosis (CF) is an autosomal recessive disorder caused by mutations of the CF transmembrane conductance regulator (CFTR) gene. It is the most common genetically inherited disease worldwide affecting more than 70,000 individuals. Airway inflammation in concert with chronic infection accounts for progressive, suppurative pulmonary disease. Platelets are critical elements in linking and modulating thrombosis, inflammation and tissue repair. Platelets orchestrate the inflammatory response by undergoing a series of highly controlled intracellular signaling events that lead to the release of its stored intracellular granules.
Aims: We therefore investigated if platelets from CF patients have an altered responsiveness to agonists compared to platelets from normal healthy donors.
Methods: Informed consent was obtained from all participants (healthy and CF) and ethical approval to carry out the study was obtained from the RCSI and Beaumont Hospital Ethics committees. Platelet aggregation was assessed by light transmittance. Alpha granule secretion was measured by flow cytometry and dense granule secretion was measured by luminometry using a luciferin/luciferase assay.
Results: CF patients ($n = 7$) and healthy control ($n = 7$) populations were equivalent in terms of age and platelet counts. There was no statistical difference in platelet aggregation betweenhealthy control and cystic fibrosis patients when stimulated with a range of concentrations of Thrombin Receptor Activating Peptide (TRAP), thromboxane mimetic, U46619, collagen related peptide (CRP) or arachidonic acid (AA) ($n = 7$). Alpha granule secretion, determined by surface expression of CD62P, was also similar between the healthy control patients and CF patients with all agonists and concentrations tested ($n = 7$). In contrast to these results, platelets from CF patients had a significantly reduced secretion from dense granules, when stimulated with strong agonists TRAP ($P < 0.01$), CRP ($P < 0.0001$), but not weak agonists U46619 and AA (P = NS). In order to confirm a dense granule secretion defect rather than reduced levels of bioactive cationic molecules present in the dense granules of CF patients, we measured the total ATP and ADP content in platelet dense granules. There was no significant difference in the total ATP or ADP levels found in healthy vs. CF patient platelets (P = NS).
Conclusion: Our results suggest that platelets from CF patients exhibit a decreased platelet dense-granule secretory response to activation signals. This may lead to a muted response in terms of inflammatory cell mobilization. Thus, drugs that suppress platelet responses such as non-steroidal anti-inflammatory drugs, should be used in a limited fashion in CF patients, as such drugs will further suppress inflammatory responses.

PB 3.25-3

Is molecular analysis of the MYH9 gene helpful in the diagnosis of May-Hegglin anomaly and related disorders?

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The autosomal dominant giant-platelet disorders, May-Hegglin anomaly, Sebastian syndrome, Epstein syndrome and Fechtner syndrome, are now collectively referred to as *MYH9*-related disease (MYH9-RD); following identification of causative mutations in *MYH9*, the gene coding for the heavy chain of non-muscle myosin-9. These disor-

ders are characterised by macrothrombocytopenia and, usually, leukocyte inclusions. There is also variable association with sensorineural hearing loss, presenile cataracts and renal disease. These non-haematological clinical manifestations may arise or worsen later in life and it is important to be aware of the possibility and offer surveillance to patients. For these reasons a definitive diagnosis is important and molecular analysis of the *MYH9* gene may help provide this. MYH9-RD is most commonly misdiagnosed as chronic ITP but may also be confused with Bernard-Soulier syndrome (BSS), Paris-Trousseau syndrome/Jacobson syndrome, X-linked macrothrombocytopenia or Gray platelet syndrome.

We have screened the *MYH9* gene in a total of 24 patients referred for possible MYH9-RD. In 13 of the 24 a causative mutation was identified, whilst in the other 11 no causative mutation was identified following sequencing of the entire coding region of *MYH9*. Of the 13 in whom a causative mutation was identified, 10 were previously reported mutations whilst three were novel (p.Leu89Arg, p.Arg1165Ser and p.Asp1941Metfs*7). Eleven different variants were identified in the 13 patients, with the previously reported p.Lys373Asn and p.Glu1841Lys mutations each being reported in two patients, confirming the heterogeneity of *MYH9* mutations. However, it is interesting to note that, despite *MYH9* being a relatively large gene (41 exons encoding a 1960 amino acid protein), there is apparent clustering of mutations at particular loci. Four different amino acid substitutions have been reported at Asp1447, three at Arg702, Arg1165 & Asp1424 and two at Trp33, Ala95 & Thr1155. That is a total of 19 different amino acid substitutions, out of a total of < 40 unique missense/nonsense mutations reported, at just seven different loci. This suggests that these regions must be either critical to function or more susceptible to mutation.

Although referral information was sometimes less than comprehensive, the referrals could roughly be categorised into Strong, Possible and Weak candidates for an *MYH9* gene mutation. Of the 11 considered strong candidates a mutation was identified in 10 with one being negative and possibly worthy of further investigation. The nine possibles resulted in three patients with an *MYH9* mutation and six negative, whilst all four patients considered to be weak candidates for a mutation were indeed negative.

A definitive diagnosis of MYH9-RD is clinically important and molecular analysis of *MYH9* can play an important role in achieving this. Our experience confirms that appropriate referral criteria are important for molecular analysis to be a practical part of the diagnostic process. However, the criteria should not be too strict or *de novo* cases, with no family history of thrombocytopenia, may be missed. For example, we confirmed the diagnosis of MYH9-RD in an 18 month old child with a *de novo MYH9* mutation. Definitive diagnosis will enable monitoring of his developmental milestones with early intervention as necessary.

PB 3.25-4

Increased total platelet TFPI and higher surface expression of TFPI in activated platelets may be involved in the pathogenesis of mild bleeding disorders (MBD) of undefined cause (BUC)

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Background: Inherited mild bleeding disorders (MBD), usually manifested with skin and mucous bleeding are heterogeneous in nature, involving different pathogenic mechanisms, including platelet function defects, decreased amount and function of plasma VWF, mild defects of clotting factors and also, increased fibrinolysis. However, up to 60% of all the patients with MBDs have abnormal bleeding of undefined cause (BUC). Platelets contain TFPI, but its function is poorly understood.

Aims: To analyze the expression of TFPI in resting and activated platelets from controls and patients with BUC.

Methods: Platelet TFPI was analyzed by flow cytometry in washed platelets (Panes et al. Blood 2007;109:5242) without previous stimulation and stimulated with VWF-Ristocetin or Thrombin Receptor Activating Peptide (TRAP). TFPI protein was measured in platelet lysates by ELISA. Measurements were performed in Control individuals and patients with inherited mucous and skin bleeding who had normal plasma VWF, normal platelet aggregation and ^{3}H-serotonin secretion and normal hemostasis screening tests. Exposure of anionic phospholipids was assessed by annexin V binding, by flow cytometry.

Results: TFPI protein measured in platelet lysates of 41 patients with BUC (mean age 19 ± 9 years, 32 women) was 36 ± 14 ng/mg protein, significantly higher than that of 40 Controls (ages 22 ± 6, 31 women) who had 25 ± 12 ng/mg protein (mean ± SD, $P = 0.0041$). In 16 of these patients and 17 Controls, platelet surface TFPI was measured. No difference in the% of labeled was observed in Patients and Controls in non-stimulated platelets (59.5 ± 15% vs. 53 ± 23%, P = NS) and in VWF-Ristocetin stimulated platelets (35 ± 24% vs. 35 ± 23%). However, stimulation with TRAP resulted in a significant fall in TFPI-labeled platelets in Controls but not in BUC patients (13 ± 13% vs. 34 ± 23%, $P = 0.0007$, non-paired '*t*'test). Annexin V binding was significantly increased after platelet stimulation with VWF-Ristocetin and TRAP, but no significant differences were observed between Patients and Controls.

Conclusions: (i) Platelets from patients with MBDs of undefined cause contain more TFPI protein than normal. (ii) TFPI is detectable on more than 50% of the platelets in normal individuals. After activation with VWF-Ristocetin, but mainly with TRAP, a significant drop to 12% of TFPI-labeled platelets is observed. In contrast, more than 1/3 of the platelets from patients still express TFPI after TRAP stimulation. (iii) No significant differences were observed in Controls and Patients regarding annexin V binding. (iv) We have observed that the mean thrombin generation in PRP in patients with BUC is decreased as compared to controls (another Abstract in this Meeting). Taken together, our current observations strongly suggest that some patients with BUC might have a defective platelet procoagulant activity, which could be pathogenically related with the bleeding symptoms. (FONDECYT 1110404 and 1130853).

PB 3.25-5

HPLC-based assay to measure platelet serotonin (5-HT) secretion in specialized hemostasis laboratories: comparison with the current 'gold standard' radioisotopic test

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Background: The underlying defect of most mild platelet function disorders (PFD) is a primary secretion defect. Moreover, a proportion of the patients with PFDs have isolated secretion defects with normal aggregation. The 'gold standard' assay for measuring platelet secretion is radioisotopic 5-HT release. However, radioactivity has been displaced from clinical labs and currently, the best assay licensed to perform both measurements is PRP-lumiaggregometry. This has been criticized because it potentiates the aggregation of platelets and the ATP release has high coefficient of variation (CV). So, new tests are being developed to improve the diagnosis of PFDs.

Aims: To measure concomitantly the aggregation and 5-HT secretion of platelets by an HPLC-based assay, comparing it with the ^{3}H-5-HT release test. Also, to measure total platelet 5-HT in the same assay.

Methods: Platelet ^{3}H-5-HT release was measured as described (Holmsen, et al. Methods: Enzymol1989;169:205). 5-HT secretion by HPLC coupled to electrochemical detection (HPLC-ED) was performed in platelet lysates, obtained after stopping the aggregation test with ice-cold EDTA, rapid centrifugation in the same cuvette and lysis of the platelet pellet by three freezing-thawing cycles. HPLC-ED assay was

done as described (Kumar, Life Sciences 1990;47:1751). Initially, secretion tests by both methods were done in separate cuvettes, assessing the variability of aggregation tracings and secretion assays. Then, we performed a study in volunteers before and 2 h after intake of 300 mg uncoated aspirin, recording aggregation in a single cuvette and measuring released ^{3}H-5-HT in supernatant plasma and endogenous 5-HT retained in the platelets by HPLC-ED. Agonists used were arachidonate, epinephrine, ADP and collagen, using saline as blank (100% 5-HT).

Results: HPLC-ED was highly sensitive (around 1×10^{7} platelets injected in the column). Retention time was 7.5 min. Mean CVs for Blank, Epinephrine and ADP were 2.99%, 2.72% and 3.7%, respectively. Parallel assays showed almost identical maximal aggregation in both aliquots (74 ± 13% and 75 ± 13%); HPLC secretion was higher than the isotopic one (46 ± 15% vs. 34 ± 11%, $P < 0.0001$, $n = 130$ samples). The same was observed in volunteers before aspirin (42 ± 12% vs. 35 ± 12%, $P < 0.0001$, $n = 78$). Spearman correlation between both secretion assays was $r = 0.913$; 95% CI: 0.8799–0.9377; $n = 141$ paired samples, including post-aspirin samples). Aspirin dampened 5-HT secretion to a median (range) of 0.0% (0.0–40) and 0.0% (0.0–33) for HPLC and isotopic determinations, respectively ($n = 69$ pairs of samples). Analysis of individual agonists was also concordant. Total 5-HT content in platelets was 586 ± 138 ng $\times 10^{-9}$ platelets ($n = 27$).

Discussion: (i) 5-HT is abundant, specific of platelets and ideal marker to assay platelet secretion. (ii) 5-HT measurement by HPLC-EC is highly specific, sensitive and accurate, has very low CV, not requiring duplicates. (iii) It allows simultaneous measurement of total platelet 5-HT content to assess dense granule content and recognition of PFDs characterized by isolated secretion defects. (iv) After an initial expensive investment, the reagents used are cheap and ideally the instrument should be a dedicated one, side by side with the aggregometer. (v) Results can be obtained overnight. (vi) Radioactive compounds are eliminated. (vii) The adapted test can measure 5-HT release for diagnosing HIT.

PB 3.25-6

Prevention of intracranial hemorrhage in fetal/neonatal alloimmune thrombocytopenia: Identification of risk factors

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Background: Fetal/neonatal alloimmune thrombocytopenia is the most common cause of severe thrombocytopenia in the fetus and newborn. The major risk is intracranial hemorrhage (ICH), with neurological sequels in 20% of reported cases or death in 10%. We previously have shown that maternal anti-HPA-1a antibody concentration measured before any treatment and before 28 weeks of gestation was predictive of the fetal status (Bertrand et al. *Blood* 2011).

Aims: Although the relationship between thrombocytopenia and hemorrhage is obvious, not every severe thrombocytopenic child is affected by ICH, and predictive parameters would be helpful.

Methods: Two cohorts of severely thrombocytopenic index cases at diagnosis were retrospectively analyzed: Group I, $n = 52$ pregnancies without ICH; Group II: $n = 27$ pregnancies with ICH. Informed consent was obtained according to the Declaration of Helsinki.

Results: Only 48% women were multigravida in Group I comparatively to 81% in Group II ($P = 0.007$). Mean neonatal platelet count was significantly lower for Group II (7.10^{9} platelets/L comparatively to Group I (14.10^{9}/L; $P = 0.018$). In the absence of ICH the outcome was favorable. In group II, ICH led to fetal/neonatal death in 59% of the cases ($P < 0.001$). Moreover a statistically significant difference between the two groups was observed for maternal alloantibody concentration measured at time of diagnosis: 40 IU/mL in Group I and 89 IU/mL in Group II ($P < 0.001$).

Conclusions: Gynecologic history (abortions or successive pregnancies) and maternal anti HPA-1a alloantibody concentrations may be considered as predictive risk factors for ICH in severe cases.

PB3.26 – Platelet Acquired Dysfunctions

PB 3.26-1

Platelet function and blood loss during coronary artery bypass surgery

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Background: Coronary artery bypass surgery (CABG) is associated to blood loss. Transfusion of platelets and plasma is often applied anticipating on heparinisation, extracorporal circulation (ECC) and use of platelet inhibitors during surgery. To prevent blood loss during surgery, clopidogrel is often stopped at least 5 days before surgery.

Aims: In this study coagulation and platelet function were monitored during the CABG procedure in a population that stopped clopidogrel use 5 days before the procedure (Group A) and in a population that continued clopidogrel through the procedure (Group B).

Methods: Patients undergoing standard CABG on ECC were included ($n = 60$, Group A 38, Group B 22). Analyses were performed pre-surgery, during surgery and within 2 h after arrival at the Intensive Care Unit. Thrombocyte function was analysed with Light Transmission Aggregometry (LTA), Multiple Electrode Aggregometry (MEA) and GPIIb/IIIa-expression with flowcytometry. Also, routine coagulation testing and thromboelastrography was performed with platelet mapping. Blood loss and transfusion was registered.

Results: No signficant difference was present in hemoglobin concentration, blood loss and transfusion between Group A and B. Platelet aggregation with ADP was reduced in Group B, while strong agonists as collagen and TRAP not significantly different. The aggregation response of all agonists was decreased during the surgical procedure and returned to pre-operative values at the time of arrival at the ICU. Coagulation assays showed no significantly different results during the procedure.

Conclusion: As most transfusions are applied at the ICU, this study shows that the use of clopidogrel during the procedure is a weak parameter for clinical decision making on transfusion. Platelet function is reduced during surgery, but is restored to pre-operation values at the time of arrival at the ICU.

PB 3.26-2

The microstructure of clots formed via the extrinsic pathway: effects of shear induced platelet activation

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Background: Platelet activation (PA) is known to be one of the earliest precursors to the formation of an incipient blood clot but little is known about its effect on clotting time and clot microstructure. Excessive PA *in-vivo* might lead to the rapid formation of a thrombus with a 'dense' microstructure and increased elasticity which is more difficult for the body to reabsorb by fibrinolysis and could therefore cause a blockage leading to thromboembolic complications and potentially death.

Aims: This study aims to find how the repression of the contact pathway affects the incipient clot microstructure and clotting time using the rheological detection of the gel point in coagulating whole blood.

Furthermore, we aim to quantify the incipient clot formation time and resultant clot microstructure in clots formed, *in-vitro*, under different levels of shear induced PA.

Methods: Whole fresh blood was mixed with Corn Trypsin Inhibitor (CTI), to inhibit the contact pathway, and immediately confined between two parallel plates (at a gap of 400 μm) of an AR-G2 Controlled Stress Rheometer. The Gel Point was found by the attainment of a frequency independent phase angle (ratio of viscous response to elastic response) during the evolution of viscoelasticity of the clot. Fractal analysis of this data provides a method of quantifying clot microstructure in terms of a fractal dimension d_f (Evans et al., 2010). Furthermore, the application of pre-shearing regimes (at different periods and levels of shear rates) allows us to manipulate levels of platelet activation prior to monitoring the formation of the clot. Flow cytometry was used to measure platelet activation on a small aliquot of sample following pre-shearing.

Results: Our preliminary data shows that the presence of CTI significantly delays the onset of the incipient clot, with clot formation time increasing approximately eight-fold compared to previous data obtained using unaltered whole blood. Furthermore, the value of d_f significantly decreased in the presence of CTI, indicative of a more 'open' or 'loose' clot microstructure. In this paper we will also report the ongoing studies of how different levels of shear induced platelet activation effect incipient clot formation time and resultant clot microstructure.

Summary: Suppression of the contact pathway of coagulation, via the addition corn trypsin inhibitor to whole blood, significantly delays the onset of incipient clot formation as measured by the rheological detection of a Gel Point. This allows us to explore the effects of platelet activation on clotting times and resultant microstructure by the application of pre-shearing regimes. The results are important for understanding clot formation *in-vivo*, in particular in instances of regions of high shear flow such as stenotic vessels, and also in ventricular assist devices.

Reference:

1. Evans PA et al. Gel point and fractal microstructure of incipient blood clots are significant new markers of haemostasis for healthy and anticoagulated blood. Blood. 2010;116(17):3341–6.

PB 3.26-3

Plasma LDL-cholesterol lowering by LDL apheresis induces acute changes in platelet properties

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Background: Arterial thrombosis is modulated by lipoproteins by altering the platelet response to activating agents. Accordingly, platelets of familial hypercholesterolemia (FH) patients who are characterized by elevated low-density lipoprotein cholesterol (LDL-C) levels due to a molecular defect in the *ldlr* gene are hyperreactive both *in vivo* and *in vitro*. For those patients who are unresponsive to or intolerant for oral lipid lowering therapy, i.e. statins, LDL-apheresis is warranted to adequately lower LDL-C levels.

Aim: To elucidate the impact of selective LDL particle removal by LDL apheresis on the responsiveness of platelets from hypercholesterolemic patients.

Methods: Experiments were performed with platelets from three statin intolerant hypercholesterolemic patients, who underwent four weekly LDL apheresis procedures performed with absorption columns of the direct adsorption of lipoprotein (DALI) system. Blood was collected prior to each session to prevent possible platelet activation induced by the apheresis procedure. Platelet reactivity after agonist stimulation was assessed in whole blood by flow cytometry by measuring fibrinogen binding and surface expression of P-selectin. The study was approved by the international review board of the Academic Medical Center, Amsterdam. All participants provided written informed consent.

Results: The mean plasma LDL-C concentration after LDL apheresis was calculated by the Kroon formula taking into account the rebound of LDL-C levels after each apheresis session. This showed a mean reduction of the plasma LDL-C level by 40 ± 6% (from 10.1 ± 2.0 to 6.8 ± 0.6 mM, respectively). The reduction of the plasma LDL-C level was associated with an increase in the platelet count from 261 ± 17 × 10^9 platelets/L to 334 ± 19 × 10^9 platelets/L ($P < 0.05$). In addition, fibrinogen binding and P-selectin expression, induced by adenosine 5′-diphosphate (ADP) or the glycoprotein (GP) VI-specific collagen peptide mimetic, cross-linked collagen-related peptide (CRP-XL), were normalized after LDL apheresis.

Summary/Conclusions: Our findings indicate that lowering of plasma LDL-C levels by LDL apheresis induces acute changes in platelet count and normalizes platelet reactivity.

PB 3.26-4

The role of platelets in vitamin K antagonist-associated bleeding

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Background: Recurrent bleeding can complicate the treatment of patients with vitamin K antagonists (VKA), even at a well-regulated level of anticoagulation. Bleeding at mucocutaneous sites is frequently reported in these patients, which may point to defects in primary hemostasis.

Aim: In this proof-of-principle study, we investigated whether defects in platelet function or von Willebrand factor (vWf) contribute to a bleeding phenotype in patients on VKA therapy.

Methods: A case–control study was performed with 33 well-regulated patients without bleeding events (controls) and 33 patients with recurrent bleeding (cases). Patients have a well-regulated anticoagulation level when the International Normalised Ratio (INR) is ≥ 65% of the time within the target range. Cases and controls were matched for age, gender and INR target range. Thrombin generation and vWf levels and function were determined in plasma. Platelet function was assessed by light transmission aggregometry and flow cytometry using a validated panel of agonists.

Results: Thrombin generation was similarly reduced in controls (median ETP: 361 nM/min) and cases (median ETP: 387 nM/min), in comparison to normal plasma. This confirmed no difference in the degree of anticoagulation between the controls and cases. The plasma level of vWf was higher than 150%, the upper limit of the normal range, in 85% of the controls and 67% of the cases. Platelet aggregation in response to ADP, epinephrine, arachidonic acid, PAR1 agonist SFLLRN, collagen or ristocetin was in the normal range for almost all patients irrespective of the type of agonist. However, in response to a low collagen dose, platelets from 21% of controls and 27% of cases showed diminished responses. Agonist-induced secretion of α- and δ-granules or integrin αIIbβ3 activation were similar in platelets from controls and cases.

Conclusion: Recurrent bleeding in well-controlled patients on VKA therapy is not explained by defective platelet or vWf function.

PB 3.26-5

An analysis of the hemostatic response using a hybrid computational and experimental approach

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Background: Recent evidence shows that the hemostatic response to penetrating vascular injuries produces a markedly heterogeneous structure in which a stable core of tightly packed, fully-activated platelets is overlaid with an unstable shell of less-activated and less densely packed platelets (Stalker, et al. Blood 2013). The development of such a structure is shaped by the interaction of biological and physical factors that include blood flow and the movement of plasma-borne solutes such as pro- and anticoagulant proteins within the complex geometry of the deposited platelets.
Aims: Our goal in this study was to apply a computational approach to understand the clinically-relevant question of how the movement of solutes affects, and is affected by the heterogeneous structure of the hemostatic thrombus that we have observed.
Methods: We started by engineering different architectures to simulate platelet accumulation from the early stages to the stable form of a hemostatic mass. Each architecture represents a hemostatic plug with a different number of platelets and different packing density. For each architecture we used fluid dynamics to simulate the flow of blood around and within the hemostatic mass. We then simulated solute transport into and out of the platelet mass using Convection-Reaction-Diffusion. The results of the simulation were compared to data obtained by observing the movement within the thrombus of albumin linked to a caged fluorophore.
Results: Our results show that the platelet mass slows solute movement, helping to facilitate its biological action. This deceleration is regulated by the platelet packing density. Based on the computational analysis, we estimated the residence time of a species. For an arteriole 30 μM wide with average blood velocity of 2 mm/s, the residence time of a protein like albumin, is about 0.5 ms in the lumen, 4 ms in the shell and 9 ms in the core, a conclusion in good agreement with the experimental evidence. We then manipulated critical parameters such as the packing density of the platelets, the production of thrombin and the release of ADP. We observed the interaction of two main effects, dilution and residence time. In the case of thrombin, for instance, we observed that a two-fold increase in production rate translates into a 50% larger area of action, defined as the region of space where the concentration of thrombin exceeds that needed to activate platelets.
Conclusions: From these theoretical findings a picture emerged in which the potency of agonists that are produced or released at the bottom of a platelet mass, decreases as they percolate through the mass. This decrease is due both to dilution and to an increase in the removal rate as the agonists encounter regions of reduced packing density, and thus the sheltering action of deposited platelets plays a fundamental role in solutes transport. In summary studying solute transport in the complex geometry of a platelet mass can teach us important lessons about the spatiotemporal function and activation of platelets. It offers a novel way to study the evolving environment surrounding a platelet during hemostasis.

PB 3.26-6

Pattern and management of bleeding complications with new oral anticoagulants. Results of the Prospective Dresden NOAC Registry (NCT01588119)

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Background: Novel oral anticoagulants (NOAC) have been approved for anticoagulation for venous thromboembolism (VTE) and stroke prevention in atrial fibrillation (SPAF). In large trials, bleeding complications were the most frequent side effect, at in general similar frequency as with respective comparators. However, little is known about the distribution pattern, management and outcome of NOAC-related bleeding complications in daily care.
Aims: To evaluate the pattern and management of bleeding complications during NOAC therapy in daily care.
Patients and Methods: The Dresden NOAC registry is a prospective, non-interventional registry. A network of more than 230 physicians from private practice and hospitals enrol eligible patients, who are centrally followed by the registry office. Inclusion criteria are: (i) indication for therapeutic NOAC anticoagulation > 3 month; (ii) age > 18 years; (iii) written informed consent; (iv) availability for follow-up. No Exclusion criteria apply. In the registry, up to 2000 patients will receive prospective follow up by phone visits at day 30 day and quarterly thereafter for up to 3 years to collect efficacy and safety data.
Results: Until December 31th 2012, 1665 patients were enrolled into the registry. Of these, 1356 patients received rivaroxaban (372 for VTE, 968 for SPAF, 16 off-label) and 309 received dabigatran (303 for SPAF and six off-label). During follow-up (635.4 patient years) a total of 400 patients reported 474 bleeding complications (61.8% minor bleeding, 32.9% non-major, clinically relevant (NMCR) and 5.3% major bleeding according to ISTH definition).
For non-major bleeding, mucosal (epistaxis, gingival, conjunctival) were the most common bleeding sites (37.8% of all bleedings), followed by bruises and traumatic skin bleeds (27.8%), genitourinary (14.1%) and gastrointestinal (11.2%). For major bleeding, gastrointestinal bleeding was the most common manifestation (2.5%), followed by intracranial (0.4%) and a variety of other manifestations.
Most patients (96.0%) either did not need any treatment or received conservative treatment with compression, tamponade or red blood transfusion (RBC) alone. Surgical or interventional treatment was used in 4% of all bleedings (0.3% of minor, 7.1% of NMCR and 28% of major bleedings).
Transfusion of RBC or fresh frozen plasma (FFP) was only observed in patients with major bleeding (4.2& and 0.8% of all bleedings; 76.0% and 16.0% of all major bleedings, respectively). Prothrombin complex concentrate (PCC) was used in 1.3 of all bleedings (24% of all major bleedings) and no patient received recombinant factor VII.
Fatal bleeding was observed in two patients (0.4% of all and 8.0% of all major bleedings), in both cases due to major intracranial bleeding.
Conclusion: Bleeding complications are common in daily care NOAC patients treated and are usually managed by conservative treatment. Only 5% of all observed bleedings fulfil the ISTH criteria of major bleeding (mainly RBC transfusion criterion) and are managed using interventions, FFP or PCC. Overall, only a very small proportion of NOAC-associated bleeding complications in daily care are fatal, indicating that available management strategies are sufficient.
For presentation at the ISTH meeting, updated results from this ongoing project will be provided.

PB3.27 – Platelet Calcium Signaling

PB 3.27-1

Simultaneous real-time imaging of fibrinogen binding and intracellular calcium reveals a role for Rac1-dependent synergy between P2Y1 and P2Y12 receptors

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Background: In platelets, ADP stimulates inside-out activation of integrin $\alpha_{IIb}\beta_3$ through co-activation of Gq-coupled P2Y1 and Gi-coupled P2Y12 receptors. These receptors operate synergistically via mechanisms that remain incompletely understood. The megakaryocyte (MK) serves as a bona fide model of platelet purinergic signalling and allows the dynamics of $\alpha_{IIb}\beta_3$ activation to be followed by imaging of fluorescently tagged fibrinogen (Tolhurst *et al.* Blood, 2005;106, 1644). We

have developed this approach further to simultaneously record Ca^{2+} waves and fibrinogen binding. We also explore the role of the small GTPase Rac1, an important mediator of positive feedback in platelets.
Aims: To explore in real time the spatiotemporal relationship between P2Y receptor-evoked elevations of cytosolic Ca^{2+} and downstream $\alpha_{IIb}\beta_3$ activation using fluorescence imaging of single primary MKs. To combine this approach with immunocytochemistry (ICC) to investigate the role of the small GTPase Rac1 in P2Y-evoked responses.
Methods: Primary rat MKs were isolated as described previously (Mahaut-Smith *et* al 1999 J. Physiol. 515, 385), loaded with the Ca^{2+} indicator Fluo-3 (2 μM Fluo-3 AM for 60 min) and imaged on a confocal microscope (Olympus FV1000) at 5.3 Hz in the presence of 60 μg/mL alexa fluor 647-tagged fibrinogen. P2Y1/P2Y12 receptors were stimulated with 100 μM ADPβS. Rac-1 was inhibited with 100 μM EHT1864. For ICC, MKs were fixed with 2% paraformaldehyde prior to staining of activated Rac1 with a mouse anti-Rac1-GTP antibody.
Results: ADPβS stimulated a series of transient Ca^{2+} increases in the form of waves spreading rapidly across the MK. Each Ca^{2+} wave was followed by a single, incremental increase in fibrinogen binding to the cell periphery. The fibrinogen bound uniformly at first, but in many cells developed a polarized appearance within 4 min that correlated with the average direction of the Ca^{2+} waves (computed from vectorial analysis). BAPTA-loading abolished the fibrinogen binding. Selective inhibition of either P2Y1 or P2Y12 receptors virtually eliminated fibrinogen binding as reported previously in murine MKs (Tolhurst et al. 2005). Although P2Y1 receptors were essential for the Ca^{2+} response, P2Y12 receptors were also required for more than a single initial Ca^{2+} spike. Inhibition of PI3K or Rac mimicked the effects of P2Y12 blockade on Ca^{2+} signalling and fibrinogen binding. Active (GTP-bound) Rac1 accumulated at the periphery of ADPβS-stimulated megakaryocytes and was abolished by inhibition of P2Y12, PI3K or Rac1.
Summary/Conclusions: In the MK, P2Y receptors generate multiple transient Ca^{2+} waves that efficiently couple to peripheral $\alpha_{IIb}\beta_3$ receptor activation. P2Y receptors also generate polarised fibrinogen binding that correlates with the direction of the Ca^{2+} waves. Sustained Ca^{2+} oscillations require co-stimulation of both P2Y1 and P2Y12 receptors, extending previous observations in platelet suspensions (Hardy *et al*. Blood, 2004;104:1745; van der Meijden *et al*. FEBS J, 2008;275:371). As described in platelets, the synergistic mechanism in the MK was blocked by PI3K inhibition suggesting the existence of a PI3K-dependent Ca^{2+} signalling pathway. We also provide evidence for the involvement in this pathway of Rac1, which is known to have a role in the development of polarity in other cell types.

PB 3.27-2

The role of plasma membrane STIM1 and Ca^{2+} entry pathways in platelet aggregation

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Background: Ca^{2+} is an essential messenger in platelet activation that is released from intracellular stores and enters from the extracellular medium. The contributions of Ca^{2+} release and Ca^{2+} entry to individual platelet activation events are not established. IP_3 receptors release Ca^{2+} from intracellular stores. STIM1 senses
Ca^{2+} in the endoplasmic reticulum and activates Orai channels following Ca^{2+} release allowing store-operated Ca^{2+} entry (SOCE). TRPC6 and P2X1 entry channels allow non-SOCE Ca^{2+} entry.
Aims; STIM1 has also been reported in the plasma membrane (PM) but its role here is not understood. The effects of SOCE and non-SOCE inhibitors on platelet activation have not been fully described.
***Methods*:** We examined the effects of the antibody GOK/STIM1, which recognises the N-terminal region of STIM1, on SOCE, agonist-stimulated Ca^{2+} entry, surface exposure, *in vitro* thrombus formation and aggregation in human platelets. In addition we determined novel binding partners of STIM1 using proteomics. We also tested the effects of SOCE and non-SOCE inhibitors (BTP2 and LOE-908 respectively) on platelet aggregation.
Results: The neat GOK/STIM1 antibody reduced thapsigargin (TG)- and agonist-mediated Ca^{2+} entry in Fura2-labelled cells, but failed to do so following dialysis of the preparation to remove preservatives. Using flow cytometry we detected surface exposed STIM1. The dialysed GOK/STIM1 antibody reduced thrombus formation by whole blood on collagen-coated capillaries under flow and platelet aggregation induced by collagen. The SOCE inhibitor BTP2 reduced TG induced platelet aggregation but not that by thrombin, collagen or OAG. The non-SOCE inhibitor LOE 908 reduced OAG and collagen induced aggregation but not that by thrombin or TG. In immunoprecipitation experiments followed by proteomic analysis, STIM1 was found to extract a number of proteins including myosin, DOCK10, thrombospondin-1 and actin suggesting novel binding partners.
Conclusion: These studies suggest that PM STIM1 may facilitate platelet activation by collagen through novel interactions at the platelet PM while the essential Ca^{2+}-sensing role of STIM1 is served by the protein in the ER. SOCE is not essential for aggregation induced by thrombin however non-SOCE contributes to aggregation induced by collagen.

PB 3.27-3

The attachment of integrin aIIbβ3 and GpIb to the platelet cytoskeleton is disrupted in the phosphatidylserine-positive platelets as a result of calpain activation

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Background: The exposure of phosphatidylserine (PS) to the surface of activated platelets is an essential hemostatic mechanism allowing binding of procoagulant proteins and dramatic acceleration of coagulation reactions. The PS-negative activated platelets have active form of integrin $\alpha_{IIb}\beta_3$ on their surface, and PS-positive platelets do not have active form of integrin $\alpha_{IIb}\beta_3$ on their membranes. In the resting platelets, integrin $\alpha_{IIb}\beta_3$ is attached to the cytoskeleton with special proteins, and their degradation is supposedly controlled by a calcium-dependent protease calpain. Early studies suggest that calpain also regulates the activation of integrin $\alpha_{IIb}\beta_3$.
Aims: We investigated the attachment of integrin $\alpha_{IIb}\beta_3$ and GpIb to the cytoskeleton in platelet subpopulations. Additionally we analyzed the effect of calpain on the activation of integrin $\alpha_{IIb}\beta_3$.
Methods: Platelets were isolated from whole blood by centrifugation and gel-filtration. Then they were activated with physiological agonists such as thrombin or collagen-related peptide. We investigated the status of intergin $\alpha_{IIb}\beta_3$ and attachment of integrin $\alpha_{IIb}\beta_3$ to the cytoskeleton in activated platelets by flow cytometry. The status of intergin $\alpha_{IIb}\beta_3$ was detected with fluorescently labeled PAC-1 binding. The attachment of membrane glycoproteins (integrin $\alpha_{IIb}\beta_3$ and GpIb) to the cytoskeleton was estimated by fixing the platelet with paraformaldehyde after activation in the presence of fluorescently labeled antibody CD61 (or CD42b) to surface $\alpha_{IIb}\beta_3$ (or GpIb) and treating fixed platelets with detergent. Additionally the status of cytoskeletal proteins was evaluated by electrophoresis.
Results: We observed a loss of membrane surface glycoproteins only in the PS-positive platelets (fixed platelets after detergent treatment), but not in the PS-negative activated platelets and resting platelets. This effect was prevented by calpain inhibitors (calpeptin and MDL28170). These results suggest that the degradation of cytoskeletal proteins that provide attachment of integrin αIIbβ3 to the platelet cytoskeleton occurred in PS-positive platelets. Preincubation of platelets with the calpain inhibitors significantly inhibited the degradation of cytoskeletal proteins proceeds at activation of platelets as shown by electrophoresis. It follows that the proteolytic degradation of cytoskeletal

proteins in platelets is controlled calcium-activated protease calpain. While preincubation of platelets with calpain inhibitors before activation did not lead to changes in the expression of the active form of integrin $\alpha_{IIb}\beta_3$ on the membrane of PS-negative platelets, or the lack of the active form of integrin αIIbβ3 in the PS-positive platelets.
Conclusions: Inhibition of calpain resulted in a significant decrease in the proteolytic degradation of cytoskeletal proteins, suggesting that calpain is involved in the regulation of the attachment of integrin $\alpha_{IIb}\beta_3$ and GpIb to the cytoskeleton of platelets. However, calpain inhibition did not lead to changes in the expression of the active form of the integrin $\alpha_{IIb}\beta_3$, this suggests that calpain is not involved in regulating the activation of integrin $\alpha_{IIb}\beta_3$.

PB 3.27-4

Reduced expression of SERCA3 isoform in platelet from patient with morbid obesity inhibits platelet functions

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Background: Obesity is a recognized cardiovascular risk factor. Platelets play an pivotal role in pathogenesis of atherothrombosis. In obese subjects, platelet activity is often described as increased and their sensitivity to antiplatelet agents is reduced. Such dysfunctions are often associated with a disruption of calcium homeostasis. Sarco/Endoplasmic Reticulum Ca^{2+} ATPase (SERCA1, 2 & 3) are proteins involved in cytosolic Ca^{2+} transport to the Endoplasmic Reticulum and regulation of calcium homeostasis.
Aims: A pilot study comparing nine subjects with severe obesity and five normal weight subjects, showed a collapse of expression of SERCA3 isoforms, whereas expression of SERCA2b remained unchanged. We hypothesized that in obese subjects, a decrease of platelet SERCA3 induced disruption of calcium signal and abnormal platelet function. To test this hypothesis, we analyzed the expression of SERCA3 in a larger group of obese subjects whose platelet functions were explored and calcium signal measured and compared to control donors.
Methods: Monocentric case/control study. Cases were women with body mass index (BMI) ≥ 35 kg/m^2, without hypertension, diabetes, dyslipidemia, cancer, sepsis or inflammation. Control subjects were women with BMI > 18.5 and < 25 kg/m^2, without the same exclusion criteria and matched to the cases for age $\pm$ 3 years. SERCA levels were measured by immunoblotting. Aggregation was examined in static conditions in washed platelets stimulated with various agonists. Activity of thrombin-stimulated platelet was measured by flow cytometry using PAC-1 (activated GPIIbIIIa). BAPTA Oregon-green (calcium fluorescent marker) loaded platelets were analyzed for calcium mobilization and influx in response to different agonists by flow cytometry and or video microscopy.
Results: We included 40 cases and 40 control subjects. Platelets from obese subjects displayed a reduced level of SERCA3 expression. This was associated to a lower calcium mobilization in response to agonists (Thrombin, collagen, PAR4-AP), while calcium influx was nearly unchanged. Such decreased sensibility was further observed in aggregation studies where higher agonist concentrations were needed to obtain similar level of aggregation. Finally, platelet activity after thrombin stimulation assessed by flow cytometry was also higher in control platelets than in platelet from subjects with morbid obesity.
Conclusion: In obese subjects, washed platelets displayed a reduced sensitivity to agonists; this was associated to a lower calcium response and a decrease in SERCA3 expression. We suggest that this SERCA3 decrease might be a physiological response to reduce the rate of spontaneous activation of platelets. Whether it is reversible after weight loss is under investigation.

PB 3.27-5

Contributing factors influencing the platelet activation time between platelet adhesion on Von Willebrand factor, detected by an increase in intraplatelet calcium ion

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Purpose: To clarify intra-platelet signaling factors contributing to platelet activation initiated by its interaction with von Willebrand factor (VWF), the time was measured between platelet adhesion on immobilized VWF to the start of an increase in intracytosolic calcium ion ($[Ca^{2+}]i$), in the presence or absence of specific inhibitors.
Method: In whole blood specimen in the presence of specific thrombin inhibitor, Argatroban (100 μM), platelets were rendered fluorescent with the Ca2+-sensitive dye fluo-3 AM. The $[Ca^{2+}]i$ of individual platelet, which adhered on immobilized VWF was measured as fluorescence changed. Whole blood was perfused on VWF in the presence or absence of phospholipase C (PLC) inhibitor U73211 (5 μM), a mixture of PI3K inhibitors (LY294002 and Wortmannin [10 μM, 100 nM respectively]), and ADP receptor $P2Y_{12}$ inhibitor AR-C69931MX (100 nM). The time from platelets adhesion to the start of increased $[Ca^{2+}]i$ was measured in 20 randomly selected platelets.
Result: In control, the time taken from platelet adhesion on VWF to the start of an increase in $[Ca^{2+}]i$ was 9.5 ± 3.8 (s). The time was significantly longer in the presence of U73211 (21.2 ± 6.6 (s), $P < 0.01$ as compared to control), however, the time taken in the presence of LY294002/Wortmannin of 9.9 ± 5.5 (s) and AR-C69931MX of 7.7 ± 5.0 (s) were not different from controlled conditions. In control, $[Ca^{2+}]i$ of platelets increased from 32.0 ± 9.4 (nM) to 397.3 ± 97.8 within 0.56 ± 0.12 s after first noting an increase in $[Ca^{2+}]i$. Maximum $[Ca^{2+}]i$ in the presence of $P2Y_{12}$ inhibitor of AR-C69931MX of 221.8 ± 115.6 (nM) was significantly lower than that in control ($P < 0.01$). The Maximuma $[Ca^{2+}]i$ in the presence of PLC inhibitor of U73211 of 328.2 ± 107.5 (nM) or the PI3K inhibitor of LY294002/Wortmannin of 332.9 ± 97.3 (nM) were not significantly different from controlled conditions.
Conclusion: It takes 9.5 ± 3.8 (s) from platelet adhesion on VWF to the start of an increase in $[Ca^{2+}]i$. This time delay is regulated by the activity of PLC, and not by PI3K since the delay was increased only when the function of PLC was blocked.

PB 3.27-6

Systems biology of platelet activation: a stepped hierarchy of responses arises from the calcium interplay between cytosol, dense tubular system and mitochondria

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Background: Activated platelets have two major functions, aggregation and acceleration of plasma coagulation. Different activation levels lead to a hierarchy of responses, three major steps of which include ability to aggregate, granule release and shape change, and phosphatidylserine (PS) exposure. The last step, which dramatically increases the rate of blood thrombin generation, leads to formation of distinct subpopulations. This stepped hierarchy of platelet responses suggests existence of one or several decision-making mechanisms in the platelet's intracellular signaling network.
Aim: The goal of this study was to investigate, by means of *in silico* systems biology analysis, how a uniform increase in the agonist concentration could be transformed into a stepped platelet response.
Methods: A mathematical model of the platelet's signal transduction was developed. As calcium signaling is at the heart of the platelet acti-

vation, we primarily targeted the structures involved in it. The model includes several compartments: the cytosol, the dense tubular system (DTS) and the mitochondria. DTS and cytosol interacts through calcium pumps (SERCA) and signal-dependent channels, and the mitochondrion has several specific calcium-regulating mechanisms, a uniporter, an exchanger and a permeability pore. The activation level was simulated by varying the concentration of the PAR1 agonist. The set of ordinary differential equations was integrated using the COPASI software (http://www.copasi.org).

Results: Computer simulation shows that the stable resting state of platelet is maintained by a balance between calcium pumps and leaks trough the DTS and plasma membranes. At very low agonist levels, the stationary concentration of cytosolic calcium is maintained at ~0.01 μM, and weak activation leads to a slight increase up to ~0.05 μM with a subsequent return to the baseline. Two-fold increase in the agonist concentration leads to rapid activation of the SERCA. More powerful pumping-in causes long-lasting oscillations in the cytosolic calcium concentrations with a magnitude of ~0.2 μM, almost independent of the agonist concentration, in good agreement with experimental observations. Another two-fold increase in the agonist level induces activation of the mitochondrial uniporter and the bulk of calcium goes from DTS into mitochondria which causes collapse of mitochondrial membrane potential. Simultaneously, the cytosolic calcium concentration becomes stationary high. This occurs only in a fraction of platelets leading to the PS exposure in a subpopulation of them.

Conclusion: For the first time, it is demonstrated how the uniform increase of the platelet activation level can be converted into a stepped response hierarchy corresponding to several distinct platelet functions. The main mechanism controlling platelet stability at low agonist concentrations and activation threshold is the intense membrane leaks. Medium activation triggers the appearance of oscillations of cytosolic calcium that are controlled by means of the DTS calcium pump. Ultimately, the interplay of DTS and mitochondria at the high activation level triggers the mitochondria collapse with the consequent divergence of subpopulations.

PB3.28 – Signal Transduction – Miscellaneous

PB 3.28-1

P21-activated kinase regulate directional migration and cytoskeletal organization in human neutrophils

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Background: Neutrophils serve as a first line of defense in innate immunity owing in part to their ability to rapidly migrate towards chemotactic factors derived from invading pathogens. As a migratory function, chemotaxis is regulated by the Rho GTPases including Rac, Cdc42 and RhoA. These Rho family members mediate the cytoskeletal rearrangements that underlie chemotaxis through the p21-activated kinase (PAK), a well-established effector of Rac and Cdc42. In neutrophils, rapid phosphorylation of PAK has been observed after the treatment with various agonists. To date, however, little is known about functional roles of PAK in primary human neutrophils.

Aims: We aimed to characterize the role of PAK downstream of Rho GTPases in cytoskeletal remodeling and chemotactic processes of human neutrophils.

Methods: Using chemotaxis assays, primary human neutrophils were allowed to migrate toward a source of the chemoattractant, f-Met-Leu-Phe (fMLP). Neutrophil directionality, morphological polarization and motility were monitored by time-lapse imaging in the presence or absence of pharmacological inhibitors of key signaling molecules including Rac, PI3K and PAK. We examined the expression of PAK isoforms in human neutrophils by Western blot. In addition, immunofluorescence microscopy was performed to characterize the activation and subcellular localizations of PAK, Rac, RhoA and cytoskeletal components such as F-actin and myosin.

Results: PAK inhibition led to a loss of directionality, increased spreading and decreased migration speed in neutrophil chemotaxis, whereas Rac or PI3K inhibition resulted in impaired directionality or polarization, respectively. PAK inhibition was associated with dysregulated intracellular Ca^{2+} release. Our data show that PAK2 is activated and accumulates at the neutrophil leading edge in response to fMLP to support Rac-mediated actin dynamics in a localized manner. In addition, PAK inhibition abrogated the subcellular localization of active RhoA and increased surface contacts via vinculin-rich complexes.

Conclusions: PAK kinase activity plays a critical role in chemotaxis of human neutrophils. Together, our data suggests that PAK establishes a 'frontness' signal by negatively regulating surface adhesion and Rho-dependent 'backness' signals in human neutrophils, thus providing a mechanism for the crosstalk between Rho-family GTPases in neutrophil cytoskeletal dynamics and cell migration.

PB 3.28-2

Correction of endothelial dysfunction by hepatocyte-specific Dyrk1a gene transfer in hyperhomocysteinemic mice

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Background: Hyperhomocysteinemia, defined by elevated plasma homocysteine level, is well recognized as an independent risk factor for cardiovascular diseases, including coronary artery, cerebrovascular, and peripheral occlusive disease even among people with normal cholesterol levels. The increased synthesis of homocysteine, a product of methionine metabolism involving B vitamins, and its slower intracellular utilization cause increased flux into the circulation. Hence, plasma homocysteine level is an important reflection of hepatic methionine metabolism and the rate of processes modified by B vitamins as well as different enzyme activity. Reduction of homocysteine levels is the key objective in treatment of hyperhomocysteinemia. Vitamin B6 in pharmacological doses in combination with folic acid or vitamin B12 or both is the treatment of choice for cystathionine beta synthase deficiency. However, approximately 50% of hyperhomocysteinemic patients due to cystathionine beta synthase deficiency are biochemically responsive to pyridoxine (vitamin B6). Therefore, effective treatments to reduce homocysteine levels are needed, and gene therapy could provide a novel approach.

Aims : We recently found a negative correlation between plasma homocysteine level and the hepatic expression of an anti-inflammatory serine/threonine kinase, Dyrk1a. We decided to use a gene transfer strategy with a specific hepatic adenoviral vector over-expressing Dyrk1a and analyzed the effect of a selective homocysteine lowering therapy on markers of hepatic and vascular function by reverse phase protein array.

Results: we found normalisation of plasma homocysteine level after hepatic Dyrk1a gene transfer in a genetically modified murine model with hyperhomocysteinemia. Specific hepatic Dyrk1a gene transfer

corrected the decreased hepatic and plasma paraoxonase 1 activities, and the decreased plasma apo A-I level which is associated with increased hepatic protein level. Paraoxonase 1 and HDL exert potent protective effects, including the prevention and correction of endothelial dysfunction. Targeted hepatic Dyrk1a gene transfer abolished the negative effect of hyperhomocysteinemia on PI3K/Akt/mTOR pathway, a cell signaling pathway that plays a key role in cellular homeostasis through its role in regulation of apoptosis, cell growth, cell cycle and angiogenesis, and the decreased protein level of cyclin D1. Commensurate with the positive effect on PI3K/Akt activation, the hepatic Dyrk1a gene transfer also resulted in GSK3 inhibition, which can prevent endothelial dysfunction in aorta of mice.

Summary/Conclusions: The positive effect on plasma homocysteine and aortic signaling pathways demonstrates that this gene therapy can constitute a useful approach for prévention of cardiovascualr diseases linked to hyperhomocysteinemia. This approach might also benefit other diseases in which hepatic homocysteine metabolism is affected and vascular alterations are prevalent.

PB 3.28-3

The novel NOX inhibitor 2-acetylphenothiazine impairs collagen-dependent thrombus formation in a GPVI-dependent manner

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Background: Besides classical agonist-induced signal transduction pathways, platelet activation is also regulated by reactive oxygen species (ROS). In particular, superoxide ions from exogenous and endogenous sources increase collagen-dependent aggregation and thrombus formation. NADPH oxidases (NOXs) play a critical role in the generation of superoxide ions in platelets and contribute to platelet activation, although their mechanism of action remains largely unknown. Therefore, NADPH inhibitors may represent novel potential candidates for the development of anti-platelet agents.

Aims: In this project, we studied the effect of the novel NOX inhibitor 2-acetylphenotiazine (2-APT) on human platelet functional responses and intracellular signalling pathways.

Methods: The generation of superoxide ions was assessed by single cell imaging on adhering platelets using dihydroethidium (DHE), while cumulative ROS generation was detected with 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (CM-H2-DCFDA). Whole blood thrombus formation, washed platelet aggregation, integrin αIIbβ3 inside-out signalling, Syk phosphorylation, and protein kinase C (PKC) activation were analysed to understand the functional consequences of NOX inhibition by 2-APT in platelets.

Results: Superoxide ion generation stimulated by platelet adhesion on collagen and fibrinogen was significantly inhibited by 2-APT in concentration-dependent manner within the submicromolar range, whereas this pharmacological agent did not affect cumulative ROS generation. 2-APT impaired washed platelet aggregation in response to collagen but not thrombin and abolished whole blood thrombus formation stimulated by collagen but not fibrinogen. The activation of integrin αIIbβ3 and protein kinase C in response to the GPVI-specific agonist collagen-related peptide (CRP) was significantly reduced, whereas the same responses to thrombin were not significantly affected by 2-APT. Finally, Syk activation in response to collagen but not thrombin was inhibited by 2-APT, which suggests a stimulatory role for NOX-generated superoxide ions in the early events of the signalling cascade of GPVI.

Summary/Conclusions: Taken together, our results suggest that 2-APT attenuates GPVI-specific signalling and is a novel inhibitor of collagen-induced platelet activation. Therefore, 2-APT can represent a novel candidate for the development of anti-thrombotic drugs and NOXs are promising new targets for anti-thrombotic drug discovery.

PB 3.28-4

The polyamines regulate intestinal epithelial barrier function through AMP-activated protein kinase and the nuclear accumulation of c-Myc

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Backgroud: Epithelial cells line the gastrointestinal mucosa and form an important barrier that protects the subepithelial tissue against a wide array of noxious substances, allergens, viruses and luminal microbial pathogens. Restoration of mucosal integrity following injury and various environmental stresses requires epithelial cell decisions that regulate signaling networks controlling gene expression, survival, migration and proliferation. Our previous studies have shown that Polyamines regulate expression of E- cadherin and play an important role in control of intestinal epithelial barrier function, but the exact mechanism is still unknown. The AMPK (AMPk- activated protein kinase), an enzyme involved in responding to metabolic stress, was recently found to be involved in the regulation of epithelial tight junction assembly.

Aims: The current study was designed to determine whether polyamines modulate the nuclear accumulation of c-Myc by AMPK and then regulate E-cadherin transcription.

Methods: The IEC-6 cell line was purchased from the A.T.C.C at passage 13. The levels of cellular polyamines were decreased as a result of inhibiting ODC with DFMO (D,L-alpha-difluoromethy lornithine). The AMPKα activity, the nuclear levels of c-myc and E-cadherin levels were detected by Western Blotting after polyamine deletion in IEC-6 cells. Then the cells were overexpressed AMPKα by infection with adenovirus-AMPKα, the nuclear level of c-Myc protein, the mRNA and protein levels of E-cadherin were detected, the E-cadherin immunostaining of IEC-6 cells were observed under confocal microscopy, and the E-cadherin promoter activity was analysis by luciferase assays. To further determine whether c-Myc had the effect on E-cadherin transcription activity, two experiment were performed. First, we used small interfering RNA(siRNA) targeting the c-Myc mRNA(siMyc) to reduce c-Myc levels and thus directly examine its role in the observed changes in E-cadherin transcription activity. Second, c-Myc has the binding site on E-cadherin promoter, so we used the different E-cadherin promoter mutation on Myc binding site to detect the effect of AMPKα on E-cadherin promoter activity.

Results: Polyamine depletion by DFMO decreased the levels of AMPKα, the nuclear level of c-Myc protein and E-cadherin. AMPK activation by overexpression of the AMPK gene increased the nuclear levels of c-myc and reversed the barrier function as indicated by increasing E-cadherin promoter activity, mRNA levels and protein levels. Decreased c-myc by transfection with c-myc siRNA could inhibit the effect of AMPK on E-cadherin promoter activity. And further researches showed that deletion and point mutations of E-pal, the binding site of c-Myc on E-cadherin promoter, prevented the increase of E-cadherin promoter activity after overexpression of AMPKα.

Conclusions: These findings confirm that polyamines regulate intestinal epithelial barrier function through AMPKα and the nuclear accumulation of c-Myc.

PB 3.28-5

Therapeutic application of activated Protein C epigenetically constrains the redox-enzyme p66shc in diabetic nephropathy

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Introduction: Mice with genetically increased levels of activated Protein C (aPC) are protected against diabetic nephropathy and have decreased

glomerular expression levels of the mitochondrial-targeted redox enzyme p66shc. In mice therapeutic application of aPC ameliorates indices of diabetic nephropathy and podocyte damage. The therapeutic mechanism and how intermittent application of a plasma protein with a half life of ~25 min modifies nephropathy remain unknown.
Methods: Unilaterally nephrectomized WT mice were STZ-injected. After 4 weeks of diabetes a subgroup was i.p.-injected every other day for 4 weeks with 1 mg/kg aPC (Elli Lilly, Xigris®) or with aPC preincubated with an antibody (HAPC1573) to block its anticoagulant function. In parallel a subgroup of aPC-injected mice were orally treated with sodium butyrate for 4 weeks. *In vitro* immortalized murine Podocytes were treated with 2 nM aPC and methylation-specific PCR (MSP) and ChIP were applied to analyze promoter methylation and acetylation of p66shc.
Results: Exogenous application of aPC and aPC-HAPC1573 ameliorates indices of diabetic nephropathy in diabetic wild-type mice. Glomerular accumulation of oxidative stress markers and of p66shc are reversed in aPC-treated diabetic mice. Renal Histone H3 acetylation and p66shc expression was increased upon diabetes and reversed with aPC-treatment. The histone H3 hyperacetylating agent sodium butyrate reversed the suppression of p66shc by aPC and abolished the beneficial effects of aPC. *In vitro* aPC reversed the glucose induced increase of the H3-acetyltransferase GCN5 and the hypomethylation and H3-hyperacetylation of the p66shc-promoter, which was abolished with sodium butyrate treatment.
Conclusion: Therapeutic application of aPC epigenetically suppresses the red-ox enzyme p66shc and through this indirect mechanism aPC has antioxidative and cytoprotective properties that are independent of its anticoagulant function.

PB 3.28-6

Protease-activated receptor-2 triggers epithelial to mesenchymal transition: potential relevance in pulmonary fibrosis and cancer

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Background: Idiopathic pulmonary fibrosis (IPF) constitutes the most devastating form of fibrotic lung disorders and remains refractory to current therapies. Fibroblast foci are the histological hallmark of IPF, and these have been shown to (partially) originate from lung epithelial cells via epithelial-mesenchymal transition (EMT). The extracellular signals and cellular receptors triggering EMT in IPF remain incompletely understood. However, aberrant reactivation of the WNT signaling pathway, which is crucial in developmental programs regulating lung development and morphogenesis, is thought to play a role in IPF.
Aims: We have recently shown that deficiency of protease-activated receptor 2 (PAR-2) affords protection in bleomycin-induced pulmonary injury. Here, we explore the role of PAR-2-induced EMT in pulmonary fibrosis.
Methods and Results: Immunostaining of lung biopsies of IPF patients revealed prominent PAR-2 expression in the fibrotic regions of the lungs of these patients, with especially strong PAR-2 overexpression by epithelial cells overlying fibroblast foci. Double immunostaining revealed that PAR-2 co-localizes with epithelial cells expressing both epithelial (cytokeratins) and mesenchymal (vimentin) markers, suggesting a role of PAR-2 in EMT in IPF. We also observed *in vitro* that, following incubation with PAR-2 agonist peptide (AP), type II lung epithelial cells (A549) acquired a fibroblast-like morphology. Moreover, western blots performed on lysates of A549 cells stimulated with PAR-2 AP, demonstrated that PAR-2 activation induces the expression of the myofibroblast markers vimentin and α-SMA, as well as collagen secretion. Additional western blots showed that PAR-2 activation of epithelial cells triggers the WNT signaling pathway, leading to β-catenin accumulation, and immunofluorescence analysis of cells stimulated by PAR-2 AP confirmed the translocation to the nucleus of β-catenin.
Summary/Conclusion: Taken together, our results strongly suggest that PAR-2 directly triggers EMT in epithelial cells via WNT signaling pathway activation. Because EMT is a mechanism common to both IPF and cancer, these results could suggest involvement of PAR-2 driven EMT in both pathologies, and inhibition of the PAR-2-coagulation axis may therefore be clinically relevant in both diseases.

PB3.29 – Megakaryocytes and thrombopoiesis – III

PB 3.29-1

Continuous platelet production in the bloodstream is regulated by von Willebrand factor

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Introduction: It is established that pre/proplatelets are formed from mature megakaryocytes (MK) as intermediates before platelet production. We have previously identified a new mechanism showing that exposure of MKs to a high shear rate (1800 s^{-1}) on von Willebrand factor (VWF) in an *ex vivo* flow model could accelerate proplatelet formation and platelet release. MK underwent microtubular rearrangementsto form proplatelets, breaking into several elements, still linked to one another via tubulin shafts; finally, pre/proplatelet reorganization could lead to the release of single, isolated platelets. Our findings therefore imply that, during the late stages of thrombopoiesis, continuous reorganization of proplatelets into platelets could be affected by their contact with VWF present on the vessel wall.
Aim: Recently, the transition of condensed platelets into multibodied proplatelets was described in circulating blood platelets in the absence of exposure to VWF and shear. The purpose of the present study was to investigate ex vivo and *in vivo* whether exposure to VWF in flow conditions was involved in the appearance of multibodied proplatelets in circulating blood.
Methods: A mouse model of severe von Willebrand disease, the *Vwf*-/- mice was used to mimick a situation of blood cells circulating in a vascular tree devoid of VWF. Cytoskeletal modifications of washed platelets from *Vwf*-/- and wild-type (*Vwf*+/+) mice were compared in an established flow model allowing the observation of rapid changes resulting from (pro)platelet adhesion to VWF. Proplatelets (designated as newly formed platelets) proportion was determined using washed platelets from murine blood, exposed to VWF at 1800 s^{-1} in a microfluidic platform and characterized by videomicroscopy, flow cytometry and activation studies.
Results: Newly formed platelets were visible within 5 min, representing up to 38% of the total number of platelets at 12 min. 1.8-fold more newly formed platelets were produced *ex vivo* in blood from *Vwf*-/- than *Vwf*+/+ mice, suggesting a potential role of VWF *in vivo*. Expression of VWF in *Vwf*-/- mice by hydrodynamic gene transfer decreased the percentage of proplatelet formation back to wild-type levels. Platelets collected at the exit of the flow chamber remained fully activable by thrombin, demonstrating that newly formed platelets were not pre-activated. Similar observations were obtained from a patient with severe von Willebrand disease. Young immature post-chemotherapy platelets also produced a 2-fold higher percentage proplatelets than control untreated mouse blood, following exposure to 1800 s^{-1} on VWF.
Conclusion: A direct role of VWF in the transition of condensed platelets into multibodied proplatelets was demonstrated *ex vivo* and *in vivo*. This suggests a new function for VWF as a regulator of bloodstream thrombopoiesis

PB 3.29-2
IL-21 promotes the expansion of primary human megakaryocytes *in vitro*

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Background: Chronic inflammatory diseases are the most frequent cause of reactive thrombocytosis. Several inflammatory cytokines (TNFα, IL-1β, IL-6 and IL-4) are known to regulate megakaryopoiesis. Gene profiling studies indicate that *in vitro* differentiated megakaryocytes express the receptor for IL-21, another cytokine produced during immune responses.
Aims: The aim of this study was to assess the role of IL-21 on *in vitro* human megakaryopoiesis.
Methods: Megakaryocytes were generated by cultivating adult peripheral blood CD34+ cells obtained from leukoreduction filters with informed consent and written agreement from the donors. In our standard culture conditions we used thrombopoietin and proliferative cytokines (stem-cell factor, IL-6 and IL-9) for 7 days (phase 1) and then, thrombopoietin alone for 5 days (phase 2). In the test experiments, IL-21 was also added at phase 1 and/or phase 2. The expression of the IL-21 receptor was confirmed by RT-PCR and flow cytometry. The effect of IL-21 was checked by evaluating cell proliferation and phenotype and measuring platelet generation. In addition, the activity of thrombopoietin and IL-21 receptors was followed by assaying the phosphorylation of Stat-3 and -5 proteins by flow cytometry.
Results: RT-PCR experiments and flow cytometry demonstrated that the IL-21 receptor was absent in peripheral CD34+ cells, but was progressively induced during the differentiation. The presence of IL-21 did not modify the expression of its receptor and appeared to be beneficial to the cell culture only when added in the second phase. The numbers of megakaryocytes differentiated after 12 days of culture was increased, provided IL-21 was added during phase 2, this, in a concentration-dependant manner, up to 1.8 ± 0.2 fold when IL-21 was used at 100 ng/mL. The phenotypes of the cells were similar, whether IL-21 was added or not, as judged by the expression of the megakaryocytic specific receptors GPIIbIIIa, GPIb and GPV, and by the ploidy of GPIIbIIIa+ cells. Morevover, platelet production was not modified by the addition of IL-21. Stimulation of the IL-21R induced Stat-3 phosphorylation in the megakaryocytes, while thrombopoietin, preferentially phosphorylated Stat-5. Thus, the two cytokines appeared to induce complementary signal transduction pathways in these cells.
Conclusion: The proliferative effect of IL-21 on megakaryocytes and the well-known implication of this cytokine in some chronic inflammatory diseases, suggest a potential role of IL-21 in reactive thrombocytosis. In addition, our observations question whether IL-21 might be useful for platelet recovery after myelodysplasia.

PB 3.29-3
Megakaryocyte regulatory pathway mediated by NMDA receptors

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Background: Novel treatment strategies are required to improve outcomes of patients with megakaryocytic malignancies. To enable progress we need better understanding of disease mechanisms and new therapeutic targets. The *N*-methyl-D-aspartate glutamate receptors (NMDARs) have arisen as potential regulators of megakaryocytic maturation but their specific roles remain unknown. Intriguingly, in other cells NMDARs are thought to help balance processes of cell death and survival and contribute to the development of cancer.
Aims: We hypothesised that similar to other cells, megakaryocytic NMDARs are involved in cell fate decisions and may therefore influence malignancy phenotype. The main aim of this study was to investigate if NMDARs regulate growth and differentiation of megakaryocytic cells.
Methods: Well-established inhibitors of neuronal NMDARs (memantine, AP5, MK-801 and DCKA; 5–100 μM) and NMDAR agonists (NMDA and glycine; 1–100 μM) were used to investigate effects on proliferation and differentiation of Meg-01 and Set-2 megakaryocytic cell lines, including after differentiation with PMA (phorbol-12-myristate-13-acetate). Phenotypic end-points were read in the MTT cell viability and proliferation assay, thymidine incorporation assay and by flow cytometry on CD41a and CD61.
Expression of all NMDAR subunits, including splice variants, was investigated in Meg-01 and Set-2 cells using RT-PCR (including real-time), Western blotting, flow cytometry and immunostaining. In a pilot study, marrow samples from patients with essential thrombocythaemia (ET), myelofibrosis (MF) and acute megakaryoblastic leukaemia (AMKL) (n = 3 of each) were tested for expression of the obligate NMDAR subunit 1 (GluN1) using immunohistochemistry. Blasts from one AMKL patient were tested for expression of all NMDAR transcripts using RT-PCR.
Results: We found that NMDAR agonists (1–100 μM) increased and NMDAR antagonists (5–100 μM) reduced proliferation of Meg-01 and Set-2 cells. NMDAR antagonists did not interfere with Meg-01 and Set-2 differentiation. Instead, mild increases in CD41a and CD61 expression were observed in Meg-01 cells treated with NMDAR antagonists.
Prototypic NMDARs in the brain are very diverse but always contain two GluN1 components that typically combine with another two (identical or different) GluN2 (A, B, C or D) subunits. The GluN3 subunits (A and B) can also be present, but are less common. We found that both Meg-01 and Set-2 cells contained transcripts for all NMDAR subunits, except for GluN2B. By real-time PCR, transcripts for GluN 2D and 3B predominated in Meg-01, and 2D and 3A in Set-2. Further, Meg-01 and Set-2 contained a similar combination of GluN1 splice variants but differed in GluN2A splicing pattern. At the protein level, we confirmed the presence of GluN 1, 2A and 2D proteins in Meg-01 and Set-2 cells, both on cell surface and intracellularly. Upon differentiation with PMA, NMDAR expression increased both in Meg-01 and Set-2, supporting receptor relevance during megakaryocytic differentiation.
Studies on human bone marrow showed that megakaryocytic cells in ET, MF and AMKL all expressed GluN1. Blasts from the singular AMKL patient who was tested by RT-PCR contained all NMDAR transcripts.
Summary/Conclusions: Human megakaryocytic cells contain complex NMDARs that influence cell phenotype. Further studies to determine how NMDARs regulate megakaryocyte biology are warranted.

PB 3.29-4
Effect of three-dimensional hydrogel scaffolds on megakaryocyte differentiation and platelet production

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Background: Hematopoietic stem cells (HSC) differentiate into megakaryocytes (MK) whose function is to terminally release platelets, which are absolutely essential for bleeding arrest. Growth in 3D conditions may replicate the bone marrow microenvironment necessary for HSC proliferation and MK differentiation. So far, information is lacking on the influence of a 3D structure on both megakaryopoiesis and thrombopoiesis.
Aim: The aim of the study was to determine the effect of porous polysaccharide-based hydrogels on MK and platelet production from

human umbilical cord blood HSC. HSC proliferation and differentiation into MK were analyzed inside 3D hydrogel scaffolds and compared to cultures in liquid conditions (2D).
Methods: Porous hydrogels composed of polysaccharides (pullulan-dextran, 3:1 ratio) with 200 μm macropores were used. This optically transparent hydrogel material allowed for a direct observation of live cells. HSC were cultured in the presence of thrombopoietin for 16 days in 2D and for 36 days in 3D scaffolds. Confocal microscopy provided identification of megakaryocytic progenitor cells by staining of CD41a and CD42b markers, and of cell ploidy by YOYO-1 staining. Following extraction of cells embedded in the scaffolds by enzymatic lysis, the differentiation markers (CD34, CD41a and CD42b) and ploidy were also quantitated by flow cytometry. Clonogenic assay was performed to study CFU-MK potential. Platelet production from mature MK exposed to physiological shear rates in Bioflux200® was studied by videomicroscopy.
Results: Rehydrated 3D scaffolds were organized in a complex network structure where proliferating cells seeded at an optimal density of 0.5×10^5 cells/cm^2 were visible inside the pores. Small spherical cells characteristic of immature cells were visible at day 2. At day 9, MK progenitors appeared as larger cells followed by proplatelet-forming MK with pseudopodial elongations at day 12. From day 12, these three cell types were observed inside pores. Finally, 36 days after initial seeding, i.e. 20 days after the end of cell survival in 2D, high cell numbers were still observed in 3D. Up to day 16, flow cytometry analysis of extracted cells grown in 3D indicated similar MK differentiation as in 2D. Between day 16 and day 36, in 3D, high proportions of immature cells were present while mature MK persisted. At day 16, the number of CFU-MK in 3D was significantly increased compared to 2D ($P < 0.05$). In 3D, most cells displayed increased 4N (30.5% ± 2.34; $P < 0.05$) and >8N (4.4% ± 0.96) ploidy classes. Functional platelet-forming mature MK were obtained for a longer period in 3D (day 36) than in 2D (day 14) as shown by separate flow experiments.
Summary/Conclusion: 3D hydrogels provide a structure to prolong the duration of HSC ability to differentiate into fully mature and platelet-producing MK and thus may mimick the bone marrow environment. Clusterization of cells in pores of scaffolds may favor their proximity and allow concentrating soluble factors. Three-dimensional hydrogels appear as a powerful tool to further unravel the physiological mechanisms of megakaryopoiesis and thrombopoiesis and the coupling with a bioreactor could strengthen this *in vitro* model of bone marrow structure.

PB 3.29-5
Impact of COX-2 deletion on platelet and megakaryocyte phenotypes

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Background: Cyclooxygenase (COX)-1 or -2 and prostaglandin (PG) synthases catalyse the formation of PGs (including PGI_2 and PGE_2) and thromboxane (TX) A_2 playing a key role in the maturation of different cell types. While mature platelets express almost only COX-1, megakaryocytes contain both COX-isoforms. *In vitro* studies showed that pharmacologic inhibition of COX-2 activity might influence megakaryocyte maturation and platelet formation. The production of platelets by megakaryocytes requires an intricate series of remodeling events; abnormalities in this process can result in clinically significant disorders.
Aims: We investigated the role of COX-2 in megakaryocytopoiesis analysing platelets and megakaryocytes from cyclooxygenase-2 knockout (COX-2KO) mice in terms of phenotype and/or function.
Methods: Platelet and megakaryocyte phenotypes were assessed by cytofluorimetry, immunohistochemistry and/or functional tests.
Results: COX-2KO platelets showed increased expression of P-selectin and GPIIbIIIa, enhanced binding to fibrinogen and adhesion to endothelial matrix compared to WT platelets. Levels of TX synthase in platelets were similar in both groups of animals, whereas levels of COX-1 were higher in platelets from COX-2KO together with an increased ability to produce TXA_2. While platelet count in COX-2KO mice was slightly lower than in WT mice (WT:1121 ± 274 vs. COX-2KO:1031 ± 263 K/μL), platelet size was larger (WT: 71.10 ± 3.87 vs. COX-2KO:91.27 ± 5.48; $P < 0.01$), and there was a higher percentage (WT: 7.62 ± 0.59 vs. COX-2KO:13.27 ± 1.01; $P < 0.01$) of reticulated, young platelets. In the bone marrows of COX-2KO mice, megakaryocyte number was significantly reduced, with phenotypic features of poor differentiation, such as high nucleus/cytoplasm (N/C) ratios and reduced positivity for lineage antigens such as CD42b (GpIb). At variance with bone marrows, megakaryocytes were significantly increased in the spleen of COX-2KO mice, with a reduced N/C ratio and hyperexpression of lineage markers, whereas few, poorly differentiated megakaryocytes were present in the WT spleens. In addition, COX-2 deletion decreased expression of the prostacylin receptor (IP) and PGE_2 production, concomitantly with increases in PGI_2 production, resulting in a reduced PGE_2/PGI_2 ratio in the bone marrow. Opposite results were obtained in the spleen, where we observed upregulation of the expression of IP, significant reduction of PGI_2 levels, and an increase in the PGE_2/PGI_2. Levels of TXA_2 measured both in the BM and in the spleen were similar in WT and COX-2KO mice.
Remarkably, splenectomy, carried out in COX-2KO and in WT mice, decreased the number of circulating platelets by about 40%, and reverted the hyper-reactive phenotype observed in COX-2KO platelets.
Summary/Conclusions: COX-2 deletion appears to delay megakaryocytopoiesis in the bone marrow, consistently with previous *in vitro* reports. The spleen megakaryocyte hyperplasia in COX-2KO mice is likely to represent a compensatory response to defective bone marrow megakaryocytopoiesis. Finally, the young and hyper-reactive platelets in COX-2KO mice, derived from compensatory spleen megakaryocytopoiesis, might contribute to an increased thrombogenicity *in vivo*.

PB 3.29-6
The cholinergic system in cord blood derived megakaryocytes and megakaryocytic lineages

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Background: In former studies we have shown that platelets and their precursors express nicotinic α7 acetylcholine receptors (nAChRα7) that are involved in platelet function. We could also demonstrate that in-vitro megakaryocytic differentiation of MEG-01 cells can be modulated via cholinergic drugs targeting nAChRα7.
Aims: In this study, we have investigated additional megakaryocytic lineages and cord blood derived megakaryocytes for the presence of additional receptors of the cholinergic family (nAChRα4, nAChRβ2, nAChRα7) and the acetylcholine esterase (AChE). Furthermore, we were interested in the effect of cholinergic drugs which are in clinical use on *ex vivo* megakaryocytic differentiation.
Methods: Expression of nAChRs and AChE was investigated in four megakaryoblastic celllines (M07, CMK, Dami and MEG-01), cord blood derived TPO-differentiated megakaryocytes at different time points of differentiation and in platelets. Expression levels of the components were investigated by quantitative real-time PCR (qRT-PCR) and Western blot analysis. Cord blood derived CD34+ cells were differentiated with TPO for up to 14 days and the effect of the AChE-inhibitor donepezil (100 nM, 1 μM, 10 μM) on the differentiation and proliferation process was investigated by flow cytometry and micrsocopy.
Results: nACHRα7, nAChRα4, nAChRβ2 and AChE gene transcripts could be identified on all cell lines and cord blood derived megakaryocytes during all stages of differentiation. This could be confirmed at

protein level. While platelets express nAChRα7, they lack expression of nAChRα4, nAChRβ2 and AChE. The TPO-induced differentiation process and proliferation was significantly inhibited by the AChE-Inhibitor donepezil in a concentration dependent manner.
Conclusions: In addition to nAChRα7, precursor cells also express nAChRα4, nAChRβ2 and AChE which are down regulated during megakaryopoesis. AChE-Inhibitors are frequently used as treatment of dementia in elderly people. Since the megakaryocytic differentiation process impaired in the presence of AChE-Inhibitors patelet function in these patients could be affected.

PB3.30 – Microparticle assays

PB 3.30-1
Towards standardized protocols for preparation and detection of microparticles

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Background: Results from studies on microparticles (MP) are difficult to compare between laboratories because no standard protocols are available for collection, handling, storage, and isolation of MP. Recently, novel technologies such as resistive pulse sensing (RPS) and single particle tracking (SPT) have become commercially available. Both technologies enable detection of *single* particles directly in solution.
Aims: In this study, we aim to develop standard protocols for collection, handling, storage, and isolation of MP. Flow cytometry (FCM), RPS and SPT are used to measure the concentration and size distribution of particles in samples prepared using these protocols.
Methods: MP were isolated from erythrocytes and platelets using different centrifugation protocols, in which the centrifugation force (18,890 ***g*** and 100,000 ***g***) and centrifugation time (0.5–2 h) were varied. Isolated MP were reconstituted in phosphate buffer saline (PBS) or in autologous vesicle-depleted human plasma (VDP) and measured directly or before snap-frozen in liquid N_2 and storage at −80 °C.
Results: RPS and SPT detect 1000–10,000-fold more particles in all MP preparations than FCM. In general, the particle concentration and diameter in all MP preparations are more affected by the single freeze/thaw cycle than by centrifugation conditions. Importantly, the effect of single freeze/thaw cycle on particle concentration and diameter are markedly different for MP prepared from erythrocytes and platelets. After single freeze/thaw cycle, the particle concentration and typical particle diameter of erythryocyte MP reconstituted in VDP are comparable to the freshly prepared samples (2.4×10^{10} particles/mL and 200 nm), but not for those reconstituted in PBS (9.9×10^{9} particles/mL and 164 nm). In contrast, regardless the reconstitution solution used, the particle concentration of samples prepared from platelets increases about 2-fold (5.8×10^{9} to 1.2×10^{10} particles/mL) and the particle diameter decreases (160–140 nm) after single freeze/thaw cycle compared to the freshly prepared samples.
Conclusions: To develop a single protocol for starting materials containing MP of different cellular origin, such as plasma, may be very challenging because each type of vesicle behaves differently especially after freezing/thawing.

PB 3.30-2
Effect of filtration on the particle concentration and diameter of microparticles

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Background: Although many investigators use filtration to remove larger particles from suspension of vesicles, the effects of filtration are not thoroughly investigated.
Aim: To investigate the effect of filtration on the detection of MP.
Methods: Microparticle (MP)-containing samples were prepared from erythrocyte concentrates. The concentrate was diluted two times with phosphate buffer saline (PBS) and centrifuged twice at 1550 ***g*** for 20 min at 20 °C. The supernatant was measured directly or filtered using 0.8 μm (mixed cellulose esters, Millex-AA; Merck Millipore, MA, USA) and 0.22 μm (polyethersulfone, Millex-GP, Merck Millipore) syringe-disc filters. Particles were measured by flow cytometry (FCM), resistive pulse sensing (RPS), and single particle tracking (SPT). PBS was used as negative control in the filtration experiments. Finally, PBS was analysed before and after filtration by transmission electron microscopy (TEM).
Results: Erythrocyte MP (CD235a-positive) are undetectable by FCM after filtration by a 0.22 μm filter or a 0.8 μm filter. In contrast, the particle concentration detected by RPS are 5.6×10^{9} particles/mL before filtration, 5.0×10^{9} after filtration by a 0.8 μm filter, and 0 particles/mL after filtration by a 0.22 μm filter respectively. Using SPT, there is a 4-fold increase in the particle concentration measured after filtration by a 0.8 μm filter. In PBS, almost no particles after filtration by 0.22 μm filter are detectable by FCM, RPS, or SPT. Surprisingly, in PBS filtered through 0.8 μm filter, there are particles detected by FCM (5.8×10^{3} particles/mL), RPS (3.1×10^{7} particles/mL), and SPT (4.6×10^{6} particles/mL). In this sample, EM also detects particles which are likely to be debris released from the filter, and such debris was not detectable in unfiltered PBS or after filtration by a 0.22 μm filter. When PBS was first filtered through a 0.2 μm filter and then a 0.8 μm filter, similar debris was present.
Conclusions: Filtration through 0.8 μm filter affects mainly the particle concentration. Filtration through a 0.8 μm syringe-disc filter is not recommended because such filter releases debris that will contaminate particle analyses.

PB 3.30-3
Effect of inflammatory cytokines and coagulation factors on endothelial microparticle formation and content

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Background: The role of microparticles in inflammatory and thrombotic disorders is still not fully understood. Endothelial microparticle formation is altered in inflammatory and thrombotic disorders like sepsis, atherosclerosis and thrombotic thrombocytopenic purpura. It is however not known if these microparticles are the cause or the consequence of these disorders.
Aims: We aimed to study the effect of inflammatory cytokines and coagulation factors as well as combinations thereof on endothelial microparticle formation and on microparticle von Willebrand factor (VWF) and its regulating protease ADAMTS-13. We also measured the microparticle thrombin generation in patients with HIV-associated TTP.
Methods: In this study microparticle formation in cultured human umbilical vein endothelial cells (HUVEC) was stimulated by different inflammatory agents IL-6 (100 ng/mL) IL-8 (100 ng/mL) and TNF-α (100 ng/mL), coagulation factors TF (2 μL/mL) and thrombin (2 U/mL) and combinations thereof. The number of endothelial microparticles formed was determined using flow cytometry. Von Willebrand factor (VWF) and ADAMTS-13 levels of the microparticles were assessed by ELISA's and the microparticle thrombin generation was measured by thrombin generation assays. Von Willebrand factor multimers were visualized by a Western blot technique. Microparticle thrombin generation was measured in the plasma of 20 patients with HIV-associated TTP.
Results: Interleukin-6 did not show any effect on HUVEC derived microparticles due to the lack of the receptor for IL-6 on these cells. Interleukin-8 only had a small increasing effect on microparticle VWF and ADAMTS-13 levels. TNF-α showed a significant effect on micro-

particle numbers and contributed to almost 80% of the thrombin generated, but has almost no effect on the VWF levels in the microparticles. Tumour necrosis factor alpha is thus a potent stimulus of endothelial cells and might be responsible for the thrombotic tendency in thrombotic and inflammatory disorders. The coagulation factor TF on the other hand showed the highest increase in microparticle VWF levels and had a huge effect on microparticle numbers. It however has no effect on the thrombin generation of microparticle. Tissue factor in combination with TNF-α also induced an increase in microparticle VWF and a little decrease in ADAMTS-13 levels. Tissue factor may thus contribute to the increased VWF levels that are commonly found in TTP patients where inflammation and thrombosis occur. To proof this, we found that microparticles contribute to more than 70% of the thrombin potential in the patients with HIV-associated TTP. Interestingly, thrombin shows a protective effect on the intact HUVEC whereby microparticle formation was prevented. The combination stimuli of thrombin and cytokines also showed a protective effect on HUVEC. This highlighted the regulatory role of thrombin in intact endothelial cells on haemostasis and in protection against thrombosis in extremely inflammatory environments.

Conclusion: Endothelial microparticles can thus be detrimental or beneficial with different stimuli and in different environments. Inflammatory and coagulation stimuli may however pose a great risk for clotting by altering microparticle quantity and content. This study contributes to the understanding of the role that endothelial derived microparticles plays in inflammation and thrombosis.

PB 3.30-4

The procoagulant phospholipids clotting time assay as a routine functional test for measuring circulating microparticle activity

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Background: Microparticles (MP) are now considered as critical effectors involved in numerous biological processes (coagulation, inflammation, vascular biology). Two major complementary approaches are available to monitor circulating MP in a clinical setting. The MP can be measured using a quantitative and descriptive approach by flow-cytometry or by a functional approach assessing MP biological activities by a FXa based clotting assay. Flow cytometry can be used to determine the cellular origin of the different MP, although there are concerns about the detection limit of this approach. Functional assays measure only the procoagulant activity of isolated MP and give no information on the cellular source or the physical properties of the MP. The advantage of the functional assays is that the assays use well-defined reagents and they are readily automated with high sensitivity and simplicity. Ideally, a combination of methods is needed to characterize MP.

Aim: Here we report a correlation between Annexin V-MP (AMP) enumeration by cytometry and the assessment of their procoagulant activity by FXa based clotting assay, in a number of different clinical settings.

Methods: This study analyzed 60 patients with active cancer of different type (M/F 30/30, age 40–92), 60 patients with a BMI >25 kg/m^2 (M/F 30/30, age 18–66), and 49 carriers of Factor V Leiden (FVL) and prior venous thromboembolism (VTE) (M/F 19/30, age 26–64). Samples were only collected after obtaining informed consents from the patients consecutive referred to our Medicine and Thrombotic Unit. All these patients had been previously enrolled in studies by our group. MPs levels were assessed by flow-cytometry (Beckman Coulter, USA) using calibrated beads of defined diameter and annexin V-fluorescein isothiocyanate staining. MP activity was assessed by a FXa based procoagulant phospholipid dependent clotting assay (Diagnostica Stago, France) using a phospholipid depleted substrate plasma. In this assay a reduction of the clotting time reflects increased procoagulant activity of the sample. Correlation analyses were performed by Spearman's rank correlation test.

Results: Flow-cytometry analysis of AMP counts was compared with MP activity measured by the procoagulant clotting assay (PPL). In all three groups of patients tested we found the same inverse correlation between the flow-cytometry analysis of AMP counts compared with PPL: in cancer patients r −0.685, $P < 0.001$, in obese patients r −0.478, $P < 0.05$ and in FVL patients who had had a previous VTE r −0.521, $P = 0.0043$.

Conclusions: The two different methods for MP detection showed good correlations with one and other even if the basis for the MP analysis was completely different. Even if flow-cytometry is considered the 'gold standard'of MP detection there are still many technical limitations reducing its wide spread use relating to the complexity of the assay and concerns about detection of the small MP. The PPL assay is much simpler to perform. The PPL assay could be a useful initial automated screening test to detect patients with abnormal MP activity. Only the abnormal samples would then need to be further evaluated by flowcytometry.

PB 3.30-5

Procoagulant phospholipid in stored erythrocyte concentrates

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Background: Transfusion of erythrocyte concentrates may cause adverse effects in the recipient, which include inflammatory and procoagulant responses. Pre-storage removal of leukocytes and platelets (leukoreduction) improves clinical outcome in the human recipient.

Aim: To evaluate the impact of leukoreduction (LR) on the development of procoagulant phospholipid (PL) in canine erythrocyte concentrates as a function of storage time.

Methods: Whole blood units were collected from healthy dogs and processed with LR ($n = 5$, using integral LR filter) and without LR (NLR, $n = 5$, using standard triple bag). Plasma was removed from all units via standard centrifugation, separation, and storage methodology. Aliquots were aseptically collected from the erythrocyte concentrate units on storage days 0, 7, 14, 28, and 35. Samples were centrifuged at 1850 $\boldsymbol{g} \times 20$ min to remove cells. Procoagulant PL in supernatants was quantified using STA Procoag-PPL on a STart4 coagulometer against a standard curve of liposomes containing 20% phosphatidylserine. PL-dependent thrombin generation was also evaluated using calibrated automated thrombography (CAT) with addition of supernatants to PL-depleted pooled normal human plasma.

Results: Procoagulant PL (range 6.9–42.1 μg/mL) was detected in all supernatant samples using the PPL assay. Concentrations were similar between LR and NLR units on storage days 0, 7 and 14, but were significantly higher in non-LR units on days 21, 28, and 35. Thrombin generation via CAT was minor in the absence of supernatants [Mean (SD) peak: 13.5 (10.9) nM]. Mean (SD) peak thrombin was similar between LR and NLR supernatants on day 0 [LR: 20.1 (7.8) nM; NLR: 23.2 (17.1) nM], day 7 [LR: 20.5 (10.2) nM; NLR: 24.4(18.6) nM], and day 14 [LR: 24.4 (7.9) nM; NLR: 25.3 (18.3) nM], but higher in NLR units on day 21 [LR: 34.3 (7.7) nM; NLR: 64.9 (35.6) nM], day 28 [LR:37.5 (14.9) nM; NLR: 138.2 (163.6) nM], and day 35 [LR: 80.3 (95.3) nM; NLR: 197.0(60.2) nM].

Conclusion: Procoagulant PL is present in stored canine erythrocyte concentrates. Pre-storage LR reduces the amount of procoagulant PL that develops as a consequence of storage, and delays the significant increase in procoagulant PL.

PB 3.30-6

In vitro microparticle-dependent clot formation mainly depends on platelet-derived microparticles and not on other subtypes

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Background: Microparticles (MP) are blebs of submicron sizes derived from various cell types including platelets, erythrocytes, leucocytes, endothelial or tumor cells (resp. PMP, Ery-MP, Leu-MP, EMP and tumor-MP). Due to the expression of procoagulant phospholipids (PPL), mainly phosphatidylserine (PS), they can be characterized by annexin V binding or/and thrombin generation. The amount of PPL can also be measured in whole plasma using chronometric assays where clotting time (CT) is related to total PS+ MP (TMP) content (1,2).
Aims: Check whether or not all MP subsets are equivalent PPL sources in MP-based clotting assays.
Methods: STA-Procoag-PPL (Stago, Asnières, F) was used to measure PPL- dependent clotting time, CAT with PRP Reagent (Thrombinoscope, NL) to monitor PPL-dependent TG and high sensitivity flow cytometry (FCM, Gallios, Beckman-Coulter) to differentially count the three major MP subsets. TMP were also measured using a capture assay coupled with TG (Zymuphen, Hyphen-Biomed, F). Platelet free plasma (PFP) samples included (i) MP-free plasma (MPFP) spiked with purified MP of various origins, (ii) PFP issued from normal citrated blood samples with/w.o. agitation prior to the 1st centrifugation (in order to artificially modulate MP content, ref. 3), (iii) PFP specifically depleted from resp. PMP, Ery-MP and/or Leu-MP using immuno-magnetic separation (IMS) (iv) PFP samples from sickle cell disease (SCD) patients and (v) PFP samples from septic patients, both as sources of distorted MP contents.
Results: In all types of samples, covering large ranges of PMP, Ery-MP and Leu-MP counts, due to either a pathology, IMS depletion or blood agitation, CT always correlates well with log [PMP]. On the contrary, Ery-MP and Leu-MP counts are poorly correlated with CT in all upper-cited plasma categories (a to e), as observed by comparison with FCM counting and confirmed by spiking experiments using pure MP, although TG levels correlate well with MP counts for all MP subtypes. In a reconstituted plasma model where MPFP was spiked with similar amounts of both PMP and Leu-MP, IMS-based removal of >90% spiked PMP clearly increased CT whereas selectively removing Leu-MP had no effect, independent of the order of the sequential IMS depletions. Re-incoporating PMP linked to IMS beads into MPFP resulted into shorter CT (e.g. 106 s down-to 35 s) whereas no effect came with Leu-MP. In pathological PFP, removing both Leu-MP and Ery-MP together had no effect on CT in contrast to removing PMP.
Conclusions: In a MP-dependent clotting assay the high correlation between PMP counts and CT was confirmed whereas low correlation values were observed with other MP subsets. Despite a tight correlation between MP counts of any type and TG, effective clotting is not only linked to PS expression but seems to require properties for clot elongation that are not provided by MP subsets other than PMP. In this respect, automatized clotting assays such as STA-Procoag-PPL may be considered as PMP-specific and used as such (4).
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PB3.31 – Endothelium: miscellaneous

PB 3.31-1

Molecular mechanisms of *S. aureus* mediated endovascular infection under fluid shear conditions

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Background: The adherence of *Staphylococcus aureus* to human endothelial cells is an important step in the pathogenesis of systemic *Staphylococcal* infections such as sepsis, meningitis and infective endocarditis. Adhesion to endothelial cells is critical in the pathogenesis and dissemination of the infective process. A major limitation in our current understanding of bacterial-endothelial cell interactions stems from the fact that previous studies have been carried out under static conditions. It has been argued in the literature that data obtained *in vitro* using static binding assays may not be relevant to the fluid dynamic environment encountered in the vasculature. The local fluid environment of the circulation critically affects the molecular pathways of endothelial cell-cell interactions and therefore may affect *S. aureus* binding.
Aim: The aim of this study was to investigate the interaction between *S. aureus* and endothelial cells subjected to physiological shear under resting and activated conditions.
Methods: Human aortic endothelial cells (HAEC's) were grown in Endothelial Cell Growth Medium MV supplemented with 5% Foetal Bovine Serum at 37 °C under either static conditions or conditions that mimic arterial shear rates experienced in the vasculature. Endothelial cells were treated with media or Human recombinant TNF-α (10 ng/mL) for 4 h followed by preincubation with heparinized plasma for 1 h at 37 °C. *S. aureus* were fluorescently labeled using a DNA stain and added to endothelial cells for 1 h.
Results: Tumour necrosis factor alpha is a major cytokine released from various immune cells and from the endothelial cells themselves in response to infection. TNF-α treatment of the HAEC's led to a significant increase in adhesion of *S. aureus* over resting HAEC's (128% increase, $P < 0.005$, $n = 3$). Clumping factor A (ClfA) is the most abundant protein expressed on the surface of *S. aureus* and has been implicated in the pathogenesis of Infective Endocarditis. Deletion of ClfA from the parent strain of *S. aureus* failed to have any effect on adhesion to the resting HAEC's (P = NS, $n = 3$), however significantly reduced adhesion to HAEC's treated with TNF-α (70% reduction compared to parent strain, $P < 0.001$, $n = 3$). We have previously demonstrated that ClfA binds plasma proteins in order to bridge the *S. aureus* to the host cell. Consistent with this removing plasma from the assay significantly reduced *S. aureus* from binding to the HAEC's (85% reduction in the absence of plasma, $P = 0.0001$, $n = 3$). Deletion of another major cell wall protein, protein A failed to have any effect on adhesion compared to parent strain (P = NS, $n = 3$)
Conclusion: Results presented here suggest that *S. aureus* have the propensity to bind to sheared endothelial cells that have been exposed to TNF-α over resting endothelial cells. The major cell wall protein ClfA bridges *S. aureus* to the TNF-α activated endothelial cell via an as yet unidentified plasma protein. A greater understanding in the area of vascular infection will aid in the development of novel therapies to treat this infection.

PB 3.31-2

Coagulation profile in patients with acute leukaemia receiving myeloablative or reduced-intensity conditioning regimens for allogeneic hematopoietic stem cell transplantation

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Background: Preparative regimens are critical elements in allogeneic hematopoietic stem cell transplantation (HSCT). Depending on intensity, preparatory regimens can be divided into myeloablative (MA) destroying host hematopoiesis with profound cytopenia and reduced-intensity (RIC) with minimal toxicities and immune mediated effects. Total body irradiation (TBI) regimens increase toxicity but improve eradication of leukaemia cells.

Blood coagulation, especially protein C anticoagulant system and inflammatory pathways are closely linked processes. There is also a relation between endothelial dysfunction and organ damage. Dysregulation of this process and endothelial injury may contribute to the pathogenesis of transplant complications.

Aims: Our aim was to evaluate the effects of the intensity of preparatory regimens on coagulation and endothelial cell activation in patients with acute leukaemia undergoing HSCT. Secondary was to identify coagulopathy abnormalities after TBI.

Methods: Fifty patients (29 males and 21 females, median age 34, range 18–63) transplanted with allogeneic stem cells after myeloablative (n = 15) or reduced-intensity (n = 35) conditioning regimen, followed by infusion of HSCs from a related donor (n = 17) or match-unrelated donor (n = 33) with peripheral blood stem cells (n = 26) and bone marrow stem cells (n = 24) were enrolled in the study. Underlying diseases included: acute myeloid leukaemia (n = 32) and acute lymphoblastic leukaemia (n = 18). TBI was applied in 36%. Graft vs. host disease prophylaxis consisted of cyclosporine A and methotrexate, while antithymocyte globulin (ATG-Fresenius) was additionally used in MUD.

We investigated citrated blood samples from 50 patients before the conditioning regimen (day −10), on the day of stem cell infusion (day 0) and on day +14 and +28 after HSCT for the following parameters: antithrombin (AT), protein S (PS), protein C (PC), thrombomodulin (TM), soluble endothelia protein C receptor (EPCR), C4 binding protein (C4BP), activated protein C resistance (APCR), factor VIII (FVIII), von Willebrand factor (vWF) and C-reactive protein (CRP). The mean values of all the parameters of both regimen groups were analyzed.

Results: There was no significant difference in the mean plasma level of the analyzed parameters between MA and RIC group at baseline (day −10 before HSCT), except for higher vWF and lower APCR ratio in RIC. On day −10, day 0 and day +14 after HSCT vWF antigen was higher in RIC than in MA group. Except for the lower PS in the RIC group (58.235 ± 21.233 vs. 76.041 ± 15.181; P = 0.0114), other parameters did not differ on day 0. In both groups, decreased PC and TM activity was shown between days 0 and +14 after HSCT, and differed significantly between the groups (P = 0.0146 and P = 0.0334, respectively). Neither the MA nor RIC group differed on day +28 with respect to coagulation and endothelial markers.

In patients conditioned with TBI, a significant decrease in PC, PS and FVIII between day −10 and day 0 was observed. Patients after ATG had significantly increased TM and AT on day 0 in comparison to patients with a related donor. CRP was similar with regard to intensity of preparatory regimen or radiation.

Summary: In our cohort, in patients with acute leukemia subjected to HSCT, a distinctive pattern of coagulation abnormalities was observed.

PB 3.31-3

Prior intracerebroventricular administration with endothelin-1A receptor antagonists reduces hypercoagulable state and systemic inflammation, and result in survival time prolongation in rat heatstroke

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Endothelin (ET)-1 was increased in the plasma and cerebrospinal fluid of patients with cerebral ischemia, stroke, brain trauma or heatstroke. Endothelial cell activation/injury also occurred in heatstroke victims. Several lines of evidence indicate that animals share with humans almost the same heatstroke syndromes. In the rodent heatstroke model, significant decrements in both mean arterial pressure (MAP) and cerebral blood flow (CBF), but increments of hypercoagulable state and systemic cytokine levels are obtained during heatstroke. ET-1 mechanisms might be involved in heatstroke-induced circulatory shock, cerebral ischemia injury, hypercoagulable state and systemic inflammation. ET-1A receptor antagonism has been shown to be of benefit in the treatment experimental cerebral ischemia and injury. In the present study, we purpose that intracerebroventricular (icv) pretreatment with ET-1A receptor antagonists act to diminish the hypercoagulable state and reduce overproduction of pro-inflammatory cytokines, and eventually improve the arterial hypotension, cerebral ischemia and damage in rats of heatstroke. The results indicate that all heat-stressed rats displayed systemic inflammation and activated coagulation, evidenced by increased tumor necrosis factor-α and interleukin-1β, prothrombin time, activated partial thromboplastin time, and D-dimer, and decreased platelet count and protein C. Biochemical markers evidenced cellular ischemia and injury/dysfunction: plasma levels of blood urea nitrogen, creatinine, glutamic oxaloacetic transaminase, glutamic pyruvic transaminase, and alkaline phosphatase, and striatal levels of glycerol, glutamate, and lactate/pyruvate were all elevated during heatstroke. In contrast, the values of MAP, plasma levels of interleukin-10, and striatal levels of local CBF were all significantly lower during heatstroke. The circulatory dysfunction, systemic inflammation, hypercoagulable state, and cerebral ischemia and damage during heatstroke were all significantly suppressed by prior icv injection with ET-1A receptor antagonist (BQ 610, 0.05 mg/mL, 10 μL) at cerebral lateral ventricle. These findings indicate that pretreatment with ET-1A receptor antagonist via icv injection may ameliorate heatstroke victims by attenuating activated coagulation, and systemic inflammation during heatstroke.

PB 3.31-4

Pulmonary embolism that causes tricuspid regurgitation is associated with impaired L-arginine metabolism

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Background: Reduced L-arginine availability to the lung can disrupt nitric oxide biosynthesis and increase pulmonary vascular resistance. Most pulmonary vasodilation comes from NO made by eNOS, with L-arginine as its substrate. L-arginine is degraded by arginase-1, which is found in high concentrations in erythrocytes. eNOS is inhibited by dimethylated arginine (DMA) metabolites. We hypothesize that pulmonary embolism (PE) with pulmonary hypertension and tricuspid regurgitation (TR+) causes intracardiac hemolysis that increases plasma arginase-1, decreases plasma L-arginine and increases total DMA in plasma.

Methods: Case-control study of normotensive patients with suspected PE who underwent CT pulmonary angiography to evaluate for PE. All patients had transthoracic Doppler-echocardiography. TR+ was defined as a jet velocity >2.7 m/s. Patients diagnosed with PE and TR+ had follow-up measurements 3 months later. Plasma L-arginine was assayed by HPLC, arginase-1 concentration by ELISA, and asymmetric dimethylarginine (aDMA) with mass spectrometry. Erythrocytic carbonic anhydrase-1 concentration was measured with ELISA as a biomarker of hemolysis.
Results: We enrolled 96 patients, including 65 with no PE on CT angiography and 31 with PE+ and TR+. The following comparisons show data first for without PE vs. patients with PE+ and TR+ (PE- vs. PE+TR+). Patients with PE+TR+ had significantly elevated mean values of arginase-1, (24 ± 8 ng/mL for vs. 42 ± 12 ng/mL, $P < 0.01$ unpaired *t*-test), elevated total DMA (0.15 ± 0.04 μM vs. 0.27 ± 0.08 μM $P = 0.04$), lower plasma L-arginine (121 ± 8 vs. 65 ± 6 μM $P < 0.01$) and elevated plasma carbonic anhydrase-1 (45 ± 12 μg/L vs. 71 ± 27 μg/L). Patients without PE but who had TR+ for other reasons, did not have increased arginase-1, decreased L-arginine or elevated carbonic anhydrase values compared with patients without PE and no TR. At 3 month follow-up, the plasma arginase-1 and L-arginine concentrations were normal or near normal in the PE+TR+ patients (16 ± 15 μg/L and 105 ± 26 μM, respectively, $P < 0.001$ paired *t*-test).
Conclusions: Pulmonary embolism that causes significant tricuspid regurgitation is associated with increased plasma arginase-1 and reduced plasma L-arginine concentrations. These abnormalities corrected after 3 months of anticoagulation. These data support the hypothesis of disrupted L-arginine availability to the lung vasculature in acute severe PE. Hemolysis from tricuspid regurgitation and intrapulmonary shear forces manifested prior to treatment remain a possible causative factor.

PB 3.31-5
Structure-function relationship and action mechanism of Krait Natriuretic Peptide (KNP)

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Background: Hemodynamics is regulated by the interplay of several hormones and neuro-transmitters. Among the factors involved in lowering blood flow or vasodilation, natriuretic peptides (NPs) are a vital and potent class of peptide hormones. NPs lower blood pressure via vasodilation and increased renal water and salt excretion. Three NP isoforms- ANP, BNP and CNP- have been identified from mammals. NPs bind to their respective membrane bound guanylyl cyclase receptors to evoke cGMP as secondary messenger to elicit their function. These peptides have similar structures with a 17-residue ring and a short (5–6 residues) or no C-tail. The ring is associated with receptor binding, while the tail confers specificity. In this report, we have described the characterization of KNP from krait venom. In contrast to mammalian NPs, KNP has an extensively long 38- residue C-tail. Compared to elapid NPs, C-tail of KNP is predicted to form an α-helix compared to elapid NPs.
Aims: To understand the structure-function relationship and action mechanism of KNP.
Methods: KNP was heterologously expressed in *E. coli* and purified. KNP or ANP was infused into euvolemic rats through the femoral vein and the mean arterial pressure (MAP) was measured using a pressure transducer introduced into the femoral artery. The urine output of measured simultaneously. Further, the ability of KNP to relax endothelium-intact and denuded aortic strips was evaluated. Various truncated KNPs were synthesized and tested for their abilities to relax aortic strips.
Results: In experimental rats, infusion of KNP caused a prolonged drop in blood pressure (8 ± 2.3 mmHg) that continued after the infusion was stopped. Recovery time was 60 min, in contrast to ANP, which showed a sudden and potent (10.83 ± 1.92 mmHg) decrease in blood pressure with a recovery time of 25 min. The ex-vivo organ bath studies showed that the ability of KNP to relax the pre-contracted aortic strips was weaker than that of ANP, and KNP acts via a different mechanism. KNP evokes vasodilation via endothelium-dependent pathways in contrast to ANP, which mediates via endothelium-independent mechanisms. Nitric oxide, prostacyclin and hyperpolarization are necessary effectors of KNP-mediated vasodilation. The putative helical segment showed an equipotent vasorelaxation in an endothelium-dependent manner, while only the ring of KNP showed similar vasorelaxation as ANP in an endothelium-independent manner. Deletion of the C-terminal helical segment abrogates KNP's activity, suggesting it has a definitive role in vasorelaxation.
Conclusion: KNP causes a prolonged reduction in blood pressure when compared to ANP. The present results show that KNP has two pharmacologically active segments- the ring and the C-tail which elicit vasodilation through distinct means. The tail of KNP confers function to the full length KNP.

PB 3.31-6
Increase of angiogenic and angiostatic mediators in patients with idiopathic pulmonary fibrosis

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Objective: Idiopathic pulmonary fibrosis (IPF) is strongly associated with abnormal vascular remodeling. The aim of the present study was to investigate a potential dysbalance between angiogenic and angiostatic factors in this disease.
Methods: Sixty-four subjects with IPF and 10 healthy control subjects (60–70 years old) were prospectively included in this multicenter study. Plasma levels of vascular endothelial growth factor (VEGF), stem cell factor (SCF) and thrombospondin-1 were determined by ELISA. Comparisons between IPF and controls were made using the Mann-Whitney test. We also analysed these soluble mediators according to IPF severity (DLCO <40% or >40% predicted) through the same test.
Results: Plasmatic levels of the angiogenic VEGF were increased in IPF vs controls ($P = 0.0008$) as well as those of the angiostatic thrombospondin-1 ($P = 0.008$), irrespective of the severity of the disease as reflected by the DLCO threshold. No difference was either observed in SCF levels.
Conclusions: Molecular factors modulating angiogenic responses are dysregulated in patients with stable IPF with increases in VEGF and thrombospondin. The serial assessment of VEGF/thrombospondin-1 during the follow-up and the search for potential relationships with the functional and hemodynamic evolution of our cohort will give us hints to clinical implication of these results.

PB 3.32-1
Novel network biomarkers profile based coronary artery disease risk stratification in Asian Indians

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Background: Multi-marker approaches for risk prediction in Coronary Artery Disease (CAD) have been inconsistent due to biased selection of specific know biomarkers. We have assessed global proteome of CAD affected and unaffected subjects and developed a pathway network model for elucidating the mechanism and risk prediction for CAD.
Aim: In this study we analyzed global proteome of CAD affected and unaffected serum samples to identify new biomarkers for risk assessment and their network in the CAD.

Methods: 252 age and gender matched subjects (112 CAD affected without family history and 140 true controls) were selected for a case control study from ongoing Indian Atherosclerosis Research Study (IARS). The information regarding inclusion, exclusion criteria and process of sample collection, and data storage have been published (Indian Heart J, 2010;6:286–95). All the blood samples were collected after overnight fasting of 12–14 h and stored at −80 °C. All the serum samples were analyzed by Surface-Enhanced Laser Desorption/Ionization time of flight mass spectrometry (SELDI-TOF-MS) using CM10 cationic chips and tagident software was used for identification of specific protein peaks. The spectra of 112 subjects with CAD and 140 controls were analyzed. 56 CAD affected and 70 control samples were used as test set and same number as validation set in blind test. Support Vector Machine based feature selection method was used for risk assessment algorithm development. ELISA assay was performed for validation of HSP27 as potential risk predictor marker.
Results: A total of 67 m/z clusters were obtained of which 36 were significantly (P value <0.05) differentially expressed in the SELDI-TOF MS analysis. The network profile analysis was performed for all 67 proteins and we found that 17 proteins did not have any network partners, suggesting that these could be new proteins identified in CAD affected subjects. The SVM base risk assessment algorithm identified nine potential biomarkers from 36 identified proteins. These nine biomarkers represent seven different pathways related to stress and immunity (HSP27, Leukocyte specific transcript-1), coagulation (Plasminogen precursor activating peptide, Vitronectin10, Pallidin gene Isoform 2), infection and inflammation (Interferon Alpha 2), mitochondrial damage (Farataxin Chain 3), Calcium binding (Calmodulin like protein 4 isoform 3) and cell cycle (Cyclin dependent kinase 4 inhibitor B). Of the nine peaks used in the risk assessment model, the m/z corresponding to 22,859 was identified as stress related protein HSP27 with an odds ratio of 3.47 (95% CI 1.41–8.56, P value 0.007).
Conclusions: Based on our data, proteome profiling with SELDI-TOF MS and SVM feature selection Methods: Can be used for biomarker discovery and risk stratification in CAD. We also validated HSP27 for potential use in risk assessment for CAD.

PB 3.32-2

Human macrophages spontaneously differentiated from blood monocytes are heterogeneous and show distinct proteome profiling

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Background: Tissue macrophages are key players in many aspects of human physiology and diseases. Their biological activities include phagocytosis, killing of pathogens, antigen presentation, chemotaxis, and release of inflammatory and healing mediators. Classically, most resident macrophages are derived from circulating monocytes that, according to the microenvironment, differentiate into subpopulations that are phenotypically distinct. As an example, macrophage subpopulations evidenced within the atherosclerotic lesions contribute in a different way to plaque development/progression and even regression. Since human tissue macrophages are not easily accessible for isolation, various *in vitro* models are currently used. These models, however, mainly represented by monocytic cell lines or by monocytes differentiated in the presence of colony stimulating factors (CSFs), do not adequately reflect the heterogeneity and plasticity of tissue macrophages. With respect to monocytic cells, the type and duration of differentiation treatment heavily impact on the functional profile. On the other hand, monocyte exposure to CSF/cytokines directs macrophages to polarization into distinct phenotypes that have been classified as classical or alternatively activated (M1-pro-inflammatory and M2-non-inflammatory, respectively). These phenotypes, however, represents the extremes of a 'continuum' in a universe of activation states and do not necessarily reflect the functional skewing that occurs '*in vivo*'. We previously reported that human macrophages spontaneously differentiated from adhered monocytes (MDM) show two dominant and distinct morphotypes, that is, round- and spindle-shaped, co-existing in the same culture.
Aim: To delineate the proteome of these distinct MDM morphotypes.
Methods: Mononuclear cells were isolated from venous blood of healthy donors by Ficoll-Paque Plus and plated on Duplex-Dish50 mm. After 90 min non-adherent cells were removed and the adherent were cultured for 7 days in medium 199 supplemented with 10% autologous serum. MDM were then fixed and the distinct morphotypes were circumdissected and dislodged from the plate by means of a laser capture microdissection system (PALM MicroLaser Technologies, Germany). Cellular proteins were extracted from a total of 6000 MDM/morphotype (n = 10 different donors) and their proteome was determined and compared, both qualitatively and quantitatively, by a label-free mass spectrometry-based method. Briefly, proteins were digested with trypsin and peptides were analyzed in triplicate, in a data independent manner, by means of a nanoUPLC-ESI-qTOF instrument (Synapt-HD, Waters, Manchester, UK).
Results: One hundred-thirty two proteins were identified and quantified. Among them, 28 were more abundant in round and 28 in spindle MDM. More in detail, 11 proteins were unique in round- and 27 were unique in spindle MDM. Of shared proteins 17 were up-regulated in round and only one in spindle MDM. Specifically, distinctive bioprofiles were observed for proteins involved in lipid metabolism, cell motility, clearance of apoptotic cells and protection against stress condition.
Conclusions: We have delineated distinct reference protein maps that discriminate between the two MDM morphotypes. In particular the protein profile of round MDM is reminiscent of an non-inflammatory and reparative phenotype. Results obtained provide a consistent basis for comparative studies of macrophages in health and disease and make available a useful tool to address the effect of environment and drugs on macrophage proteome.

PB 3.32-3

Detection of protein S in necrotic core of atherosclerotic plaque and of lipoprotein-associated protein S in plasma

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Background: The internalization of lipoproteins in the vessel wall is an important step in the atherosclerotic plaque formation. Plasma PS is characterized by high affinity for negatively charged and oxidized phospholipid surfaces. Nevertheless, experimental evidence for interaction between PS and HDL and LDL is scanty, mainly due to methodological limitations.
Aims: To investigate the presence of PS in the atherosclerotic plaque, and the association of PS and plasma lipoproteins.
Methods: PS from the necrotic core of plaques obtained from patients who underwent carotid endarterectomy was investigated after (i) detergent-based extraction; (ii) liposome (80/20% and 100/0% phosphatidylcoline/phosphatidylserine) -and Hepes buffer- mediated recovery. LDL- and HDL-associated PS was obtained from fresh plasma samples (10 patients with chronic obstructive artheriopaty and 10 healthy subjects) by immunodepletion in microplates coated with antibody against ApoB100 (LDL) or SAA2 (inflammatory HDL). Plaque PS or lipoprotein-associated PS was evaluated by Western blot analy-

sis (WB) with polyclonal Ab. PS plasma levels were measured by ELISA.

Results: PS was clearly detected by WB in the necrotic core of plaques either after detergent extraction or phosphatidylserine rich-liposome recovery. Improved recovery of PS using liposomes displaying negative charged phospholipids may suggest the presence of not grossly misfolded PS molecules. Interaction of PS with plasma LDLs and HDLs, hypothesized to carry circulating PS into the vessel wall, was investigated. Levels of LDL- and inflammatory HDL-associated PS showed ample variation among patients (LDL-PS = 2.49 ± 3.19 μg/mL; HDL-PS = 3.19 ± 2.37 μg/mL) and healthy subjects (LDL-PS = 0.87 ± 0.59 μg/mL; HDL-PS = 1.13 ± 0.81 μg/mL). In patients, positive association with lipoprotein levels (LDL, $r^2 = 0.85$, $P = 0.0002$) was observed. Total-PS plasma levels did not differ between patients and healthy controls (55.2 ± 10.0 μg/mL and 52.5 ± 14.9 μg/mL). With the limitation of the assay we roughly estimated that lipoprotein-associated PS was a small fraction of total circulating PS in patients (mean PS-LDL = 4.4%, mean PS-HDL = 5.6%) and in healthy subjects (mean PS-LDL = 1.7%, mean PS-HDL = 2.1%).

Conclusions: These methodological approaches permitted to detect PS protein (i) from the necrotic core of plaques and (ii) associated with circulating lipoproteins. In this experimental setting a small and variable amount of total PS appeared to be associated to LDL and inflammatory HDL, and positive relation with lipoprotein levels was detectable in plasma of atherosclerotic patients. The hypothesis that alteration in the composition/oxidative state of the lipids might influence the amount of lipoprotein bound PS, and its delivery within plaque, deserves further investigation.

PB 3.32-4

Fractalkine is expressed in early and advanced atherosclerotic lesions and supports monocyte recruitment via CX3CR1

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Background: Rupture of carotid artery atherosclerotic plaque causes transient ischemic attack and stroke. Chemokines play a central role in atherogenesis and support the development of an unstable plaque phenotype in manifest lesions. The membrane-bound chemokine fractalkine (CX3CL1, FKN) is expressed in the inflamed vascular wall and absence of FKN reduces atherogenesis.

Aim: Whether FKN is expressed throughout all stages of atherosclerotic disease and whether it directly contributes to monocyte recruitment to atherosclerotic lesions is not known and therefore addressed in the present study.

Methods: We collected human atherosclerotic plaque material and blood samples from patients with carotid artery disease undergoing endarterectomy. Plaques were analyzed by immunohistochemistry and qPCR. Soluble FKN levels were measured by ELISA. To determine the role of the FKN-CX3CR1 axis for monocyte adhesion *in vivo* we then performed intravital videofluorescence microscopy of the carotid artery in ApoE−/− mice.

Results: We found that FKN is expressed at all stages of atherosclerotic lesion formation, and that the number of FKN-expressing cells positively correlates with the number of CX3CR1-positive cells in human carotid artery plaques. In the circulation, soluble FKN levels are significantly elevated in the presence of high-grade (sub-occlusive) stenosis. Notably, FKN-CX3CR1 interactions are critical for recruitment of circulating monocytes to the injured atherosclerotic vascular wall.

Conclusion: The soluble form of FKN appears to be a useful biomarker for patients with unstable atherosclerotic disease of the carotid artery. In addition, FKN is directly involved in the recruitment of monocytes to unstable, subocclusive atherosclerotic lesions, which renders this chemokine dyad an attractive therapeutic target.

PB 3.32-5

UPA/UPAR and vascular repair in atherosclerotic vessel wall

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Migration of vascular smooth muscle cells (VSMC) within the arterial wall is a crucial event for progression of atherosclerotic plaques as well as in vascular repair. LDL infiltrated in the vessel wall become aggregated (agLDL) and retained in the intima by binding matrix proteoglycans. agLDL are internalized by VSMC in a LRP-1 mediated process impairing vascular remodeling due to changes in cytoskeleton-dynamics and migration-kinetics. The urokinase (UPA)/UPA-receptor (UPAR) system plays a relevant role in human VSMC function during remodelling. The aim of this study was to investigate whether UPA-ligand binding is involved in the mechanism underlying the impairing effects of the agLDL in VSMC migration.

The effect of high LDL levels on VSMC migration were analyzed in aortic explants of rats fed high-fat diet in presence/absence of angiotensin II (angII, 200 ng/kg/min). An *in vitro* model of wound repair was used to investigate the role of UPA in the migration of human VSMC exposed to atherogenic levels of agLDL (100 μg/mL). UPA expression was knocked down by small interfering RNAs (siRNA) and UPA function blocked by specific antibodies. Protein subcellular localization during cell migration was assessed by confocal microscopy. For analysis of gene and protein expression real-time PCR and western blot were carried out.

Rats fed a high fat diet for 14 days had 10-fold higher cholesterol-LDL plasma levels, >60% decrease in aortic UPA protein expression ($P < 0.05$ vs. normolipemic group), and VSMC with significant lower capacity to migrate from the aortic explants (50% reduction vs. control group after 6 days in culture, $P > 0.05$). The administration of angII increased UPA-aortic levels and accelerated VSMC migration from the vascular explants. Using the *in vitro* model of wound repair we showed that agLDL inhibited human VSMC migration by a UPA-dependent mechanism. Exogenous native UPA (2 μg/mL) increased >2 fold ($P < 0.01$) cell migration of agLDL-VSMC without affecting migration kinetics of control VSMC. UPA-gene silencing reduced migration of control cells to the levels of agLDL-VSMC (>50% reduction vs. random siRNA transfected cells, $P < 0.05$). UPA levels (mRNA, protein) were significantly reduced in migrating VSMC exposed to agLDL. Confocal microscopy showed a 52% decrease in UPA-labelling at the leading edge of the migrating agLDL-VSMC compared to controls ($P < 0.05$), and a decrease from 32% to 23% in the colocalization with its receptor UPAR ($P > 0.05$). Rescue experiments showed that UPA (either by enhanced endogenous expression or by exogenous additions) acting as UPAR-ligand restored migration capacity of lipid-loaded cells to control levels. UPA effect on migration of agLDL-loaded cells occurs through an LRP-1 mediated mechanism.

UPA-ligand binding regulates VSMC migration, a process that is interfered by LDL. Thus tissue infiltrated LDL, through the abrogation of UPA function reduces VSMC-regulated vascular repair.

PB 3.32-6

Immunological tolerance to a combination of ApoB and HSP60 peptides decreases markers known to be associated with plaque instability in mice model of atherosclerosis

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Background: Atherosclerosis is a progressive, inflammatory disease characterized by accumulation of lipids, inflammatory cells and cytokines in the arterial wall. Clinical complications of atherosclerosis occur due to rupture of unstable plaques, responsible for 90% of fatal heart attacks and represent the most urgent unmet clinical need. Current treatments including lipid lowering drugs are not totally effective in stabilizing a vulnerable plaque and thus preventing their rupture. An effective immune based therapy will be the one that can stabilize the plaque in addition to reducing its development and progression. Vulnerable plaques are characterized by thin fibrous cap, higher expression of biomarkers such as tissue factor, calprotectin (Mrp8/14), matrix metalloproteases (MMPs), inflammatory cytokines, and apoptosis of intimal cells, leading to an expansion of a lipid-laden necrotic core. Mice models have provided valuable insights into pathological mechanisms of atherosclerosis but the occurrence of spontaneous plaque rupture is very rare in mice. We studied expression of markers associated with plaque instability in mice after prolonged hyperlipidaemia.

Aim: The objective of the present study was to assess the efficacy of mucosal tolerance to a combination of ApoB and HSP60 peptides on markers associated with plaque instability in double-gene knockout ($Apob^{tm2Sgy}$ $Ldlr^{tm1Her}$/J) mouse model.

Methods: Groups of $ApoB^{tm2Sgy}$/$Ldlr^{tm1Her-}$ mice were fed a high-fat diet for 10 weeks, to establish atherosclerotic lesion. In the last 2 weeks, animals were orally dosed with combination of ApoB and HSp60 peptides or KLH and continued on high fat diet for another 10 weeks. Quantification of atherosclerotic lesions was carried out in the aortic sinus sections stained with Elastic van Geison (EVG). Plaque necrosis was quantified by measuring the size of the hematoxylin and eosin-negative acellular area. Immunohistochemical analysis were carried out by indirect immunofluorescence, and gene expression was quantified using real time polymerase chain reactions.

Results: Prolonged hyperlipidaemia for 20 weeks resulted in significant increase in the serum lipid concentrations in control as well as treated animals. Oral tolerance to peptides resulted in a 60.8% reduction in the necrotic core area in lesion compared with KLH (6.5 ± 1.4% vs. 16.6 ± 2.9%, $P = 0.012$) though the total lesion area did not change significantly in both the groups. Expression of biomarkers of plaque instability and rupture MMP9 ($P = 0.035$), tissue factor ($P = 0.028$), and Mrp8/14 ($P = 0.045$) were significantly decreased in the tolerized animals compared to control. Oral tolerance to peptides was associated with increased number of smooth muscle cells (SMC) and collagen content in the lesion, while SMC apoptosis found to be significantly lower in peptide treated mice (16.55 ± 0.95 vs. 31.15 ± 2.97, $P < 0.001$), suggesting features of plaque stabilization. Tolerance was associated with increase in Treg cells and significant reduction in inflammatory cytokines in the lesion suggesting immune regulation.

Conclusions: Our results suggest that oral tolerance to combination of ApoB and HSP60 peptides promotes multiple features of atherosclerotic plaque stability, including increased plaque SMC and collagen content and reduction in markers associated with plaque instability and induce regulatory immune response to control inflammation

PB3.33 – ADAMTS13: Basic – I

PB 3.33-1

Pharmacokinetics of a recombinant ADAMTS13 in mice, rats and macaques

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Deficiency of ADAMTS13, a von Willebrand factor (VWF)-cleaving protease, is the key factor in the pathogenesis of thrombotic thrombocytopenic purpura (TTP), a life-threatening thrombotic microangiopathy. Baxter is developing a recombinant ADAMTS13 (rADAMTS13, a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13) for potential prophylaxis and treatment of TTP. The presented studies evaluated the pharmacokinetics of rADAMTS13 in mice, rats and macaques.

Ten ADAMTS13 ko mice (B6.129-$ADAMTS13^{tm1Dgi}$) per time point were administered rADAMTS1340 at 80 or 200 U/kg as an intravenous bolus injection and were bled by cardiac puncture 5 min to 42 h after dosing; Sprague Dawley rats ($n = 10$) received 80, 200 or 400 U/kg. Blood samples were taken from the tail artery before and 5 min to 72 h after dosing. Macaques ($n = 8$) received 200 U/kg followed by a dose of 1790 U/kg (limit dose) after a washout period of 2 weeks. Blood samples were taken from a suitable vein before and 5 min to 60 h after each dosing.

Citrated plasma samples were analyzed for ADAMTS13 activity (FRETS-assay) and ADAMTS13 antigen (ELISA). $AUC_{0\text{-tlast}}$ (the area under the concentration vs. time curve from 0 to the last measured time point), *in vivo* recovery (IVR), mean residence time (MRT) and terminal half-life (HL) were evaluated.

Results for activity and antigen were similar. For ADAMTS13 activity, dose-adjusted $AUC_{0\text{-tlast}}$ [hmkg] ranged from 0.098 in mice dosed with 40 U/kg to 0.401 in macaques dosed with 1790 U/kg. In mice, IVR was 51.9–62.8%, HL was 10.0–17.3 h and MRT was 13.6–21.1 h. In rats, IVR was 60.2–65.7%, HL was 16.7–25.6 h and MRT was 22.8–30.8 h. In macaques, IVR was 80.9–90.0%. Terminal HLs (24.6–27.9 h) and MRTs (29.6–36.2 h) were comparable between day 1 and day 15 for combined sexes for both analytes. The dose-adjusted pharmacokinetic curves of ADAMTS13 activity after doses of 200 or 1790 U/kg overlap well, suggesting a dose-proportionality in macaques for this dose range. The preclinical pharmacokinetic profile of Baxter's rADAMTS13 suggests that it is a promising drug candidate for future clinical trials.

PB 3.33-2

Preclinical safety of Baxter's recombinant ADAMTS13

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Baxter has developed a recombinant ADAMTS13 (rADAMTS13, a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13) product for treatment and routine prophylaxis of acute episodes of thrombotic thrombocytopenic purpura (TTP) in ADAMTS13 deficient patients. TTP is caused by excessive aggregation of platelets and accumulation of unusually large von Willebrand factor (VWF) multimers in small organ vessels, resulting in platelet thrombosis in the circulation followed by ischemic organ damage.

The preclinical safety of Baxter's rADAMTS13 was characterised in a 1-month repeat dose toxicity study in rats, an escalating dose and pilot 4-week repeat dose toxicity study in macaques, and a 1-month repeat dose toxicity study including cardiovascular/pulmonary safety pharmacology in macaques. Local tolerance was assessed in rabbits. Species suitability was demonstrated by VWF analysis *in vitro* and *in vivo*

under native conditions to present a worst case scenario for repeat dose toxicity studies.
Baxter's rADAMTS13 was well tolerated in rats at intravenous doses of 80, 400 and 800 FRETS-U/kg administered every third day for 1 month; thus, the NOAEL was the highest dose of 800 FRETS-U/kg. Intravenous treatment with rADAMTS13 at 800 FRETS-U/kg once weekly for 5 weeks (pilot repeat dose study) showed no adverse effects in macaques; the NOAEL for the escalating dose phase of the study was 1790 FRETS–U/kg. Treatment of macaques with rADAMTS13 at doses of 80, 200, and 400 FRETS-U/kg via bolus injection once weekly for 29 days did not reveal adverse findings; the NOAEL was the highest dose of 400 FRETS-U/kg. No adverse effects on respiratory and cardiovascular function in macaques were observed up to and including a dose of 400 FRETS-U/kg/day/week. As expected for a heterologous human protein drug, repeat doses of rADAMTS13 resulted in the formation of anti-drug antibodies specific for human ADAMTS13, and in neutralising human ADAMTS13 activity in animal models. No injection site reactions were observed in animals of either species. In rabbits, rADAMTS13 was well tolerated after intravenous (intended clinical administration route), intraarterial and paravenous administration.
In conclusion, no toxicity was observed for Baxter's rADAMTS13 in rats, macaques and rabbits even at the highest dose levels tested.

PB 3.33-3

Human umbilical vein endothelial cells, platelets and human tumor cell lines express isoforms 2 and 3 of ADAMTS13

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Background: ADAMTS13 contains 29 exons and spans 37 kb on chromosome 9q34. It has 1427 amino acids, 180 kDa of molecular weight and consists of a signal peptide, a propeptide, a metalloprotease and a disintegrin-like domain, a thrombospondin type 1 (TSP1) motif, a cysteine-rich domain, seven TSP1 repeats and two CUB domains. ADAMTS13 appears to be synthesized in the liver, particularly in hepatic stellate cells. Recent reports have shown that ADAMTS13 is synthesized and released from human megakaryocytes and platelets. ADAMTS13 mRNA is detected in almost every organ tissue by reverse transcriptase-polymerase chain reaction (RT-PCR), suggesting that the vascular endothelium may also be the source of plasma ADAMTS13. Given the large surface area of vascular endothelial beds, even a few endothelial cells producing ADAMTS13 at any moment may produce a significant contribution to plasma levels of ADAMTS13 activity. Therefore, any small change at the site of ADAMTS13 syntheses may be crucial in the pathogenesis of TTP and possibly other thrombotic diseases.
Aims: We analyzed alternative splicing of ADAMTS13 in Human Umbilical Vein Endothelial Cells (HUVEC), platelets and human tumor cell lines.
Methods: Experiments were performed in primary HUVEC obtained with 0.1% collagenase type 1. Platelets were isolated by differential centrifugation from total blood of healthy donors. Human cancer cell lines: Hep3b hepatoma and breast cancer cell lines (MCF-7 and MDA-MB-231).
RNA extraction and RT-PCR. Total RNA was extracted from cells using TRIzol reagent. The integrity of RNA was verified by 260/280 optical density ratios. The RNA sample was treated with DNase I, followed by phenol/chloroform extraction. The RNA was reverse transcribed into cDNAs using random primers. In our PCR experiments, we used the primers to amplify the portion of ADAMTS13 that is present in all the known isoforms, the expected size of ADAMTS13 isoform 1 was 610 bp, isoform 2 and 3 were 442 bp, and isoform IR25 was 1075 bp. We used Hep3b as a positive control for isoforms 2 and 3 mRNA expression.
The ADAMTS13 and β-actin mRNA was amplified for 30 cycles in the same reactions. The PCR products were analyzed on an agarose gel containing SYBR Safe.
Results: All PCR performed, except the controls, were positive for β-actin, showing the presence of amplifiable cDNA. We have detected, in our view for the first time, isoforms 2 and 3 in HUVEC and human platelets. In both breast cancer lines isoforms 2 and 3 mRNA were found to be strongly expressed in comparison to those of HUVEC and platelets, although they didn't reach the expression levels of Hep3b cells.
Conclusion: The presence of isoforms 2 and 3 in HUVEC and platelets from normal donors that we detected will enable us to assess the importance of the presence of ADAMTS13 alternative splicing products in tumor line cells and whether there is a correlation between the levels of expression of the isoforms and thrombotic events.

PB 3.33-4

FRET rather than CBA reflects ADAMTS13 proteolytic activity in thrombotic thrombocytopenic purpura patients with discordant measurements

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Background: Collagen binding assay (CBA) and fluorescence resonance energy transfer (FRET) are two widely adopted methods for the measurement of the plasmatic activity of ADAMTS13, the von Willebrand factor (VWF) cleaving-protease. Accurately measuring ADAMTS13 plasmatic activity is essential in the management of thrombotic thrombocytopenic purpura (TTP), a thrombotic microangiopathy (TMA) often characterized by severely reduced ADAMTS13 activity. The finding of severely reduced ADAMTS13 activity (activity below 10%) has important prognostic implications, being associated with a 10-fold increased risk of disease recurrence. Despite a good agreement between the two assays in the measurement of normal plasmas, discrepant results have been reported in up to 10% of TMA cases. The cause for the observed discrepancies is unknown, but it has been suggested that the use of a denaturing agent (<i.e. urea) in CBA may dissociate complexes between ADAMTS13 and anti-ADAMTS13 antibodies, thus generating erroneously high ADAMTS13 activity results.
Aims: To determine whether FRET or CBA results reflect ADAMTS13 activity levels *in vivo* in samples from autoimmune TTP patients with discordant results between the two assays. To evaluate the role of denaturing agents in the determination of discrepant results.
Methods: We analyzed up to 20 discordant samples (FRET <10% and CBA >20%) and 11 concordant samples (FRET and CBA <10%) with anti-ADAMTS13 antibodies from patients with autoimmune TTP, selected from the Milan TTP Registry (URL: http://www.ttpdatabase.org/). The analysis of the VWF multimeric pattern on discordant samples collected during disease remission (n = 13) and the measurement of ADAMTS13 activity under flow conditions (n = 10) were performed. FRET assay in presence of urea 1.5 M was performed in 19 discordant and 11 concordant samples.
Results: All discordant samples showed a ratio of high molecular weight VWF multimers higher than normal, due to the presence of ultra large VWF multimers. Under flow conditions, all tested samples showed reduced ADAMTS13 activity (range: 0–29%). In FRET experiments performed in presence of urea, ADAMTS13

activity level became detectable and/or overcame the 10% activity cut-off in 11 out of 19 discordant samples, whereas it remained undetectable in all 11 concordant samples tested (Fisher's exact test, $P = 0.0016$).

Summary/Conclusions: The presence of ultra large VWF multimers and the reduced ADAMTS13 activity observed under flow conditions in discordant TTP samples indicate a deficiency of the enzyme, supporting FRET over CBA results. This study showed that the presence of urea in CBA could lead to spurious and misleading results in measuring ADAMTS13 activity. ADAMTS13 activity assays, which do not require any denaturing agents, should be considered more reliable when assessing the presence of severe ADAMTS13 deficiency in patients with autoimmune TTP.

PB 3.33-5
The research of ADAMTS13 binding to human microvascular endothelial cells

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Background: ADAMTS13, as a specific VWF cleaving protease, could prevent the microvascular microthrombus formation caused by regulated release of ultra-large vWF multimers (UL-VWF), Previous studies mostly research ADAMTS13 function base on the large and medium-sized vascular endothelial cells. Our recent study found that ADAMTS13 could bind to the surface of human microvascular endothelial cells. Which maybe play an important role in the microvascular microthrombus formation. However, the binding mechanism remains unclear.

Aims: our study is aim to preliminary investigate that which domains of ADAMTS13 participate in the binding to the surface of human microvascular endothelial cells.

Methods: We used Hela cells to transfect and permanent express the full-length and C-terminal domain truncated types of human ADAMTS13 recombinant protein. Collected and concentrated cell supernatants, and purified recombinant proteins, then we used flow cytometry to detect the binding between recombinant ADAMTS13 proteins and human microvascular endothelial cells. We also used thrombopondin-1(from 0 to 200 nM) as a block factor, observed whether thrombospondin-1 influence the binding.

Results: We found that full-length ADAMTS13 protein could bind to the surface of human microvascular endothelial cells, the binding rate was up to 49.1%, While, the binding of full-length ADAMTS13 protein to these cells would drop to 26.5% with 200 nM thrombospondin-1 blocking. If we truncated C-terminal domain of ADAMTS13 (from tsp8), the binding was just down 4.8%.

Conclusions: The study suggests that ADAMTS13 could bind to the surface of human microvascular endothelial cells, and this binding maybe rely on the N-terminal and thrombospondin-1 repeats domains of ADAMTS13 protein.

PB 3.33-6
Characterization of IgG anti-ADAMTS13 autoantibodies isolated from patients with acquired TTP and a healthy donor pool

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Background: ADAMTS13-neutralizing IgG autoantibodies are the major cause of acquired thrombotic thrombocytopenic purpura (TTP). So far, the properties of such antibodies have only been assessed using plasma samples of patients with acquired TTP.

Aim: We sought to functionally characterize affinity purified anti-ADAMTS13 antibodies of the IgG class from patients with acquired TTP, and a healthy donor pool as control.

Methods: Plasma collected after plasma exchange from three patients with acquired TTP and a plasma pool from 45 randomly selected healthy blood donors (HD) were used to isolate anti-ADAMTS13 IgG antibodies. Purification was carried out by a two-step procedure using an ADAMTS13 affinity matrix followed by protein G chromatography. Total IgG antibody yield, isotype distribution and ADAMTS13 specificity were determined by commercial or in-house enzyme-linked immunosorbent assays (ELISA). The binding kinetics of the isolated IgGs to ADAMTS13 were evaluated by BIAcore and ELISA. Their inhibitory activity towards ADAMTS13 was analyzed using FRETS-VWF73 assay and residual collagen-binding activity assay (CBA). The ADAMTS13-VWF binding assay was employed to assess the competitive inhibition constants (Ki) of the purified antibodies. Interaction studies with fragments of ADAMTS13 were used to determine the domains of ADAMTS13 that these IgGs target.

Results: IgGs specific to ADAMTS13 were recovered from the three TTP patients as well as from the HD plasma pool. All four subclass isotypes were present, with IgG2 being the dominant subclass, followed by IgG1 and either IgG3 or IgG4. Biacore measurements led to calculated K_D values for the IgGs isolated from TTP patients towards ADAMTS13 of around 1 nM. Anti-ADAMTS13 IgG subclass-specific ELISA revealed apparent K_D values in the nanomolar range for all IgG subclasses of these IgGs. Significantly lower affinities were determined for the IgGs from the HD pool. The TTP-associated IgGs displayed inhibitory activity ranging from 0.1 to 0.6 BU/μg IgG whereas the HD IgG did not inhibit ADAMTS13 activity in either of the two methods applied. Furthermore, only the IgGs from the TTP patients interfered with the interaction of ADAMTS13 and VWF, with a mean Ki of 0.35 nM. Epitope mapping revealed a polyclonal nature for all of the antibodies tested.

Conclusions: We present the first comprehensive study on affinity-purified anti-ADAMTS13 IgG autoantibodies from patients with acquired TTP. We also provide evidence that a subpopulation of healthy individuals possesses non-inhibitory ADAMTS13-specific IgG autoantibodies, suggesting that preexisting natural anti-ADAMTS13 B cell clones could be expanded during the development of acquired TTP.

PB3.34 – Fibrinolytic system: Basic – III

PB 3.34-1
Fractal kinetic models of plasmin-catalyzed dissolution of fibrin

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Background: Intravascular fibrin clots are resolved through the proteolytic action of plasmin acting at the interface of gel-phase substrate and fluid-borne enzyme. The classic Michaelis-Menten (MM) kinetic scheme cannot describe satisfactorily this heterogeneous-phase proteolysis, because it assumes homogeneous well-mixed conditions. A more suitable model for these spatial constraints, known as fractal kinetics, includes a time-dependence of the Michaelis constant $Km = Km0.(1 + t)\exp(hF)$, where hF is a fractal exponent of time, t. Furthermore, a realistic kinetic model should take into account

sequestration of plasmin due to kringle binding to C-terminal lysines (CTL), newly exposed during fibrin degradation.
Aim: The aim of the present study is to build up and experimentally validate a mathematical model that adequately describes the kinetics of plasmin-catalyzed fibrin dissolution and thus contributes to a better understanding of the factors that influence plasmin efficiency at the fluid-gel interface.
Method: Two modifications of the basic MM scheme were introduced: a term reducing the enzyme concentration through rapid equilibrium binding of plasmin to continuously increasing unproductive sites including an association constant *Ka* and a fractal exponent, *hF* resulting in apparent *Km*, which accounts for the time-dependent clustering of the enzyme. A broad range of biochemical (fibrin turbidimetry, densitometry of electrophoretic samples, amidolytic assay on synthetic plasmin substrate) and imaging (atomic force microscopy, AFM; confocal laser microscopy, CLM) techniques were applied to test the predictions of the fibrinolytic model. The power of the predictions was assessed using known modifiers of fibrinolysis; ε-amino caproic acid (EACA) which blocks the kringle-dependent binding and carboxypeptidase B (CPB) which removes CTLs. The clustering of plasmin was evaluated with AFM using nanogold-labeled anti-plasmin antibodies in an experimental setup where plasmin was applied to a mica surface decorated with fibrinogen using microcontact printing and with CLM using fluorescent protein-fusion derivative of plasminogen.
Results: Using a range of fibrin and plasmin concentrations, variants of the kinetic model were fitted to the turbidimetric data for lysis of fibrin by plasmin applied to the surface of the clots. A global fit to 32 time-course curves with 90 measured points each yielded four model parameters with optimal values $Km0 = 1.5\ \mu M$, $hF = 0.25$, $Ka = 1.3\ \mu M^{-1}$ and $k_{cat} = 32.4\ min^{-1}$. Addition of 1 mM EACA or 8 U/mL CPB increased the lysis rate, which could be satisfactorily explained with unchanged *Km0* and k_{cat} model parameters accompanied by a decrease in *hF* to 0.031 and in *Ka* to $0.001\ \mu M^{-1}$ in line with the interpretation of these parameters as measures of spatial clustering (*hF*) and sequestration of enzyme molecules in solution (*Ka*). This concept gained further support from imaging techniques. AFM images revealed significant changes in plasmin distribution on the patterned fibrinogen surface: the ratio of surface occupied by plasmin/intact substrate area decreased by 25% over a 5-min interval in line with the time-dependent clustering of fluorescent plasminogen in CLM.
Conclusion: These data, from multi-faceted, complementary approaches, support a mechanism for time-dependent loss of plasmin activity resulting from spatial redistribution in this heterogeneous system. Thus, plasmin-CTL binding retards lysis, opposing the stimulatory effect of CTLs in plasminogen activation.

PB 3.34-2
DNA methylation and regulation of t-PA gene expression

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Background: Tissue-type plasminogen activator plays an important role in the removal of intravascular fibrin deposits and has several important physiological roles and pathological activities in the brain. Its expression by many other cell types and the diversity of regulatory mechanisms impacting on t-PA gene expression suggests that t-PA has many roles outside the vascular and central nervous system. For an understanding of these roles it is important to better understand the relationship between cell-type specific epigenetic gene modifications of the t-PA gene and regulation of t-PA gene expression.
Aim: To determine whether there is a relation between basal (unstimulated) t-PA gene expression and DNA methylation at the proximal t-PA promoter and at the multi-hormone responsive enhancer (MHRE) located 7 kbp upstream.
Methods: The methylation state of 16 CpG residues in the proximal t-PA gene promoter (from −647 to +94 with respect to the transcription initiation site) and of 12 residues in the MHRE (from −7466 to −7227) was determined by direct sequencing of bisulfite treated DNA, obtained from five primary human cell types (umbilical vein endothelial cells, monocytes, astrocytes, fibroblasts, and hepatocytes, and from five human transformed cell lines (Bowes melanoma, HT1080 fibrosarcoma, NB4 acute promyelocytic leukemia and HepG2 and HuH7 hepatoma). Basal t-PA expression was assessed by measuring t-PA antigen in 24 h cell conditioned media.
Results: Expression of t-PA by Bowes melanoma cells was 50 fold higher than in the second highest t-PA producer cell type. Melanoma DNA was fully unmethylated both in the proximal t-PA gene promoter and the MHRE region. Expression of t-PA was low in monocytes, hepatocytes, HuH7 hepatoma cells and NB4 cells and DNA of these cells was mostly methylated in the proximal promoter. Intermediate levels of t-PA expression were observed with the other cell types. In these cells, the nine CpG's in the promoter region from −121 to +94 were unmethylated, whereas the seven CpG's from −647 to −366 were in general methylated, except in HT1080 cells, in which CpG's −366 and −537 were methylated and the intervening two CpG's (at −452 and −421) unmethylated. The pattern of CpG methylation in the MHRE was more complex, with highly methylated CpG's often adjacent to unmethylated CpG's. No relation between basal t-PA gene expression and CpG methylation at the MHRE was evident.
Conclusions: Our results suggest that an unmethylated state of the promoter region from −121 to +94 is required for enabling t-PA gene expression. An unmethylated state of the upstream promoter region (from −647 to −366) was associated with extremely high t-PA expression in melanoma cells. The methylation state of the MHRE is very complex and may change from one CpG residue the other. There is no clear-cut relation between basal t-PA gene expression and methylation state of individual CpG's in the MHRE.

PB 3.34-4
Covalently linking heparin to antithrombin diminishes the protective effect of fibrin on plasmin

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Background: Antithrombin (AT) is the major regulator of coagulation enzymes *in vivo*. We have previously demonstrated that AT, in the presence of unfractionated heparin (UFH), can inhibit free plasmin, as well as reduce generation of plasmin from purified plasminogen. Additional studies utilizing a covalent complex of AT and UFH (ATH) revealed that plasmin generation in the presence of a fibrin clot was reduced to a greater degree by the ATH compared to AT + UFH. Furthermore, it is well known in literature that fibrin protects plasmin from inhibition by inhibitors such as α2-antiplasmin. However, little is known about the ability of AT + UFH, or ATH, to inhibit plasmin in the presence of fibrin.
Aim: To determine the rate of inhibition of plasmin in the presence of fibrin by AT + UFH vs. ATH.
Methods: Discontinuous second order rate constant assays were used to determine the overall *k2* values for inhibition of plasmin ± fibrin by AT + UFH vs. ATH. Briefly, 200 nM fibrinogen was converted to fibrin by reaction with 0.6 U/mL Ancrod for 15 min at room temperature. Fibrin was then reacted with 10 nM plasmin in six different wells of a 96-well plate, followed by addition of inhibitors (100 nM AT + 3000 nM UFH or 100 nM ATH) at specific time intervals (buffer was added to the last well as a control). The reactions were neutralized by the simultaneous addition of polybrene and S-2236 in buffer. Residual plasmin activity was measured at 405 nm for 10–30 min, and *k2* values determined from plots of $\ln(v_t/v_o)$ vs. time.
Results: The *k2* values ($x10^6$ M/min) for inhibition of free plasmin by AT + UFH and ATH were 5.74 ± 0.28 and 6.39 ± 0.59, respectively.

However, when fibrin was placed in the system, a mild protective effect by the fibrin was observed for ATH reactions ($k2$ value = 3.28 ± 0.36, $P < 0.006$). Whereas for AT + UFH, the $k2$ values were barely measurable when fibrin was present ($k2 < 0.1$). This suggests that fibrin almost completely blocks plasmin from inhibition by AT + UFH.
Summary/Conclusion: From our study, we demonstrate that fibrin almost completely protects plasmin from inhibition by AT in the presence of excess UFH. Moreover, covalently linking UFH to AT, as in ATH, diminishes the protective effect and restores the inhibition rates to approximately 50% that of free plasmin. Similar to our previous work, this suggests that the UFH in AT + UFH can dissociate and form non-productive complexes with fibrin. Such interactions may result in either repulsive effects that prevent additional AT + UFH from inhibiting plasmin or the AT being left in a non-activated state. However covalent linkage of UFH to AT, assists access and subsequent inhibition of plasmin bound to fibrin.

PB 3.34-5

Zinc inhibits fibrinolysis by attenuating plasminogen activation and fibrin degradation

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Background: Tissue plasminogen activator (tPA) initiates fibrinolysis by converting plasminogen (Pg) to plasmin (Pn), which then degrades fibrin (Fn). This process is regulated by Fn, which promotes Pg activation, and by plasminogen activation inhibitor 1 (PAI-1) and antiplasmin, which inhibit tPA and Pn, respectively. Zinc (Zn), which circulates at a concentration of 10–20 μM, modulates platelet activation and coagulation. Whether Zn affects fibrinolysis is uncertain.
Aim: To examine the effect of Zn on fibrinolysis.
Methods: To determine the affinity of Zn for fibrinolytic proteins, Zn was bound to FluoZin-1 and fluorescence was monitored as the mixture was titrated with the protein of interest. Chromogenic or fluorogenic assays were used to examine the effect of Zn on tPA and Pn activity, and the rates of their inhibition by PAI-1 and antiplasmin, respectively. Turbidity was monitored as fibrinogen (Fg) was clotted with thrombin and degraded by tPA/Pg, Pn or trypsin in the absence or presence of Zn. A Pn-directed substrate was included to examine the effect of Zn on the kinetics of Pg activation.
Results: Zn bound Fg, tPA, and Pn with K_d values of 0.91, 0.14, and 0.05 μM, respectively, but did not bind Pg. Zn had no effect on Pn or tPA chromogenic activity, or on rates of Pn or tPA inhibition by antiplasmin and PAI-1, respectively. Nonetheless, Zn produced a significant ($P < 0.05$) 2-fold reduction in the catalytic efficiency of Pg activation by tPA in the absence or presence of Fg or Fn, mainly reflecting a decrease in k_{cat}. Time to lysis of Fn clots by Pg/tPA or by Pn was significantly ($P < 0.05$) prolonged by 24% and 17%, in the presence of 2 μM Zn compared with that determined in its absence. In contrast, Zn had no effect on lysis times with trypsin.
Summary and Conclusions: Although Zn binds tPA and Pn, it does not affect their chromogenic activity nor does it impair their inhibition by PAI-1 and antiplasmin, respectively. However, Zn inhibits tPA activation of Pg and delays lysis of Fn clots by tPA/Pg. Lysis of Fn clots by Pn also is delayed in the presence of Zn. This likely reflects modulation of Pn activity rather than altered Fn structure because Zn has no effect on Fn lysis by trypsin. Thus, Zn modulates fibrinolysis; an effect that may be particularly important at sites of injury where Zn is released from activated platelets.

PB 3.34-6

Plasma proteins and soluble proteins from red blood cells cooperatively regulate wound healing process

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Background: The wound healing process is triggered by the restoration of endothelium and is regulated by various factors including plasma proteins and blood cells. The process involves endothelial cells, blood vessel constituting cells, such as smooth muscle cells and fibroblast cells, and extracellular matrices. We have previously reported that plasminogen accelerated the recovery of endothelial cells from scratch wounding using perfusion cell culture system. And this process was controlled by a temporal interaction between the endothelial cells and plasminogen. Other recent studies showed that the lysate of red blood cells affected the wound healing process by modulating the expression levels and activities of extracellular matrix components.
Aims: To investigate the effect of proteins from red blood cells on the process of wound healing, we examined the effects of fractionated proteins derived from red blood cells on the growth and motility of endothelial cells, smooth muscle cells, and fibroblast cells. We also investigated the behavior of smooth muscle cells and fibroblast cells in responding to the fractionated proteins under perfusion cell culture system.
Methods: Soluble proteins from red blood cells were subjected to ammonium sulfate precipitation to eliminate hemoglobin, and the resultant solution was fractionated by ion exchange column. The fractions were applied to the culture media, then the proliferative effect on each cells were examined. Motility activities on those cells were measured by Modified Boyden Chamber assay and the collagen gel contraction assay was also performed. In addition, proteinase activities of cultured media were measured by use of synthetic substrates and gelatin zymography. We also performed perfusion cell culture to investigate the effects of the fractions on smooth muscle cells.
Results: We found that one of the fractions suppressed the growth and motility activities of endothelial cells, on the other hand the same fraction extensively and weakly promoted those activities on smooth muscle cells and fibroblast cells, respectively. The same fraction stimulated the collagen gel contraction induced by fibroblast cells, meanwhile it inhibited the recovery of endothelial cells from wounding under perfusion culture conditions. These bioactive fractions also influenced protease activities such as matrix metalloproteinases. By using perfusion culture system, co-culture of smooth muscle cells and endothelial cells resulted in activation of protease activities including matrix metalloproteinases. In addition, the proteins from red blood cells induced the morphological changes of smooth muscle cells under perfusion culture conditions. This suggests that smooth muscle cells play a role in reconstitution of blood vessel.
Conclusions: Our result suggests that red blood cells contain some proteins which regulate the process of wound healing by affecting the function of blood vessel constituting cells, namely, endothelial cells, smooth muscle cells and fibroblast cells. And these processes are influenced by protease activities of various plasma proteins.

PB3.35 – Haemophilia A: Clinical – IX

PB 3.35-1

DDAVP treatment: clinical efficacy and molecular mechanisms of response in mild haemophilia A patients. A single center experience

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Background: Although desmopressin (DDAVP) is considered the treatment of choice for a large number of patients with mild hemophilia A for its safety and effectiveness, and several thousands of them have been treated with this synthetic compound worldwide during the last 35 years, several aspects of DDAVP therapy are still unclear such as the rate and type of response and the molecular and clinical determinants of its efficacy.

Aims: In order to elucidate these issues, we have conducted a retrospective study on all mild hemophilia A patients followed at the hemophilia treatment center (HTC) of Parma and treated with DDAVP.

Methods: We included in the study all consecutive patients with mild hemophilia A (basal FVIII:C levels >5 to 40%) regularly examined at the HTC of Parma since 1985 and with DDAVP test. Age of patients at DDAVP test was evaluated and the disease characteristics included the basal level of the deficient factor and molecular diagnosis (F8 mutation).

Our DDAVP test protocol consisted in a test dose of 0.3 mg/kg body weight administered i.v. from 1984 to 1995 and since 1996 by subcutaneous route. Patients were defined as complete responders with FVIII:C 50 IU/dL or higher after DDAVP; partial responders with FVIII:C lower than 50 IU/dL but increased at least 3-fold and with levels >30 IU/dL; and non-responders with neither criterion.

The definition of clinical efficacy of DDAVP treatment was defined as follows: *complete efficacy* resolution of bleeding after one DDAVP treatment; *partial efficacy*: resolution of bleeding after 2–4 DDAVP treatments; *no efficacy*: need for FVIII concentrate use. All side effects related to DDAVP infusion were also recorded. Statistical analysis was performed using Student *t* or Chi-square tests. A P value <0.05 was considered significant.

Results: Seventy-five patients were enrolled and underwent to DDAVP test with a complete or partial response in 76% (57/75) of them. The response to DDAVP was significantly correlated with patients' age (median age of responders and non-responders: 24 years and 18 years, respectively; $P = 0.04$), with the type of mutation (all the 10 patients with the mutation in the promoter region were non-responders) and with basal FVIII:C levels (complete responders had higher median than partial responders 0.18 IU/mL vs. 0.11 IU/mL). During the 12-years follow-up period, 82 of the 237 (35%) bleeding episodes occurring in 27 responder patients were treated with 246 DDAVP infusions with an overall complete or partial efficacy of 92% (75/82). Overall 142 events (30 traumas, 11 surgeries, 67 dental procedures, 17 invasive procedures, 17 home treatment post-FVIII concentrates) were managed with 253 prophylaxis DDAVP infusions with an hemostatic efficacy in 96% of the cases. No severe adverse reactions to DDAVP administration were recorded during the study period.

Conclusions: In conclusion, the results of our study clearly document the safety and efficacy of DDAVP as treatment or prevention of bleeding in mild hemophilia A patients and encourage the even more widespread use of such therapeutic strategy among physicians operating at the HTCs.

PB 3.35-2

Clearance during continuous infusion of recombinant factor VIII products to 27 patients with hemophilia A at Nagoya University Hospital

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Background: Coagulation factor products are usually administered to hemophiliacs as a bolus injection. However, for cases of massive bleeding and major surgery, these drugs are increasingly being administered by continuous infusion to maintain coagulation factor activity at a constant level.

Aims: Determination of the clearance (CL) value is very important for undertaking continuous infusion more safely and ensuring reliable hemostasis management. We reviewed factor that influenced CL value.

Method: We used recombinant factor VIII (FVIII) products for continuous-infusion hemostatic therapy in 33 cases of hemophilia A (27 patients). We calculated CL values and analyzed factors impacting CL values. The equation used for the calculation was CL = infusion rate (U/kg/h)/FVIII factor level (U/mL). Because the drug concentration needs to be assessed in the steady state, we calculated CL using activity values measure at ³70 h after starting continuous infusion. Factors that were analyzed for possible impacts on CL were the type of administered FVIII product, patient age, severity of hemophilia, presence/absence of infection (Hepatitis C Virus [HCV] and Human Immunodeficiency Virus [HIV]) and duration of continuous infusion. Fifteen cases were administered second-generation full length recombinant products, while 18 received third-generation full length recombinant products. For age strata, 1 case was in the 20s, 3 in their 30s,10 in their 40s, 9 in their 50s, 7 in their 60s, 2 in their 70s and 1 in their 80s. Disease severity was severe in 27 cases, moderate in 2 and mild in 4. Twenty cases were HCV-positive, including nine with cirrhosis, and 13 were negative. Nine cases were positive for HIV, while 24 were negative.

Result: Adequate hemostatic management was achieved in all cases. No cases developed adverse experiences, such as bacterial infection, thrombophlebitis, etc. In the analysis of factors possibly impacting CL, a significant difference ($P < 0.05$) in mean (±standard deviation) CL was found between administered drug products: 2.9 ± 1.0 mL/kg/h for second-generation full length recombinant products; and 4.1 ± 1.9 mL/kg/h for third-generation full length recombinant products. No other analyzed factors showed any significant impact. For three cases, the pharmacokinetics were analyzed and CL values were calculated and compared with CL values during continuous infusion. In each case, CL values obtained by pharmacokinetic analysis were lower than values during continuous infusion.

Summary: These findings reveal a large difference in CL as a function of the specific FVIII product used for continuous infusion treatment of hemophiliacs. We conclude that there is a need to set continuous infusion dosages. Moreover, CL values determined by pharmacokinetic analysis may not accurately reflect CL values during continuous infusion.

PB 3.35-3

NO-PEACKS: Non-interventional observations of practical implementation, efficacy, and safety of continuous infusion with full-length recombinant factor VIII formulated with sucrose in surgery

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Background: Continuous infusion (CI) of full-length recombinant factor VIII formulated with sucrose (rFVIII-FS) during surgery has been shown to be effective in preventing high peaks and low troughs of FVIII levels in a clinical trial setting. However, there is controversy regarding whether CI along with surgery or surgery in general would trigger FVIII inhibitors and whether CI is feasible in real practice.
Aims: The objective of this non-interventional, observational, prospective study was to collect data on the feasibility, efficacy, and safety of CI with rFVIII-FS in the real-practice setting, and to monitor for inhibitor formation.
Methods: Patients with severe hemophilia A (FVIII <1%), >150 exposure days to FVIII, and no positive inhibitor history from seven European countries who were administered CI with rFVIII-FS during and after surgery were enrolled. The observation period covered the entire course of CI and up to 3 months after surgery. Information such as infusion rates, number of used vials, additional blood product use, and FVIII measurements were recorded. Efficacy and tolerability of CI with rFVIII-FS were assessed. All adverse events (AEs) and results of inhibitor assays were documented. Informed consent was obtained by all study participants. The study was approved by the relevant ethics committees or authorities.
Results: Twenty-eight surgery cases were analyzed from 25 enrolled patients (median age, 49.4 years; range, 10–75 years). Of these patients, 64% had comorbidities. Median in-hospital stay due to the surgery was 9.0 days (range, 1–23 days). Median surgery duration was 120 min (range, 20–331 min). Twenty-six surgeries were elective; two were performed for emergency reasons. The majority (n = 21, 75%) were orthopedic operations. Median blood loss during surgery was 200 mL (orthopedic surgeries, 300 mL; non-orthopedic surgery, 0 mL). Median duration of CI was 128 h (range, 9.0–430.8 h). Median total rFVIII-FS consumption with CI was 376.0 IU/kg (range, 157.9–3605.6 IU/kg). One or two additional bolus injections were required during six orthopedic surgeries (21.4% of all surgeries). During three orthopedic surgeries (10.7% of all surgeries), a maximum of two blood transfusions was required. A median of nine FVIII level measurements per surgery were performed (range, 2–23). For all cases, the proportion of all FVIII level measurements in expected range per case was higher than 50%. Efficacy was considered good or excellent in 89.3% of cases, and tolerability was good or excellent in 100% of cases. Four non-drug-related AEs were the only AEs reported. Of these, two were considered serious AEs: a post-procedural hemorrhage and a peripheral artery aneurysm. None of the patients developed inhibitors during or after surgery.
Summary/Conclusions: The results of the study indicate that CI with rFVIII-FS was feasible, effective, and safe in the analyzed surgical cases. No inhibitors or drug-related AEs occurred in this CI study.

PB 3.35-4

Knowledge, attitudes, and behaviors of adolescents in developing world hemophilic population: a survey

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Background: Hemophilia patients must receive care from healthcare workers with expert knowledge of the bleeding disorder. In developing country like India many barriers have to be overcome to improve quality of life in hemophiliacs.
Aim: This survey focuses on knowledge about, attitudes towards and behaviors associated with key prevention activities among adolescents with hemophilia in India.
Methods: A team composed of experts working directly in hemophilia care conducted various hemophilia awareness camps for general population as well as for medical fraternity in previous 2 years. A survey form which included a set of questionnaire including both structured and open-ended questions were filled by these patients.
Results: The 167 (Hemophilia A and B-150 and 17 respectively) adolescents survey respondents ranged in age from 10 through 14 years were 109 (65.26%) and 15–19 years were 68 (40.7%) patients. Severe, moderate and mild hemophilia were 120 (71.8%), 31(18.56%) and 10 (5.9%) patients respectively. 82 patients had family history of hemophilia 36/167 (21.5%) patients did not know their blood group. Joint disease (target joint) in 116 (69.4%) followed by bleeding episodes in 12.5% were the primary concerns of most of the adolescents. Only two patients reported hepatitis C as a primary concern and none showed any concern for HIV or Hepatitis B. More than a three fourth (80%) of the adolescents believed that joint disease is not preventable; only 10% said it is an extremely preventable. Although hepatitis was a clear threat to this hemophilic cohort, majority of adolescents (90%) were unaware about the transmission route of Hepatitis B and C. Only10% of the respondents treated bleeding episodes within 1 h. Only 4.1% had facility of factor at their home in case of emergency situation. Majority (>90%) of being treated on demand therapy, that is also for severe bleeds only. They did not believe the episode was serious enough to require treatment. This finding was also related to the most prevalent response among the adolescents: that they did not always recognize a bleeding episode when it occurred.12 (7.1%) patients were on secondary prophylaxis and none was on primary prophylaxis. Icepacks were used by 115/167 (68.8%) of patients for their joint bleeds and tranexemic acid for mucosal bleeds by 60% of patients. Majority of adolescents managed hemophilia by avoiding physical activity and did not engage in any strenuous or moderate physical activity on aregular basis. More than 90% knew the diagnosis (name) of their disease and had informed their respective schools and collages about the same, but hardly anybody knows about the course of their disease.
Conclusions: Adolescents with hemophilia need help understanding that they can prevent complications especially joint damage. We emphasize messages for a hemophilia prevention campaign, including course of disease, recognition of bleeds, exercising to ensure healthy joints and treating bleeding episodes early and adequately.

PB 3.35-5

Bleeding risks in male patients and carriers with the valine to alanine 2016 form of mild hemophilia A

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Background: The phenotypic expression of mild hemophilia A differs significantly with moderate-severe form of hemophilia A. The difference in clinical impact of the disease has led to delays in diagnosis and treatment neglect resulting in potential serious morbidity and mortality. The valine to alanine 2016 (Val2016Ala) form of mild hemophilia A is an X-linked recessive disease with a high prevalence in Newfoundland and Labrador.

Aims: To determine the clinical manifestations and bleeding risks of affected male patients and carriers with the Val2016Ala form of hemophilia A.
Methods: 61 affected male patients with the Val2016Ala mutation, 44 unaffected males, 88 carriers and 65 unaffected females were recruited for analysis. Participants were scored based on a modified bleeding score from the previously published MCMDM-1 VWD score. Each category was scored as per data collected from a bleeding history questionnaire and chart audit. Additional data for health care utilization such as emergency room (ER) visits for bleeding, amount of packed red blood cell (PRBC) transfusion and number of hospitalizations for bleeding were also recorded. Separate analysis was carried out for each gender group.
Results: Affected males had significantly higher mean cumulative (27.64 vs. 82) and non-cumulative bleeding scores than control males (14.31 vs. 0.84) ($P < 0.01$). They also had a much higher likelihood of requiring surgical hemostasis, blood transfusions and/or replacement therapy for the following symptoms: (i) tendency to bruise (21.3% vs. 0%), (ii) gum bleeds (21.3% vs. 0%), (iii) gastrointestinal (GI) bleeds (18% vs. 0%), (iv) muscle hematoma (13% vs. 0%), (v) hemarthrosis (17% vs. 0%) and (vi) abnormal blood loss from dental work (55.7% vs. 0%), (vii) surgery (42.6% vs. 2.3%), and (viii) injury or trauma (50.8% vs. 0%). There were no significant differences for central nervous system (CNS) bleeds (3.3% vs. 0%) ($P = 0.4$). Affected males were also more likely to require hospitalizations, ER visits and PRBC transfusions for bleeding ($P < 0.05$). Carriers did not have a statistically significant difference in comparison with control females in any of the bleeding categories.
Summary: With the usage of a modified previously published bleeding score, we were able to evaluate the clinical characteristics of patients with the Val2016Ala from of hemophilia A. The affected male cohort unequivocally had significantly higher rates of bleeding as compared to the unaffected male cohort. Contrary to prior studies on mild hemophilia A, carriers status does not seem to confer any higher risk of bleeding in our cohort.

PB 3.35-6
Retrospective analysis of 1226 Chinese patients with haemophilia in a single medical centre

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Background: Haemophilia is an X-linked congenital disorder caused by deficiency of coagulation factor. Being the most populous country, China also has a large potential population of haemophilia patients. No studies have been published regarding the hemophilic epidemiology and medical situation of contemporary China.
Aims: The primary aim of this research was to achieve contemporary information of PWH in China, describe the prevalence, complications, as well as replacement therapy-related side effects of haemophiliacs for this decade.
Methods: A retrospective study was conducted in patients with haemophilia (PWH) visited in Tianjin haemophilia centre between 2002 and 2012. We evaluated the correlation between the severity of haemophilia and bleeding, analyse the inhibitor development in PWH of each severity group and treated with different clotting factor products.
Results: Over the past decade, a total of 1226 PWH (1019 HA and 207 HB) were identified in Tianjin single centre. Most of them came from north and northeast regions of China. Although a trend was observed towards lower Hb among more severe HA, the difference was not statistically significant ($P > 0.05$). APTT, age at initial bleeding and diagnosis showed association with the disease severity, but no correlations in the time of delayed diagnosis was found in each severity group of HA and HB. Our data did not offer sufficient evidences of any relationship between disease severity and risk or site of haemorrhage. Haemophilia related hemarthroses occurred in 65.7% HA (669/1019) and 56.5% HB (117/207). The most involved joints in HA are, in order, knee, elbow and ankle. Additionally patients with HB usually suffered from pain of hip. The overall incidence rate of inhibitors was 15.4% in HA and 9.8% in HB, and the prevalence of inhibitor was 18.8% in severe HA and 18.8% in severe HB. The prevalence of inhibitor and HTI were both associated with the severity haemophilia. In HA the incidence of inhibitor of using single each generation of rFVIII product was 10.5%, 8.8% and 2.9%, respectively. While the rate of inhibitor was 15.0% in PWH treated with pdFVIII, 8.1% in patients treated with both formulations simultaneously. In 21 haemophiliacs with HB by using single rFIX in a clinical trial, we found that three patients developed inhibitor following usage of recombinant factor.
Conclusions: We assessed the prevalence and treatment of haemophiliacs identified in Tianjin haemophilia centre. Although the present study highlights some of stepwise improvements in diagnosis and therapy of PWH, also reflects some challenges in contemporary China.

PB3.36 – Haemophilia A: Clinical – X

PB 3.36-1
FVIII neutralization kinetics – a complimentary tool for individual assessment of inhibitor patients

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Background: Treatment of Hemophilia A patients with inhibitors is challenging, as correlation between inhibitor level and hemostatic response to therapy may be limited. The Bethseda unit (BU) titer assay reflects the inhibitory capacity for FVIII neutralization following long incubation of patient's plasma but does not express the kinetic behavior of the inhibitor. Furthermore, standard plasma derived FVIII is used for the test whereas most patients are currently treated by recombinant FVIII products.
Aims: We aimed to define the inhibitor affinity against various FVIII sources and asses FVIII inhibitory kinetics as a potential tool for therapy tailoring.
Methods: Blood was taken from 14 hemophilia patients aged (0.8–68 years) with inhibitors. All subjects or their guardians gave their informed consent to blood drawing for study purposes. The study was approved by the institutional ethical committee in accordance with the declaration of Helsinki. Inhibitor activity was determined against human rFVIIII (Kogenate), plasma derived FVIII (pd FVIII-Optivate) or recombinant porcine FVIII (pFVIII-Inspiration Biopharm Inc.) and compared to the titer measured by the BU. To test FVIII neutralization profile, patients' PPP was spiked with 2 U/mL rFVIII, pdFVIII and pFVIII and sequential measurements of residual FVIII activity was measured over 120 min. FVIII activity was measured by one stage PTT assay with Sysmex CA-1500 (Siemens Healthcare Diagnostics). All inhibitor (including the BU assay) and kinetics assays were performed on the same sample simultaneously.
Results: Variability of inhibitor activity against different FVIII sources was noted for all patients and did not necessarily correlate with the BU assay. For example, in a patient whose inhibitor titer was measured as 22BU, inhibitor activities of 16.8, 10 and 1.8 were detected for rFVIII, pdFVIII and pFVIII, respectively. All patients demonstrated individual FVIII source dependent neutralization curves. When rFVIII neutralization kinetics was analyzed over time, no obvious correlation was found between the measured BU titer and the rate of rFVIII neutralization. Interestingly, for some patients with high responding

inhibitors slow and incomplete FVIII neutralization was noted at 2 h after incubation, whereas some patients with low responding inhibitor titer showed fast and complete FVIII neutralization within 15 min.
Conclusion: Our assays suggest that FVIII source dependent neutralization kinetics may be applied as a complimentary tool for future therapy tailoring in inhibitor patients. The assay may depict the inhibitor patients that may benefit from FVIII therapy (regardless of inhibitor BU titer) and identify the best potential FVIII source for treatment.

PB 3.36-2

Low inhibitor incidence in previously untreated patients with severe haemophilia A treated with octanate – Update from the PUP-GCP clinical trial

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Background: Octanate is a highly purified, double virus inactivated, human plasma-derived factor VIII (FVIII) concentrate with all coagulation FVIII bound to its natural stabilizer VWF in a VWF:RCo/FVIII:C ratio of approximately 0.4. Five prospective GCP studies with octanate were conducted in 77 previously treated patients (PTPs) with severe haemophilia A. None of these 77 PTPs developed an inhibitor.
Aim: To assess the immunogenicity in previously untreated patients (PUPs), a prospective clinical trial has been initiated in 2000. This included 48 PUPs with severe hemophilia A after treatment with octanate for an observational period of 100 exposure days and at least 6 months.
Methods: Patients with severe haemophilia A without previous exposure to FVIII or FVIII containing products were enrolled. Efficacy and tolerability are assessed by a 4-point verbal rating scale. Inhibitor assay, according to modified Bethesda method is tested pre-treatment, every 3–4 exposure days (ED 1-20) and every 10 EDs (ED 21-100) but at minimum every 3 months.
Results: Two of 48 (4.2%) subjects receiving treatment developed clinically relevant inhibitor titers over the course of the study. Another two displayed inhibitors that disappeared spontaneously without change of dose or dosing interval. All inhibitors developed under on-demand treatment and before ED 50. From the 48 subjects, 42 had exceeded 50 EDs at the time of this analysis. octanate was well-tolerated and the adverse event profile was consistent with the population studied. The haemostatic efficacy in prophylaxis and treatment of bleeding were generally rated as 'excellent' and no complication was reported for any surgical treatment.
Conclusion: Despite frequent inhibitor testing and predominant on-demand treatment, octanateshowed a low rate of clinically relevant inhibitor formation (4.2%) in this cohort of patients.

PB 3.36-3

A European certification system for haemophilia centres

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Background: There are 409 facilities in Europe, of widely varying size and expertise calling themselves 'Haemophilia Centres'. Although a small number of countries have accreditation systems the majority do not. The European haemophilia community of professionals and patients has agreed the principles of haemophilia care. Care delivery is challenging due to the rarity and expense of treatment of the disease. We set to provide EU Member States (MS) with guidelines on a European certification system (CS) for Haemophilia Centres (HCs), in order to improve haemophilia care and to ensure equity of treatment throughout Europe.
Aims: To draw up a Guideline document establishing quality standards for European HCs and setting criteria for their certification and designation as European Comprehensive Care Haemophilia Centres (i.e. expert) and European Haemophilia Treatment Centres (i.e. non-expert).
Methods: The drafting of the Guideline document was performed by a panel composed of haemophilia experts and consultant methodologists. The methodology includes the following steps: (i) collection of the available MS regulations on HCs certification system by administering a questionnaire to key European stakeholders (physicians, patient representatives, institutions) (ii) systematic review of literature and MS regulations on HCs certification (iii) definition of a proposal of criteria and principles to be adopted for the production of the standards and launch of a consultation process involving key European stakeholders (iv) validation of criteria and principles for the production of the standards (v) standards production (vi) design of a HCs evaluation and certification system (vii) launch of a consultation process on standards and HCs evaluation and certification system (viii) approval of standards and evaluation/certification system.
Results: The first draft sets the standards for the designation of European HCs of two levels: European Haemophilia Comprehensive Care Centres, that provide specialized and multidisciplinary care and function as tertiary referral Centres, and European Haemophilia Treatment Centres, that provide local routine care. The standards apply to both adults and paediatric patients. They focus on organizational issues and cover: (i) general requirements (facility; general policy and objectives; information about the Centres; organization and staffing; policies and procedures; record-keeping and data collection; personnel appraisal and continuing education; supply and management of therapeutic products, reagents and medical devices; quality planning, evaluation and improvement; participation in registries; participation in clinical research), (ii) patient care (awareness, information and education of patients and their families; diagnosis and therapy of haemophilia and other related bleeding disorders and all the forms of acquired haemophilia; periodic clinical and multi-disciplinary review; genetic services; outcome indicators), (iii) advisory service, (iv) laboratory; (v) networking of clinical and specialised service.
Conclusions: The haemophilia standards have been developed following consultation and in 2013 all European haemophilia centres will be invited to apply for certification. A panel of physicians, patients and nurses will assess the applications. Once implemented across EU MS, the certification system will contribute to the reduction of health inequalities through the standardization of quality of care provided by HCs.

PB 3.36-4

Patient and parent preferences for efficacy, inhibitor risk, safety, and infusion frequency associated with prophylactic Factor VIII treatment

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Background: As new options become available for Factor VIII prophylaxis treatment, research is needed to understand a patient's willingness to accept tradeoffs among treatment attributes such as effectiveness, inhibitor risk, safety, and infusion frequency.
Aims: To quantify patient and parent preferences for prophylactic Factor VIII treatments and compare the relative importance of treatment attributes.

Methods: Adult patients and parents of children with severe hemophilia A in the United States were recruited and completed a web-enabled, discrete-choice experiment survey. The survey presented a series of 10 choice questions, each including a pair of hypothetical prophylactic Factor VIII treatment profiles. Each profile was defined by average number of bleeds per year, risk of developing an inhibitor, infusion frequency, and track record of product safety. The majority of levels for each attribute were identified based on the literature or clinical trial results. Choice questions were based on a predetermined experimental design with known statistical properties. Preference weights for attribute levels were estimated using random-parameters logit. The survey was tested to help inform attribute selection and ensure respondent comprehension in 10 face-to-face interviews. All respondents provided online informed consent and the study complied with the Declaration of Helsinki, receiving ethics board approval.
Results: The final sample included 76 adult patients and 86 parents. Mean age for adult patients was 32 years and the mean age for children was 8 years. 47% of adult patients and 41% of parents reported prophylactically infusing three times a week. Statistical tests indicated that the two samples could not be pooled due to different preferences ($P < 0.01$). For parents, the most important attribute was average number of bleeds per year and was assigned a mean relative importance score of 10.0. The remaining attributes in descending order of importance were track record of product safety (8.1), risk of developing an inhibitor (8.0), and infusion frequency (6.0). For adult patients, risk of developing an inhibitor was the most important attribute (10.0). The remaining attributes in descending order of importance were average number of bleeds per year (7.3), infusion frequency (6.3), and track record of product safety (4.5). Both patients and parents significantly ($P < 0.05$) preferred 0–1 bleeds per year over five bleeds per year and a track record of 4–10 years of product safety over a product new to market. There were significant ($P < 0.05$) differences between a 0%, 1% and 2% risk of inhibitor development as there were between infusions three times per week and infusions two times per week. Parents and adult patients revealed that achieving zero bleeds per year (relative to five bleeds) was 2.4 and 1.7 times more important than being able to infuse two times per week over three times per week, respectively.
Conclusion: While these results suggest that adult patients and parents of children with severe hemophilia A have different preferences, both were not willing to trade off having several bleeds per year to infuse less frequently. Healthcare providers are encouraged to take these preferences into account when prescribing Factor VIII prophylaxis.

PB 3.36-5

Comprehensive assessment of hemorrhagic phenotype and cardiovascular risk profile in carriers of severe haemophilia

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Background: There are few studies about bleeding phenotype in haemophilia carriers. All of them describe haemophilia carriers with low factor VIII (FVIII) levels without bleeding problems and on the other hand an haemorrhagic tendency in some of these women even with factor VIII (FVIII) plasma levels almost in a normal range.
Aims:
- To evaluate hemostasis in severe hemophilia carriers.
- To identify symptomatic carriers.
- To descrige this group bleeding profile.
- To exclude other concomitant haemostasis disorders supporting this bleeding profile.
- To evaluate this population cardiovascular risk profile and its influence on the bleeding profile.
- To analyze the quality of life of this population.

Method: This is a descriptive cross-sectional, non-interventional, single center study. Ethics Committee evaluation and Informed Consent are requested. The target population are severe Haemophilia A carriers from our center, there is no exclusion criteria. We will evaluate family bleeding, ischemic and thrombotic antecedents, individual hemostasis profile using Tosetto bleeding score (TBS) and pictorial blood assessment chart (PBAC), FVIII genetic study, complete blood count, basic biochemistry, haemostasis (aPTT, PT, fibrinogen, platelet function tests, FVIIIc, FvWAg and FvWRCo, FXIII, homocysteine, resistence to activated protein C, antithrombin, protein C and S, 20210A prothrombin mutation), cardiovascular risk throughout Framingham score and Systematic Coronary Risk Evaluation Project. We will study quality of life using SF-36 questionary. Anxious and depression disorders will be evaluated with Goldberg score. To describe continuous variables we will use mean, median, standard deviation, maximum and minimum. For categorical variables, it will be used the percentage of every category. Statistical techniques are used to ensure compliance with statistical assumptions to use non parametric tests.
Results: Since julio 2012, we have studied 31 severe haemophilia A carriers. Median age is 43 years-old (range, 14–73 year-old). Media FVIII: C level is 82 ± 32% (range, 35–152%). There is no difference in FVIII levels with regard to bleeding profile or FVIII gene mutation. Tosetto bleeding score is higher than 3 in 42% of the group (13/31). Abnormal bleeding in surgery, postpartum or menorrhagia were described in 26% (8/31) of cases. PBAC is higher than 100 in 70% of the group (22/31), a higher rate than general population. Three of these patients have low levels of VWF: RCo and VWF: Ag. Farther studies let us classify them as von Willebrand disease patients appart from haemophilia carriers. We find no other associated haemostatic disorder. There is no thrombophilia, nor correlation between cardiovascular risk score and bleeding profile, although serie is still short.
Conclusion: Severe haemophilia A carriers can suffer from abnormal bleeding because of low FVIII levels or association of other haemostatic difects. There is short information on general population bleeding events incidence apart from metrorrhagia. This makes difficult to verify and to prove that the incidence of bleeding in haemophilia carriers with FVIII levels over 40% is higher, particularly since these seem to occur with bleeding hemostatic levels of FVIII and no other hemostasis defect.

PB 3.36-6

Do inhibitors, treatment regimen and bleed frequency impact haemophilia treatment centre and provider utilisation: an analysis of adults with haemophilia in the HERO study

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Background: Congenital haemophilia is primarily treated through comprehensive care at a haemophilia treatment centre (HTC) by a variety of specialised practitioners. Little is known about the relationship of HTC utilisation to the presence of inhibitors, on-demand vs. prophylactic treatment or bleed frequency.
Aims: To describe the relationship between HTC and healthcare professional utilisation, inhibitor status, treatment regimen and bleed frequency in adults with haemophilia in the Haemophilia Experiences, Results and Opportunities (HERO) Study.
Methodology: A post-hoc descriptive analysis of data from adults ≥18 years in eight countries with home treatment. Annual bleed frequency was categorised for analysis (0, 1–5, 6–10, 10–20, >20 bleeds).
Results: Overall, 515 adults (431 without inhibitors, 84 with inhibitors) responded, with treatment distributed between on-demand (without

inhibitors 141, with inhibitors 39) and prophylaxis (without inhibitors 174, with inhibitors 29). The distribution of adults among each annual bleed frequency category (0/1–5/6–10/10–20/>20) was 54/144/63/57/75. Median age (years) of those on prophylaxis was younger than those patients on on-demand for adults without inhibitors (36 vs. 41) but not for adults with inhibitors (35 vs. 35). There was no consistent trend in median age across bleed frequencies (45/40/36/35/40). Overall, 64% of adults were full-time, part-time or self-employed; rates of employment were similar in adults without inhibitors (on-demand 65%, prophylaxis 58%), with inhibitors (on-demand 76%, prophylaxis 73%) and with increasing annual bleed rate (65%/63%/74%/63%/53%). Higher risk activities were reported more commonly by those on prophylaxis (without inhibitors 16%, with inhibitors 10%) vs. on-demand (without inhibitors 10%, with inhibitors 3%) and by those with lower annual bleed rates (15%/10%/17%/7%/9%). Mean/median bleeds per year were higher for adults without inhibitors on prophylaxis (14.9/5) vs. on-demand (10.3/2) but were lower for adults with inhibitors on prophylaxis (5.6/2) vs. on-demand (15.6/7). Mean/median number of HTC visits per year were higher for adults on prophylaxis (without inhibitors 4.5/2, with inhibitors 5.4/4) vs. on-demand (without inhibitors 3.5/2, with inhibitors 4.8/3). There were no differences seen in relation to bleed rates. Nurses were more frequently involved in haemophilia care in adults with inhibitors on prophylaxis (without inhibitors 55%, with inhibitors 69%) vs. on-demand (without inhibitors 45%, with inhibitors 44%) and with increased bleed frequency (37%/54%/54%/65%/57%), while social worker involvement was more common in adults without inhibitors on prophylaxis (20% vs. 8% on-demand) but less common in adults with inhibitors on prophylaxis (17% vs. 44% on-demand), and increased with bleed frequency (9%/15%/19%/30%/32%). Physiotherapist involvement was similar in adults on prophylaxis (without inhibitors 39%, with inhibitors 41%) and on-demand (without inhibitors 31%, with inhibitors 46%) but increased based on bleed frequency (20%/38%/41%/35%/37%).

Conclusions: For adults, employment was relatively high irrespective of the presence of inhibitors, current treatment regimen and increasing bleed frequency. Reported bleed frequency was higher in adults without inhibitors on likely secondary prophylaxis but lower in those with inhibitors on prophylaxis. HTC utilisation was slightly higher in adults on prophylaxis vs. on-demand. Bleed frequency did not seem to impact utilisation. Involvement of physiotherapy and social work in comprehensive care was generally less than expected.

PB3.37 – Haemophilia A: Clinical – XI

PB 3.37-1

Outcomes of total knee and hip arthroplasty for hemophilic arthropathy

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Background: Hemophilic arthropathy results in joint pain and dysfunction and significant disability in people with bleeding disorders. Total joint arthroplasty (TJA) carries substantial bleeding risk, but may also be associated with increased risk for venous thromboembolism (VTE). Our practice is to ensure normal hemostasis using coagulation factor concentrates perioperatively while administering pharmacologic thromboprophylaxis concurrently to prevent VTE. Our center is one of the few to employ this management strategy for TJA in this population; the aim of the current study was to assess safety and efficacy of this practice.

Methods: We performed a retrospective chart review to identify patients with congenital bleeding disorders who underwent TJA between 1987 and 2012. We collected data on range of motion (ROM) and pain before and after surgery and on early and late complications (bleeding, infection, thrombosis). Data are presented descriptively using median values and ranges where appropriate.

Results: We identified 38 procedures (29 knees (TKA) and 9 hips (THA) in 28 patients (26 male, 2 female) with hemophilia A ($n = 21$), hemophilia B ($n = 4$), factor 11 deficiency ($n = 1$) and von Willebrand disease ($n = 2$). Median age at operation was 42 years (range, 17–74) for TKA and 45 years (range, 18–71) for THA. Inhibitors were present in one patient with hemophilia A (1.5 B.U.) and one patient with factor 11 deficiency (0.5 B.U.). All patients were treated with hemostatic agents appropriate to their disorders for up to 4–6 weeks post-operatively. Complete data at 2 months post-operatively are available for 27 TKA patients, of whom, 7 (23%), demonstrated improvement in ROM (median 15 degrees, range 5–25). At 1.5 years post-operatively, 17/29 (59%) TKA patients showed improvement in ROM (median 15 degrees, range 4–58) and 100% reported decreased knee pain. All nine THA patients demonstrated improved ROM at 2 months post-operatively. Eight (89%) demonstrated gains in internal rotation (median, 45 degrees, range 15–45), 9 (100%) in external rotation (median 30 degrees, range 15–45), 5 (56%) in flexion (median 35 degrees, range 27–55), 7 (78%) in extension (median 15 degrees, range 3–95), and 7 (78%) in abduction (median 15 degrees, range 10–25). Low molecular weight heparin was administered post-operatively in 29 of 38 procedures (76%). Thromboprophylaxis was discontinued in three patients for non-joint bleeding (one hematuria, two cases of hypotension and anemia). There were no symptomatic VTE. Early complications included five cases of cellulitis and two hemarthroses in patients not receiving thromboprophylaxis. Late complications included two patients with aseptic loosening in prosthetic knees leading to TKA revisions, one with a subsequent joint infection requiring surgical debridement and one patient with a worsening flexion contracture requiring TKA revision.

Conclusions: While there are risks associated with TJA in patients with bleeding disorders, our data suggest they are outweighed by the benefits manifesting as decreased pain and improved function. Pharmacologic thromboprophylaxis appears safe in this population; whether it is necessary is unknown and should be a subject of future trials.

PB 3.37-2

European Haemophilia Network (EUHANET)

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Background: Inherited bleeding disorders are rare and care for patients is provided by specialist haemophilia centres. The quality of care varies enormously between countries and in different centres within countries. It is difficult for patients to select which centre to attend due to lack of information about the quality and level of care offered. There is limited independent information available for patients about their disorders and there is no uniform system to collect adverse events of their treatment.

Method: EUHANET is a European Commission funded project with additional support from industry. There are five main partners and 84 collaborating haemophilia centres participating. The project uses information technology extensively and centres collaborate on several projects including the certification of haemophilia centres, the development of a new website, the expansion of the adverse event reporting

system (EUHASS) and a prospective registry of patients with afibrinogenemia and FXIII deficiency and their management.
Results: (a) Certification
We have identified 409 facilities that call themselves haemophilia centres in Europe. After review of the currently available literature and guidelines from individual countries, we have proposed the standards required for European Comprehensive Care Haemophilia Centres (expert) and European Haemophilia Treatment Centres (non-expert) certification. All European centres will be able to apply for certification from 1st June 2013.
(b) Haemophilia Central website
A new publically available website (www.haemophiliacentral.org) contains frequently updated news stories as well as information on guidelines, clinical trials, concentrates and a locator listing all haemophilia centres and organisations in Europe. Patients can find the nearest haemophilia centre from anywhere in Europe.
(c) Adverse event reporting
The EUHASS adverse event reporting system has been expanded to include platelet disorders and acquired bleeding disorders due to inhibitors. So far in the first 4 years of the system 952 individual reports from centres have been submitted. These included 101 allergic or acute reactions, 197 first ever inhibitors, 26 recurrent inhibitors, 103 thromboses (of which 62 occurred within 30 days of concentrate administration), 207 malignancies and 310 deaths.
(d) Prospective registry for afibrinogenemia and FXIII deficiency
A new project has started where all cases of afibrinogenemia and FXIII deficiency in the participating centres are registered and data on number of bleeds and concentrate usage are prospectively provided every 6 months.
Conclusion: Although in its early days, the EUHANET project aims to improve the collaboration of European haemophilia centres and the success of the ongoing EUHASS project is a demonstration of what can be achieved with such collaboration.

PB 3.37-3

Osteoporosis and osteopenia in patient with severe and moderate type hemophilia A

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Background: Hemophilia patients have been found to have lower bone mineral density (BMD) as compared to normal healthy subjects. The pathophysiology of the development of osteoporosis in hemophilia patients is not exactly known.
Aim: The aims of this study were to realize the percentage of osteoporosis in hemophilia patients and to analyze the correlation of osteoporosis with some clinical factors and a biomarker in hemophilia A patients.
Patients and Methods: We prospectively enrolled 30 consecutive adult hemophilia A patients (23 severe and seven moderate type) with hemophilic arthropathy from January to December, 2011 for this study. The median age of patients was 38.5 years old with a range of 21–66 years. Each patient's clinical informations including age, disease severity, body mass index (BMI), anti-HCV positivity, Pettersson score of the X-ray of six index joints and dual-energy X-ray absorptiometry (T score) for femoral neck and lumbar spine and tartrate-resistant acid phosphatase isoform 5b level were collected for correlation analyses.
Results: The percentage of osteopenia determined by T score of femoral neck and lumbar spine in our cohort patients was 70% and 28%, respectively. The percentage of osteoporosis in femoral neck and lumbar spine was 12% and 4%, respectively. There were good correlation between patients' BMI and T score of either femoral neck ($r = 0.635$, $P = 0.0046$) or lumbar spine ($r = 0.690$, $P = 0.002$). The BMD of femoral neck and lumbar spine between patients with and without HCV were not significantly different.
Conclusion: Our study demonstrated there was a high percentage of osteopenia measured by T score of femoral neck and BMI has significantly good correlation with BMD of femoral neck and lumbar spines in this cohort of hemophilia patients.

PB 3.37-4

Haemate® P for the treatment of vWD and haemophilia A: new results of a pharmacovigilance project

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Haemate® P(CSL Behring, Marburg) is a factor concentrate that contains von-Willebrand-Factor (vWF) and Factor VIII (FVIII). It is indicated for the treatment of von Willebrand disease (vWD), haemophilia A and ITT. This pharmacovigilance surveillance should collect data on the long-term efficacy, tolerability and safety of Haemate® P in clinical routine situations.
All patients treated with Haemate® P could be enrolled (vWD and haemophilia A-patients, PUPs and PTPs). Patients are routinely screened every 3–12 months. The following parameters were documented (non-interventional design): overall clinical response, bleeds, adverse drug reactions including the incidence of inhibitors, laboratory safety parameters, virus safety, relevant concomitant diseases, and relevant concomitant medication.
Up to now, 91 patients were included into this study and data from 330 visits were available for this analysis. 87 patients suffered from vWD (57 patient with Type 1, 18 patients with Type 2, and 11 patients with Type 3), and four patients from haemophilia A. The median age was 36 years (range 0.4–84.3).
In all vWD patients the median number of bleeds per year was 2.34 (in the vWD-Type 1-group median 7.74 bleeds, in the vWD-Type 2-group median 1.76 bleeds, in the vWD-Type 3-group median 0.97 bleeds). This is correlated to a higher percentage of patients with 'on demand' treatment in Type 1 patients.
Four patients with haemophilia A are enrolled in the study. One patient with known risk factors (afro-american origin) developed a low titer inhibitor, an ITI was started and successfully completed. The results included in this interim analysis confirm the very good efficacy, tolerability and safety of Haemate® P.

PB 3.37-5

Safety and pharmacokinetics of a recombinant fusion protein-linking coagulation factor VIIa with albumin (rVIIa-FP) in healthy volunteers

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Background: Development of neutralizing antibodies remains the most problematic complication in the treatment of patients with congenital haemophilia. Therapy with the bypassing agent rFVIIa is limited by the short half-life of the commercially available product. Here, we report on pharmacokinetics and safety of a novel, recombinant fusion

protein linking coagulation factor VIIa with albumin (rVIIa-FP) in a placebo controlled, first-in-man study in healthy male subjects that have been anti-coagulated with an oral vitamin K antagonist (OVKA).

Aims: To investigate safety andpharmacokinetics of rVIIa-FP in healthy volunteers.

Methods: A total of 40 healthy male volunteers between 18 and 35 years of age were included in the study and were dosed in five consecutive cohorts of eight subjects each. In each cohort, six subjects were randomized to a single dose of rVIIa-FP (140, 300, 500, 750 and 1000 μg/kg) and 2 to placebo. All subjects received anticoagulation with OVKA to reach a stable INR between 2 and 3 prior to dosing with rVIIa-FP/placebo. Dosing with OVKA was continued at a fixed dose for 5 days after injection of rVIIa-FP and then antagonized with Vitamin K.

Results: Tolerance of rVIIa-FP was excellent at all dose levels. No serious adverse events were observed. None of the subjects developed anti-drug antibodies or inhibitors. FVIIa baseline-corrected mean (SD) C_{max} plasma activity increased in a dose-proportional manner, from 9240 (515) mU/mL for the 140 μg/kg dose to 63520 (13515) mU/mL for the 1000 μg/kg dose. Across the dose range, the median half-life (t1/2) was consistent, ranging from 6.1 h to 9.7 h. At the highest dose of 1000 μg/kg, the median FVIIa activity-based was 8.5 h. Clearance (CL) was consistent across the dose levels, ranging from 7.62 to 12.74 [mL/h/kg].

Conclusions: Compared to the commercially available rFVIIa product, the t1/2 of rVIIa-FP is increased and clearance reduced by approximately 3–4 fold. Tolerance and safety in this first-in-man study were excellent. The study has been registered on clinicialtrials.gov (Registration No: NCT01542619).

PB 3.37-6

Adherence to hemophilia treatment in the Dutch pediatric population; reliability and validity of the VERITAS-Pro questionnaire

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Background: Patients' adherence is strongly associated with the cost-benefit of hemophilia treatment. It is therefore important to be able to quantify adherence and to formulate interventions aiming to modify patient adherence.

Aims: To assess the reliability and validity of the cross-culturally adapted Dutch version of the Validated Haemophilia Regimen Treatment Adherence scale (VERITAS-Pro) designed to quantify adherence in patients with hemophilia on prophylactic clotting factor replacement therapy. This first evaluation focuses on the test equivalence of the psychometric properties of the original and the adapted VERITAS-Pro.

Patients and Methods: In this study, children from three Dutch Hemophilia Treatment Centers with severe and moderate hemophilia on prophylactic treatment were included. Exclusion criteria were: prophylactic treatment <1 year, presence of inhibitors, language difficulties. Written informed consent and ethical approval was obtained. Parents and adolescents aged 10–18 years filled out the study questionnaire.

The original VERITAS-Pro is a validated adherence scale of prophylactic clotting factor replacement therapy, applied in the USA. The scale consists of 24 items on six (four item) subscales (Time, Dose, Plan, Remember, Skip, Communicate). Lower scale scores reflect higher adherence. The VERITAS-Pro was translated into Dutch according to a predefined protocol with two forward and two backward translations. Psychometric properties (construct validity including Cronbach's alpha and item correlations per subscale, discriminative validity according to the infusion logbooks, test-retest reliability) were empirically assessed.

Results: A total of 60 parents and 30 adolescents filled out the questionnaire with a response rate of 87%. Median age of children was 11 years (IQR 8–14). Test-retest reliability was measured in a subgroup ($n = 58$). Construct validity: mean Cronbach's alphas were adequate (>0.70) for the total score, and the subscales 'skip' and 'communicate', but not for subscales 'time' (0.42), 'dose' (0.34), 'plan' (0.62), and 'remember' (0.50). Two scales had floor effects (>50%), not previously reported. The item-own subscale correlations within all scales were considerably higher than most of the item-other subscale correlations. Test-retest correlations were statistically significant for all scales, except for the subscale 'time'. Discriminative validity: overall total scores were significantly higher for non-adherent respondents according to the infusions logbooks ($n = 48$, $P < 0.05$).

Conclusions: This suggests that the total score of the VERITAS-Pro is useful to quantify adherence to treatment in the Dutch population; subscales are however less applicable. Equivalence of psychometric properties of the adapted VERITAS-Pro was reached on most items.

PB3.38 – Haemophilia A: Clinical – XII

PB 3.38-1

Clinical features and management of hemophilic pseudotumors: a single US center experience

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Background: Hemophilic pseudotumor is an uncommon but potentially life-threatening complication occurring in 1–2% of hemophilia patients. It is thought to occur more frequently in developing countries that have limited access to factor replacement therapy, and is rarely seen in developed countries. Given the rarity of this condition, consensus on the management of pseudotumors in hemophilia patients is lacking and largely confined to case reports or small series.

Aims: We describe the clinical features and management of hemophilic pseudotumors in a single US institution.

Methods: We retrospectively reviewed the medical records of hemophilia patients with a diagnosis of pseudotumor seen at the UNC Hemophilia Center from 1981 to 2011. We recorded the following data: type and severity of hemophilia, documented etiological antecedent, localization of the pseudotumor, presenting symptoms, management and outcome.

Results: We identified 12 pseudotumors in 11 hemophilia patients in the age range of 23–69 years (median: 47 years) at the time of diagnosis. Ten patients had hemophilia A (severe, $n = 5$; moderate, $n = 4$; mild, $n = 1$) and one patient had moderate hemophilia B. Six patients had known inhibitors or a history of inhibitor.

An etiological antecedent leading to the development of pseudotumor was reported in nine cases (75%): prior trauma to the affected area in six cases and prior spontaneous bleed in the affected area in three cases. Localization of the pseudotumor was confined to soft tissue in three cases (25%), and affected the bones in eight cases (66.7%). The remaining case was a right lung pseudotumor.

Localized pain from the pseudotumor was present in seven cases, of which additional neurological compromise (paresthesia) and vascular compromise (arterial ulcers) occurred in one case each. Two patients presented with a pathological fracture while the patient with right lung pseudotumor presented with hemoptysis. One patient presented with obstructive renal symptoms from his retroperitoneal pseudotumor, subsequently progressing to end stage renal disease, requiring hemodi-

alysis. Six of the 12 pseudotumors (50%) were not diagnosed at the time of initial presentation, with the delay in time to diagnosis ranging from 6 weeks to 6 years.

In eight cases, surgical intervention (incision and drainage [I&D], $n = 2$; excision, $n = 4$; amputation, $n = 2$) was the initial treatment choice, with complete resolution obtained in six cases. Both I&Ds were unsuccessful, with one case subsequently undergoing successful surgical excision, while the other case was followed by an unsuccessful arterial embolization; the patient declined further intervention.

Conservative management with close monitoring occurred in three cases, with one case subsequently requiring surgical resection. The patient with a right lung pseudotumor was treated with multiple arterial embolizations without resolution. He was subsequently placed on biweekly factor replacement therapy to reduce the frequency of hemoptysis.

Conclusion: This is the largest single-US institution case series of hemophilic pseudotumors. Our series demonstrates that hemophilic pseudotumors still occur sporadically, and the diagnosis is frequently delayed. Surgical intervention seems to be a safe and effective treatment, although conservative management may be appropriate in selected cases.

PB 3.38-2

The relationship between specific annual bleed rates and health outcomes among children with severe hemophilia A

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Background: Recurrent bleeding suffered by children with severe hemophilia A has been shown to lead to chronic pain, disability, arthropathy and lower health-related quality of life (HRQOL). Little has been published to understand what level of bleeding can be tolerated before patients report an impact on their health outcomes.

Aims: The objective of this analysis was to assess the health outcomes of pediatric patients reporting a zero annual bleed rate (ABR) and identify the incremental impact of higher ABRs on these outcomes.

Methods: A multi-national, cross-sectional survey of severe hemophilia A patients was administered in collaboration with hemophilia associations or hemophilia treatment centers across 10 countries. A centralized ethics review board approved the study. Eligible, consenting caregivers of hemophilia patients under 18 years of age completed a detailed questionnaire during one of two phases: from October-November 2009 (Argentina, China, Russia, US) and January-August 2011 (Chile, Colombia, Malaysia, Mexico, Philippines, Singapore). HRQOL was measured using the Pediatric Quality of Life Inventory (PedsQL) and the EQ-5D. Treatment regimen, ABR, number of target joints and days missed from school were also assessed. Median results of those who reported a zero ABR were compared to those reporting ABR categories of 1–2, 3–4, 5–10, 11–20, 21–30, 31–50 and >51 using Wilcoxon Rank-Sum Test.

Results: 470 parents/caregivers of severe hemophilia A children completed the survey. The mean age of pediatric patients was 9.7 years. 48% of the pediatric patients were on an on-demand treatment regimen while another 48% were on prophylaxis (defined as regular infusions to prevent bleeds from occurring), and the remainder did not specify. A pattern emerged between an increase in ABR and significant worsening of HRQOL scores. Compared to patients with a 0 ABR who reported a median PedsQL Total score of 82.6, patients with ABR categories of: 3–4, 5–10, 11–20, 21–30, 31–50, 51 or more showed significantly worse median PedsQL Total scores of: 71.2, 65.3, 68.7, 67.4, 64.0 and 56.5, respectively (all $P < 0.05$). This statistically significant pattern held for all PedsQL summary scores (physical, psychosocial, school) as well as the EQ-5D Index and EQ-5D VAS scores (all $P < 0.05$). Additionally, differences in number of target joints and missed days from school all showed the same statistically significant trend of progressively worse scores when comparing 0 ABR to ABR categories of 3–4 and beyond. There were no significant differences between patients with zero compared to 1–2 ABR on these health outcomes.

Summary/Conclusions: This analysis suggests that even 3–4 bleeds per year may have a negative impact on a patient's joint health, days missed from school and HRQOL. Efforts to maintain a 0 ABR among pediatric patients with severe hemophilia A may help ensure optimal outcomes.

PB 3.38-3

Comparison of factor VIII half-lives in severe haemophilia A following switch to Refacto AF

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Background: Haemophilia A patients on prophylaxis therapy have pharmacokinetic studies performed as part of routine clinical management to confirm that their circulating factor VIII level is being maintained between doses at an appropriate level to reduce the risk of spontaneous bleeding. Concerns have been raised regarding pharmacokinetic performance of B-domain-deleted recombinant factor VIII (BDD rFVIII) compared to full-length factor VIII (FL FVIII) replacement therapy. This is retrospective analysis of laboratory data collected from patients treated with recombinant full length Factor VIII concentrate under a prophylactic regime and switched to B-domain deleted Refacto AF on an equivalent dosage regime and frequency as part of national procurement.

Aims: To compare factor VIII half-life measurements and thrombin generation in patients with severe haemophilia A since switching to BDD rFVIII from full-length factor VIII (FL FVIII) replacement therapy.

Methods: Factor VIIIC levels were measured on the Destiny Max analyser (Stago) using TriniCLOT aPTT HS, reference plasma (Stago) for FL FVIII replacement therapy and Refacto laboratory standard for BDD rFVIII, FVIII deficient plasma (Technoclone) for 1-stage assay and chromogenic FVIII (Technoclone). Thrombin generation was measured using CAT analyser and reagents (Thrombinoscope). Citrated plasma samples from patients ($n = 9$) were collected during pharmacokinetic studies pre and post FL FVIII or BDDrFVIII replacement therapy at four time points over 32 h.

Results: There was no significant difference detected in the levels of FVIII measured by 1-stage or chromogenic assay for FL FVIII or BDD rFVIII ($P = 0.0.94$ and $P = 0.65$ respectively. There was no significant difference in half-lives between treatment with FL FVIII or BDD rFVIII $P = 0.94$. There was no significant difference in thrombin generation between FL FVIII or BDD rFVIII as measured by endogenous thrombin potential (ETPnM*r), $P = 0.60$

Summary/Conclusions: Laboratory data has not identified any difference in the half-life of circulating levels of factor VIII or thrombin generation in haemophilia A patients treated with either FL FVIII or BDD rFVIII replacement therapy. Pharmacokinetic studies in these patients have not identified any difference in laboratory performance between FL FVIII or BDD rFVIII.

PB 3.38-4

Differential patterns of dynamic whole blood platelet aggregation in haemophilia A following *in vitro* addition of rFVIII and by-passing agents

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Background: Haemostatic interventions in haemophilia A vary in mode of action and this may be reflected on platelet aggregation (PA).
Aims: This study examined the PA patterns after *in vitro* addition of recombinant factor VIII (rFVIII) or bypassing agents.
Methods: The study was approved by the appropriate medical ethics committee. Following informed consent citrated whole blood with corn trypsin inhibitor (100 μg/mL) was obtained from 10 patients with severe haemophilia A. Dynamic whole blood PA was evaluated applying a novel not standardized assay using endogeneously generated thrombin as agonist. PA was recorded by impedance aggregometry after addition of tissue factor. Fibrin polymerization was inhibited and WB was spiked with buffer, rFVIII, plasma-derived activated prothrombin complex concentrate (pd-aPCC), recombinant factor VIIa (rFVIIa), or rFVIIa analogue (vatreptacog alfa).
Results: Baseline total PA was reduced in haemophilia A (980 ± 332 AUxmin, [mean]) with the absence of a second wave. All *in vitro* additions induced a healthy PA pattern introducing a second wave of PA. Total PA was increased following pd-aPCC (3124 AUxmin) and particularly rFVIIa analogue (3678 AUxmin) compared to rFVIII (2099 AUxmin) and rFVIIa (2224 AUxmin). Earlier onset of thrombin induced PA was provided by rFVIIa (7.1 min), rFVIIa analogue (2.2 min), and pd-aPCC (5.3 min) compared to rFVIII (11.5 min). Recombinant FVIIa analogue (0.125–2 μg/mL) showed significant dose-response, whereas rFVIIa (1–4 μg/mL) did not.
Conclusion: The haemostatic interventions showed varying potential to support platelet aggregation *in vitro*. This may have implications optimizing patient treatment, however, the potential clinical impact of these observations needs to be addressed in future studies.

PB 3.38-5

Qualitative findings contributing to the development of a hemophilia-specific caregiver burden instrument: caregiver and health care professional insights

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Background: The majority of children who have congenital hemophilia with and without inhibitors are treated at home, mainly by their parents; older children treat themselves. Data describing the caregiver experience among this population are limited. There are no disease-specific instruments that have been developed and validated for this population.
Aims: The purpose of this study was to develop an instrument to measure the burden of caring for pediatric patients with hemophilia based on qualitative findings among caregivers and health care professionals (HCPs).
Methods: Item generation was based on two approaches (focus groups, questionnaire evaluation). Two focus groups with caregivers of children with hemophilia were held at RUSH HTC by an experienced psychologist. A semi-structured interview guide was developed for discussions. Notes from discussions were transcribed by an independent note-taker. Qualitative findings were assessed by the psychologist to determine concepts that emerged during focus groups for inclusion in the draft instrument. Statements were thematically categorized and items formulated based on caregivers' statements. A literature review was undertaken to identify existing validated caregiver burden instruments for the general population or chronic/acute diseases. Seven instruments covered aspects suitable for hemophilia caregivers. Approval to use these questions were obtained from the developers or paraphrased when developers did not respond. HCPs were asked to rate each identified question for its importance to hemophilia caregivers on a 5-point Likert scale ranging from '*not important at all'* to *'very important'*. Questions were included in the draft instrument if more than 73% of all HCPs agreed they were '*very important'*. In addition HCPs were asked what the main burden of hemophilia in their opinion is for children, caregivers and HCPs. IRB approval was obtained for both focus groups and HCP assessments.
Results: 11 caregivers participated in focus group discussions. All were caring for children with hemophilia A; three (27%) were caring for children with inhibitors. Caregivers reported patients ranged in age from 2 to 26 years. Discussions with caregivers generated a total of 92 questions organized into eight domains: emotional stress ($N = 21$), medical management ($N = 10$), financial ($N = 6$), personal sacrifice ($N = 6$), work ($N = 11$), interactions with others ($N = 10$), perception of child ($N = 11$), coping with hemophilia ($N = 17$). A total of 16 HCPs completed the HCP evaluation; of these were seven physicians (43.8%), six nurses (37.5%), one social worker (6.3%) and two were employed in 'other' functions (pharmacy, dental hygienist). A total of nine questions were ranked as '*very important'* by HCPs (VI) and eight additional aspects emerged (E) based on HCPs opinions about caregivers' burden: emotional stress: VI = 2, medical management: E = 5, financial: VI = 4, personal sacrifice: VI = 2 and E = 2, interactions with others: VI = 1, coping: E = 1. The draft instrument for pilot-testing contains 109 questions.
Summary: Qualitative findings among hemophilia caregivers and HCPs contributed to the development of a draft instrument to assess caregiver burden specific to hemophilia. Next steps in the process include a pilot test to inform formal psychometric testing, item reduction, and comprehensibility assessments via interviews among a subgroup of pilot test participants.

PB 3.38-6

Findings from a conjoint analysis with hemophilia A patients: clinical characteristics and patient preferences for treatment

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Background: We conducted a multi-country, cross-sectional survey of patients with severe hemophilia A to assess their preferences for different treatment attributes.
Aims: To present clinical characteristics of patients participating in a discrete choice experiment (DCE) about differences in preferences for treatment by current treatment regimen
Methods: An international, cross-sectional, web-based DCE was conducted in 2012 with patients with hemophilia A. Participants completed a series of clinical questions about their hemophilia and treatment regimen. The DCE examined eight attributes of factor VIII (FVIII) treatment (dosing frequency, treatment administration time, duration of protection, time required to stop a breakthrough bleed, number of infusions required to stop a breakthrough bleed, physical activity level, infusion volume, and joint protection and preservation). Participants were presented with two hypothetical FVIII profiles described by different levels of the eight attributes. Preference weights for the attributes were estimated using random effects multinomial models. Relative importance (RI) values, which represent the total

proportion of the variance that is accounted for by an attribute relative to the other included attributes, were calculated.
Results: A total of 117 male hemophilia A patients with a mean (SD) age of 35.6 (16.3) completed the survey. Respondents were from the United States, UK, France, Germany, Italy, Spain, and Sweden. The majority were on a prophylaxis treatment regimen (62%) using a recombinant factor product (92%). Of the few respondents with an on-demand regimen, the most frequently cited reason for using on-demand treatment was an acceptable bleeding frequency without prophylaxis. Respondents reported experiencing no breakthrough bleeds per month (40%), one per month (36%), two (11%), three (9%), or four (3%). Thirty-seven percent of patients used one infusion to stop breakthrough bleeds, 14% needed two, and 6% needed three infusions. Sixty percent of respondents reported at least one joint bleed per month. We compared the preferences of prophylaxis and on-demand participants. The most important attribute identified by both groups was joint protection and preservation, with an RI of 25.2 (prophylaxis) and 22.8 (on demand), meaning that on average, prophylaxis and on-demand patients based approximately 25% and 23% of their decision, respectively, on the joint preservation attribute. Prophylaxis patients rated physical activity level as the second most important attribute (RI 19.1), and on-demand patients rated it only fifth (RI 9.5). On-demand patients rated time required to stop a breakthrough bleed as the second most important attribute (RI 20.9), whereas prophylaxis patients rated it as third most important (RI 15.0).
Summary/Conclusions: Patients with severe hemophilia A use treatment regimens that allow them to participate more fully in life through sports and other physical activities. Both prophylaxis and on-demand patients prefer treatments that are effective in protecting joints. Ranking of the remaining treatment attributes illustrates the trade-offs patients are willing to make for a treatment that is effective in preserving joints and maintaining activity levels. These results demonstrate a continued need to develop better factor treatments that provide sustained protective levels of FVIII.

PB3.39 – Heparin and heparinoids – I

PB 3.39-1

Anticoagulant profile studied by thromboelastography of multiple batches of branded enoxaparin and a US generic version of enoxaparin

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Introduction: Enoxaparin is the most widely used low molecular weight heparin (LMWH) in the US and has been approved for clinical use in multiple indications. Enoxaparin is a complex biological product with multiple known activities relevant to its antithrombotic effects, and variations in different forms of enoxaparin may have important clinical implications. This study aimed to compare the physiological anticoagulant activity of branded and a generic enoxaparin, using thromboelastography (TEG) to evaluate their effect on the dynamic formation of blood clots as quantitated by interactions between coagulation factors and inhibitors, fibrinogen, platelets and the fibrinolytic system.
Methods: Blood was obtained from 12 healthy volunteers after giving written informed consent. Five batches each of branded (Sanofi-aventis; Bridgewater, NJ) and generic (Sandoz US; Princeton, NJ) enoxaparin were studied. Drugs were purchased through hospital pharmacies as pre-filled syringes containing 40 mg drug. Immediately after venipuncture whole native (no preservative) blood was mixed with various concentrations of branded or generic enoxaparin and TEG (Haemoscope; Niles, IL) was performed without addition of exogenous activators.
Results: Initial TEG analysis of one concentration of all LMWH batches to all 12 donors revealed greater inter-individual variation of the anticoagulant response for the generic product. Investigations revealed a greater batch-to-batch variation for the generic enoxaparin within individual donors and among individual donors. Across all donors both the branded and generic enoxaparin produced a concentration-dependent anticoagulant effect in the TEG; however, the greater degree of variability for the generic LMWH resulted in a less predictable linear response as drug concentration increased. Of note was that some individuals responded with a higher than expected anticoagulant response to given concentrations of the generic enoxaparin. When the increase in TEG R-time was plotted vs. concentration for all donors, a lower overall anticoagulant effect ($P = 0.05$; no overlap of 95% confidence intervals) with a wider inter-individual variation for generic enoxaparin in comparison with branded enoxaparin was demonstrated.
Conclusion: By using only human blood in its native state and evaluating the full dynamic hemostatic process of coagulation, this study was able to demonstrate differences in the anticoagulation response of branded and generic enoxaparins, clinically used LMWHs that have similar anti-FXa, anti-FIIa and average molecular weight. The test system used here more closely mimics the innate *in vivo* anticoagulant action of LMWH than what can be demonstrated by pure buffer system anti-FXa and anti-FIIa singular activities or other single endpoint assay systems commonly used to determine an anticoagulant response. The findings of this study suggest that other pre-clinical and clinical investigations should be performed to validate the clinical interchangeability between branded and generic LMWHs.

PB 3.39-2

Tissue culture based approach to discriminate branded and generic low molecular weight heparins

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Background: Pharmaceutical preparations of heparin and its congeners consist of a heterogeneous mixture of long polyanionic molecules. In addition to their anticoagulant properties, heparins are pleiotropic through a variety of mechanisms of action including modulation of the action of cytokines, chemokines and proteases. With the introduction of generic forms of established pharmaceutical LMWHs, there is increasing need for assays that take into account the often subtle differences in structure of LMWHs, which arise according to the method of manufacture. Simple assays of anticoagulant activity are unlikely to be adequate in this respect.
Aim: It was the aim of the present study to investigate if the ability of heparin to modulate the protein expression by cultured cells could be used to generate a proteomic signature that was unique and discriminatory.
Method: Cultures of HUVECs were treated with 400 nM thrombin in medium containing 2% serum for 3 h at 37 °C (24 well plate). Cells were washed in medium containing 10% FCS and medium containing heparin was then added together with VEGF (20 ng/mL). After 24 h, medium was removed and cells lysed in U9 buffer (9M urea, 2% CHAPS, 10 mM Tris, pH 7.0). 50 μL of the mixture was added to 450 μL of 50 mM Na acetate pH 5.0 and centrifuged at 10,000 ***g*** for 2 min. 150 μL of supernatant was added to CM-10 SELDI chips equilibrated with 50 mM NaAc pH 5.0. After incubation for an hour, the chip was washed and dried before the addition of matrix (SPA). Analysis was carried out in a Ciphergen Biomarker analyser and the data collated and analysed using Biomarker Wizard.
Results: Two series of experiments were carried out in sextuplet; in the first series, we compared the activity of Lovenox with two generic copies (L1 and L2) and in the second series two different batches of Lovenox (A v B), in both sets testing against untreated cells.
Series 1, Lovenox, L1 and L2: Each of the heparins was tested at a final concentration of 0.001, 0.01, 0.1 and 1.0 mg/mL. A dose related

effect was observed with each of the heparins and the results of the analysis showed a difference in intensity of 13 peaks (selecting those according to a $P < 0.05$) was found between Lovenox and L1 and nine peaks comparing Lovenox and L2. There was only one peak different between L1 and L2. Series 2, Lovenox batches A and B: There were no significant differences in peaks when batches A and B of Lovenox were compared at 1 mg/mL suggesting that this approach is unable to detect differences between batches of the same heparin.

Conclusion: These results suggest that the effect of individual forms of LMWH on protein expression by cultured cells is unique and can be used to develop a 'fingerprint' to discriminate each heparin from generic forms using statistical approaches such as decision tree analysis or neural networks as are currently used in the diagnostic field.

PB 3.39-3

Pharmacodynamic response to unfractionated heparin used for initial treatment of acute deep vein thrombosis in elderly patients with renal impairment. A substudy of the IRIS clinical trial

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Background: The 'Innohep® in Renal Insufficiency Study' (IRIS) was an international, open, randomized, clinical trial comparing the safety of tinzaparin and activated partial thromboplastin time (aPTT)-adjusted unfractionated heparin (UFH) in terms of clinically relevant bleeding (CRB) in elderly patients with moderate to severe renal impairment, treated for initial treatment of acute deep vein thrombosis (Leizorovicz *et al*., 2011). While UFH is usually preferred in these frail patients, little data exists on UFH dose management in very elderly with venous thromboembolism (VTE) in controlled trials.

Aims: In the IRIS patients receiving UFH, we conducted a substudy in order to: i/analyse the characteristics of UFH treatment; ii/identify individual factors determining the UFH dose; iii/evaluate quality of anticoagulation during UFH treatment and its relationships with VTE recurrence and CRB.

Methods: Patients received an IV bolus UFH 50 IU/kg followed by an initial dose (Day1) of 400–600 IU/kg/day given subcutaneously (SC) twice daily; UFH doses were further adjusted by aPTT ratio according to local practice (scheduled at least once daily in the midinterval). Patients received UFH for at least 5 days. Vitamin K antagonist was initiated between Day1 and Day3. Clinical, laboratory (aPTT ratios) and UFH doses were prospectively collected. We classified patients as being in a subtherapeutic, therapeutic or supra-therapeutic range according to each therapeutic range center.

Results: Of the IRIS study population who received UFH ($n = 270$), 259 patients with complete data were analysed: mean age was 83 ± 6 years (70–98 years), mean creatinine clearance (CrCl) (Cockcroft-Gault) 39.7 mL/min (SD 11.9, range 11–59); 25% had severe renal impairment. The mean daily UFH dose was 372 IU/kg (SD 88) over the SC treatment period (mean 6.8 – SD 2.7 days) and decreased from 447 IU/kg (SD 80) on Day1 to 308 IU/kg (SD 152) on Day5. Body-weight ($P < 0.001$), age ($P = 0.013$), sex ($P = 0.03$), and CrCl ($P = 0.049$) were independent predictors of the daily dose (IU), explaining 50% of the dose variability. Over the five first days, 163 patients (63%) had at least three dose adjustments; 104 (40%) had at least one 12 h-interruption of treatment (range 1–8) due to overdosage. The lower limit of the therapeutic aPTT range was reached in 55% of patients within the first 48 h and maintained for four consecutive days in only 17% of patients. The proportion of patients with an overdosage varied from 10% to 42%, between Day1 and Day5, with underdosage from 70% to 42%. High body-weight was associated with overdosage ($P = 0.009$). During SC treatment, 15 patients presented with CRB: six had aPTT ratios above 5.0, among whom five had major bleeding.

Conclusions: Our results confirm the low UFH dose requirements in the elderly with renal impairment with important individual variability. Body-weight, age, sex, and CrCl were the main predictors of the UFH dose in these patients. While the initial dose of 500 IU/kg/day was found adequate to reach the therapeutic range within the first 48 h, this study highlights the difficulties in maintaining the aPTT in the therapeutic range in this population.

PB 3.39-4

High risk of nadroparin to induce cutaneous delayed-type IV hypersensitivity reactions (DTH)

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Background: Heparin is widely used for prophylaxis and treatment of thromboembolic events. Recently attention has been drawn to heparin-induced skin lesions, as those are observed more frequently than initially reported; e.g. 7.5% of all medical patients and 19–39% of pregnant patients develop heparin-induced skin lesions. These may be the only clinical manifestation of immune-mediated heparin-induced thrombocytopenia, but are commonly caused by a delayed-type hypersensitivity response. Several risk factors, such as molecular weight of heparins, sex, obesity, age, duration and previous heparin therapy have been implied as risk factors for development of heparin-induced delayed-type hypersensitivity.

Aim: To identify possible risk factors of delayed-type IV hypersensitivity reactions (DTH) by a meta-analysis of three prospective studies.

Methods: We have recently performed three independent clinical trials to determine the incidence and causes of heparin-induced skin lesions. Here, data from all trials were used for multivariate logistic regression analysis to determine possible risk factors.

Results: Regression analysis confirmed obesity and prolonged anticoagulant therapy as independent risk factors for development of heparin-induced hypersensitivity. Interestingly, the choice of anticoagulant preparation had the greatest influence. Comparing dalteparin, enoxaparin, the pentasaccharide fondaparinux and nadroparin, the latter was associated with a significantly increased risk to elicit heparin-induced hypersensitivity (log OR 2.2; 95% CI [0.5–4.3], $P = 1.44e^{-5}$).

Summary/Conclusion: As determination of risk factors had not been an endpoint of the initial studies, and investigators did not influence the choice of anticoagulant, a possible selection bias cannot be excluded. Given, the high risk of nadroparin is validated in necessary controlled trials, this would underscore the uniqueness of individual heparin preparations, and should thus be considered for an individualized anticoagulant treatment.

PB 3.39-5

Low incidence of heparin-induced skin lesions in orthopedic surgery patients

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Background: Heparin-induced skin lesions occur with an incidence of 7.5% in medical and up to 19.8% in pregnant patients during s.c. hep-

arin treatment. Mostly they are due to allergic delayed-type hypersensitivity reactions (DTH), rarely due to HIT.
Aim: The aim was to determine the incidence and causes of heparin-induced skin lesions in orthopedic surgery patients. Secondary outcome measures were: rate of cross-allergies, frequency of thromboembolic and bleeding complications and HIT.
Methods: Patients were prospectively screened for cutaneous adverse effects during prophylactic or therapeutic s.c. heparin treatment for ≥7 days. Further procedures (skin biopsy, subcutaneous provocation, clinical assessment for thrombosis, bleedings and HIT, laboratory HIT-diagnostics) were initiated if a heparin-induced skin reaction was suspected.
Results: 316 patients were recruited between May 2010 – September 2011. The median duration of heparin treatment was 10 days (range 7–331). We observed heparin-induced skin reactions in 6 (1.9%) patients. All lesions could be identified histologically and/or allergologically as DTH reactions. HIT-diagnostics were negative in all six patients. The lesions in 5 of 6 patients occurred in combination with the use of the low-molecular weight heparin nadroparin. Cross-allergies with other heparins were noted in 1 of 6 (16.7%) patients. Three thromboembolic events and four major bleeding events occurred.
Summary/Conclusion: Heparin-induced skin lesions in surgical patients are less frequent compared to medical and pregnant patients. The incidence is 1.9%. They were all due to allergic DTH reactions. Thus, different patient populations seem to carry distinct allergy risk profiles. It is tempting to speculate that in the current cohort of surgical patients postoperative inflammatory states or steroid hormone levels might contribute to the lower incidence of skin lesions. Furthermore, our data support findings from previous studies that different heparin preparations, i.e. nadroparin, show distinct differences in their sensitisation potential.
Investigations and procedures were approved by the local ethics committee of the J. W. Goethe University(16/07). The study was registered at clinicaltrials.gov (NCT00510432) and performed in accordance with theDeclaration of Helsinki.

PB 3.39-6
Incidence of heparin-induced skin lesions in postmenopause

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Background: Heparin-induced skin lesions occur with an incidence of up to 19.8% in pregnant patients during s.c. heparin treatment, and occur 7–9 times more frequently in women than in men. Because this suggests a hormonal impact on allergy development.
Aims: The aim of our study was to determine the incidence and causes of heparin-induced skin lesions in postmenopausal women. Secondary outcome measures were: rate of cross-allergies, frequency of thromboembolic and bleeding complications and HIT.
Methods: Patients were prospectively screened for cutaneous adverse effects during prophylactic or therapeutic s.c. heparin treatment for ≥7 days. Further procedures (skin biopsy, subcutaneous provocation, clinical assessment for thrombosis, bleedings and HIT, laboratory HIT-diagnostics) were initiated if a heparin-induced skin reaction was suspected.
Results: 193 patients were recruited between January 2011 – January 2012. The median duration of treatment with low-molecular weight heparins was 11 days (range 7–52). Enoxaparin was used in 92.8%, nadroparin in 6.2%, and dalteparin in 0.5% of patients. We observed heparin-induced skin reactions in 4 (2.1%) patients, and all occurred during anticoagulation with enoxparin. All lesions could be identified histologically and/or allergologically as DTH reactions. None of the lesions was associated with HIT. No thromboembolic events and no major bleeding events occurred.
Summary/Conclusion: Heparin-induced skin lesions in postmenopausal women are less frequent compared to pregnant or medical female patients. The incidence is 2.1%. They were all due to allergic DTH reactions. Our study results indicate that the risk of sensitization after menopause is lower, possibly due to decreasing estrogen or progesterone levels. Future studies might investigate the role of selective estrogen receptor blockers on the modulation of type IV allergies.
Investigations and procedures were approved by the local ethics committee of the J. W. Goethe University(16/07). The study was registered at clinicaltrials.gov (NCT00510432) and performed in accordance with theDeclaration of Helsinki.

PB3.40 – Massive Blood Loss

PB 3.40-1
Contribution of the plasminogen activation system during hyperfibrinolysis in trauma-induced coagulopathy

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Background: Traumatic injury is the third leading cause of death in the US. One quarter of all severe trauma patients experience some degree of prolonged hemorrhage due to impaired blood clotting, a phenomenon known as coagulopathy. The etiology behind trauma-induced coagulopathy (TIC) remains unknown. Hyperfibrinolysis, or the inappropriate and rapid breakdown of hemostatic clots, contributes to TIC and is associated with increased fluid resuscitation, hemorrhagic shock, and mortality. Under physiologic conditions, fibrinolysis is primarily regulated by the plasminogen activation system. We hypothesize that clot stability is weakened by excessive plasminogen activation caused by dysregulation of the plasminogen activation pathway during TIC.
Aims: The aims of this study were to (i) determine the relationship between fibrinolysis measured by thrombelastography (TEG) and mortality following trauma, and (ii) characterize changes in proteins of the plasminogen activation system in hyperfibrinolytic vs. non-hyperfibrinolytic trauma patients.
Methods: Whole blood was collected from severely injured trauma patients upon admission to the emergency department. This study was performed with patient informed consent and Institutional Review Board approval. Patient demographics were collected at the time of presentation and from hospital records. Fibrinolysis was determined by TEG (LY30%). Plasma was analyzed for circulating levels of fibrinogen, antithrombin, in addition to active and total tPA, uPA, and PAI-1 by ELISA.
Results: Data was collected on 381 consenting trauma patients. The population contained 69% blunt, 24% penetrating, and 6% burn injuries. Ly30% values ranged from a minimum of 0 to maximum of 92.9 (median 1.6, IQR 0.3–3). Mortality was positively associated with LY30% ($R^2 = 0.91$, $n = 381$). Hyperfibrinolysis for this study was defined as LY30% ≥5 ($n = 19$) where we observed a doubling of the morality rate in this population. These patients were compared to those with an LY30% = 0 ($n = 19$) to dichotomize the experimental population. While plasma levels of fibrinogen, antithrombin, tPA, and uPA are similar between the two groups, hyperfibrinolytic patients display a reduced ability to upregulate plasma levels of PAI-1 from healthy baseline (4 ng/mL total, 10 U/mL active) in response to injury compared to non-hyperfibrinolytic trauma patients (active PAI-1 median 10.0, IQR 18.8–171.1 ng/mL vs. 30.3, IQR 3.2–29.6 ng/mL; $P < 0.05$) (total PAI-1 median 2.4, IQR 1.8–5.4 U/mL vs. 4.5, IQR 3.5–20.1 U/mL; $P = 0.06$).

Conclusions: Excessive plasminogen activation due to inhibition or insufficient expression of antifibrinolytic PAI-1 is a potential contributing mechanism behind hyperfibrinolysis during TIC and offers a point for therapeutic intervention.

PB 3.40-2

Principal roles of platelets and fibrinogen in whole-blood fibrin clot formation in dilutional coagulopathy determined by thromboelastometry

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Background: Patients undergoing cardiothoracic surgery are massively infused with crystalloids and colloids, resulting in blood dilution and hemostatic insufficiency. A major consequence is diminished elastic fibrin clot formation, which can be measured by rotational thromboelastometry (TEM) in whole blood. Upon dilution, the plasma fibrinogen level is a key limiting factor for clot formation, and this can be readily detected by the TEM assay. How other blood components determine this process, as measured with TEM, is largely unknown.
Aims: To investigate the contribution of blood cells (platelets or red cells) and plasma coagulation factors to fibrin clot formation with TEM under conditions of dilution.
Methods: Control blood was diluted *in vitro* by 20–80%, and then supplemented with blood cells, fibrinogen or prothrombin complex concentrate, and assayed for fibrin clot formation. Plasma was diluted, supplemented with platelets or factor concentrates, and used for measurement of fibrin clot formation (TEM) and thrombin generation (Calibrated Automated Thrombogram method). Blood was obtained from 48 patients before and after cardiothoracic surgery. Blood loss and fluid transfusion during surgery resulted in a 43–49% reduction in platelet and coagulation factor levels. Whole blood and plasma samples obtained before and after dilution were assayed for TEM. Experiments were approved by the medical ethics committee.
Results: In control blood or control plasma, diluted *in vitro*, supplementation of platelets reversed the decreased fibrin clot formation (maximal clot firmness and rate of clot formation). Addition of fibrinogen was partly effective, while addition of red cells or prothrombin complex concentrate was without effect. In diluted plasma, addition of platelets or prothrombin complex concentrate, but not fibrinogen concentrate, reversed the decreased thrombin generation (thrombin peak height and endogenous thrombin potential). In whole blood from patients, dilution due to surgery caused a diminished fibrin clot formation by 19% (maximal clot firmness and rate of clot formation), which was explained by reductions in platelet count and fibrinogen level. Correlation analysis indicated that platelet count was an independent predictor of the maximal clot firmness, measured by TEM. In plasma, reduced fibrin clot formation after surgery was most evident in the absence of platelets. Thrombin generation in plasma was also less affected after surgery with platelets present, and it normalized by addition of prothrombin complex concentrate but not by added fibrinogen concentrate.
Conclusions: Under conditions of dilution, whole-blood fibrin clot formation, as measured by TEM, is primarily determined by the platelet and fibrinogen concentrations. The platelet count should be considered as independent variable of whole-blood TEM assays. Levels of coagulation factors controlling thrombin generation are less critical for the extent of fibrin clot formation.

PB 3.40-3

The use of preoperative erythropoiesis-stimulating agents (ESAs) in patients who underwent knee or hip arthroplasty a meta-analysis of randomized clinical trials

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Introduction: Erythropoiesis-stimulating agents (ESAs) have been used preoperatively in orthopedic patients to reduce the need for allogeneic blood transfusion. However, their use for this indication is still not widely practiced. The purpose of this review is to evaluate the efficacy of preoperative administration of ESAs on hemoglobin level at discharge and frequency of allogeneic blood transfusion in patients undergoing hip or knee surgery.
Methods: This is a systematic review of comparative randomized clinical trials that compared preoperative ESAs to other interventions or placebo in reducing the need for allogeneic transfusions and increase in hemoglobin levels at discharge.
Results: Pooled results of 26 trials with 3560 participants showed that the use of preoperative ESAs significantly reduced the need for allogeneic blood in patients undergoing hip or knee surgery [Relative Risk (RR): 0.48, 95% CI: 0.38–0.60, $P < 0.00001$)]. Hemoglobin mean difference between ESA and control groups was 7.16 (g/L) [95% confidence interval (CI) of 4.73–9.59, $P = 0.00001$]. There was no difference in the risk of developing venous-thromboembolism between ESA group and the control groups [Risk Difference (RD): 0, 95% CI: −1 to −2%, $P = 0.95$; I2 = 0%].
Conclusion: ESAs offer an alternative blood conservation method to avoid allogeneic blood transfusion in patients undergoing hip or knee surgery.

PB 3.40-4

Predictive value of the HAS-BLED score in patients with atrial fibrillation and chronic kidney disease using vitamin K-antagonists

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Background: The HAS-BLED score enables a risk estimate of major bleeding in patients with atrial fibrillation (AF) on vitamin K-antagonists (VKA) treatment, but has never been validated in patients with chronic kidney disease (CKD).
Aim: We analyzed the predictive value of the HAS-BLED score in CKD compared with non-CKD patients.
Methods: Medical records of 416 CKD(eGFR0-30 mL/min, 30–60 mL/min) and 300 non-CKD patients starting VKA treatment for AF between 1997 and 2005 were searched for items on the HAS-BLED score (hypertension; renal or liver disease; stroke; major bleeding or anemia; labile INR, (time within therapeutic range <60%), age >65 years; use of NSAIDs, anti-platelet therapy or alcohol) and major bleeding events, according to the ISTH criteria. Areas under the curves (AUC) of the receiver operating characteristic (ROC) were calculated for the total population and CKD patients.
Results: Mean HAS-BLED score in CKD patients was 3.1 vs. 2.6 in non-CKD patients ($P < 0.01$). Major bleeding occurred in 115/716 (16.1%, 95% CI 12.8–19.9%) patients. The AUC of the ROC analysis in the total population was 0.50 (95% CI 0.44–0.56); 0.53 (95% CI 0.43–0.62) in patients with an eGFR 30–60 mL/min, and 0.35 (95%

CI 0.21–0.49) in patients with an eGFR <30 mL/min. In a Cox regression analysis performed in all patients, renal impairment, labile INR and age >65 years were predictive of major bleeding, hazard ratios 2.5 (95% CI 1.2–5.2), 2.2 (95% CI 1.2–3.7) and 5.2 (95% CI 1.6–16.8), respectively.

Conclusion: Performance of the HAS-BLED score was limited in the total population and further reduced in patients with an eGFR <30 mL/min. Further research is needed before the HAS-BLED score can be used in CKD patients.

PB 3.40-5

Development of an electronic identification tool for in-hospital bleeding

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Background: In recent years hospital medicine has seen a transition towards patient-physician partnership, with emphasis on quality of care and patient safety. Spontaneous bleeding and peri-operative bleeding are potentially preventable events. There has been little research on the epidemiology of such adverse incidents. A real time identification tool would enable further analysis, to identify potentially preventable causes of bleeding.

Aims: To develop an electronic identifying trigger for peri-operative and spontaneous inpatient bleeding events.

Methods: This was a retrospective study. A search of the hospital ICD 10 coding identified 250 patient admissions during 2011–2012 coded for both bleeding and surgery, 250 coded for bleeding and no surgery, and 500 admissions without a bleeding code. The hospital charts were hand searched, blinded to the ICD 10 coding, to establish whether the admitting team had identified the patient as bleeding, and to extract data on potential surrogate markers of bleeding (type of surgery, drop in haemoglobin, number of units blood transfused, return to operating room, readmission, anticoagulant medication, endoscopy and comorbidity). Receiver operating characteristic (ROC) curves were constructed to evaluate the area under the curves (AUC) for continuous variables, and to identify optimal cut-points. Peri-operative bleeding and inpatient hospital bleeding events were combined into a single bleeding outcome. Variables found to be significant predictors of bleeding on univariate logistic regression analysis were included in multivariate analysis.

Results: 156 patients were documented by their inpatient team as having had a peri-operative bleed and 29 patients had a spontaneous bleed during their hospital stay. 28% of the total cohort were taking an antiplatelet medication, 41% received prophylactic anticoagulation and 19% were fully anticoagulated. 70% of the cohort underwent surgery, the most common surgeries being arthroplasties, neurosurgeries, laparotomies, gynaecological, coronary interventions and bypass grafting. 20% had a diagnosis of cancer. The AUC for the measured drop in haemoglobin was 0.69 (95% CI 0.64–0.74) and for number of units blood transfused, 0.73 (95% CI 0.69–0.78). The optimal model for predicting bleeding included number of units blood transfused, endoscopy procedure, return to the operating room following surgery, readmission and concomitant antiplatelet and anticoagulant therapy. The C-staistic for the model was 0.88.

Summary/Conclusions: Identification of in-hospital bleeding is the first step towards implementing improved systems to reduce preventable bleeding. Our analysis has identified five potential surrogate electronic markers for in-hospital bleeding. Our next step is to validate the performance of this electronic trigger real time within the hospital.

PB 3.40-6

Evolution of plasma fibrinogen levels in trauma patients during the first 7 days of hospital stay is not influenced by initial treatment with fibrinogen concentrate

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Background: Coagulopathy is present in approximately one third of all trauma patients on admission to the emergency room and is primarily associated with acquired fibrinogen deficit. Due to early and individualized goal-directed therapy for trauma-induced coagulopathy, fibrinogen concentrate (FC) is increasingly used in some trauma centers. It has been questioned whether the use of FC leads to higher plasma fibrinogen levels (FIB), thus possibly carrying a prothrombotic risk.

Aims: We evaluated FIB on seven consecutive days in acute trauma patients with or without FC therapy in an Austrian trauma centre.

Methods: Retrospective study of patients in the hospital trauma database, admitted to the AUVA Trauma Centre of Salzburg, between 2005 and 2011, in whom FIB was documented on admission to the emergency room and 48 h thereafter. For analysis patients were categorized into two groups: Treatment group (TFC) with patients that received at least 1 g FC during the first 24 h after hospital admission; Control group (CTR) with patients that did not receive FC.

Results: 402 patients were enrolled in this study, with a median (interquartile range) age of 42 years (26–56). 327 patients (81%) were male, overall ISS was 26 (18–38) with 357 patients (89%) classified ISS ≥16. 209 patients (52%) were grouped into TFC, 193 patients (48%) into CTR.

ISS was higher in TFC patients with 34 (25–45) than in CTR patients with 21.5 (16–27), $P < 0.0001$. Mortality was 10% in the TFC group vs. 4.1% in the CTR group, $P = 0.03$. Massive transfusion (≥10 red blood cell units in 24 h) was provided in 30.6% of TFC patients vs. 0.5% in CTR patients, $P < 0.0001$.

FIB (mg/dL) on admission was lower in TCF with 148 (108–192) as compared with CTR with 225 (178–271), $P < 0.0001$.

Mean FC administration was 5 g (3.5–9) with a minimum of 1 g and a maximum of 26 g administered in the first 24 h.

FIB (mg/dL) on admission to the ICU and on day 1 (after admission) was still lower in the TFC group as compared with the CTR group: 155 (124–194) vs. 170 (136–224), $P < 0.01$ and 240 (208–294) vs. 262 (214–312), $P < 0.05$, respectively.

FIB values did not differ significantly between groups on all following days 2–7, reaching the highest values on day 6, in TFC with 680 (521–832) and a minimum/maximum of 228/1292 vs. CTR with 664 (527–781) and a minimum/maximum of 266/1392, $P = 0.82$.

Summary/Conclusion: FIB evolution in severe trauma patients does not depend on initial treatment with FC in the acute phase of trauma care.

PB 3.41-1

Founder effect for a novel GPIBB mutation in Bernard-Soulier patients from La Réunion island

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Background: Bernard-Soulier syndrome (BSS) is a rare hereditary bleeding disorder due to mutations in the genes coding the platelet

GPIb-IX complex. In a recent survey within the consortium led by Savoia et al (Blood, 2011; 118: 707), 64 different mutations have been reported in over 128 families which indifferently affected the GPIBA, GPIBB and GP9 genes. These are mainly sporadic cases with only rare occurrence of founder effects. Two examples of the opposite are the GP9 p.Asn61Ser and GPIBA p.Ala175Val mutations which have been found in several distantly related individuals of European and Italian origins, respectively.

Aims: Our aim was to study at the genetic level several patients from La Réunion island suspected of presenting with a BSS.

Methods: All patients were recruited at the Groupe Hospitalier Sud Réunion site St Pierre with the exception of one case studied at the Centre Hospitalier Universitaire de Bicêtre. Platelet counts were determined in an automated counter and manually, and platelet size by blood smears observation. Platelet expression of the GPIb-IX complex was monitored by FACS analysis. Sequences of the GPIBA, GPIBB and GP9 genes were obtained after PCR amplification of leukocyte DNA and automated sequencing.

Results: We report on 14 patients from eight different families all originating from l'Ile de La Réunion, a French island in the Indian Ocean. BSS was established on the presence of mucocutaneous bleeding, giant platelets, decreased platelet counts (33–70 G/L), absent GPIba and GPIX, and an autosomal recessive inheritance. Despite the fact that these families did not know of any familial relationship, all 14 patients harbored the same novel GPIBB c.265A>G transition leading to a p.Asn89Asp amino acid change in the GPIbbeta subunit (p.Asn64Asp for the mature protein). Interestingly, this well conserved residue has been previously reported to be mutated to Thr in an isolated BSS case leading to impaired GPIbbeta folding, GPIbalpha maturation, GPIX stability and GPIb-IX surface expression (Strassel C et al, Biochemistry 2003;42:4452; McEwan et al, Blood 2011;118:5292). Prevalence of this mutation suggested that it resulted from a founder effect. This hypothesis was supported by the reconstruction of a genealogy tree dating back 10–14 generations which allowed connecting all the affected individuals to a common ancestor born in 1671 and originating from India.

Conclusion: Although founder mutations in genetic isolates have already been reported in another platelet hereditary disorder, Glanzmann's thrombasthenia, this represents the first such case for BSS, an even rarer disorder. It is expected that the number of patients with this same mutation is substantially larger justifying more extensive screening to improve on genetic counseling.

PB 3.41-2

Identification of novel mutations causing congenital factor XIII deficiency in Pakistan

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Background: Rare bleeding disorders (RBDs) represent 3% to 5% of all congenital coagulation deficiencies, factor VII deficiency being the most common. However, in Karachi, Pakistan, we report a high number of congenital factor XIII (FXIII) deficiency in comparison to other RBDs.

Aims: This study aims to give a first insight into *F13* gene mutations in Pakistani population where the ritual of consanguineous marriages is practised.

Methods: The medical histories of congenital FXIII deficiency patients were recorded in a questionnaire and bleeding score was determined according to RBD database. 5M urea clot solubility test served as a first indicator of FXIII deficiency. FXIII A-subunit and B-subunit antigen levels were determined in citrated plasma by ELISA. FXIII activity was measured with an incorporation assay. Direct sequencing of all exons and intron/exon boundaries of *F13A* was performed. Novel splice site defect was confirmed by RT/PCR analysis.

Results: Patients in our cohort belong to six different families. Molecular analysis of 12 homozygous patients and six heterozygous family members revealed five different mutations in *F13A* gene, four of them being reported for the first time. Most common bleeding manifestations were epistaxis, gum bleeds, bruises, circumcision and umbilical cord bleeding; female patients often experienced menorrhagia and repeated abortions. Mean bleeding score (±SD) in homozygous patients was 13.8 ± 9.6, while heterozygous were mainly asymptomatic (2.0 ± 0.6). Majority (80%) of patients have a history of cousin marriages in their parents.

We identified two mutations localized on the exon/intron boundary (c.1460 + 1G>A and c.2045 G>A). Mutation c.1460 + 1G>A, IVS11 + 1G>A is being reported for the first time, and we confirmed that it leads to a splicing defect. It was found in nine related family members, homozygotes presented with severe FXIIIA deficiency, the FXIII antigen levels and activity in heterozygotes were variable. The second splice site mutation, c.2045 G>A in the last nucleotide of exon 14, was identified in five patients from two different families. This mutation has previously been reported in patients of Indian or Pakistani origin. Two novel missense mutations in exons 8 and 9 (c.1126 C>T, p.Trp375Arg and c.1040C>A, p.Ala346Asp) were identified in single cases and both patients were homozygous for the mutation. Both patients presented with severe FXIIIA deficiency with FXIIIA antigen levels below the detection limit, suggesting that the hypothetical protein would be rapidly degraded. A novel nonsense mutation in exon 4, c.567T>A, p.Cys188X, was identified in two related patients, both being homozygous for this allele. Both patients presented with a complete lack of FXIII.

Conclusion: We have reported four novel mutations leading to congenital FXIII deficiency in a cohort of 18 patients. Early diagnosis of FXIII deficiency enables parental counseling and identification of related family members at risk, which is critical for specific treatment of the patients and for prevention of bleeding complications in the future. We plan to continue to characterize other severe affected patients in our centre to verify if these variants could be recurrent mutations in our specific geographic area.

PB 3.41-3

Intracranial hemorrhage in factor XIII deficiency

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Background: Intracranial hemorrhage as a one of clinical manifestations in severe congenital FXIII deficiency is a life-threatening situation and major cause of death in untreated patients .

Aims: The aim of study was to collect data on patients with FXIII deficiency who had an intracranial hemorrhage.

Methods: In this case series, from January to May 2012, 35 patients with severe F XIII deficiency in Southeast of Iran were evaluated. The ICH episodes, family history, general physical examination, detailed neurological complication, incidence of recurrence, management strategies, survival status were noted.

Results: Out of 35 patients, the site of ICH in 32 individuals was intraparenchymal (91.4%). subdural and epidural hemorrhage were observed in 2 (5.7%) and1 (2.9%) patients, respectively.

The anatomic regions in patients with intraparenchymal hemorrhage was as follow: temporal in 10 patients (28.6%), occipital in 9 (25.7%), temporo-occipital in 4 (11.4%), subdural with temporal in 1 (2.9%), subdural with occipital in 1 (2.9%), parietal in 1 (2.9%), and diffused intraparenchymal hemorrhage in 6 (17.1%). Neurologic complications was also observed in 20 patients (57.1%) including behavioral disorders (28.6%), developmental disorders (11.4%), aphasia (8.6%), ophthalmic complications (5.7%), and hemiplegia (2.9%). Age at diagnosis of FXIII deficiency was significantly correlated with age of CNS bleeding ($r = 0$ .542, $P = 0.001$). Thirty four patients (97.2%) response to treatment response except one patient who had experienced one episode of recurrent. Prophylaxis was started with a dose of 10 IU/kg every 4–6 weeks for all patients. No episode of recurrence was observed in patients after that. Death was recorded in in 44 first degree family members of patients. The causes of death was in 10 cases FXIII deficiency, 17 suspicious death primarily associated with FXIII deficiency disease and eight unknown causes.

Summary/Conclusions: It seems that optimal management of ICH in patients with FXIII deficiency could be depend on immediate recognition and prophylactic treatment. Due to higher risk of such a catastrophic bleeding episode in younger Patients, therefore these patients should be more considered.

PB 3.41-4

Severe FVII-deficiency- a study of geno-phenotype relationship using thromboelastography and thrombin generation assay

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Background: FVII deficiency is a rare autosomal recessive inherited bleeding disorder characterized by a wide heterogenicity in the bleeding phenotype. The correlation between a bleeding tendency and the plasma FVII activity level is weak. Currently, there exist no laboratory test that can predict the bleeding risk accurately.

Aims: We aimed to study whether thromboelastography (TEG) and thrombin generation assay (TGA) are suitable.

Methods: To predict the bleeding risk and to investigate whether there is any association between the plasma level of free tissue factor pathway inhibitor (TFPI), platelet aggregation capacity or other relevant coagulation parameters and the bleeding phenotype in persons with severe FVII deficiency.

Materials and methods: Twelve patients ($n = 12$) with severe FVII deficiency (FVII: activity level <1%), aged between 19 and 69, were included. Eleven of those were homozygous for Q100R mutation, whereas one was compound heterozygous. Clinically, 10 had increased haemorrhagic diathesis whereas two were asymptomatic. Blood sampling was performed at baseline for TEG and TGA analysis. TEG was performed using ROTEM, Coagulation Analyzer (Pentapharm®, Munich, Germany) while TGA by calibrated automated thrombin generation method (CAT) (Thrombinoscope BV® Maastricht, The Netherlands) on platelet poor plasma (PPP) and platelet rich plasma (PRP). Furhermore, platelet aggregation was performed, TFPI and haemostatic parameters were measured.

Results: We found no difference in TEG and TGA results between FVII deficient persons with an increased bleeding tendency and persons without. Compared with healthy normals clotting time (CT) and time to peak (ttpeak) was twofold increased (2394 vs. 1095 s for CT and 2966 vs. 1407 s for ttpeak) whereas Maximum Velocity (MaxV) was reduced (5 vs. 3). The TGA data showed that there was a minimal coagulation activation and thrombin generation in all FVII deficient subjects compared with normal (186 vs. 1308 nM thrombin in PRP and 209 vs. 658 in PPP). The level of free TFPI was similar in symptomatic as well as in asymptomatic persons and was within normal range, 8.3 ± 2.6 ng/mL (±SD). None of the participants were tested positive for FV Leiden mutation, prothrombin genmutaion or had abnormal level of anticoagulant inhibitors. A normal platelet aggregation was also found in the asymptomatic persons.

Conclusion: TEG and TGA, when tested in severe FVII deficiency, show results corresponding to the severity of factor deficiency but do not correlate with the bleeding phenotype. Either plasma TFPI level or other known anticoagulant inhibitors are associated with the clinical phenotype. We postulate that an unidentified biologic factor with procoagulant function is responsible for initiating and maintaining haemostasis in the asymptomatic patients and that this factor is probably located in the vessel wall, therefore not measurable with either TEG or TGA. Identifying such a factor will extend our knowledge concerning the coagulation system and the haemorrhagic/thrombotic tendency, providing novel diagnostic and therapeutic opportunities.

PB 3.41-5

Overcoming barriers to diagnosis of bleeding disorders including Acquired Hemophilia (AH) by healthcare practitioners (HCP): The Coags Uncomplicated iPhone/Android/Web application

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Background: Characterized by a prolonged aPTT that does not correct with mixing, Acquired Hemophilia is a rare and often fatal acquired bleeding disorder caused by autoantibodies to factor VIII. Multi-specialty case-based research around an actual case of delayed AH diagnosis (Reding et al 2012) suggested diagnostic delays can result from lack of recognition of the significance/etiology of an abnormal aPTT and focus on site of bleeding by emergency medicine, critical care and other non-hematologists. The Coags Uncomplicated app was initially designed as a collaboration between the hematology community and industry to assist healthcare practitioners (HCPs) in the diagnosis of these cases. Since its introduction, however, the scope has broadened considerably to encompass all congenital and acquired bleeding disorders.

Methods: Based upon gaps around interpretation of PT/aPTT abnormalities and bleeding disorders with normal PT/aPTT, an updated iPhone/android/web application (www.coagsuncomplicated.com) was expanded to include 60+ bleeding disorders. The *Lab Value Analyzer* first screens for medication-related abnormalities, pattern matches lab values entered with disease profiles, and lists potentially matching diagnoses. The *Diagnostic Algorithm* guides HCPs in obtaining additional lab tests. Published algorithms for isolated abnormalities of PT and aPTT were reorganized to include also an algorithm for common pathway (abnormal PT/aPTT) and disorders with a normal PT/aPTT. The *Neonatal* module compares entered lab values with age-adjusted normal values and provides a tailored algorithmic approach including reference age-adjusted normal values. Educational components provide.

Background: On all assays, diseases and medications. Third party analysis of de-identified utilization data are monitored to assess diagnoses reached and educational needs.

Results: Since January 2011, 5259 downloads resulted in 3744 registrations (Heme/Heme-Onc 1620, ER 702, Internal Medicine 244, Critical Care 217, Pediatrics 111, Surgery and obstetrics/gynecology 111). Common lab value analyzer matches were disseminated intravascular coagulopathy (6%), dysfibrinogenemia (5%), lupus anticoagulant (5%) and monoclonal gammopathy (5%). Common diagnostic algorithm endpoints were DIC (23%), liver disease effect (17%), vitamin

K deficiency (15%), lupus anticoagulant (5%), and warfarin/heparin effects (4%/4%). AH diagnoses were reached 73 times (2%) in diagnostic algorithm and 143 times (2%) in lab value analyzer.
Conclusions: Diagnosis of bleeding disorders first and foremost requires HCP recognition of abnormal laboratory values (including awareness of age-related normal values) that can prompt inquiry into the cause of the bleeding, not just its location. Monitoring demonstrated medication effects (lab value analyzer) and common disorders (lab value analyzer and diagnostic algorithm) were most accessed. This innovative approach helps to bridge the knowledge gap for non-hematologists and to facilitate initiation of prompt appropriate referrals to hematology.

PB 3.41-6

A literature review on the burden of illness and management of congenital FXIII deficiency

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Background and Aims: Factor XIII congenital deficiency (FXIII CD) is a serious bleeding disorder caused by a lack of clotting factor XIII. This disease results in lifelong bleeding diathesis, defective wound healing, and spontaneous abortions. The aim of this study was to review available literature on the burden and management of FXIII CD in order to provide insight into how it affects the lives of patients and their families, and to evaluate the current treatment paradigm.
Methods: A broad literature search was conducted in PubMed, Embase and Cochrane databases using disease specific search terms for FXIII CD. All studies reporting data on the humanistic and economic burden of FXIII CD and available treatments were included in the final selection. No language or time restrictions were applied.
Results: Out of 551 records, 13 studies met the inclusion criteria. Three of the studies reported the occurrence of bleeds and intracranial hemorrhage (ICH) in patients with FXIII CD; the remaining 10 publications reported efficacy and safety outcomes of treatment with FXIII concentrates. No reports on the economic consequences of FXIII CD were identified. In selected literature, FXIII CD is described as one of the most severe forms of a congenital coagulation disorder, primarily due to severe bleeding events and a high risk of ICH. Current literature suggests over 50% of untreated FXIII CD patients experience severe bleeding symptoms, with ICH reported in 20–60% of patients and regarded as a major cause of death and morbidity. Data on health-related quality of life (HRQoL) pertaining to the impact of such complications in patients with FXIII CD is sparse. Only one prospective questionnaire-based study including five FXIII deficient patients was found, demonstrating that the severity of bleeds is a factor which influences HRQoL. However, from other rare coagulation disorders, like Von Willebrand disease, it is known that severe bleeds have a detrimental impact on HRQoL. Therefore, very severe complications, such as ICH should be prevented where possible. It is widely accepted practice to prophylactically treat patients with FXIII CD, as early as possible in their lives, to prevent the occurrence of bleeds, including potentially life-threatening ICHs.
Summary and Conclusions: Limited data is available on the humanistic and economic burden related specifically to FXIII CD. However, the high risk of severe bleeds, including ICH, is widely accepted to result in a high level of burden in patients with bleeding disorders. Therefore, potential bleeds should be prevented with a safe and effective prophylaxis treatment. Until recently the treatment of FXIII CD was limited to plasma or plasma-derived products, bearing the risk of pathogen contamination, as well as time consuming, high volume infusions. Recently, a recombinant FXIII has been developed enabling an efficient and safe prophylaxis in patients with FXIII A-subunit CD with no risk of contamination with viral agents, prions and other infectious agents. Further research is required to gain insight in how specifically FXIII CD affects HRQoL and to fully understand associated economic consequences.

PB3.42 – Von Willebrand disease: CLINICAL – IV

PB 3.42-1

Analysis of sequence variation reported within the von Willebrand factor gene locus

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Background: The von Willebrand factor (*VWF*) gene consists of 52 exons; exons 2–52 encoding the VWF protein. Mutation of *VWF* results in von Willebrand disease (VWD), the most common inherited bleeding disorder. VWD is divided into three distinct types; types 1 (VWD1) and 3 (VWD3) caused by mild-moderate or severe reduction in circulating plasma VWF respectively, and type 2 (VWD2A, VWD2B, VWD2M, VWD2N) caused by specific defects influencing normal VWF function. The ISTH-SSC on VWF online database (VWFdb) provides access to VWF/VWD information for diagnostic and research laboratories. This information includes a searchable registry of *VWF* sequence variation (derived from investigator submissions and literature reports), recently moved to the Leiden Open (source) Variation Database (LOVD) system (http://www.LOVD.nl/VWF), including new fields (VWF:Ag, VWF:RCo, FVIII:C, bleeding score, multimer profile) providing access to substantial phenotypic information for each variant. VWFdb also lists variants by patient highlighting additional information, for example homozygosity/heterozygosity for recessive mutations. Unlike VWFdb, the Human Genome Mutation Database (HGMD) only catalogues *VWF* variation reported in the literature, with no additional phenotypic information.
Aims: (i) To evaluate the extent of *VWF* sequence variation currently reported. (ii) To investigate any patterns in sequence variation based on VWD type.
Methods: The VWFdb registry and HGMD (accessed January 2013) were examined for unique sequence variants and these were classified as either mutations or polymorphisms. Mutations were further grouped by VWD type. Variant location vs. VWD type was mapped according to VWF protein domains.
Results: VWFdb contains information on 585 unique *VWF* variants; 424 mutations and 161 polymorphisms. Analysis of HGMD data highlighted an additional 173 variants, the majority of which resulted in missense/nonsense mutations (n = 120, 69%). Mutations represented all VWD types, the majority associated with VWD1 (n = 135, 23%), VWD2A (n = 107, 18%) and VWD3 (n = 176, 29%) with VWD2B (n = 30), VWD2M (n = 32) and VWD2N (n = 30) mutations each accounting for 5%. VWD1 and VWD3 mutations occurred throughout VWF (30% and 23% D3-A2 domains respectively; 15% and 14% C1-CK domains respectively). VWD1 mutations also clustered around A3-D4 (22%), whereas VWD3 mutations clustered around D1-D2 (35%). VWD2 mutations predominantly occurred in specific domains (VWD2B/2M: A1; VWD2N: D'/D3). VWD2A mutations were located throughout VWF, however hotspots (D2, D3-A2 and CK) demonstrated subtype specificity, for example VWD2A(IIE) in the D3 domain. Interestingly, the combined data contained 87 (15%) mutations either associated with multiple VWD types or unclassified, many of which (47%) were located in exons 25–28.
Conclusions: There are currently 758 unique variants reported in *VWF* (585 in VWFdb; 173 in HGMD), 597 (79%) associated with VWD. Those associated with VWD mostly account for VWD1, VWD2A and VWD3, and occur throughout VWF. VWD mutations located in exons 25–28 (D3/A1 domain NH_2 boundary) may result in a pleiotropic phenotype making them difficult to classify. Reports of *VWF* variation

on HGMD not currently entered on VWFdb highlights the requirement for investigators to submit both their published and unpublished data.

PB 3.42-2

Phenotypic and genotypic characterization of 10 Finnish patients with von Willebrand disease type 3: discovery of two main mutations

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Background and aims: Severe von Willebrand disease (VWD) type 3 is a rare autosomal-recessively inherited bleeding disorder, showing considerable genotypic heterogeneity. We investigated the phenotype in correlation to the genotype in Finnish type 3 VWD patients.

Methods: Ten patients (40% out of total of 28 patients recorded in Finland) from different families previously diagnosed with VWD type 3 treated at the Coagulation Disorder Unit in Helsinki University Hospital were re-evaluated for bleeding tendency and treatment. Phenotypic characterization included coagulation and platelet function testing confirming the diagnosis. Multimer study was performed for all the patients. Bleeding history was evaluated using a bleeding assessment tool modified from the Tosetto score and also with regard to specific bleeding symptoms. Detailed genealogical information was obtained from patients. The genotype was assessed by initial screening for the common c.2435delC mutation and subsequently if needed, by analyzing all 51 coding exons of the von Willebrand factor (VWF) gene.

Results: The diagnosis of type 3 VWD was confirmed for all patients. The patients presented with surprisingly heterogeneous bleeding phenotype: seven patients with severe spontaneous bleeding tendency required prophylactic treatment with replacement therapy whereas three patients on on-demand treatment presented with relatively mild bleeding phenotype. Clinical symptoms included hemophilia-like hemarthropathy in 8/10 (80%) patients studied. In addition, all patients presented with epistaxis and all female patients with menorrhagia. Two patients (20%) had a history of alloantibody development. In genotype analysis we discovered two common mutations among the patients: nine of the 20 (45%) alleles were found to carry the c.2435delC frameshift mutation, previously described to be frequent in countries surrounding the Baltic Sea. The nonsense mutation c.4975C>T (p.R1659X) was found on 8/20 (40%) of the alleles. Additionally, three novel mutations, a potential splice site mutation (c.874 + 2T>C) and two frameshift mutations (c.1668delC and c.2072delCCinsG) were found. Seven patients were homozygous and three compound heterozygous for the reported mutations.

Conclusion: Our study indicates that mainly two mutations (c.2435delC and p.R1659X) are causing the majority of type 3 VWD in Finland, unlike the reports from most of the previously studied populations. c.2435delC has been commonly reported at high frequency in countries surrounding the Baltic Sea, 50% in Sweden, 20% in Germany, 75% in Poland and, in addition 12% in Hungary. A common origin can be suggested for this mutation and our study supports this theory. Our genotype results in this group of patients set future standards for the genetic testing among the type 3 VWD population in Finland.

PB 3.42-3

Bleeding caused by acquired von willebrand syndrome (AVWS) in adult patients with congenital heart disease (CHD)

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Background: In acquired heart disease espec. aortic valve stenosis AVWS is typical. However, little is known about the role and prevalence of von Willebrand syndrome in congenital heart disease in adults. We hypothesized that VWS may play a causative role in surgical as well as non-surgical bleeding in different types of CHD.

Aims: We evaluated the prevalence and relevance of AVWS in CHD including various defects.

Methods: AVWS was evaluated in 192 patients in relation to the underlying cardiac defect, operative procedures and presence of Eisenmenger Syndrome. PT, aPTT, PFA-100, VWF:Ag, VWF:RiCof, VWF:CBA, FVIII:C, repeated multimeric analysis and MCMDM bleeding score were performed in addition to tests evaluating heart, liver and kidney function.

Result: The overall prevalence of AVWS in this study was 20.8%. It was found across all cardiac defects, with the highest prevalence in defects of great complexity (38%) in contrast to 9% in patients with CHD of simple/moderate complexity ($P < 0.001$), Eisenmenger Syndrome ($P < 0.001$) and more severe heart failure symptoms (NYHA III/IV vs NYHA I, $P < 0.001$; NYHA III/IV vs. NYHA II, $P = 0.044$). Only 2/11 patients (18%) with aortic stenosis showed AVWS. Among lab results, a combination of multimeric analysis, VWF:Ag/VWF:CBA ratio (sensitivity: 77.5%, specificity: 93.3%) and PFA-100 (Col/Epi sens.: 77%, spec.: 52%; Col/ADP sens.: 75%, spec.: 74.3%) were suitable to detect AVWS. The use of the MCMDM bleeding score does not seem to be appropriate in these patients to objectify the bleeding tendency (sens.: 22.5%, spec.: 92.7%).

Summery/Conclusions: Here we present the largest study on patients with congenital heart disease and acquired von Willebrand syndrome to date. This study demonstrates that AVWS occurs in patients with various congenital cardiac defects, but the highest prevalence is in patients with more complex CHD. We, therefore, suggest preoperative screening for AVWS in all patients with CHD to prevent intra- and postoperative bleeding complications using a panel of specific lab tests.

PB 3.42-4

Type 2N von Willebrand disease in the population of Little Poland

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Background: Won Willebrand disease (VWD) is the most prevalent inherited bleeding disorder. Differential diagnostics of type 1 and type 2 VWD requires access to an experienced coagulation laboratory. Type 2N VWD, first identified in 1989 in Normandy, is characterized by decreased von Willebrand factor (VWF) binding to factor VIII (FVIII), resulting in disproportionally low plasma FVIII levels. VWF activity and antigen level can be decreased or normal, type 2N VWD is therefore often misdiagnosed as mild hemophilia A (HA) or HA car-

rier state. To identify this condition VWF:FVIII binding assay (VWF:FVIIIB) has to be performed.

Aims: We present the process of identification and laboratory diagnosis of type 2N von Willebrand disease in our reference hemophilia treatment center in Little Poland (circa 3,300,000 inhabitants, 165 VWD patients and 111 mild HA patients registered).

Methods: In the years 2009–2012 twenty individuals were selected with clinical or laboratory suspicion of type 2N VWD (all VWD patients with FVIII:VWD ratio lower than 0.7 and all HA patients with strong tendency to mucosal bleeds or unusual mode of inheritance). FVIII and FV activity was measured by the one-stage assay using Siemens reagents (BSC XP, Siemens Germany), VWF activity (VWF:RCo) was measured by turbidymetric method using BCS XP analyzer and VWF antigen level (VWF:Ag) with enzyme linked fluorescent assay (ELFA, BioMerieux, France). VWF:FVIIIB was performed in the MEDILYS Laboratory Hamburg (Germany) with an established inhouse method (Schneppenheim et al. 1996).

Results: Among our 20 patients and their four symptomless relatives VWF:FVIIIB was significantly decreased in five individuals (7–22%, mean 13.4%, normal range 60–180%) and moderately decreased in other five (43–55%, mean 46.6%), all belonging to six families. In one other patient type 2N VWD was diagnosed based on family history, bleeding symptoms and slightly decreased FVIII activity (45%, *n*. 50–150%). In the remaining 13 patients type 2N VWD was excluded. Combined FVIII and FV deficiency was excluded in all patients.

In all six type 2N patients FVIII activity was lowered to 31–45% (mean 36.6%). VWF:RCo was decreased only in one patient (47–132%, mean 82%, *n*. 50–150%) and the FVIII/VWF:Ag ratio was 0.34–0.63 (mean 0.43). Five of them show a history of mucosal, post-surgery and/or postpartum bleeds. In five 2N VWD carriers all parameters were within the normal range. In one carrier with lifelong history of posttraumatic joint/muscle bleeds FVIII activity was 11%, indicating coinheritance of mild HA.

Summary/Conclusions: We identified six cases of type 2N VWD (3.6% of all VWD patients registered in our region). To assess the true prevalence of type 2N VWD in Little Poland all mild HA patients should be screened with the VWF:FVIIIB assay. Implementation of this measurement in at least one reference laboratory in Poland would facilitate this task. Molecular testing should be available to identify the underlying genetic defect in female patients with mild HA and no male HA patients within family.

PB 3.42-5

The usage of pediatric bleeding questionnaire in the diagnosis of von willebrand disease and thrombocyte function defects among Turkish children

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Background: The diagnosis of mild bleeding disorders is not easy in the pediatric population as most of the 'healthy' subjects also report bleeding symptoms. In order to get a precise bleeding history Pediatric Bleeding Questionnaire (PBQ) has been developed previously.

Aim: The aim of the study was to determine the relevance of PBQ in the diagnosis of von Willebrand disease and thrombocyte function tests and to determine a 'clinically significant' bleeding score cut off for our population to differentiate 'hemostatically normal bleeders' and 'children with mild bleeding disorders'.

Methods: In our study, Turkish children previously diagnosed with von Willebrand disease (VWD), thrombocyte function defect (TFD) and healthy children without any symptoms (Control group 1) and healthy children with symptoms but found hemostatically normal (Control group 2) were analysed with PBQ.

Results: Von willebrand disease group consisted of 46 patients (27 VWD Type 1, 5 VWD Type 2, 14 VWD Type 3), TFD group consisted of 65 patients (7 Glanzmann Thrombastenia, 9 Bernard Solier syndrome, 4 Hermansky Pudlak syndrome and 45 thrombocyte secretion defects). Control 1 group had 38 and Control Group 2 had 32 patients. The ages of all groups were between 6 months and 18 years. The median bleeding scores were four in VWD Type 1, seven in VWD Type 2, 16 in VWD Type 3, nine in Glanzmann thrombasthenia, 10 in Bernard Solier Syndrome, 7.5 in Hermansky Pudlak syndrome, four in thrombocyte secretion defect, 0 in Control one group and 1 in Control two group. The patient and control subjects were compared as four major groups (VWD, TFD, Control 1 and 2 groups) with PBQ. The most frequent clinically significant bleeding symptoms that can differentiate VWD/TFD and control groups were found to be epistaxis, oral cavity bleeding, bleeding after tooth extraction, menorrhagia and post circumsicion bleeding. The cut off level for 'clinically significant bleeding score' was found to be ≥3 (AUC: 0.785%95 CI: 0.718–0.852). The sensitivity, specifity, PPV and NPV of PBQ to define VWD vs. Control group 1 was %93.5; %97.4; %97.7; %92.5; VWD vs. Control group 2 was %93.5; %87.5; %91.5; %90.3; TFD vs. Control group 1 was % 89.2; %97.4; %98.3; %84.1 and TFD vs. Control group 2 was %89.2; %87.5; %93.5; %80 respectively.

Conclusion: In conclusion, PBQ was found to be useful in the diagnosis of VWD and TFD in Turkish children, however to reach an exact diagnosis hemostatic laboratory tests are stil warranted.

PB 3.42-6

Characterization of Von Willebrand Disease type 2A mutation G1579R in the Brno-VWD study

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Von Willebrand Disease (VWD) is an autosomally inherited bleeding disorder caused by a quantitative or qualitative defect of von Willebrand factor (VWF). The Von Willebrand Factor mutation c.G1579R in the A2-domain is known to be responsible for a 'classic' type 2A (group 2) VWD with normal multimerization and secretion, but with increased susceptibility to degradation by ADAMT13.

In the Brno-VWD study blood was collected from 205 patients representing 95 families with suspected VWD. FVIII:c, VWF:Ag, VWF:RCo, VWF:CB, VWFpp, VWF-FVIII binding (if indicated), VWF multimers and molecular analysis were performed in all patients.Within the Brno-VWD study we analyzed 17 patients from five families with the c.G1579R mutation.

As expected, the results show that the c.G1579R mutation is characterized by a very low VWF:RCo/VWF:Ag ratio (mean 0.23, CI_{95} 0.17–0.30), a very low VWF:CB/VWF:Ag ratio (mean 0.31, CI_{95} 0.26–0.36) reflecting decreased platelet binding, and an increased VWFpp/VWF:Ag ratio (mean 1.57, CI_{95} 1.43–1.70) showing increased elimination through proteolysis by ADAMTS13. Multimeric analysis shows a type 2A pattern with loss of high molecular weight multimers and a pronounced first sub-band. Unexpectedly, because classic VWD type 2A is mostly linked with platelet aggregation abnormalities there is also a relatively low FVIII:c/VWF:Ag ratio (mean 0.59, CI_{95} 0.50–0.67) which cannot easily be explained by the mechanism behind this mutation. The difference with the overall mean FVIII:c/VWF:Ag ratio in the full Brno-VWD population of1.13 (CI_{95} 1.04–1.21) is striking.

Although the VWD mutation c.G1579R is conventionally classified as a 'classic' type 2A (group 2) mutation the results of the FVIII:c/VWF:Ag ratio indicate that other mechanisms may be present which may lead to reclassification of this mutation.

PB3.43 – Von Willebrand factor – IV

PB 3.43-1

Resistance of C2362F von Willebrand factor to ADAMTS13-induced proteolysis

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Background: The triplet-organized multimer pattern of circulating von Willebrand factor (VWF) is the result of the proteolytic action of the metalloprotease ADAMTS13. VWF carrying the C2362F mutation (C2362F-VWF) characteristically lacks the normal oligomer structure, which is replaced by a diffuse smear extending to the origin of the separating gel, suggesting the presence of unusually large species. We suggest that this picture might stem from C2362F-VWF being ADAMTS13-resistant.
Aims: We aim to demonstrate that the C2362F mutation alters the susceptibility of VWF to the proteolytic action of ADAMTS13.
Methods: The effects of recombinant ADAMTS13 on C2362F-VWF were explored in patients' plasma VWF and recombinant VWF fragments. Given the very low circulating VWF levels in patients homozygous for the C2362F mutation, blood samples were enriched with VWF by cryoethanol precipitation. Normal and mutated VWF fragments extending from domains A3 to B3 were produced using the pEXP5-NT/TOPO$^{(R)}$ vector and Origami E. coli strain. ADAMTS13-induced proteolysis experiments were performed under static conditions in the presence of urea, with a constant amount of normal and mutated plasma VWF and increasing concentrations of ADAMTS13. Again under static conditions, the binding of recombinant biotinylated ADAMTS13 to VWF was explored in the presence of EDTA by means of an ELISA assay, using both plasma and recombinant normal and mutated VWF fragments.
Results: Patient's plasma C2362F-VWF was found completely ADAMTS13-resistant. Whatever the concentration of recombinant ADAMTS13, there was no sign of the abnormally-large VWF multimers of C2362F-VWF disappearing, nor any increase in the representation of triplet multimer bands. At the same ADAMTS13 concentrations, in normal VWF the large VWF multimers disappeared and the satellite bands became more strongly represented, in a pattern resembling type 2A-II von Willebrand disease. Binding experiments were carried out to ascertain whether the ADAMTS13 resistance of C2362F-VWF is due to an abnormal interaction between the two molecules. ADAMTS13 binding to C2362F-VWF was found significantly impaired (around 40% lower than the normal counterpart), and this defective binding was confirmed using the C2362F A3-B3 recombinant VWF fragment.
Conclusions: These findings demonstrate that the loss of cysteine 2362 makes VWF resistant to proteolysis by ADAMTS13. This abnormality seems to be at least partially due to a defective binding of ADAMTS13 to VWF. The C2362F-VWF mutation thus brings to light a new abnormality in the VWF-ADAMTS13 relationship, i.e. the existence of an ADAMTS13-resistant VWF.

PB 3.43-2

Chicken glycoprotein Iba and Ibβ form both GPIb and non-GPIb complexes: implications for the evolution of GPIb-IX

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Background: The association of various subunits in human glycoprotein (GP) Ib-IX-V complex has been characterized extensively in transfected cells, as the surface expression level of GPIb-IX correlates with its proper assembly. The interaction among GPIba, GPIbb and GPIX transmembrane helices, in cooperation with those between GPIbb and GPIX extracellular domains, provides the driving force for GPIb-IX assembly. GPV associates weakly with GPIb-IX. However, it is not clear if the assembly of GPIb-IX from other species is the same as that of human GPIb-IX and how GPIb-IX has evolved into its highly integrated form.
Aim: This study characterizes the assembly of chicken (*G. gallus*) GPIb-IX complex and its difference from human complex.
Methods: Chicken GPIbα, GPIbβ and GPIX genes were cloned from total mRNA extracted from fresh chicken blood and appended with a N-terminal immuno tag. The same tags were also appended to corresponding human paralogs. The plasmids containing human or chicken GPIb-IX genes were transiently transfected, in various combinations, into Chinese hamster ovary (CHO) cells. Surface expression levels of GPIb-IX subunits, and the association among them, were measured by flow cytometry, and immunoprecipitation. Disulfide formation between GPIba and GPIbb was analyzed by SDS-PAGE under non-reducing conditions.
Results: While the sequences of cloned chicken GPIbb and GPIX cDNAs are the same as predicted in the NCBI database, the N-terminal sequence of the cloned chicken GPIbα cDNA differs, due to incorrect prediction of a splicing site. Like human GPIb-IX, efficient expression of chicken GPIb-IX in the plasma membrane of transfected CHO cells required all of its subunits. However, in addition to the GPIb complex (i.e. GPIbb-GPIba-GPIbb), nearly half of chicken GPIba connects with chicken GPIbb to form a non-GPIb complex (i.e. GPIbb-GPIba-GPIba-GPIbb). Domain swapping analysis showed that surface expression of human GPIX could be enhanced by coexpression of human GPIbb, or a human/chicken GPIbb chimera (GPIbb-hTM) that contains the human-derived transmembrane domain, but not chicken GPIbb. Surface expression chicken GPIX could be enhanced by coexpression of chicken GPIbb, but not human GPIbb nor GPIbb-hTM. Coexpression of chicken GPIbα, GPIbb-hTM and human GPIX produced GPIb and very little non-GPIb complex. Further sequence analysis identified residues in the GPIbb transmembrane domain that are critical to specific GPIb formation and conserved across mammalian species, but not in birds, reptiles, amphibians or fishes.
Conclusions: Chicken GPIba, GPIbb and GPIX form human-like GPIb-IX complex but also a non-GPIb complex. The interfaces between GPIbb and GPIX extracellular domains are largely conserved between human and chicken, but differences in the interface between transmembrane domains are observed. That such difference coincides with the division of platelet-bearing and thrombocyte-bearing vertebrates suggests that the non-GPIb complex plays a functional role in thrombocytes.

PB 3.43-3

Characteristics and angiogenic properties of blood outgrowth endothelial cells from a type 3 von Willebrand disease patient

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Background: Blood Outgrowth Endothelial Cells (BOECs) from von Willebrand Disease patients (VWD) have been used as an effective tool for studying von Willebrand factor (VWF) storage and secretion defects. Isolation of these cells from peripheral blood from both type 1 and type 2 VWD patients has proven to be efficacious. However, successful isolation of BOECs from type 3 VWD (complete absence of VWF) patients has not been reported yet. VWF plays a major role in

blood coagulation as it is involved in platelet adhesion and functions as a carrier protein for factor VIII. Besides playing a role in coagulation, it has recently been demonstrated that endothelial VWF regulates angiogenesis and that BOECs from type 1 and 2 VWD patients seem to display increase *in vitro* angiogenic capacity. Whether this also holds for type 3 BOECS is unknown.

Aims: We aimed to isolate BOECs from a type 3 VWD patient in order to investigate whether angiogenesis is also increased in BOECs from patients who have a complete lack of VWF.

Methods: BOECs were derived from peripheral blood of a type 3 VWD patient compound heterozygous for a nonsense mutation p.Arg2535X and an unknown mutation on the other allele. Informed consent and ethical approval was obtained. Endothelial phenotype of the cultured cells was evaluated by fluorescence activated cell sorting (FACS). Angiogenic capacity of these cells was analysed *in vitro* by matrigel assay for the tube formation and migration assay for wound healing capacity. Immunofluorescence staining was performed to visualize VWF.

Results: We successfully isolated BOECs from a type 3 VWD patient. FACS analysis confirmed the presence of endothelial markers including CD31, CD144 (VE-Cadherin) and KDR/VEGFR-2. Cells were negative for the monocyte markers CD45 en CD14. Immunofluorescence staining showed cells express PDI and β-catenin, but do not express VWF or P-selectin. No Weibel-Palade bodies (WPB) were observed. Preliminary data on the angiogenic properties of these cells revealed a higher migration rate for the type 3 VWD BOEC compared to BOECs from three healthy individuals (0.64 μm/min ±0.06) vs. 0.49 μm/min ±0.07). When seeded onto matrigel the patient's BOECs formed more capillary-like structures than healthy BOECs (35,214 μm vs. 32,486 μm) and these structures contained more branching points (138 vs. 115) and loops (54 vs. 45).

Conclusion: We established that it is feasible to obtain BOECs from a type 3 VWD patient. Preliminary results indicate that BOECs obtained from a type 3 VWD patient have enhanced angiogenic potential compared to BOECs from healthy individuals as evidenced by faster migration and enhanced capillary formation. The pro-angiogenic state is possibly caused by constituent release of angiogenic factors normally stored in the WPB like angiopoietin-2 and insulin-like growth factor binding protein 7 (IGFBP7).

PB 3.43-4

Characterisation of observed changes in vWF target disposition following repeated administration of the anti-vWF Nanobody caplacizumab

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Background: Caplacizumab is a bivalent Nanobody that targets the von Willebrand Factor (vWF) A1 domain and blocks the interaction of any sizes and activation states of vWF with the platelet glycoprotein (Gp)Ib-IX-V receptor. Nonclinical and clinical data revealed manageable and transient reductions in vWF antigen (vWF:Ag) and Factor VIII clotting activity (FVIII:C) levels upon repeated administration of caplacizumab, indicating a reversible effect of the Nanobody on vWF disposition.

Aims: The objective of this study is to characterise and identify the molecular mechanism of vWF:Ag and FVIII:C changes in nonclinical and clinical species.

Methods: vWF:Ag and FVIII:C levels were measured in nonclinical studies (baboon efficacy/safety study and 13-week/26-week toxicity studies in cynomolgus monkey) and Phase I multiple-dose clinical study. Biostatistical analysis compared the vWF disposition in cynomolgus monkey vs. human. A pharmacokinetic/pharmacodynamic (PK/PD) model was developed to characterise the disposition of caplacizumab and to describe the decreases in vWF:Ag levels in humans. vWF biosynthesis was evaluated by studying vWF release by endothelial cells *in vitro*, and by *ex vivo* analysis of vWF propeptide (vWFpp) levels in toxicity studies.

Results: Data from Phase I studies in human and data from the 13-week and 26-week repeated-dose toxicity studies in cynomolgus monkey indicated decreases in vWF:Ag and FVIII:C after repeated administration of caplacizumab. The observed changes are dose-dependent but saturable. Values never reached clinical significant levels: maximal changes in vWF:Ag levels ranged from ±25–40% of baseline in the 26-week toxicity study, and from ±35–40% of baseline in the Phase 1 study (i.e. lowest observed mean levels of 0.36–0.47 U/mL), with proportional decreases in FVIII:C. Statistical analysis demonstrated that the changes in vWF:Ag and FVIII:C levels observed in humans were comparable or slightly less pronounced compared to cynomolgus monkey. vWF:Ag and FVIII:C levels were also reversibly decreased in a baboon model of acquired thrombotic thrombocytopenic purpura (TTP). The effect was not linked with clinical symptoms, even in conditions of severe thrombocytopenia. Values rapidly normalised to predose levels after stopping treatment in all studies.

Experimental evidence points towards an altered clearance of vWF in complex with caplacizumab, rather than a change in vWF biosynthesis: (i) the ratio of vWFpp to processed vWF was changed upon treatment with caplacizumab in toxicity studies. A decrease of vWF:Ag was observed, while vWFpp was unchanged compared to control animals. (ii) *in vitro* data did not show an effect of caplacizumab on the stimulated or constitutive release of vWF by endothelial cells. (iii) PK/PD modelling indicated that the elimination rate of the drug-target complex is decreased compared to free drug in humans.

Summary/Conclusions: Repeated administration of caplacizumab results in mild and transient reductions of vWF:Ag and FVIII:C in the clinic. These changes are mirrored by nonclinical *in vivo* and *in vitro* models. Experimental evidence and PK/PD modelling suggest a change in the elimination rate of the vWF-drug complex due to target binding of the compound. The changes are expected to lead to manageable symptoms in the clinic, even in conditions of TTP. Caplacizumab is currently in Phase II clinical development.

PB 3.43-5

Acquired Von Willebrand Disorder in Pediatric Extracorporeal Membrane Oxygenation

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Extracorporeal membrane oxygenation (ECMO) can provide lifesaving temporary hemodynamic and/or respiratory support in critically ill patients. The use of ECMO has been rapidly increasing worldwide. However, life-threatening bleeding remains a major complication with ECMO. Acquired von Willebrand disease (VWD) while on ECMO has been suggested to occur in small case series. Increased consumption via ADAMTS-13 proteinolysis of von Willebrand factor (VWF) has been proposed as one of the mechanisms of acquired VWD. However, the characteristics of acquired VWD in pediatric patients on ECMO have not been fully studied. The aim of this study was to describe the VWF hemostatic pathway in pediatric patients on ECMO. Plasma samples of pediatric patients on ECMO were collected from a single center. We measured VWF antigen (VWF: Ag), VWF ristocetin cofactor assay (VWF: CoF), VWF collagen binding assay (CBA), VWF multimeric analysis, VWF propeptide (VWF: PP), Factor VIII activity, and ADAMTS-13 activity. We also calculated and normalized the various biomarkers to VWF: Ag such as CBA to VWF: Ag ratio (VWF: CBA/Ag). We also calculated the percent large plasma VWF multimers by using radio optical densitometry on the VWF multimeric gels. We defined the percent large plasma multimers

as the multimeric bands above the three smallest molecular weight bands. We analyzed plasma from 12 patients on days 1 and 5 of ECMO. VWF: Ag were (297%, 112–333) on day 1 and (261%, 198–350) on day 5 (median, interquartile range). VWF: CoF/Ag were (0.6, 0.5–0.7) and (0.5, 0.4–0.5) and VWF: CBA/Ag were (0.43, 0.39–0.69) and (0.44, 0.35–0.60) on day 1 and day 5, respectively. VWF: PP/Ag were significantly higher on day 1 (2.2, 1.67–2.85) then on day 5 (0.94, 0.72–1.37) ($P < 0.05$). FVIII were (153%, 69–180) and (156%, 115–253) and ADAMTS-13 were (52%, 48–60) and (75%, 57–85) on day 1 and day 5, respectively. The percent large plasma VWF multimers were (79%, 73–85) on day 1 and (69%, 19–79) on day 5. VWF multimeric gel analyses revealed loss of large plasma VWF multimers in three patients. Seven patients had life-threatening bleeding events. VWF: CoF/Ag were (0.45, 0.30–0.50) vs.(0.50, 0.50–0.65) and VWF: CBA/Ag were (0.41, 0.38–0.51) vs.(0.60, 0.32–0.66) in patients with life-threatening bleeding compared to no bleeding on day 5, respectively. VWF: PP/Ag were (1.06, 0.72–1.63) vs. (0.94, 0.21–1.37) in patients with life-threatening bleeding compared to no bleeding on day 5. The percent large plasma VWF multimers were (64%, 29–74) vs. (79%, 19–83) in patients with life-threatening bleeding compared to no bleeding on day 5, respectively. In conclusion, pediatric patients on ECMO had evidence of acquired VWD with loss of VWF function as indicated by low ratio (<0.7) of VWF: CBA/Ag and VWF: CoF/Ag. Furthermore, our study is the first to show that the VWF: PP/Ag, a marker for synthesis, was significantly decreased from day 1 to day 5 on ECMO. This decrease in VWF: PP/Ag ratio suggests that decreased synthesis could also play a role in acquired VWD on ECMO. Further studies are warranted to better understand the mechanisms of VWF pathway coagulopathy on ECMO.

PB 3.43-6
Physical fitness determines the von willebrand factor response to exhaustive physical exercise

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Background: Physical stress triggers the endothelium to release von Willebrand Factor (VWF) from the Weibel Palade bodies. Since VWF is a risk factor for arterial thrombosis, it is of great interest to discover the determinants of the VWF response to physical stress. Recently it has been shown that three important genes determine VWF:Ag levels: Syntaxin Binding Protein-5, Syntaxin-2 and the VWF promoter. Since the SNARE proteins may be involved in Weibel Palade body exocytosis, we hypothesize that these genetic variations may be associated with the exercise induced VWF:Ag rise.
Aims: We aimed to investigate the effect of exhaustive physical exercise on VWF levels and to identify its main mediators.
Methods: 105 healthy individuals (18–35 years) free from cardiovascular risk factors were included in this study. Each participant performed an incremental exhaustive exercise test on a cycle ergometer. Respiratory gas exchange measurements were obtained while cardiac function was continuously monitored using ECG. Venous blood was drawn from the forearm at baseline and directly after exhaustion. VWF antigen levels (VWF:Ag) were determined with an in-house ELISA. DNA was isolated for genotyping of common variations in Syntaxin Binding Protein-5 (*STXBP5*, rs1039084 and rs9399599), Syntaxin-2 (*STX2*, rs7978987) and VWF (promoter, rs7965413).
Linear regression analysis was used to analyse the relationship between performance related determinants and VWF levels. Informed consent was obtained and the study was approved by a recognised medical ethics committee.
Results: The median VWF:Ag level at baseline was 0.94 IU/mL [IQR 0.8–1.1] and increased with 47% [IQR 25–73] after exhaustive exercise to a median maximum VWF:Ag of 1.38 IU/mL [IQR 1.1–1.8] ($P < 0.0001$). The strongest determinants are performance related ($P < 0.0001$), namely the peak power output per kg bodyweight ($\beta = 0.6$ [95% CI 0.4;0.7]), the ratio between the power output at the ventilatory threshold and the peak power output in Watt ($\beta = -2.6$ [95% CI −4.00; −1.2]), VO_2 peak per kg ($\beta = 0.05$ [95% CI 0.03;0.06]) and the maximum respiratory exchange ratio at peak power output ($\beta = 3.7$ [95% CI 2.0;5.5]). We observed a gender difference in VWF: Ag response to exercise (females 1.2 IU/mL [1.1;1.6] and males 1.7 IU/mL [1.2;2.1], $P = 0.001$), which was associated by a difference in performance. Genetic variations in *STXBP5*, *STX2* and the VWF promoter were not associated with VWF:Ag levels at baseline nor with the VWF:Ag increase.
Summary/Conclusion: VWF:Ag levels strongly increase upon exhaustive exercise and this increase is strongly determined by physical fitness level and the intensity of the exercise, while there is no clear effect of genetic variation in *STXBP5*, *STX2* and the VWF promoter.

PB3.44 – Von Willebrand factor – V

PB 3.44-1
Defining the molecular basis underlying the physiological interaction between Von Willebrand factor and galectins in normal plasma

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Background: Von Willebrand Factor (VWF) is abundantly glycosylated with 12 N-linked and 10 O-linked glycans, which are heavily sialylated. Importantly, these structures influence VWF functional properties, including plasma clearance and susceptibility to ADAMTS13 proteolysis. Although the molecular mechanisms through which glycans modulate VWF biology remain poorly understood, recent studies have demonstrated that plasma VWF circulates in complex with specific members of the galectin family. Moreover, these galectin-interactions modulate VWF-mediated thrombus formation *in vivo*.
Aims: In this study, we sought to define the molecular basis underlying the interactions between VWF and galectins -1 and -3 respectively.
Methods: VWF was purified from human plasma (pdVWF) by cryoprecipitation and 2BCL gel filtration. VWF glycosylation was then modified using specific exoglycosidases. Blood group specific VWF was purified from pooled group AB, O or Bombay plasmas. Mutated full length VWF and a series of truncated VWF domain fragments were transiently expressed in HEK293T cells. Recombinant galectin-1 and -3 were expressed in *E. coli* and purified via nickel affinity chromatography. Binding interactions were characterized via modified immunosorbent assay.
Results: Both galectin-1 and galectin-3 bound to pdVWF in a dose-dependent manner. Pre-incubation with PNGase F markedly decreased binding to both galectin-1 and galectin-3 ($13 \pm 1\%$ and $57 \pm 2\%$, $P < 0.001$). Moreover, removal of both N- and O-linked glycans (PNGase F and O-glycosidase treatment) further attenuated galectin-3 binding ($21 \pm 1\%$, $P < 0.0001$). ABO blood group antigen expression significantly influenced interaction with both galectins. In particular, group AB VWF bound to both galectin-1 and galectin-3 significantly better compared to group O VWF. In keeping with its lack of AB antigen expression, platelet-derived VWF bound both galectins with significantly reduced affinity compared to pdVWF. Terminal sialic acid and sub-terminal galactose expression on VWF also modulated galectin interaction. For example, enzymatic desialylation of pdVWF with α2-3,6,8,9 neuraminidase markedly enhanced binding to galectin-1 and galectin-3 ($231 \pm 6\%$ and $136 \pm 6\%$, $P < 0.05$). Importantly, both galectins −1 and −3 bound with higher affinity to HMWM VWF multimers compared to LMWM of the same blood group. The relative importance of specific VWF domains in regulating galectin interaction was investigated using a series of recombinant truncated domain fragments. Differential galectin binding was evident across the individual domains. Interestingly, a key role for the VWF A domains was observed. In keeping with these findings, incubation with

ristocetin significantly enhanced pdVWF binding to both galectin-1 and galectin-3 (914 ± 50% and 205 ± 23%, $P < 0.05$). Moreover, both galectins bound to the VWD type 2B mimic R1450E with significantly enhanced affinity (165 ± 10% and 117 ± 4%, $P < 0.05$). Finally, targeted removal of the individual N-linked glycan located at N1515 in the VWF A2 domain via site directed mutagenesis lead to dramatically decreased galectin-3 binding (23.86 ± 3%, $P < 0.0001$).
Conclusions: These novel data define the molecular basis underlying the physiological VWF-galectin interaction. In particular, we have demonstrated that both N- and O-linked glycan determinants modulate VWF-galectin binding through terminal sialic acid and ABO blood group expression, with an additional role for specific N-linked glycans. Furthermore, we have identified a critical role for the VWF A domains in modulating these interactions.

PB 3.44-2
Free thiol groups in von Willebrand factor (VWF) are required for its proper function under physiological flow conditions

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Background: The multimeric plasma glycoprotein von Willebrand factor (VWF) is exceptionally rich in cysteine, and its structure is largely determined by inter- and intramolecular disulfide bonding. Additionally, VWF was shown to contain unpaired cysteine residues potentially affecting protein function. The significance of free thiols on the surface of plasmatic VWF has been confirmed previously with respect to platelet binding under pathologically high shear stress. Furthermore their potential involvement in functional VWF self-association occurring at elevated shear stress has been suggested.
Aims: Objective of the present study was to investigate whether free thiol groups of plasma VWF contribute to the physiological VWF function under high physiological arterial shear stress conditions. Furthermore, we aimed to elucidate possible underlying mechanisms involved in this regulation.
Methods: Free and accessible thiol groups of plasma-derived VWF were blocked with N-ethylmaleimide (NEM). Derivatization was followed by detailed structural and functional examination including multimer analysis (MMA) and Fourier transform infrared spectroscopy (FTIR). Functional differences between the NEM-derivatized sample and the control sample were detected using an *in vitro* flow chamber system with respect to VWF-mediated platelet adhesion to collagen. Interactions with collagen type III and platelet glycoprotein (GP)Ib receptor were investigated using surface plasmon resonance (SPR). Identification of accessible cysteine residues was accomplished using biotin-linked maleimide (MPB) followed by analysis of multimer and domain incorporation as well as mass spectrometry.
Results: Blocking free thiol groups provoked substantial loss of VWF activity with respect to platelet recruitment to collagen type III under flow. The lowered platelet adhesion to collagen type III was shown to be a combined effect of inhibition of (i) the initial VWF binding to collagen type III as well as (ii) VWF-platelet GPIb interaction. Free thiol groups were accessible for derivatization solely on the surface of coiled multimers. Domain incorporation studies revealed a high level of derivatization in VWF *N*- and *C*-terminus. This was in accordance with the mass spectrometric analysis, where 19 MPB-derivatized peptides, predominantly located at the *N*- and *C*-terminus, could be identified.
Conclusion: Blocking free thiol groups in VWF significantly impaired mediation of platelet adhesion under physiological shear stress conditions. This result suggests an essential functional role of free thiol groups in VWF regarding binding to subendothelial matrix as well as platelet recruitment.

PB 3.44-3
Detecting the inner-interaction sites of VWF A1 domain and A3 domain

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Background: VWF is a multimeric glycoprotein composed of multiple functional domains and it interacts with the platelet membrane receptor GPIb-IX-V complex through its A1 domain and with collagen fibers through its A3 domain. Several researches indicate that the A1-A2-A3 triple domain has a more complicated secondary structure rather than the three single domains. Recently, we demonstrated that there was interaction between A1 and A3 domain. The interaction generally will be interrupted by conformational change of A1 and A3 each.
Aims: To unravel the mechanism of inner-interaction of VWF A domain and map the interaction binding site in detail.
Methods: We introduced 11 amino-acid mutations of A1 domain and 13 mutations of A3 domain. The mutants were expressed as recombinant VWF A1, VWF A3 as well as full-length VWF. The role of mutation site in A1-A3 interaction was evaulated in a binding assay using snap/clip-tag technology.
Results: The Asp(506), Tyr(508), Lys(549) of A1 domain and Arg (1026), Pro(1027) of A3 domain significantly reduced binding affinity of VWF A1-A3 interaction. Arg(632), Lys(643), Leu(964) and Gln (966) has weak impact on A1-A3 binding affinity. In addition, the reduction of the affinity enhanced the binding of platelet GPIb to VWF A1 domain.
Conclusion: It was predicted using bioinformatics methods that some amino-acid sits on VWF A1 domain or A3 domain were related with interaction of these two domains. Our results confirmed that some sites of them can indeed interfere the affinity of VWF A1-A3 interaction. Interestingly, several sites were in line with VWD mutaion site. These results help us to reveal the function of VWF A domain and the pathogenesis of VWD.

PB 3.44-4
Oxidized von Willebrand factor is associated with thrombotic micro- and macro-angiopaties in diabetes mellitus

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Background: The thrombogenic activity of Von Willebrand factor (VWF) is proportional to its molecular size and is inversely related to its proteolysis by ADAMTS-13. Oxidation of VWF at Met1606 with formation of sulfoxy-methionine severely impairs its proteolysis by the metalloprotease. Thus, oxidative stress may favor accumulation of high molecular weight VWF multimers, which can have prothrombotic effects. Moreover, diabetes mellitus, especially type 2, is characterized by high production of reactive oxygen and nitrogen species.
Aims: This study was aimed at assessing in patients with type 1 and type 2 diabetes whether protein carbonyls, marker of oxidative stress, are associated with both the level and oxidation status of VWF as well as with micro- and macroangiopathic complications.
Methods: Eighty-three diabetic patients (41 type 1 and 42 type 2 diabetic subjects) and their respective 83 healthy controls were studied after verifying the availability, through institutional databases, of clinical biochemistry records spanning at least 3 years. Plasma proteins and VWF-bound protein carbonyls were measured by immunoassays, whereas VWF multimers were studied by SDS-agarose gel electrophoresis.

Results: Type 2 diabetic subjects had higher levels of VWF antigen (VWF:ag), VWF activity (VWF:act) and plasma proteins' carbonyls in comparison with both their controls and type 1 diabetic subjects. Moreover, high molecular weight VWF multimers and specific VWF-bound carbonyls were significantly increased in subjects with micro- and macro-angiopathic complications. By contrast, in both type 1 and type 2 diabetic subjects, ADAMTS-13 activity was in the normal range. In a multivariable analysis, only VWF-bound carbonyls were significantly associated with any form of thrombotic angiopathy, considering the entire group of T1- and T2 diabetic patients.
Summary/Conclusion: These data provide first evidence that not only high VWF levels but also its oxidation status and the presence of high molecular weight VWF multimers, not observed in SDS-agarose gel electrophoresis of normal subjects are associated with thrombotic angiopathies in diabetes mellitus. These findings may help identify diabetic patients particularly at risk for these complications and elucidate a pathogenetic mechanism, centered on exalted activity of VWF, which contributes to the genesis of thrombotic angiopathies in this clinical setting

PB 3.44-5

A single finger prick for the assessment of Von Willebrand factor binding to platelets

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Background: It has been shown that blood collection via finger prick can be used for platelet function measurements. Significant correlations have been found between whole blood aggregometry (WBA) in venous blood and platelet reactivity time in capillary blood. However, there have been no publications that report a finger prick method to assess the binding of Von Willebrand factor (VWF) to platelets. We have developed a finger prick assay which requires only 40 μL of blood to measure the ristocetin induced binding of VWF to platelets. This could provide a new simple and less invasive method to assess binding capacity in disease (e.g. Von Willebrand disease VWD) and in response to therapy such as DDAVP or VWF infusion.
Aim: Our aim was to develop a finger prick based method for the assessment of VWF binding to platelets.
Methods: This study was approved by the Medical Ethics Committee of the University Medical Center Utrecht. The procedures followed were in accordance with the Helsinki Declaration. After informed consent, blood samples were obtained from 30 healthy controls. Blood sample collection procedure is slightly altered when compared to the conventional method; the hand is not warmed and a small tourniquet is placed around the base of the finger, total 'ligature time' is <1 min 30 s. An appropriate sized sterile safety lancet is used and capillary blood is collected in a 200 μL EDTA Microvette. The assay consists of a serial dilution of ristocetin (0–0.75 mg/mL) containing a fixed concentration of FITC labelled anti-VWF antibody. 5 μL of citrated blood was added to each well and VWF binding was assessed with fluorescent activated cell sorting. Results were plotted in a dose response graph.
Results: Mean age was 29.7 years, 12 subjects (40.0%) were male. 16 (53.3%) subjects had blood type 0, 11 (36.7%) type A and 3 (10.0%) Type B, none were type AB. We have recently validated this assay in healthy donors and VWD patients using *venous* blood. The inter-donor variability in the finger prick assay is comparable to the venous assay. The intra-donor variability, comparing the dose response curves in one donor on the same day, is low. We observed a strong learning curve in collecting blood, this could be due to the slightly altered finger prick method.
Summary/Conclusion: This is the first report on a finger prick method for the assessment of ristocetin induced VWF binding to platelets. Once experience in the specific finger prick method is gained, it is a simple, minimally invasive and reproducible method. Further research into the effect of disease and therapy on this assay will be performed. If these effects can be quantified, the assay would ideally be further developed into a Point of Care test.

PB 3.44-6

Quality specifications for imprecision, bias and total error to be used for measures of von Willebrand Factor antigen, activity and multimerisation

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Background: Reduced quantity or quality of von Willebrand Factor (VWF) is responsible for the symptoms of the most frequent hereditary bleeding disorder: von Willebrand disease (VWD). Increased VWF level or activity is linked to thrombotic thrombocytopenic purpura (TTP), various vascular disorders, liver diseases and cancers and also seems to be useful biomarkers of these diseases as indicators of endothelial damage.
Aim: Our aim is to provide quality specifications for imprecision, bias and total error to be used for measures of VWF antigen (VWF:Ag) level, activity (VWF collagen binding, VWF:CB) and multimerisation (MMW25). To analyze function structure relationship, we also aim to relate these VWF parameters to each other. Furthermore, we aim to correlate the quantity measures of functional test results to disease symptoms and genotype to better understand structure-function relationships.
Methods: Polyclonal antibody based VWF:Ag assay, VWF:RCo assay, five commercial and two freshly isolated human collagen type I and type III based VWF:CB assays, SDS-agarose multimer analysis and quantitative densitometry with a software developed by us for numerical expression of the VWF multimerisation, genetic analysis by direct sequencing of exon 28 (primers were designed at the basis of Penas et al 2005). Statistical quality tools (fishbone diagram, check sheet, control chart, histogram, Pareto chart, scatter diagram and stratified sampling).
Results: Specimen handling, storage and processing and all assay conditions were standardized. The limit of detection of the VWF:Ag and VWF:CB assays are 1.5 and 3.1 ng/mL respectively. Using control samples from different sources assessed accuracy and analytical error was 5.2 and 3.5% respectively. Analytical measurement range was 3.1–185 ng/mL (VWF:CB) and 3.1–218 ng/mL (VWF:Ag) at a standardized 200-fold plasma dilution. Clinical reportable range was 5–1653 and 3–1862 ng/mL at different dilutions of samples (50–3200). Total errors of the methods were 7–19%. Bland-Altman plots were used to evaluate agreement between VWF:Ag and VWF:CB assays applied for 1218 samples. Diagnostic sensitivity and specificity evaluated by applying the methods for 45 genotyped VWD patients.
Summary/Conclusion: Specimen handling, storage and processing is one of the critical factors, the other is the dilution buffer in the different assays. Using validated assays, VWF parameters became valuable tools as endothelial damage indicators and promising diagnostic or prognostic markers of many other diseases.

PB3.45 – Anticoagulant agents – XIII

PB 3.45-1

Prothrombin complex concentrate use for the emergency reversal of vitamin K antagonists

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Introduction: The need for normalisation of coagulation because of major bleeding or the requirement to perform invasive procedures are the primary indications for prothrombin complex concentrate (PCC) in patients receiving vitamin K antagonists (VKA).

Methods: We retrospectively audited the use of PCC over a year in two hospitals in the West Midlands Region of the UK.

Results: One hundred and eleven patients, all receiving warfarin, received PCC (either Beriplex® or Octaplex®). The mean age of the patients was 77 years, with 58.6% men. Ninety four percent of patients were discussed with a haematologist and 91% of patients were felt to have appropriate indications for PCC. The majority of patients had atrial fibrillation (74.8%) with other indications including DVT/PE (15.3%) and heart valve replacement (7.2%). Indications for PCC were major bleeding (73.0%) (gastrointestinal 46.9%, intracranial 42.0%, subcutaneous/intramuscular 6.2%) or an interventional procedure (26.2%) (abdominal surgery 42.3%, hernia repair 11.5%, orthopaedic 15.4%, urology 30.8%, central venous access 11.5%) or sepsis 0.9%.

There was a considerable time delay between it being recognised that the patient required warfarin reversal and requesting PCC (median 1 h 10 min, range 10 min–5 h 14 min) and from issue of the product by the Blood Bank and it being infused (median 1 h 46 min, range 10 min–6 h 55 min). This meant that the median time from the need for warfarin reversal being recognised to infusion of PCC was 3 h 25 min (range 45 min–11 h 14 min). All patients had their warfarin withheld but only 92.8% received vitamin K (with variable dosing).

Sixty seven percent of patients (who had a post-PCC INR available) achieved a normal INR. Mortality and morbidity was significant with 22 patients (19.8%) of patients dying in the first 30 days. Causes of death were bleeding (36.4%), sepsis (36.4%), cardiac events (22.7%) and cancer (4.5%). Time of death ranged from 10 h, due to an intracranial haemorrhage, to 21 days (due to sepsis). Of those surviving the rate of thrombotic events was 12.4% with six cardiac events, three PEs and two ischaemic strokes.

Conclusion: The two factors stand out from this audit. Firstly the excessive time from when it becomes evident that the patient requires warfarin reversal until they receive the PCC. Intravenous vitamin K works within 6–12 h therefore several of the patients could have been managed with vitamin K alone thus reducing the need for PCC and the extra risk associated with this.

The second finding is the high rate of thrombotic events, of which five were deaths, this further reinforces the need to use PCC only in those who either have life, limb or sight threatening bleeding or require emergency surgery that cannot wait for VKA reversal with vitamin K.

PB 3.45-2

Validation of a new questionnaire measuring Satisfaction with Medical Care in Non Valvular Atrial Fibrillation patients (SAFUCA study)

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Background: Treatment satisfaction measurement is a very relevant issue in the clinical follow-up of patients and their adequate handling. Although other generic instruments are already available we intend to develop a specific instrument for those patients treated for non-valvular atrial fibrillation (NVAF).

Aims: To validate a new questionnaire developed to measure satisfaction with medical care (antithrombotic treatment effectiveness and control, daily living interference) in patients with NVAF.

Methods: The 25-items instrument, reduced from a 37-items initial version, was administered to 254 NVAF patients under antithrombotic treatment, along with the Treatment Satisfaction Questionnaire for Medication (TSQM) concurrent questionnaire, and five visual analogic scales (VAS) measuring: Quality of Life (patient), perceived effectiveness (patients and clinician), general satisfaction (patient), and tolerability (clinician). Dimensional structure was assessed through inter-rater agreement and Confirmatory Factor Analysis, and convergent and divergent correlation with other measurements.

Results: An expert panel composed of six specialists rated a high correspondence with the theoretical construct attaining high values on concordance indexes (0.53–0.81) for items on their target dimension. A high goodness-of-fit was obtained (χ^2/df = 1.55, CFI = 0.71, RMSEA = 0.049) for the 7-dimensional structure (item #): 1- Effectiveness (3), 2- Convenience (3), 3- INR interference (4), 4.- Impact on HRQoL (6), 5- Undesired Effects (3), 6- Satisfaction with care (3) y 7- Overall Satisfaction (3). Correlations with TSQM convergent dimensions were moderate/higher (0.328–0.647) than those with divergent dimensions. Correlation pattern with VAS scores was concordant with that expected

Conclusions: The reduced questionnaire presents good construct, structural, convergent and divergent validity properties. The seven proposed dimensions are stable and well defined in the 25-item version.

PB 3.45-3

Reversal of the oral direct thrombin inhibitor dabigatran captured by visco-elastic and thrombo-imaging techniques

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Background: In the near future large numbers of patients are expected to be on antithrombotic treatment with the direct oral thrombin inhibitor dabigatran. It is also expected that a small percentage of the patients will experience serious bleeding or be in need of urgent surgery. In those instances the clinicians need guidance how to treat the patient in order to arrest or prevent bleeding. At present no specific antidote is available.

Aim: The aim of the project is to provide the medical community with data to support decision-making by testing different *in vitro* procedures for improving the coagulation in blood or plasma spiked with dabigatran

Methods: Whole citrated blood was spiked with dabigatran (Boehringer-Ingelheim), 200 μg/L and 400 μg/L for viscoelastic measurements of clotting (ReoRox instrument from Medirox, Nyköping, Sweden) with coagulation initiated with tissue factor (0.24 nM) or platelet-rich plasma (dabigatran only 200 μg/L) for measurement of clot initiation on a tissue factor coated surface and clot propagation velocity utilizing video-recording (Thrombodynamics Analyzer T-2 instrument from HemaCore LLC, Moscow, Russia). Different candidate drugs; fibrinogen (Riastap®, CSL Behring), activated prothrombin complex concentrate (ACCP; FEIBA®, Baxter AG), prothrombin complex concentrate (PCC; Ocplex®, Octapharma), recombinant factor VII (rVIIa; Novoseven®, Novo Nordisk), with potential to reverse the dabigatran effects on haemostasis, was be added at therapeutic concentrations used in patients for other indications at present and then reanalyzed.
Results: In viscoelastic measurements fibrinogen had small effects, if any, on clotting time but increased clot elasticity. APCC shortened clotting time considerably, for the lower concentration of dabigatran even to shorter times than the control. PCC partially reversed the prolongation caused by dabigatran. rVIIa had no significant effects. A limitation of this study is the relatively high tissue factor concentration used (0.24 nM) which might obscure the effects of platelets on enhancement of coagulation and thus also a potential positive effect of rVIIa. As measured by video-recording dabigatran did not influence the initial rate of coagulation at the surface, but attenuated the propagation of coagulation and decreased clot size (measured at 30 min). APCC not only reversed the dabigatran attenuation of clot propagation but increased above the value for the control for initial rate of coagulation, PCC and rVIIa had only minute effects.
Conclusion: APCC and PCC seem to have some potential for reversal of dabigatran effects on coagulation, but APCC in to high doses might induce too much coagulation and eventually cause thrombotic complications. Clinical studies are needed to evaluate the potential of these drugs for reversal of dabigatran effects on haemostasis in patients.

PB 3.45-4

Simulation of the international normalized ratio during switching therapy from rivaroxaban to warfarin and its potential clinical implications

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Background: The oral anticoagulants warfarin and rivaroxaban are options for the management of several thromboembolic diseases. In patients receiving long-term anticoagulation therapy, switching from rivaroxaban to a vitamin K antagonist (e.g. warfarin) may be required in some patients, which necessitates co-administration of both drugs initially because of the slow onset of action of warfarin. However, both drugs affect prothrombin time and the international normalized ratio (INR), complicating warfarin dosing during the transition (co-administration period).
Aims: The objective of this study was to use a computer model of blood coagulation to evaluate the influence of rivaroxaban 20 mg once daily on INR during the co-administration period of a switch from rivaroxaban to warfarin.
Methods: A model of coagulation that included the formation and clearance of the vitamin K-dependent coagulation factors was used. Factor clearance was implemented as an exponential decay to reflect physiological conditions. To validate the model, warfarin decay simulations were conducted first. To calibrate the model, warfarin concentration fit by best match of modelled and experimental Factor II and Factor VII concentrations was conducted, and prothrombin time and INR values were simulated by use of a phospholipid concentration that matched Neoplastin Plus®. To simulate the combined effects of both anticoagulants on INR during a switch from rivaroxaban to warfarin, warfarin initiation was simulated by adjusting the warfarin effect magnitude to reach desired target INRs in a sufficient time scale (21 days); the warfarin effect values obtained every 6 h from this simulation and the desired rivaroxaban plasma concentrations (derived from pharmacokinetic modelling of phase II dose-ranging studies in patients being treated for deep vein thrombosis) were used in the coagulation model. The increases in the simulated INR caused by rivaroxaban were used to generate nomograms. Simulations were done by emulating Neoplastin Plus and Innovin® reagents.
Results: Warfarin decay simulations showed that the simulator had good prediction quality. Rivaroxaban-induced increases in the total INR from the warfarin-attributed INR were seen in simulations emulating Neoplastin Plus. There was an approximately linear dependence on rivaroxaban plasma concentration. However, the rivaroxaban-induced deviation of INR was also dependent on the magnitude of the warfarin-induced basal INR. Simulations emulating Innovin showed smaller rivaroxaban-induced increases in INR from the warfarin-attributed INR compared with Neoplastin Plus. At median trough rivaroxaban plasma concentrations (38 μg/L), the INR contribution of rivaroxaban was 0.5–1.2 with Neoplastin Plus and 0.3–0.6 with Innovin if the warfarin-only INR was 2.0–3.0. At a high trough concentration of rivaroxaban (160 μg/L), the contribution of rivaroxaban had a maximum increase in INR of approximately 1.0 if the overall INR was 2.0–3.0 when Innovin was used.
Summary/Conclusions: Based on the simulations, the best time to measure the effect of warfarin on INR would be at the time of trough rivaroxaban concentrations (24 h after rivaroxaban dosing) during the co-administration period when switching from rivaroxaban to warfarin. Furthermore, the use of Innovin would be preferred to Neoplastin Plus because of its substantially lower sensitivity to rivaroxaban, thereby reducing the influence of rivaroxaban on measured increases in INR.

PB3.46 – Anticoagulant agents – XIV

PB 3.46-1

Pharmacodynamics and pharmacokinetics during the transition from warfarin to rivaroxaban in healthy subjects: a multicentre, randomized, placebo-controlled study

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Background: In clinical practice, patients receiving long-term anticoagulation therapy for the prevention and treatment of thromboembolic disorders may require switching between anticoagulants, for example, from vitamin K antagonists (e.g. warfarin) to rivaroxaban, or vice versa. Rivaroxaban and warfarin both affect coagulation tests, and information on the pharmacological changes that occur during such transitions would be useful to physicians.
Aims: The primary objective of this study was to investigate the relevant pharmacodynamic parameters during the transition from steady-state warfarin to rivaroxaban 20 mg once daily in healthy volunteers.
Methods: This multicentre, randomized, placebo-controlled, parallel-group study enrolled 96 healthy men, aged 18–45 years. All subjects provided written, informed consent prior to enrolment and the study was registered with EudraCT (2008-005540-16). Subjects were randomized into three treatment groups. In group A, warfarin was administered in varying doses to achieve a steady state with an international normalized ratio (INR) range of 2.0–3.0; rivaroxaban 20 mg once daily was then given for 4 days (days 0–3) starting 24 h after warfarin was stopped. In group B, the same warfarin regimens as treatment group A were used to achieve an INR of 2.0–3.0, followed by placebo once daily for 4 days (days 0–3). In group C, rivaroxaban 20 mg once daily was given for 4 days (days 0–3) without prior warfarin treat-

ment. Blood samples were collected at prespecified time points for pharmacodynamics and pharmacokinetic analyses. The following pharmacodynamic parameters were assessed: anti-Factor Xa activity, inhibition of Factor Xa activity, prothrombin time (PT), activated partial thromboplastin time (aPTT), HepTest, prothrombinase-induced clotting time (PiCT), Factor VIIa activity, Factor IIa activity, and endogenous thrombin potential (ETP).

Results: The pharmacodynamic profiles during the transition from steady-state warfarin (INR 2.0–3.0) to rivaroxaban 20 mg once daily varied and depended on the type of test used. An additive effect was observed on the prolongation of PT/INR during the initial transition period. The mean maximum prolongation of PT (in seconds) was 4.39-fold in warfarin followed by rivaroxaban group (group A) compared with 1.88-fold in warfarin followed by placebo group (group B) and 1.57-fold of baseline value in the rivaroxaban alone group (group C). However, at trough concentrations (i.e. 24 h after rivaroxaban dosing) rivaroxaban had minimal influence on the PT/INR. Inhibition of Factor Xa activity, aPTT and ETP were also enhanced during the transition from warfarin to rivaroxaban, but to a lesser extent compared with the PT. In contrast, the effects of rivaroxaban on anti-Factor Xa activity, HepTest and PiCT were not affected by pretreatment with warfarin. There was no pharmacokinetic interaction between the two drugs during the transition.

Conclusions: Changes in pharmacodynamic parameters during transitioning from warfarin to rivaroxaban vary depending on the tests used. The data suggest that INR monitoring should be stopped once rivaroxaban is initiated during the transition from warfarin to rivaroxaban. Conversely, when switching from rivaroxaban to warfarin, INR monitoring of warfarin therapy should be performed at trough rivaroxaban levels during the co-administration period.

PB 3.46-2

Combination effects of edoxaban, an oral direct factor Xa inhibitor, and P2Y12 receptor antagonists on ADP plus tissue-factor induced thrombin generation in human platelet-rich plasma

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Background: Activated platelets and tissue factor participate in arterial thrombosis by forming platelet aggregates that initiate and propagate blood coagulation. Adenosine diphosphate (ADP) is one of the major agonists for platelet activation. Edoxaban is an oral, once daily, direct, selective and reversible factor Xa (FXa) inhibitor in late-stage clinical development for the prevention of stroke and systemic embolic events in atrial fibrillation and the treatment and prevention of recurrence of venous thromboembolism.

Aims: To determine the effects of (a) edoxaban; and (b) the combination of edoxaban and a P2Y12 receptor antagonist (clopidogrel or ticagrelor) on thrombin generation induced by ADP plus tissue factor in human platelet-rich plasma (PRP).

Methods: Citrated human PRP from healthy subjects was stimulated with 10 μM ADP plus 0.25 pM tissue factor in the absence or presence of edoxaban and/or P2Y12 receptor antagonists. Thrombin generation was measured by means of calibrated automated thrombography with the thrombinoscope software. Lag time, peak, time to peak, endogenous thrombin potential (ETP), and maximum rate parameters were calculated from thrombin generation curves.

Results: Combination of ADP (10 μM) and low concentration tissue factor (0.25 pM) induced reproducible and steady thrombin generation in human PRP. Edoxaban (40 and 80 ng/mL), active metabolite of clopidogrel (AM-Clo, 10 and 20 μg/mL), and ticagrelor (3 μg/mL) alone inhibited ADP plus tissue factor-induced thrombin generation. Edoxaban significantly suppressed all five parameters (lag time, peak, time to peak, ETP, and maximum rate) of thrombin generation. AM-Clo inhibited lag time, peak, time to peak, and maximum rate, but not ETP. Ticagrelor suppressed peak, time to peak, and maximum rate, but not lag time or ETP. Concomitant presence of edoxaban (40 and 80 ng/mL) and AM-Clo (10 and 20 μg/mL) or edoxaban (40 and 80 ng/mL) and ticagrelor (3 μg/mL) produced a significant and additive inhibition of ADP plus tissue factor-induced thrombin generation compared to the single agents.

Conclusions: ADP plus tissue factor induces thrombin generation in human PRP. Both edoxaban, a FXa inhibitor, and P2Y12 receptor antagonists (clopidogrel and ticagrelor) inhibit this effect; however only edoxaban had a significant effect on all five thrombin generation parameters and especially ETP. Moreover, significant additive effects were observed with the concomitant presence of edoxaban and a P2Y12 receptor antagonist. The present results suggest that ADP plus tissue factor-induced thrombin generation assay may be a useful biomarker to quantitate and monitor the effects of combination therapy with FXa inhibitors and P2Y12 receptor antagonists.

PB 3.46-3

Efficacy and safety of weight-adjusted extended duration tinzaparin for prevention of post-operative venous thromboembolism after bariatric surgery

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Background: Bariatric surgery patients are at moderate to high risk for venous thromboembolic events (VTE) with pulmonary embolism representing the most common cause of post-operative death. The majority of VTE occur at a median of 14 days after hospital discharge. The optimal dosing strategy and duration of anticoagulant thromboprophylaxis have not been established.

Aim: To evaluate the efficacy and safety of post-operative thromboprophylaxis using weight-adjusted tinzaparin continued for 7 days following hospital discharge in patients undergoing bariatric surgery.

Methods: We conducted a retrospective chart review of 366 consecutive patients undergoing bariatric surgery from July 2009 to December 2010. Patients received thromboprophylaxis with weight-adjusted tinzaparin 4500–14,000 units daily (75 μ/kg, rounded to the nearest prefilled syringe). The primary efficacy outcome was the frequency of VTE within 30 days of surgery. The primary safety outcome was the frequency of major bleeding (International Society on Thrombosis and Haemostasis definition) within 30 days of surgery. Patients with a baseline indication for therapeutic dose anticoagulation were excluded from analysis. Trough anti-Xa levels were measured in 190 patients at approximately 7 days following surgery.

Results: Mean age was 44.8 years (SD 9.4). Median body mass index was 45.9 (range 30.9–71.0). A total of 351 and 342 patients were included in the efficacy and safety analyses, respectively. Outcome data were unavailable for nine patients (2.4%). VTE occurred in two patients (0.6%; superior mesenteric vein thrombosis, pulmonary embolism). Major bleeding events occurred in eight patients (2.3%) who received at least one dose of tinzaparin. Trough anti-Xa levels were undetectable in 143 patients. In the remaining 47 patients the median trough anti-Xa level was 0.12 (range 0.10–0.41).

Conclusions: Weight-adjusted extended duration tinzaparin appears to be an effective and safe thromboprophylaxis strategy in bariatric surgery patients. We found no evidence of drug accumulation despite use of large doses of tinzaparin.

PB 3.46-4

Effective inhibition of proteases of the coagulation cascade by di-cationic pentamidine-like molecules

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Background: There has been a recent surge of interest in the replacement of heparin and warfarin with more targeted antithrombotic agents that would reduce thrombosis but also lower the incidence of bleeding found with warfarin. Progress has been made in this arena with the introduction of several new drugs to the market and early reports suggest that these drugs are effective at inhibiting thrombosis. However, the risk of major bleeding with these drugs was greater than existing treatments in some clinical trials, with only a minimal benefit in lowering thrombosis. There has been recent interest in creating small molecule inhibitors against factor IXa and factor VIIa, which may be superior therapeutic targets to thrombin and fXa. Di-cationic, 'pentamidine-like' compounds have been investigated for therapeutic use as anticoagulants, platelet inhibitors, contraceptives, anti-virals, anti-inflammatories, anti-cancer agents, anti-fungals and anti-protozoan agents. While the mechanism of action of these compounds is not known for all of the above listed uses, the anticoagulant effect of these compounds appears to be due to inhibition of serine proteases of the coagulation pathway. Recent work has produced a library of over 2800 mono- and di-cationic molecules and several have shown promise in clinical trials in treating tropical diseases, but the majority of this library has not yet been screened for potential anticoagulant therapies.
Methods: We screened the anticoagulant potential of a subset of 15 compounds from the di-cationic library using prothrombin time (PT), activated partial thromboplastin time (aPTT), calibrated automated thrombography (CAT), coagulation factor inhibition assays, and an *in vivo* ferric chloride model of thrombosis.
Results: We find that 5 of the initial 15 compounds selected for screening (1RRT 063, 279OXD 032, 25DAP 013, 150OXD 230 and 1MCC 182) showed prolonged PT and aPTT, increased time to thrombin generation, and decreased total thrombin generated. Of these five compounds, two were effective fXa inhibitors (279OXD 032 and 1MCC 182) while two were effective thrombin inhibitors (1RRT 063 and 25DAP 013). In an *in vivo* thrombosis model, 150OXD 230 and 1RRT 063 significantly prolonged the time to occlusion while 25DAP 013 had no effect on time to occlusion.
Conclusions: These results suggest that our initial screening strategy for the entire di-cationic library is valid, and that this library may yield novel, targeted anticoagulant molecules that have the potential as therapeutic anticoagulants. Additionally, the *in vivo* results highlight the need to utilize an *in vivo* model in order to verify that compounds which may exhibit anticoagulant properties *in vitro* are also effective *in vivo*.

PB 3.46-5

Adherence to oral anticoagulants in an outpatient setting

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Background: Patient's compliance to therapy is crucial for good quality treatment. Literature reports that 50% of patients may cease to be adherent for drugs prescribed in chronic conditions that are left unmonitored. The introduction of a new generation of oral anticoagulants (NOACs) with a short half-life and for which no regular laboratory controls are necessary, may therefore harbor an increased risk of treatment failure due to non-adherence. Insight into patient factors that may influence treatment outcomes is lacking.
Aim: To explore the risk factors that may affect treatment compliance in the outpatient setting, and to evaluate the adherence to oral anticoagulants (OAC) and the relation to emotional status
Methods: This is a cross-sectional study of patients visiting the Thrombosis Service of the Maastricht University Medical Center in The Netherlands between December 2012 and January 2013. Patients were invited to complete an anonymous questionnaire including questions on: patient characteristics, indication for anticoagulant treatment, comorbidity, medication adherence and emotional status. Medication adherence was measured by application of a self-assessed Dutch version of the Morisky Medication Adherence Scale (MAMS-8); emotional status was measured with the Dutch self reported depression (CES-D) scale. Completed questionnaires from patients ≥18 years old, treated with OAC therapy for at least 2 months were included in the analysis. Self-reported adherence rates were classified into three categories: low, medium and high adherence. Descriptive statistics were performed with SPSS 19.0.
Results: In this study 316 questionnaires were retrieved, of which 275 were included in the analysis. The mean age of the patients in this cohort was 69.5 years (range 18–89 years), 48.7% of patients was ≥70 years old. 38.2% of the patients were female, 57.1% male. Education level of patients was similar to that of the Dutch population; 22.9% had an elementary education, 65.6% tertiary or higher and 11.5% a university education. The most common clinical indications for OAC use were atrial fibrillation (45.9%), the presence of artificial heart valves (20.4%) and myocardial infarction (19.7%). Most patients reported on comorbidities of which cardiovascular diseases (61.1%), hypertension (60.7%), hypercholesterolemia (46.9%) or diabetes (16.7%) were common. Self-reported adherence rates were categorized as low in 8%, medium in 80% and high in 12% of patients. No correlation was found between depressive symptoms and medication adherence ($r = -0.175$).
Conclusions: In contrast to reported adherence rates in literature, 92% of patients had medium or high adherence. This could be due to the positive effect of structured care with regular visits and treatment monitoring, but this may also be suggestive for reporting bias. The levels of adherence were found not to be significantly associated with any of the patient characteristics. The emotional status as measured by the CES-D scale, did not influence the adherence rate in this sample. Further studies are needed to identify risk factors for non-adherence and further explore the effect of structured care.

PB 3.46-6

Rivaroxaban superior to Nadroparin for thromboprophylaxis in patients receiving hip or knee arthroplasty

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Background: Deep-vein thrombosis (DVT) and subsequent pulmonary embolism (PE) are major complications in total joint arthroplasty of the lower limbs. Low molecular weight (LMW) heparins have been the gold standard for prophylactic treatment. In this regard, however, studies on the usage of new oral anticoagulants and especially rivaroxaban have shown promising results. Nonetheless, very few independent studies have been published and practically no study investigated the usage under normal clinical conditions.
Aims: Aim of this study was to evaluate the efficacy of rivaroxaban vs. nadroparin for thromboprophylaxis in patients receiving either total hip or total knee arthroplasty in an 'every day life' clinical setting.
Methods: In a mono-centric non-randomized controlled study performed from 2007 until 2013, Patients receiving hip or knee arthro-

plasty were administered nadroparin body-weight adapted 5700–7600 I.E. subcutaneously, beginning during surgery, or oral rivaroxaban 10 mg daily, starting 8 h after surgery. Anticoagulation was continued for 35 days. Mobilization was initialized the first day after surgery. All patients were screened for DVT or muscle-vein thrombosis (MVT) using bilateral compression sonography combined with color doppler sonography between days 5 and 9 after surgery.

Results: DVT or MVT occurred in 13 (6.0%) of 218 patients given nadroparin and in 24 (3.0%) of 810 patients given rivaroxaban representing an absolute risk reduction of 3.0% (95% confidence interval 0.1% to 5.8%, $P < 0.035$) while no significant difference in bleeding was reported by the surgeons.

Conclusions: While overall complications were quite low compared to published data, a daily oral dose of 10 mg rivaroxaban was more effective than a daily weight-adapted subcutaneous dose of nadroparin of 5700–7600 I.E. in preventing DVT, MVT or PE after arthroplasty.

PB3.47 – Anticoagulant agents – XV

PB 3.47-1

A low fixed dose of Prothrombin Complex Concentrate is cost effective in emergency reversal of vitamin K antagonists

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Background: Prothrombin Complex Concentrates (PCC) are commonly recommended for treatment of major vitamin K antagonist (VKA) associated bleeds. While their efficacy and safety have been well studied, a well defined dosing strategy is still lacking.

Recently, we studied the effectiveness of a low fixed PCC dose regimen of 1040 IU F IX compared to the commonly applied variable PCC dosing strategy based on body weight, baseline INR and target INR in a prospective, two-cohort design. This study showed that the low fixed dose of PCC was non-inferior to the variable dose in terms of clinical outcome. In reaching the target INR, defined as INR <2, the fixed PCC dose was non-inferior in patients with a baseline INR below 7.5. An important question from both a clinical and costing point of view is whether additional interventions were needed in the fixed dose cohort to reach the non-inferior outcome as observed in our study.

Aims: In the present study we assessed the cost-effectiveness of a low fixed PCC dose strategy of 1040 IU IX vs. the variable PCC dosing strategy.

Methods: A decision tree model was used in which target INR and clinical outcome were taken into account. Clinical outcome and resource utilization were obtained from our prospective, non-inferiority study in patients admitted through the emergency room. Only direct medical costs that were made during hospitalization were included. Analyses included Monte Carlo simulations and base-case analysis to assess the mean treatment costs, as well as costs per successfully treated patient (mean costs per patient/probability to obtain a patient with INR decrease below the target INR and a positive clinical outcome).

Results: The mean treatment costs were €5774 (Sd 294) for the fixed dose ($N = 59$) and €7408 (Sd 365) for the variable dose strategy ($N = 78$). PCC costs accounted for 13% and 17% of the treatment costs in the fixed dose and the variable dose cohorts, respectively.

In the majority of patients target INR was reached with a positive clinical outcome (N fixed dose: 50/59, N variable dose: 64/78). Costs per successfully treated patient were €6929 (Sd 352) and €9029 (Sd 445), for the fixed dose and variable PCC dose strategy, respectively ($P < 0.001$). Sensitivity analyses confirmed the robustness of these findings.

Conclusions: Our cost analyses showed that a cost reduction in PCC with a low fixed dose strategy did not coincide with a cost increase due to utilization of other treatment options for VKA associated bleedings. Furthermore, by treatment of these bleeding emergencies with a low fixed PCC dose strategy, on average €1634 per patient to €2100 per successfully treated patient was saved compared to a variable dosing strategy.

Taking into account the effectiveness of the low fixed dose of PCC in our previous study, we conclude that a low fixed dose of 1040 IU IX PCC is more cost-effective in emergency reversal of VKA than a higher variable dosing strategy.

PB 3.47-2

Interaction of metformin with the vitamin K antagonist phenprocoumon

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Background: Anticoagulant therapy with vitamin K antagonists (VKA) like warfarin, phenprocoumon and acenocoumarol is hampered by the interaction of the VKA with a great number of other drugs. Metformin is generally not considered as an interacting drug. We observed however a number of patients using VKA with a decreased INR after starting metformin. Therefore we performed this study to systematically investigate the influence of metformin on phenprocoumon, the VKA mostly used in our anticoagulation clinic.

Aims: In this cohort study we investigated the influence of metformin on the dosage of phenprocoumon in stable anticoagulated patients.

Methods: All patients of the Anticoagulation Clinic Leiden using phenprocoumon and metformin in the period January-March 2009 were screened. Patients starting metformin within this period and continuing metformin for at least 2 months were included. The first delivery date of metformin was checked with the pharmacist. Patients were excluded when they started another drug that is known to interfere with phenprocoumon or when they were having medical interventions requiring adjustment of the anticoagulation treatment during the observation period. We compared the average daily dosage of phenprocoumon in mg and the INR at three specific moments: before, 6 weeks after and 3 months after the start of metformin. In addition the number of patients having an INR below the therapeutic range was compared at these three moments. Statistics were performed using a paired t-test and a McNemar's test.

Results: 369 Patients were using metformin next to phenprocoumon within the observation period. Of these, 27 started metformin within this period and met the inclusion criteria.

The average phenprocoumon dosage increased from 2.13 mg/day before the start of metformin to 2.37 mg/day at 6 weeks (mean increase 0.23 mg, 95% CI: 0.12–0.34) and 2.49 mg/day at 3 months (mean increase 0.36 mg, 95% CI: 0.24–0.48).

The average INR decreased from 2.88 before the start of metformin to 2.26 (mean decrease 0.63, 95% CI 0.41–0.85) at 6 weeks and 2.54 (mean decrease 0.35, 95% CI 0.24–0.48) at 3 months after the start of metformin.

One patient had an INR below the therapeutic range before the start of metformin which increased to 10 patients (OR 10, $P = 0.0004$) at 6 weeks and two patients (OR 2, $P = 1.0$) at 3 months after the start of metformin.

Conclusions: Starting with metformin in patients using phenprocoumon resulted in a significant increase of the mean daily dosage of phenprocoumon. Despite the increased dosage of phenprocoumon, mean INR-values decreased in nearly 40% (10/27) of patients below the therapeutic range.

Therefore, clinicians should be aware that the dosage of phenprocoumon should be increased once metformin is started. Further research is necessary to investigate whether warfarin and acenocoumarol are also influenced by metformin. With the increasing prevalence of diabetes and the increasing popularity of metformin in the treatment of dia-

betes the combination of metformin with VKA will be encountered much more frequently in the future.

PB 3.47-3

Inhibition kinetics of plasma-derived and recombinant activated protein C in human plasma

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Background: The half-life of plasma activated protein C (APC) and its regulation and functional manipulation are important basic information for both, APC assay development and estimation of APC-mediated *in vivo* effects. To date, however, detailed information on the impact of heparins, APC-targeting ligands, and plasma levels of Protein S on APC half-life are lacking.
Aims: The aim of this study was to assess the impact of heparins of different molecular weights, APC-targeting DNA-aptamers, and protein S (PS) levels on the inhibition kinetics of recombinant (rAPC) and plasma-derived APC (pAPC) in plasma.
Methods: The half-life of plasma APC in the absence or presence of the different agents was determined using exogenously added or previously immobilized APC. Reactions were stopped at different time points using aprotinin that targets the active site of APC. Levels of residual free APC in the samples were measured using a recently described oligonucleotide-based enzyme capture assay (J Thromb Haemost. 2012; 10: 390–8). Inhibition kinetics were monitored by either loss of APC amidolytic activity or formation of APC-PCI-complexes over time.
Results: Recombinant and pAPC showed no significant differences in inhibition patterns or corresponding formation of APC-PCI-complexes. The presence of negatively charged molecules like heparins or DNA-aptamers enhanced the inhibition of APC in a size- and concentration-dependent manner. Interestingly, the pentasaccharide fondaparinux (Arixtra®) showed no influence of APC inhibition patterns, even at highest concentrations. In the presence of molecules that accelerate APC-inhibition, addition of aprotinin still proved to be reliable for the prevention of further inactivation of plasma APC, being an important information with respect to the development of aptamer-based assays for plasma APC measurements. In contrast to heparin or aptamers, plasma concentrations of PS showed no consistent effect on APC inhibition patterns.
Summary/Conclusions: The inhibition of plasma APC was found to be accelerated by negatively charged molecules that target the basic exosite of APC. This effect depends on size and concentration of these molecules. Plasma concentrations of Protein S did not correlate with APC half life times, indicating, if at all, only a minor effect of PS on serpin-mediated inactivation of APC. The results shown here mainly question the implications of low and high-molecular weight heparins on APC functional activity *in vivo*.

PB 3.47-4

The clinically relevant interaction of warfarin and capecitabine

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Background: Capecitabine is an orally administered prodrug of 5-fluorouracil and is frequently used in patients with metastatic colorectal or breast cancer. The stability of anticoagulation in patients with metastatic cancer undergoing chemotherapy is often poor. The elevation of INR in patients treated simultaneously with warfarin and capecitabine has been repeatedly reported, and inhibition of S-warfarin biodegradation during capecitabine therapy has been described.
Aims of the study: (i) To determine the average daily warfarin dose and the average interval between INRs before, during and after concomitant capecitabine therapy. (ii) To detect the differences in percentage of time in therapeutic INR range (TTR) before, during and after capecitabine therapy. (iii) To detect the differences in percentage of time above therapeutic range.
Patients: Ten consecutive patients (six men and four women) treated with warfarin, in which capecitabine therapy was administered.
Methods: The patients were prospectively followed before, during, and after capecitabine therapy, the dose of warfarin was adjusted according to the INR. The average daily dose of warfarin, average interval between INRs, percentage of TTR and percentage of time above therapeutic range before, during, and after capecitabine therapy was calculated.
Results: The average daily dose of warfarin before, during and after capecitabine therapy was 4.62 (2.63–8.5) mg, 2.56 (1.03–4.31) mg and 3.76 (1.4–7.2) mg, respectively. The average warfarin dose during capecitabine therapy was significantly lower, than before capecitabine ($P = 0.003$), the average warfarin dose after capecitabine therapy was higher, than during capecitabine therapy, the difference was not significant ($P = 0.06$). The average daily warfarin dose was reduced after initiation of capecitabine therapy by 39.7 (15.0–72.3)%. The average interval between INRs before, during, and after capecitabine therapy was 11.10 (5.17–25) days, 8.41 (5.53–10.9) days, and 11.19 (7.3–13.5) days, respectively. The interval after capecitabine therapy was significantly longer, than the interval during capecitabine therapy ($P = 0.024$), the difference of intervals before and during capecitabine therapy was not significant ($P = 0.138$). The percentage of TTR before, during, and after capecitabine therapy was 78.2% (95% CI 69.4–87.1%), 56.6% (95% CI 40.8–72.4%), and 71.4% (95% CI 52.8–90.0%), respectively. The percentage of TTR was significantly higher before capecitabine treatment than during capecitabine therapy ($P = 0.035$), the difference between percentage of TTR during and after capecitabine therapy was not significant ($P = 0.356$). The percentage of time above therapeutic range before, during, and after capecitabine therapy was 11.3% (95% CI 0.3–19.9%), 33.4% (95% CI 16.4–50.3%), and 2.4% (95% CI 0–5.5%), respectively. The percentage of time above therapeutic range was significantly higher during capecitabine therapy, than before capecitabine therapy ($P = 0.038$), and than after capecitabine therapy ($P = 0.018$).
Conclusion: The capecitabine therapy in patients treated with warfarin results in the enhanced effect of warfarin and increased risk of warfarin overdose and bleeding complications. The stability of anticoagulation is poor during the capecitabine therapy and the frequency of INR monitoring should be increased. When the stability of INR is unachievable, an alternative anticoagulant therapy should be considered.

PB 3.47-5

Prothrombinase induced clotting time (PICT) for the monitoring of new oral anticoagulants

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The new oral anticoagulants such as Rivaroxaban (Bayer Healthcare) (R) Apixaban (BMS/Pfizer) (A) and Dabigatran (Boehringer Ingleheim) (D) have been approved for Several indications in theUS and Europe. Initially it was suggested that these agents did not require monitoring at the approved dosages for specific indications, however there have been reported bleeding complications with some of these agents that warrants the monitoring of these drugs to optimize therapy in some patient populations. A new clot-based assay, the prothrombinase induced clotting time (PICT) (Pentapharm, Basal, Switzerland) has been developed to monitor the anticoagulant effect of these new oral anticoagulants. In the assay plasma is mixed with FXa, phospholipids, calcium and RVV-V from the venom of the Daboia russelli. The prothrombinase complex formed and the Xa and IIa generated is inhibited by the test agent.

Materials: Citrated blood was drawn from five donors and spun at 800 rpm to obtain platelet rich plasma (PRP). The PRP was removed and spun at 3000 rpm to obtain platelet poor plasma (PPP). The PRP was frozen at −80 °C for 24 h and the PPP and PRP were supplemented with A, R and D in a concentration range of 0–2.5 μg/mL. The plasma samples were analyzed using three PT/INR reagents (Innoven, Dade-Behring, Germany; Recombiplastin, Instrumentation Laboratories, Bedford, MA; Neoplastin, Stago, Parsippany, NJ), two APTT reagents (Platelin, TCoag, Ireland; Actin FSL, Instrumentation Laboratories, Bedford, MA), Heptest and the one stage PICT. All assays were performed on the ACL 300 Plus (, Instrumentation Laboratories, Bedford, MA). The PICT was also run on the ST4 (STago, Parsippany, NJ).
Results: In the PPP system, the A, R and D showed assay differences in the clotting times which demonstrated good sensitivity to D and R compared to A. No individual assay except for PICT demonstrated good sensitivity to all three drugs. In the PT/INR assay, all reagents were sensitive to D and the Innovin and recombiplastin were sensitive to R and showed a weaker response to A. Similar responses were observed in the APTT and Heptest assay. In the PICT, all reagents showed a concentration dependent increase in the PICT test. D was strongest followed by R, and A showed the weakest effect. In the PRP supplemented system, all agents showed a weaker effect on the clotting times in all tests, however PICT demonstrated a concentration dependent response for all agents. Comparable results for the PICT were obtained on both instruments used for the PICT.
Conclusions: These results demonstrated that neither the PT/INR, APTT nor heptest can be solely used to monitor the effects of all of the new oral anticoagulants. The one stage PICT is a simple, fast, automated or semi-automated test which can be performed on any mechanical or optical coagulation analyzer to monitor the anticoagulant effects of these agents. In addition, the one stage PICT can also be used to monitor the effects of these agents in other matrices such as PRP. These studies warrant clinical validation of this test in patients treated with the newer oral anticoagulant drugs.

PB 3.47-6
Ex vivo monitoring of Fenprocoumon reversal by prothrombin complex concentrate using thrombography

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Background: The vitamin K antagonist (VKA) Fenprocoumon is an oral anticoagulant (OAC) used to treat and prevent thromboembolic diseases. The prothrombin complex concentrate (PCC) is used as an emergency treatment to reverse OAC anticoagulation when excessive bleeding occurs or to prevent patients on OAC treatment from excessive bleeding during surgery. PCC dosing strategies are based on body weight and clotting time INR values, but it is not unusual in clinical practice that more PCC is required than initially calculated.
Aim : The aim of the present study was to assess the added value of thrombography in monitoring patients' haemostatic status and subsequent PCC dosing calculations.
Methods: Blood samples were collected from patients on Fenprocoumon anticoagulation before and within 15–30 min after PCC administration. Venous blood samples were collected into siliconized tubes containing trisodium citrate (0.129 M, 1/9 v/v) and platelet poor plasma was prepared and frozen within 2 h after venipuncture. Plasma samples from hospitalized Fenprocoumon treated patients undergoing surgery and plasma samples from Fenprocoumon treated individuals that presented themselves with bleeding complications were included in the study. Plasma samples were subjected to INR measurements and thrombography (CAT method). The present study was conducted with the approval of the ethical committee of the involved medical institution and plasma samples were included in the study after patients had given their informed consent.
Results: In all patients on Fenprocoumon treatment we observed rapid normalization of both INR and thrombin generation lag time upon PCC addition. However, for the bleeding arrest in a subpopulation of patients more PCC was required. Cumulative dosing revealed a linear relationship between PCC dose and the thrombin generation parameters peak thrombin and area under the curve. Normalization of these parameters as well as bleeding arrest in these patients needed at least twice the amount of PCC as needed for INR and lag time normalization.
Conclusion: These data point towards thrombography as a potential beneficial tool in calculating PCC dosing.

PB3.48 – Anticoagulant agents – XVI

PB 3.48-1
Impact of pre-injury warfarin use on hospital mortality in elderly united states residents with torso trauma

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Background: More than 2 million Americans are started on warfarin annually for indications including atrial fibrillation, venous thromboembolism, peripheral vascular disease, and coronary artery disease, resulting in more than 30 million prescriptions per year. As the US population ages, the elderly are becoming an increasingly large proportion of the trauma population, giving rise to concern about the risk of injury-related bleeding complications due to warfarin. Relatively little is known about the impact of anticoagulation on elderly patients suffering from chest, abdominal, and/or pelvic trauma. These extracranial injuries can be severe and potentially fatal. We studied a cohort of elderly Americans with torso injury to determine the effects of warfarin exposure on outcomes in patients with torso trauma.
Aims: To determine the effect of pre-injury warfarin use on the outcomes of US patients admitted with torso trauma.
Methods: A retrospective review of a 5% random sample of Medicare claims data from 2009 to 2010 was performed for enrollees with at least 1 year of Medicare eligibility. Torso trauma cases were identified using ICD-9 codes for abdomen, chest, or pelvic injuries. Using Part D Prescription drug claims, warfarin exposure was defined as two or more warfarin prescriptions filled in the 60 days prior to injury. Characteristics and outcomes (mortality, length of stay, ICU days) between warfarin patients and patients not on warfarin were compared using univariate tests of association. Multivariable models adjusted for age (10-year increments), concomitant head injury, need for ICU care, co-morbidity index, sex, and race were run to measure the independent effect of warfarin exposure on mortality.
Results: We identified 6820 torso trauma patients, of which 5.7% were treated with warfarin. Patients on warfarin were more likely than non-users to be white non-Hispanic (94% vs. 88%, $P = 0.04$), older (15% vs. 22% for 65–74 years, 42% vs. 37% for 75–84 years, and 44% vs. 41% for >84 years, $P = 0.004$), and have more co-morbidities (57% vs. 55% for Elixhauser index 0, 4.4% vs. 5.4% for Elixhauser index 1, 6.5% vs. 8.4% for Elixhauser index 2, and 33% vs. 32% for Elixhauser index 3 $P = 0.53$). In univariate analyses by age group, mortality rates differed between warfarin patients and those not on warfarin in the oldest age group (>84 years) (10.7% vs. 4.7%, $P = 0.001$). There was no statistically significant effect on hospital length of stay or ICU days between the warfarin patients and those not on warfarin. In multivariable models, all co-variates, except race and co-morbidity index, were independent predictors of mortality. Warfarin exposure increased the odds of death nearly two-fold (odds ratio 1.9, 95% confi-

dence interval 1.3, 2.9) after adjusting for potential confounders, including age.

Conclusion: Anticoagulation with warfarin increases risk of mortality after torso trauma in elderly patients, driven largely by the oldest patients on warfarin, suggesting that physicians should exercise caution when giving chronic anticoagulation to the oldest (>85 years) elderly patients. As newer agents are developed for chemical anticoagulation, such added risks must be considered prior to initiating therapy.

PB 3.48-2

Reversal of anticoagulant effects of apixaban with non-specific prohaemostatic agents: an *in vitro* study

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Background: As a potent anticoagulant agent, apixaban an oral factor Xa inhibitor exposes to a risk of haemorrhagic complications. In the absence of specific antidote, bleeding management is challenging.

Aim: To investigate the efficacy of three prohaemostatic agents, recombinant activated factor VII (rFVIIa), prothrombin complex concentrate (PCC) and activated prothrombin complex concentrate (aPCC) to neutralize, *in vitro*, the anticoagulant effects of apixaban on several laboratory assays.

Methods: Blood samples were collected into 0.105 M sodium citrate from healthy volunteers from the local blood bank (Etablissement Français du Sang, Paris, France). Whole blood (WB) was spiked with apixaban at therapeutic (200 ng/mL) and 3-fold higher concentrations and either rFVIIa (equivalent to 90 and 120 µg/kg), PCC (25 and 50 UI/kg), or aPCC (80 and 160 UI/kg). Reversal was assessed on WB with thromboelastography (ROTEM®, TEM innovation), but also on platelet poor plasma (PPP) using standard laboratory assays (PT, aPTT) and thrombin generation test (TGT). Clot turbidity, as a function of time and measured on a spectrophotometer was added to explore the dynamics of fibrin polymerization[1].

Results: Apixaban concentration-dependently lengthened coagulation times (PT, aPTT, ROTEM® Coagulation Time [CT]). It also increased lag time (LT) and decreased endogenous thrombin potential (ETP) and peak height. On turbidity curves, fibrin polymerization was delayed and slower compared to control suggesting a network made of thinner fibers.

rFVIIa had predominant effects on kinetics parameters. It overcorrected coagulation times (PT, aPTT, CT) at apixaban therapeutic concentration and only partly restored them in overdose situation. It also decreased LT. At therapeutic concentration only, it accelerated fibrin polymerization.

PCC increased peak height and ETP, which was up to 50% higher compared to apixaban alone in therapeutic situation. They shortened PT and CT whereas paradoxically lengthened aPTT. Turbidity curves were not modified.

With aPCC, almost all parameters were improved: PT, aPTT, LT were shortened, thrombin peak was higher, ETP was overcorrected and fibrin polymerization started earlier and was faster compared to apixaban alone suggesting improvement of fiber thickness. Surprisingly, on ROTEM®, CT was never modified.

Conclusions: The non-specific prohaemostatic agents tested, partially or completely corrected several laboratory assays. aPCC was the most seducing one as it improved almost all parameters. Several points need to be discussed: reversal effects of aPCC were not confirmed in WB, increasing concentration of prohaemostatic agents had no additive effect and the putative efficacy was strongly dependent on the observed parameter (rFVIIa best option on CT, PCC on ETP). This underlines the need for a more robust endpoint and for clinical trials.

1. Chernysh I. Blood 2008.

PB 3.48-3

Determination of dabigatran, rivaroxaban and apixaban using UPLC-MS/MS and comparison with coagulation assays for therapy monitoring

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Background: Over the last several years, novel oral anticoagulants (NOACs) have been developed. Monitoring of these new drugs was postulated to be obsolete, because of improved pharmacokinetics and pharmacodynamics. However, it soon became evident that monitoring can be important for patients having deviating posture, diminished renal function or in emergency (bleeding) situations. Since conventional coagulation assays are not suitable for adequate monitoring, many new coagulation assays have been developed. At this moment, there is no agreement about which assays are preferred for the measurement of NOACs. Additionally, peak and trough concentrations in stable situations have not been unequivocally determined.

Aims: Our goal is the development of a reference UPLC-MS/MS technique for the quantification of dabigatran, rivaroxaban and apixaban, comparison with several coagulation tests and determination of target values for peak and trough plasma concentrations.

Methods: Plasma and full blood were spiked with dabigatran, rivaroxaban and apixaban for calibration and quality control. Labeled internal standards of the NOACs were added, followed by protein precipitation. Analysis was done with UPLC-MS/MS using a two-step Multiple Reaction Monitoring (MRM) monitoring mode. Validation of the method was done by determining specificity, matrix effects, precision, LLOQ, LLOD, carry over, recovery and stability. Several coagulation assays (PICT (Pentapharm), ECA-T (Stago) & Hemoclot (Hyphen) for dabigatran and PICT & STA Liquid anti-Xa (Stago) for apixaban and rivaroxaban) were validated with standard protocols. Target values were determined in an in-clinic orthopedic population and out-clinic cardiologic population. Samples were taken 2, 4 and 12 h after taking the drug to establish peak and trough concentrations. Different coagulation assays were be evaluated for their trueness by comparison with UPLC-MS/MS.

Results: All calibration lines were good ($R^2 > 0.99$) and no significant difference could be seen between plasma and full blood calibration lines. Stability tests showed adequate stability during sample storage and freeze-thaw cycles. Specificity was good and LLOD/LLOQ were <1 ng/mL. Matrix effects and carry over were absent. For dabigatran, rivaroxaban and apixaban, the recovery was respectively 73%, 78% and 104%; bias was respectively 4.6%, 4.17% and 4.9%; total precision was respectively 4.7%, 4.8% and 4.10%. The studied coagulation assays showed adequate reproducibility and bias.

Conclusion: An adequate UPLC-MS/MS method for the measurement of dabigatran, rivaroxaban and apixaban was developed and validated. Target values were determined and compared to different coagulation assays in an in-clinic orthopedic and out-clinic cardiologic patient population.

PB 3.48-4

Measurement of anti-Xa activity of apixaban in plasma

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Background: Apixaban is a new oral anticoagulant, a direct reversible Factor Xa (FXa) inhibitor approved in thromboprophylaxis after major orthopaedic surgery and for stroke prevention in patients with non-valvular atrial fibrillation.

Although no coagulation monitoring is required, measurement of apixaban anticoagulant activity may be required in some special clini-

cal settings: emergency surgery, bleeding, thrombosis, reversal with pro-hemostatic drugs, compliance, and suspect co-medication interference.

Aims: Recently it was reported that anti-FXa measurement was preferable to PT and APTT. We have furthered the investigations of the influence of apixaban on anti-FXa activity using two different techniques in order to measure its concentration in plasma.

Methods: Apixaban, kindly provided by Bristol-Myers Squibb (New York), was dissolved according to manufacturer's recommendations. Pooled human platelet poor normal plasma (Cryocheck) was supplemented with apixaban at concentrations covering the expected clinical therapeutic range, from 10 to >600 ng/mL, then assayed with two anti-FXa methods. Measurements were performed in duplicate, with two series a day, on three consecutive days ($N = 12$). Two controls were analyzed in each series: C1 at 300 ng/mL, C2 at 150 ng/mL.

Samples were analyzed with the Rotachrom® assay (Diagnostica Stago, Asnieres, France) and with the Biophen® DiXaI assay (Hyphen Biomed, Neuville-sur-Oise, France), using the STA-R instrument (Diagnostica Stago, Asnières, France).

The DiXaI assay is a specific method for direct FXa inhibitors insensitive to heparin, low molecular weight heparin (LMWH) or fondaparinux. Plasma is assayed diluted at 1:50. The DiXaI assay shows intra-assay ($N = 10$) and inter-assay ($N = 20$) coefficient of variations from <3% to <10%.

Results: Rotachrom, designed for measuring LMWH anti-FXa activity, is not appropriate for apixaban since concentrations >200 ng/mL cannot be determined. A variant technique, using plasma diluted 1:4 (rather than 1:2) and a plasma sample of 20 µL rather than 50 µL gives a linear dose-response curve from about 5–400 ng/mL.

- The DiXaI method gave an inverse linear dose-response relationship for rivaroxaban concentrations from 5 ng/mL to 500 ng/mL, and the same with apixaban. Therapeutic concentrations of heparin, LMWH or fondaparinux are not measured. On six independent series, performed over 3 days, with two series per day, recoveries for the two controls were of 103% for C1 and of 101% for C2, and inter-assay CVs <5%.

Summary/Conclusions: Clinicians must be aware that PT and APTT can be normal in apixaban treated patients with a significant hypocoagulant activity. Measurement of anti-FXa activity is preferable when clinically indicated. Our results are in agreement with a recent work showing that the anti-FXa measurement is preferable for the measurement of direct FXa inhibitors. Moreover, a specific anti-FXa assay (DiXaI), insensitive to heparins or fondaparinux, is useful in patients switching from heparin therapy to apixaban. Standardization of the anti-FXa measurement of apixaban is in progress by a working group of the ISTH.

PB 3.48-5

Patient outcomes with anticoagulation therapy after hip and knee replacement: comparison of two models of care

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Background: Warfarin is commonly used for venous thromboembolism (VTE) prophylaxis of patients undergoing hip and knee replacement surgery. Management of patients on warfarin therapy has been a challenge, especially in inception phases of therapy. Pharmacist-managed anticoagulation clinics have been reported to attain better anticoagulation control compared to routine medical care (RMC). However, the two models of care have not been compared for patients receiving post-surgery VTE prophylaxis which requires short-term therapy.

Aim: To compare quality of anticoagulation control between specialized care (pharmacist-managed antithrombosis clinic [ATC]) and RMC (orthopedic clinic) in an inception cohort receiving short-term post-surgery VTE prophylaxis with warfarin.

Methods: We conducted a retrospective, observational study of patients who underwent total hip or total knee replacement surgery at University of Illinois Hospital and Health Sciences System between the years 2000 and 2009, and were referred to either the ATC or RMC for post-surgical anticoagulation prophylaxis. Means for continuous variables were compared by using *t*-tests and frequencies for categorical variables were compared by using chi-squared tests. Propensity scores were used to adjust for potential confounding on observable risk factors. Propensity score was defined as the predicted probability of being managed by ATC compared to RMC. The average treatment effect (ATE) and average treatment effect for treated (ATT) for ATC compared to RMC was expressed as the% change in anticoagulation control (expressed as the time in therapeutic international normalized ratio range [TTR]) using inverse probability weighting and regression adjustment.

Results: A total of 873 patients were included in the study cohort, of which 294 were referred to ATC and 579 to RMC. The average age of the study cohort was 60 ± 12.3 years, and 68% were female. The majority of patients was African Americans (53.8%), followed by Caucasians (19.7%), followed by Hispanics (19.6%) and other race (6.9%). Before applying the propensity score method, several covariates were imbalanced between the two groups. For example, the average length of warfarin therapy (46.7 ± 20.3 vs. 31.9 ± 10.7; $P < 0.05$) and inpatient length of stay in days (7.0 ± 4.6 vs. 6.3 ± 3.3; $P < 0.05$) were significantly higher in ATC compared to RMC. After balancing the groups using inverse probability weighting and performing regression adjustment, TTR remained significantly higher in ATC compared to RMC via both ATE (7.1% higher in ATC vs. RMC, $P < 0.001$) and ATT (9.08% higher in ATC vs. RMC, $P < 0.001$).

Summary: Patients managed in the specialized pharmacists-led ATC had better anticoagulation control compared to routine medical care. Our study is among the first to evaluate and show benefit of a specialized systematic anticoagulation management model on quality of anticoagulation in an inception cohort receiving short-term post-surgery thromboprophylaxis.

PB 3.48-6

Psychological influence of the media on patients commencing oral anticoagulation in atrial fibrillation; a qualitative analysis

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Background: Oral anticoagulation (OAC) reduces stroke risk in patients with atrial fibrillation (AF); however it is still underutilized and sometimes refused by patients. Two inter-related studies were undertaken to understand the experiences and what influences this underutilisation of warfarin treatment in AF patients. These studies explored physician and patient experiences of AF and OAC treatment. The paper focuses on specific sub-themes from the study that explored patients' experiences will be discussed.

Aim: The study in question aimed to explore the experiences which influence patients' decisions to accept, decline or discontinue OAC.

Methods: Semi-structured individual interviews with patients were conducted. Three sub-groups of patients ($n = 11$) diagnosed with AF were interviewed; those who accepted, refused, and who discontinued warfarin. Interpretative phenomenological analysis (IPA) was used to examine the data. IPA is a qualitative method that focuses on how participants make sense of an experiences phenomenon.

Results: Three over-arching themes comprised patients' experiences: (i) the initial consultation, (ii) life after the consultation, and (iii) patients' reflections. In the last theme, patients reflected on their perceptions of aspirin and warfarin. Aspirin was perceived as a natural wonder-drug while warfarin was perceived as a dangerous drug usually given to people at the end of their life. Interestingly they perceive both drugs as 'old'. However, for aspirin it had a positive association, old meaning tried and tested. While for warfarin, old meant 'has been around for too long'.

Conclusion: Media had an important role in how patients' perceptions of these two drugs were influenced. Literature shows that framing techniques, i.e. using certain words or phrases such as 'rat poison', are processes adopted by media to alter medical knowledge into lay person's language. Patients in turn form negative cognitive schemas, between the word 'poison' and warfarin, leading to the negative perception of warfarin which could influence non-adherence to treatment. This qualitative research highlighted the potential influences of the media on AF patient perceptions commencing OAC treatment. The association between media stimuli and patient perceptions on OAC should be further explored. The influential power of lay-media could also be instrumental in disseminating appropriate educational material to the public.

PB3.49 – Coagulation factor IX – II

PB 3.49-1

The activity of GlycoPEGylated recombinant FIX (N9-GP) can be measured in two-stage chromogenic and one-stage clotting assays

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Introduction: N9-GP is a 40K glycoPEGylated recombinant human FIX derivative. The PEG group is selectively attached to an N-linked glycan in the activation peptide of recombinant FIX (N9), which upon activation is released and generates activated FIX (FIXa) similar to native FIXa [1]. In haemophilia B patients the half-life of N9-GP is 93 h which is approximately five-fold prolonged compared to BeneFIX® and MonoNine® [2].

The aim of this study is to evaluate the performance of N9-GP relative to non-PEGylated rFIX in two-stage chromogenic and one-stage clot assays.

Method: Different FIX preparations (N9-GP, N9, MonoNine® and BeneFIX®) were spiked into Haemophilia B plasma and tested in two different two-stage FIX chromogenic assays (Biophen® and Rossix®) using the WHO FIX international standard. The same FIX preparations were analysed in the FIX one stage clotting assay with different aPTT reagents using the WHO FIX international standard and a N9-GP standard. The N9-GP standard was calibrated against the WHO FIX international standard using SynthAFax® as the APTT reagent.

Results: The activity of N9-GP, N9, MonoNine® and BeneFIX® was comparable when spiked into Haemophilia B plasma and measured in the two different two-stage chromogenic assays calibrated against WHOFIX international standard. The PEG group of N9-GP does not appear to interfere with the reagents in the chromogenic assays.

APTT reagents SynthAFax®, Cephascreen® and DAPPTIN® provided full recovery of N9-GP activity (100% ± 25%) in the one-stage clotting assay using the WHO FIX international standard. Some reagents resulted in activity below 75% and other reagents resulted in values above 125% when calibrated against WHO FIX international standard. In general, aPTT reagents containing ellagic acid provided activities within target range (100% ± 25%), whereas reagents containing silica gave values above target, indicating some interaction of the silica based material with the PEG group of N9-GP. Full recovery was achieved for all APTT reagents when using the N9-GP standard.

Conclusion: As for MonoNine® and BeneFIX®, the activity of GlycoPEGylated recombinant FIX (N9-GP) can be reliably measured in two-stage chromogenic and one-stage clotting assays using ellagic acid based APTT reagents against the WHOFIX international standard. Other APTT reagents provided either too low or too high activity, probably because of interaction with the PEG group of N9-GP. The reagent specific variations can be normalised by introduction of an assay specific conversion factor or by using a N9-GP standard. The data illustrate the need for evaluating performance of new FIX products in various activity assays using different reagents.

References:

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PB 3.49-2

Comparative field study evaluating the activity of recombinant Factor IX – Fc fusion protein (rFIXFc) in plasma samples at clinical haemostasis laboratories

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Background: The recombinant coagulation factor IX Fc fusion protein (rFIXFc) consists of a single molecule of human factor IX (FIX) covalently linked to the dimeric Fc domain of human immunoglobulin G1 (IgG_1). rFIXFc utilizes the FcRn receptor-mediated immunoglobulin cycling pathway to extend its plasma half-life. The rFIXFc product was studied in a global, multi-center, Phase 3 clinical study (B-LONG) with prophylactic dosing intervals ranging from 7 to 21 days.

Aims: To evaluate the performance of commercially available one-stage clotting assay reagents used at clinical laboratories for monitoring of rFIXFc activity in patients, we conducted a field study to assess the accuracy and inter-lab variability in measuring rFIXFc activity in spiked plasma samples at 30 clinical haemostasis laboratories.

Methods: Human hemophilic donor plasma was spiked with either rFIXFc or a rFIX comparator product at 80, 20 or 5 IU/dL based on the label potency. Laboratories were blinded with respect to the drug product and concentration in each vial and were asked to test three sets of samples on different occasions using their routine one-stage clotting assay and in-house FIX plasma standard. The results were evaluated for accuracy, intra- and inter-laboratory variation, and possible product-specific assay discrepancies.

Results: The median spike recovery for rFIXFc was 68.7 IU/dL, 19.5 IU/dL, and 5.7 IU/dL at the nominal 80, 20 and 5 IU/dL concentrations, respectively, among the 30 laboratories. For rFIX, the corresponding spike recoveries were 97.8 IU/dL, 27.8 IU/dL and 7.9 IU/dL. Intra-laboratory variation was typically below 15% for both products. Among the three major activators used in clotting assays, ellagic acid ($n = 8$ labs) generally resulted in the highest observed activities, with median spike recoveries and inter-laboratory variation (%CV) of 93.3 IU/dL (13%), 28.3 IU/dL (18%) and 8.2 IU/dL (41%) for rFIXFc and 104.5 IU/dL (13%), 31.7 IU/dL (19%) and 8.7 IU/dL (35%) for rFIX at the 80, 20 and 5 IU/dL concentrations, which were well above the nominal concentrations. In comparison, silica ($n = 18$ labs) and kaolin ($n = 3$ labs) typically resulted in lower observed recoveries, with median results of 63.6 IU/dL (24%), 18.0 IU/dL (32%) and 5.3 IU/dL (35%) for rFIXFc and 97.7 IU/dL (13%), 27.6 IU/dL (19%) and 7.7 IU/dL (28%) for rFIX at 80, 20 and 5 IU/dL samples, respectively.

Summary/Conclusions: Our field study revealed a combination of laboratory- and reagent-specific assay variabilities, with progressively higher variability at lower FIX concentrations and non-linearity against the FIX plasma standard observed with both rFIXFc and the comparator (rFIX) product. This lead to consistent over-estimation of FIX activity at the lower levels and overall higher than expected inter-laboratory variability for both products. The differential reagent-dependent effects on rFIXFc and rFIX observed in the field study were

reproducible in side-by-side comparisons at a central laboratory. For both products, the assay results were generally consistent when compared between laboratories using the same reagents and calibrators. The rFIXFc product can be monitored in patients using existing methods and current plasma standards in the majority of haemostasis laboratories with reasonable accuracy and without the need for a product-specific standard.

PB 3.49-3

The effect of different APTT reagents on the potencies of plasma-derived and recombinant factor IX concentrates in one-stage clotting assays

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There are several new Factor IX (FIX) therapeutics in development, including recombinant and modified products. Currently, the one-stage clotting assay is employed for potency labelling of all FIX products. Although the current plasma derived International Standard (IS) has served well as a calibrant for licensed FIX concentrates, it is not clear whether it can provide valid potency estimates for the new generation recombinant products. There are suggestions of assay discrepancy related to Activated Partial Thromboplastin Time (APTT) reagents used when these products are assayed against the plasma derived IS. The aim of this study is therefore to evaluate the effects of choice of APTT reagent on the potencies of both plasma-derived (pdFIX) and recombinant (rFIX) concentrates.

Frozen aliquots of pdFIX and two different rFIX (rFIX1 and rFIX2) products were assayed against either the 4th IS FIX Concentrate (07/182) or an in-house rFIX reference (07/142) by the one-stage clotting assay as described by the European Pharmacopoeia, using incubation times as recommended for each of the APTT reagents tested. Several different APTT reagents covering a range of phospholipid sources and activators were used: Actin FS, APTT-SP, SynthAFax, Siron, CK Prest and Cephascreen. Potencies were determined by parallel line assay. Four assays were performed on each material. Data were calculated using combination of potencies and differences were deemed significant at the 5% level by Tukey's analysis of variance.

All assays were statistically valid. Against the 4th IS (07/182), the pdFIX showed no significant differences in potency estimates between APTT reagents (average potency 89 IU/mL, GCV 7.2%). This was not the case for either rFIX. rFIX1 showed significantly higher potencies with Actin FS and APTT-SP (average 125 IU/mL, GCV 0.55%) than with the other four reagents (average 93 IU/mL, GCV 4.8%). rFIX2 behaved similarly, with Actin FS and APTT-SP producing significantly higher potencies than all other APTT reagents (average 97 (GCV 1.14%) vs. 76 (GCV 6.2%) IU/mL). Against the rFIX reference (07/142), the pdFIX showed reasonable agreement in potency estimates between APTT reagents, with some significant differences, particularly with SynthAFax, which generated significantly higher potency estimates than the others (114 IU/mL compared to 84 IU/mL, GCV 12.9%). In contrast, both rFIX materials showed good agreement in potency estimates relative to the rFIX reference, with rFIX1 showing no significant differences between APTT reagents (average 104 IU/mL, GCV 7.1%), and rFIX2 showing only a small difference between SynthAFax and Actin FS ($P = 0.02$).

Different APTT reagents therefore yielded significantly different potencies when rFIX was assayed relative to a pdFIX standard, and vice versa. These differences were reduced when a 'like for like' standard was used. There are many different commercial APTT reagents and several new recombinant or modified FIX products in development that may assay differently to the current IS. The combination of these two factors could result in discrepant potencies between different laboratories. The data presented here suggest that assaying like against like reduces the discrepancy and highlights the importance of choice of standard.

PB 3.49-3

Population pharmacokinetic model for a novel recombinant fusion protein linking coagulation factor IX with albumin (rIX-FP) in hemophilia B patients

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Background: rIX-FP is a recombinant fusion protein linking coagulation FIX with human albumin intended for prophylaxis and on-demand treatment of hemophilia B. Results from the first-in-human dose escalation trial have demonstrated improved pharmacokinetics of this product with prolonged half-life. Thus, there is a great interest in evaluating less frequent administration of this product to prevent bleeding.

Objectives: The objective of this analysis was to evaluate pharmacokinetics of rIX-FP by means of population pharmacokinetic (PK) approach.

Methods: A population pharmacokinetic model was built using FIX activity levels from 37 subjects which were measured using a validated 1-stage clotting method after administration of three dose levels of 25, 50 and 75 IU/kg rIX-FP. The influence of potential covariates such as age, body weight, and baseline value was evaluated. Model robustness was assessed using nonparametric bootstrap and visual predictive check approaches.

Results: A two compartment model appropriately described the rIX-FP pharmacokinetics. A covariate analysis identified baseline as influencing the volume of distributions of rIX-FP. The visual predictive check indicated that the final pharmacokinetic model adequately predicted observed concentrations.

Conclusions: The parameter estimates of the population PK model were similar to the estimates from the traditional non-compartmental approach. The selected pharmacokinetic model accurately characterized rIX-FP pharmacokinetics and integrates information across all dose groups. This model was utilized as a tool to simulate various dosage regimens to instruct future clinical trials.

PB 3.49-4

Efficacy and safety of a novel rFIX (BAX326): phase III study in previously treated patients with severe or moderately severe hemophilia B undergoing surgical or other invasive procedures

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Background: Factor IX (FIX) replacement with regular prophylactic infusions has substantial benefits for patients with hemophilia B, a congenital X-linked bleeding disorder associated with a decrease or lack of circulating FIX. BAX326 is a recombinant FIX manufactured with two viral inactivation steps (solvent/detergent treatment and 15 nm nanofiltration) and without addition of any human or animal materials.

Aims: This first prospective clinical trial was conducted to assess the safety and efficacy of BAX326 in previously-treated patients with hemophilia B, 12 to 65 years of age with severe (FIX level <1%) or moderately severe (FIX level ≤2%), as well as to characterize the PK profile of BAX 326 and determine equivalence with comparator rFIX.

Methods: Safety was evaluated by adverse events, clinical and immunological assessments. PK parameters were compared between BAX326 and a commercial rFIX in a crossover design. Hemostatic efficacy after twice weekly prophylaxis with BAX326 was determined in terms of annualized bleeding rate compared with a historical control group treated on-demand. The study was approved by relevant medical ethics committees and informed consent/assent was obtained from each subject prior to enrollment.
Results: BAX326 was equivalent to the comparator rFIX in terms of AUC $_{0–72\ h}$/dose. BAX326 is safe and well tolerated in hemophilia B, with no signs of immunogenicity or thrombotic events. Compared with the annualized bleeding rate (ABR) in the historical control group, there were significantly fewer bleeds in subjects who received twice weekly prophylaxis with BAX326 over at least 3 months during the present study with a bleed reduction of 79%. Joint bleeds (major joints: wrist, elbow, shoulder, hip, knee, ankle) occurred at a mean ABR of 2.85 (4.25) compared with 1.41 (2.87) in non-joint bleed sites. Of the 32/56 subjects with bleeds, 90.6% (29/32) had arthropathy at screening and only 28.1% (9/21) did not have target joints, as compared to subjects without bleeds, of whom 79.2% (19/24) had arthropathy and 50% (12/24) did not have target joints at screening. The ABRs by bleeding site (i.e., joint vs. non-joint) and cause were further analyzed by subjects with (N = 46) and without arthropathy (N = 8), as well as with target joint (N = 35) and without (N = 21). Higher mean ABRs were observed in subjects with arthropathy vs. without arthropathy (4.54 vs. 2.57 for all bleeds, 3.16 vs. 1.02 for joint bleeds, and 1.97 vs. 0.25 for spontaneous bleeds). A similar pattern was observed for the ABRs of joint bleeds and spontaneous bleeds in subjects with target joints (N = 35) (mean ABR: 2.41 ± 3.79) and those with no target joints (mean ABR: 0.58 ± 1.63). Most bleeds, irrespective of location, cause, or baseline status, were controlled with 1–2 infusions of BAX326 and with an efficacy rating of 'excellent.'
Summary: BAX326 has a positive safety profile and is efficacious in treating bleeds and in routine prophylaxis in PTPs aged ≥12 years with hemophilia B. The results also demonstrate that subjects with target joints and hemophilic arthropathy receiving secondary prophylaxis tend to have higher ABRs as compared to those without these underlying conditions.

PB 3.49.6

Prospective study of a novel recombinant factor IX in previously treated patients with hemophilia B

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Background: Factor IX (FIX) replacement with regular prophylactic infusions has substantial benefits for patients with hemophilia B, a congenital X-linked bleeding disorder associated with a decrease or lack of circulating FIX. BAX326 is a recombinant FIX manufactured with two viral inactivation steps (solvent/detergent treatment and 15 nm nanofiltration) and without addition of any human or animal materials.
Aims : This first prospective clinical trial was conducted to assess the safety and efficacy of BAX326 in previously-treated patients with hemophilia B, 12–65 years of age with severe (FIX level <1%) or moderately severe (FIX level ≤2%), as well as to characterize the PK profile of BAX 326 and determine equivalence with comparator rFIX.
Methods: Safety was evaluated by adverse events, clinical and immunological assessments. PK parameters were compared between BAX326 and a commercial rFIX in a crossover design. Hemostatic efficacy after twice weekly prophylaxis with BAX326 was determined in terms of annualized bleeding rate compared with a historical control group treated on-demand. The study was approved by relevant medical ethics committees and informed consent/assent was obtained from each subject prior to enrollment.
Results: BAX326 was equivalent to the comparator rFIX in terms of AUC $_{0"\blacklozenge 72\ h}$/dose. BAX326 is safe and well tolerated in hemophilia B, with no signs of immunogenicity or thrombotic events. Compared with the annualized bleeding rate (ABR) in the historical control group, there were significantly fewer bleeds in subjects who received twice weekly prophylaxis with BAX326 over at least 3 months during the present study with a bleed reduction of 79%. Joint bleeds (major joints: wrist, elbow, shoulder, hip, knee, ankle) occurred at a mean ABR of 2.85 (4.25) compared with 1.41 (2.87) in non-joint bleed sites. Of the 32/56 subjects with bleeds, 90.6% (29/32) had arthropathy at screening and only 28.1% (9/21) did not have target joints, as compared to subjects without bleeds, of whom 79.2% (19/24) had arthropathy and 50% (12/24) did not have target joints at screening. The ABRs by bleeding site (i.e., joint vs. non-joint) and cause were further analyzed by subjects with (N = 46) and without arthropathy (N = 8), as well as with target joint (N = 35) and without (N = 21). Higher mean ABRs were observed in subjects with arthropathy vs. without arthropathy (4.54 vs. 2.57 for all bleeds, 3.16 vs. 1.02 for joint bleeds, and 1.97 vs. 0.25 for spontaneous bleeds). A similar pattern was observed for the ABRs of joint bleeds and spontaneous bleeds in subjects with target joints (N = 35) (mean ABR: 2.41 ± 3.79) and those with no target joints (mean ABR: 0.58 ± 1.63). Most bleeds, irrespective of location, cause, or baseline status, were controlled with 1–2 infusions of BAX326 and with an efficacy rating of 'excellent.'
Summary: BAX326 has a positive safety profile and is efficacious in treating bleeds and in routine prophylaxis in PTPs aged ≥12 years with hemophilia B. The results also demonstrate that subjects with target joints and hemophilic arthropathy receiving secondary prophylaxis tend to have higher ABRs as compared to those without these underlying conditions.

PB 3.50-1

Tyrosine phosphorylation of macrophage factor XIII-A: a potential mechanism for controlling intracellular enzyme activation and localization

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Background: In addition to the plasma, coagulation factor XIII A-subunit (FXIII-A) is present in hematopoietic cells including macrophages. Both plasma and macrophage pools have been implicated in cardiovascular repair processes but the mode of action of the macrophage enzyme is uncertain. Cross-linking of intracellular proteins by FXIII-A has been reported and it has been proposed that secretion of the enzyme from macrophages could occur to cross-link the extracellular matrix. However the mechanisms mediating intracellular FXIII-A activation and secretion remain unclear. Unlike in plasma, cellular FXIII-A is not activated by proteolysis and the calcium concentration required for activation *in vitro* is supra-physiological. Further, FXIII-A lacks an ER-Golgi leader sequence and little evidence exists for secretion from macrophages.
Aims: To gain insight into the function of macrophage FXIII-A we examined for enzyme secretion and activation under a range of conditions. We also investigated whether post-translational modification of FXIII-A could regulate these processes.
Methods: Human macrophages were generated from $CD14^+$ monocytes by culture for 6d in the presence of M-CSF. Murine macrophages were generated by bone marrow culture with CSF-1 for 7d. FXIII-A was measured in lysates and medium by immunoprecipitation/immunoblotting. Cell-surface proteins were labelled on live unpermeabilized cells at 4 °C and cells were imaged by confocal microscopy.

Transglutaminase activity was assayed by incorporation of dansylcadaverine into cellular proteins. Tyrosine phosphorylated FXIII-A was detected by immunoprecipitation using anti FXIII-A antibody followed by immunoblitting with anti pan-phosphotyrosine antibody. Proximity ligation assays also utilized this antibody combination and were performed using commercially available reagents

Results: No cell-surface or secreted FXIII-A was detected from resting or activated macrophages. However, protein cross-linking was detected upon PMA treatment and cross-linking also occurred in $TG2^{-/-}$ macrophages. Tyrosine phosphorylation of FXIII-A in transformed cells has been reported so we investigated this modification in macrophages. Immunoprecipitation/immunoblotting showed FXIII-A tyrosine phosphorylation in these cells but not in plasma or in platelets. Proximity ligation assays confirmed tyrosine phosphorylation and showed accumulation in the perinuclear region. An increase in phosphorylation was detected upon PMA treatment and both phosphorylation and cross-linking greatly increased when cells were preincubated with orthovanadate.

Summary/Conclusions: We have demonstrated that FXIII-A can be tyrosine phosphorylated in macrophages and that phosphorylation is increased by the protein kinase c activator PMA. We have also shown that PMA induces intracellular protein cross-linking in wild-type and in $TG2^{-/-}$ cells, strongly implicating FXIII-A in this process and suggesting a link between FXIII-A phosphorylation and enzyme activation. Elevation of tyrosine phosphorylation and intracellular cross-linking by orthovanadate treatment provides further evidence for this link. We therefore propose that macrophage FXIII-A could mediate reparative processes by cross-linking of intracellular proteins upon activation by phosphorylation leading to alteration of cell function. Potential substrates include the cytoskeletal proteins actin, vinculin and tubulin and the cell surface receptor AT1. Cross-linking of these or related substrates could modulate macrophage adhesion/migration, phagocytosis and intracellular signalling.

PB 3.50-2

Biochemical and numerical simulation of thrombin decay

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Background: Human plasma contains various thrombin inhibitors such as antithrombin (AT), α2Macroglobulin (α2M) and a group of miscellaneous serpins. Thrombin decay during thrombin generation (TG) is predominantly caused by AT (~65%) and α2M (~25%) with a small role for the other inhibitors. Thrombin decay is slowed down in the presence of fibrin(ogen), because fibrinogen contains thrombin binding sites, which compete with thrombin inhibitors for thrombin binding. Mathematical simulation models of TG at present do not take into account the interaction of thrombin with α2M or fibrin (ogen).

Aims: As a first step in a larger project in which we aim to arrive at congruence between mathematically simulated and experimentally obtained TG curves, we want to obtain a quantitatively correct description of thrombin decay in plasma.

Methods: The AT and α2M concentrations were determined in the control normal pool plasma (NPP) and a biochemical model was prepared, which consisted of purified human AT and α2M in Ringer buffer containing albumin and citrate. Thrombin decay was determined in this model system and in non-recalcified defibrinated NPP by the addition of purified human thrombin and the subsequent measurement of the disappearance of thrombin activity in time. Thrombin decay was measured in absence and the presence of various concentrations of fibrinogen.

The AT dependent decay was described as a bimolecular reaction (k_1) and the α2M and remaining thrombin inhibitor dependent decay as pseudo-monomolecular reactions (k_2 and k_3). Thus, a simple mathematical model for thrombin decay was obtained using ordinary differential equations.

$-dT/dt = k_1*AT_t*T_t + k_2*T_t + k_3*T_t$ and $d(\alpha2MT)/dt = k_2*T_t$

Results: The biochemical model consisting of AT and α2M (1.94 mM and 3.03 mM, respectively, as measured in NPP) could satisfyingly mimic total thrombin decay in plasma (1.122 and 1.168 μM/min, respectively), whereas thrombin decay in a biochemical model consisting of AT alone could not (0.961 μM/min, $P < 0.001$). The rate of thrombin decay by α2M was the same in defibrinated NPP and the biochemical model (0.21 μM/min). For each condition, a satisfying fit could be obtained between the biochemical model, the numerical model and defibrinated NPP.

The addition of fibrinogen (3 mg/mL) reduced the velocity of total thrombin decay in the biochemical model system (16%, $P = 0.004$) as well as in plasma (37%, $P = 0.010$). This was primarily due to its effect on thrombin decay by α2M (53%, $P = 0.004$; 43%, $P = 0.011$, respectively). In the biochemical system, the presence of fibrinogen primarily affects thrombin decay by α2M (fibrinogen reduced k_2 by 70%; $P = 0.004$) more than thrombin decay by AT (k_1 was 10% reduced; $P = 0.065$).

Conclusions: Our results suggest that in addition to AT, both α2M and fibrinogen are of great importance in the biochemical simulation of thrombin decay and are therefore indispensable in any representative model of thrombin generation.

PB 3.50-3

Hemostatic status pre – post intracoronary injection of peripheral blood stem cells in patients with recent myocardial infarction

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Background: Cardiovascular disease has increased in the last half century, it is becoming one of the major cause of morbidity in all hospital admission. Myocardial infarction (MI) constitutes half of all coronary heart disease cases. Current guidelines emphasize early coronary reperfusion on acute myocardial infarction (AMI) to alleviates mortality rates. However these conventional therapy sometimes cannot reverse the damage to infarcted myocardium. Even with timely executed rapid reperfusion, there are still patients with AMI surviving with significant LV dysfunction.

On the other hand patients undergoing PCI procedure are at risk for further ischemic events not only because procedure-related platelet activation occurs, but also due to the persistent platelet hyperreactivity and enchanced thrombin generation associated with ACS. This hypercoagulable state, in addition to vascular injury and platelet activation from PCI may lead to abrupt vessel closure, restenosis, and subacute stent thrombosis. It is not known how the acute coronary thrombosis alter the kinetics of the systemic coagulation system.

In the recent researches, Stem cell therapy has been highlighted as new therapy for patient population at risk for heart failure. Meta-analysis showed safety and favorable effects of stem cell transplantation in patients with AMI. In our latest research we successfully demonstrate the effect of injecting Peripheral Blood Stem Cells (PBSCs) in improving cardiac functions in patients with ST-segment elevation AMI. Intercorrelation between arterial and venous thrombosis risk factor 'vice versa' have not been established yet.

Aims: The purpose of this study is to report hemostatic parameter changes, such as *platelet aggregation, blood and plasma viscosity*, prothrombin time, APTT, CRP and fibrinogen, before and after (3 months) intracoronary administration of stem cell therapy.

Methods: A total of 24 patients diagnosed with anterior ST-segment elevation AMI who had successful percutaneous coronary intervention (PCI) with drug-eluting stent implantation within 15 days after onset of symptom were enrolled. PBSCs were harvested and injected into the infarct-related artery after five consecutive days of G-CSF administration. Recombinant human erythropoietin was administered at the time of intracoronary PBSCs injection.
Results: This research produce surprising results. Some of the patients' hemostatic parameter show 'normalization', which is is rarely achieved even by optimal AMI treatment. There were no significant difference between baseline vs. 3 months in spontaneous aggregation ($P = 0.350$), PT ($P = 0.793$), APTT ($P = 0.255$) and TT ($P = 0.254$). There were also no significant difference between baseline vs. 3 months in plasma viscosity ($P = 0.442$) and blood viscosity ($P = 0.843$). Nevertheless the patient who has their blood and plasma viscosity above or below normal laboratory range return to normal point after the treatment. Both PT and APTT also show normalization value, as seen in table 4. Both Fibrinogen and CRP level show significant decrease between baseline and 3 months after treatment ($P = 0.009$) and ($P = 0.04$) respectively.
Conclusion: PBSC mobilization with G-CSF and EPO based-intracoronary injection, showed normalization and improvement of hypercoagulable state beyond optimal conventional treatment post AMI.

PB 3.50-4
Successful use of recombinant activated factor VII for a major surgery in a patient with severe FXI deficiency and severe allergic reaction to fresh frozen plasma

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Severe congenital FXI deficiency is the commonest of the rare bleeding disorders. In contrast to hemophilia A or B, bleeding tendency in FXI deficiency do not correlate with FXI level. Therefore, optimal management of the patients with FXI deficiency is difficult. In addition, the bleeding risk for a surgery depends upon the type and the site of surgery and increases in high fibrinolytic areas such as the mouth, and nose. Several therapies such as fresh frozen plasma (FFP), FXI concentrate, fibrin glue, antifibrinolytic drugs are available for these patients. In the last years, the successful off-label use of recombinant activated factor VII (rFVIIa) has been reported in a very limited number of cases, including those with inhibitors. However, the dose and the duration of rFVIIa treatment has varied greatly between these case reports, and the development of thrombosis is a major concern. In this case report, we present our successful experience with the off-label use of rFVIIa for repairing the alveolar cleft and anterior palatal fistula in a 7-year-old boy with severe congenital FXI deficiency and severe allergic reaction to FFP. The patient was born to first degree consanguineous parents with the clefts of lip and palate. He was diagnosed as a severe congenital FXI deficiency (FXI:C level 0.2%) after the bleeding from the cleft lip repair surgery when he was 6 months old. First successful cleft palate surgery was done with FFP concentrates and tranexamic acid, when he was 18 months old baby. He was followed-up in our unit. The repairing surgery of alveolar cleft and anterior palatinal fistula was planned when he was 6.5 years old. However, severe allergic reaction developed during FFP infusion at the dental extraction before surgery. For this reason, we planned to use of rFVIIa for his surgery (FXI concentrate is not available in Turkey). Tranexamic acid was started orally every 8 h the day before surgery, and was given intravenously 2 h before surgery, and rFVIIa was given 40 μg/kg 1 h before surgery. Secondary alveolar bone grafting technique was performed under general anesthesia for the patient who was in his period of mixed dentition. Ilium was chosen as the donor site for obtaining autogenous cancellous bone grafts. The second dose of rFVIIa was given 4 h later after the first dose, and the dose of rFVIIa was reduced to 20 μg/kg, and continued at 6 hourly intervals for the first day, 8 hourly for the second day, 12 hourly for the 3rd and 4th day, and once a day for 5th and 6th day at the same dosage. Tranexamic acid was given until to 10 days after the surgery. No excessive bleeding or thrombosis were observed. In conclusion, low-dose rFVIIa therapy was successfully used for a major surgery in our patient with severe FXI deficiency and severe allergic reaction to FFP. However, treatment safety is a major concern and the best dosing regimen of this off-label drug is still to be defined for the selected patients.

PB 3.50-5
The 78 kDa glucose response protein (GRP78) interacts with ATP to inhibit a modified prothrombin time

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Background: We have previously described an interaction between the LLD domain of thrombomodulin (TM), a multi-domain glycoprotein expressed primarily on vascular endothelial cells and GRP78, the 78 kDa glucose regulated protein. GRP78, an ER chaperone, is found on the surface of endothelial and several tumour cells. We generated recombinant GRP78 and analysed its effect on haemostasis *in vitro* and *in vivo*. We previously showed GRP78 has anticoagulant, antithrombotic and antiplatelet properties. GRP78 prolongs TF dependent clotting (in a modified prothrombin time, PT) but not TF independent clotting. GRP78 also inhibits FXa generation (Xa spectrozyme assay) while showing no effect on thrombin time. We also demonstrated for the first time that recombinant GRP78 (8–10 μg/mL) can inhibit platelet aggregation up to 80% (compared to buffer) in response to collagen (1 U/mL), TRAP (1 μM) and ADP (10 μM). Pre administration of GRP78 also prolonged mouse tail bleeding time and GRP78 conferred significant protection in a platelet dependent model of acute venous thrombosis, induced by collagen (1.2 μg/g) into the jugular vein of anaesthetised mice.
Aim: Building on the work performed in our laboratory, we aimed to further explore the anticoagulant effects of GRP78 by investigating interactions of GRP78 with one of its substrates, ATP. GRP78 is known to bind ATP via the ATPase domain (aa 1–391) and exhibits a low level of ATPase activity. The binding of ATP to GRP78 is known to change tertiary confirmation of the latter and enhance the binding avidity of GRP78 to other substrates.
Method/Results: Using a high sensitivity phosphate assay (EasyRad [TRADEMARK] phosphate assay Biochem Kit[TRADEMARK]) we showed that our purified GRP78 had a very low rate of ATPase activity of 0.02 nmole ATP hydolyzed/min/mg.
We performed a modified PT using recombinant thromboplastin as a source of tissue factor and 20 μg/mL of GRP78 with 12 mM $CaCl_2$. We found that preincubation of GRP78 with calcium and ATP (1 mM) for 180 s resulted in a three fold increase in clotting time when compared with GRP78 and calcium alone ($P < 0.05$).
In an attempt to find a point in the pathway where GRP78 may inhibit coagulation, we performed a modified FXa (activated factor X) chromogenic assay (1/12 dilution of liquid Xa in STA™-Rivaroxaban anti FXa assay) to assess FXa inhibition by GRP78. GRP78 at 18 μg/mL did not inhibit FXa activity.
Conclusion: The addition of ATP to GRP78 prolongs a modified prothrombin time and may do so by enhancing the binding of GRP78 to a clotting factor or inhibitor involved in the coagulation cascade. GRP78 does not appear to inhibit FXa but we have shown it reduced FXa generation suggesting the point in the pathway at which it inhibits coagulation is above this level.

PB 3.50-6

Post-operative bridging therapy in a tertiary hospital in Singapore

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Background: Perioperative management of surgical patients who require temporary discontinuation of Warfarin involves, weighing the risk of thrombosis during the interruption against the risk of bleeding when such therapy is used in close proximity to a surgical procedure. There is a paucity of randomized clinical trials and practice is based on consensus guidelines and narrative expert reviews.
Aims: To look at the risk of thrombosis and bleeding in patients who underwent bridging therapy during their attendance at the Anti Coagulation Clinic (ACC).
Methods: This retrospective study amongst patients who attended ACC for 'perioperative bridging' looked at the risk of bleeding (patients given bridging therapy) and thrombosis (patients whose anticoagulation was interrupted). Data were collected for the reason for anticoagulation, nature of surgery, number of days Warfarin was discontinued and Heparin given, post-operative day on which anticoagulation was restarted and the incidence of thrombosis and bleeding post-operatively upto a period of 3 months.
Results: Fifty-four patients were analyzed, of which nine patients had procedures with a low bleeding risk for whom Warfarin was not interrupted. Amongst the patients with a high bleeding risk for whom Warfarin was interrupted ($N = 45$), all received instructions for post operative bridging with LMWH and Warfarin. Postoperatively the average time to attain therapeutic anticoagulation (INR >2) while on Warfarin was more than 7 days.
Amongst these, only 40% patients complied to low molecular weight heparin while the INR was sub-therapeutic. 20% were not advised anticoagulation due to major bleeding.
However, despite there being no contraindication for full therapeutic anticoagulation only one patient had an episode of Thromboembolism (ischemic CVA in Atrial fibrillation) in the immediate post operative period.
Conclusions: The risk of major bleeding is about 20% in patients undergoing interventions with a high bleeding risk while on bridging therapy. This is independent of the level of anticoagulation at the time of bleeding and more dependent on the nature of the surgical procedure. Delay in achieving therapeutic anticoagulation (initially with LMWH and subsequently with Warfain -therapeutic INR) in the post operative period is due to a combination of factors- smaller loading doses of Warfarin, interaction of Warfarin with diet and medications, reluctance on part of the Surgeon and the Clinic to ensure compliance of the regimen. Delay in achieving therapeutic anticoagulation is not associated with an increase in the risk of Venous Thromboembolism in the post-operative period.

PB3.51 – Blood coagulation tests – XI

PB 3.51-1

Sensitivity of various aPTT reagent – instrument combinations to dabigatran concentrations

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Background: It is known that the activated partial thromboplastin time (aPTT) is sensitive to the oral thrombin inhibitor dabigatran and may be useful in determining the relative intensity of anticoagulation in urgent clinical situations. However, the variation in sensitivity of different aPTT reagent-instrument combinations to dabigatran is not well studied.
Objective: To assess the responsiveness of the common aPTT reagent-instrument combinations in use in Ontario, Canada, to dabigatran concentrations across the therapeutic range.
Methods: Six aPTT reagents and three coagulation analyzers were used in this study. The aPTT reagents were CK Prest and PTT A from Diagnostica Stago, SynthASil and APTT-SP from Instrumentation Laboratories (IL) and ActinFS and ActinFSL from Siemens. The analyzers were Diagnostica Stago – STAR-Evolution, IL – ACL TOP and Siemens – CS 2000i. All aPTT reagents were reconstituted and used as per individual manufacturers' assay protocols. The Dabigatran plasma calibrator kit (HYPHEN BioMed) containing a set of three calibration plasmas at concentrations of 0.04 μg/mL, 0.28 μg/mL and 0.51 μg/mL spanning the therapeutic range of dabigatran was used. Additionally, the 0.28 μg/mL and 0.51 μg/mL calibrators were diluted with pooled normal plasma (Precision Biologic Cryocheck) to make concentrations of 0.10, 0.15, 0.35 and 0.45 μg/mL to provide additional time points. The pooled normal plasma was also run as a zero concentration. An aPTT was performed on each of the dabigatran concentrations as well as the pooled normal plasma (zero concentration) using each aPTT reagent on each coagulation analyzer generating individual aPTT results at the seven different dabigatran concentrations for the reagent-instrument combinations.
Results: All six aPTT reagents resulted in aPTTs ranging from 26 to 35 s with pooled normal plasma (zero dabigatran concentration) across the three different analyzers. For the 0.04 μg/mL concentration (corresponding to the trough dabigatran concentration) the aPTT range for the six aPTT reagents on the IL-ACL TOP analyzer was 41–53 s, on the Stago analyzer was 45–52 s and on the Siemens analyzer was 40–56 s. For the 0.51 μg/mL concentration (corresponding to the upper end of the dabigatran therapeutic range) the aPTT range for the six aPTT reagents on the IL-ACL TOP analyzer was 84–112 s, on the Stago analyzer was 83–111 s and on the Siemens analyzer was 73–120 s. There was overlap between the aPTT results for the 0.28 μg/mL concentration and the 0.51 μg/mL concentration for all reagent-instrument combinations. While all reagents exhibited a curvilinear dose response at high dabigatran concentrations, CK Prest and PTT-A reagents were the most sensitive to dabigatran on all the coagulation analyzers while Actin FSL was the least sensitive across all the analyzers.
Conclusions: This study provides an evaluation of the varying sensitivity (dose-response) of six different aPTT reagents run on three different coagulation analyzers commonly used across Ontario, Canada, to varying concentrations of dabigatran across the therapeutic range. This information may be clinically useful for assessing the relative intensity of coagulation specific to the reagent-instrument combination used to determine the aPTT.

PB 3.51-2

Bleeding disorders in children presenting with different bleeding symptoms or abnormal coagulation tests-First evaluation results in an out-patient clinic

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Mild bleeding symptoms (easy bruising, epistaxis, etc.) are common in the general population, and are reported in up to 25–45% of healthy people. Many of them do not have any identifiable bleeding disorders. In this study, our aim was to determine the clinical and laboratory features of 102 children referred for the first evaluation of a suspected bleeding disorder in an out-patient clinic of a university hospital. The medical records of 26,737 children (between 31-Oct-2011 and 31-October-2012) at the Department of Ambulatory Pediatrics were assessed, and 111 children with suspected bleeding disorders who did not have a known bleeding disorder were identified. Nine children were excluded from the study due to lost to follow-up. The patients with ITP were also excluded. Bleeding symptoms, duration of bleeding symptoms,

any bleeding history at newborn period, family history of bleeding, physical exam findings, and laboratory evaluation (complete blood count, PFA100 test, and coagulation tests) were analyzed, retrospectively. All coagulation tests were studied in the Pediatric Hematology&Oncology Hemostasis Laboratory. The mean age of the 102 patients (52 M, 50 F) was 8.9 ± 4.1 years (range 2–18 years). The most frequent bleeding symptoms were epistaxis in 36, easy bruising in 33, and menorrhagia in seven patients. The range of duration of bleeding symptoms was 2 days–6 years. Nine patients were referred for the evaluation of prolonged PT and/or aPTT levels, and two for pre-op evaluation. After the first evaluation at the Department of Ambulatory Pediatrics, 63 patients (61.8%) did not have a diagnosed coagulopathy, and 39 patients(38.2%) were diagnosed as a congenital bleeding disorder. The diagnoses included vWD type 1 in eight, vWD type 2 in four, vWD and FXI deficiency in three, mild hemophiliaA in four, moderate hemophiliaA in two, hemophiliaA carrier in two, hemophiliaB carrier in one, FXI deficiency in two, FV deficiency in three, FVII deficiency in two, viral infection induced thrombocytopenia in two, FX deficiency, combined FII, VII, IX, X and FXII deficiency, combined FV and FVIII deficiency, combined FVII and FX deficiency, FXII deficiency, platelet function disorder in each patient. Acquired coagulation inhibitor was not detected in any patient. Nine of 39 patients had a family bleeding history. Twenty-eight percent of the patients with epistaxis (10/36; mild hemophilia(3), vWD type 1(3), vWD + FXI deficiency(1), FVII deficiency(1), FV deficiency(1), FII, VII, IX, X + FXII deficiency(3), 30.3% of the patients with easy bruising (10/33; vWD type 1(3), vWD type 2(1), moderate hemophilia(1), hemophiliaA carrier(1), FV deficiency(1), FXI deficiency(1), FVII + X deficiency(1), viral infection induced thrombocytopenia(1), and 57.1% of the patients with menorrhagia (4/7; hemophiliaB carrier(1), vWD + FXI deficiency(1), FX deficiency(1), platelet function disorder (1) were diagnosed as a bleeding disorder after the first clinical and laboratory results. All 39 patients were referred to the Pediatric Hematology&OncologyUnit for the last evaluation and diagnosis.

In conclusion, about 40% of children presenting with bleding symptoms were diagnosed as a bleeding disorder in the out-patient clinic after the first evaluation. Menorrhagia may be the first clinical manifestation for the patients with bleeding disorders, and if a child with menorrhagia is referred, an underlying bleeding disorder should be considered.

PB 3.51-3

Studies on thrombin generation and thrombelastometry in haemophilic plasma show good correlation at low FVIII levels and the feasibility to use frozen samples of PRP

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Introduction: Replacement therapy in haemophilia A and B is currently based on plasma FVIII or IX activities even though it is known that FVIII and FIX plasma levels not always correlate to bleeding phenotype. In fact, in one study 10% of patients with severe haemophilia had a very mild phenotype with practically no spontaneous joint bleeds (Aledort LM et al 1994, Beeton K et al 2006). Neither genotype nor APTT based assays can identify these patients.

The last decade new methods aiming to evaluate the total capacity of the hemostatic system has been developed, namely thrombin generation (TGA) methods and viscoleastic assays (rotational thrombelastometry, ROTEM and thrombelastography, TEG). TGA measures the total amount of thrombin that is released in platelet poor plasma (PPP) or platelet rich plasma (PRP) over time, after activation with small amounts of tissue factor or kaolin. ROTEM/TEG measures the time to clot formation as well as the viscoelastic properties of the clot.

Correlation between TGA and the bleeding phenotype has been shown in patients with heamophilia (Santagostino et al. 2010, Trossaert et al. 2008), but so far no data have been published on the correlation between ROTEM/TEG parameters and tendency to bleed.

Aim: The aim of this study was to investigate if TGA parameters correlate to ROTEM parameters in patients with severe heamophilia at (i) baseline and (ii) after injection of replacement therapy.

Methods: Blood samples were taken from patients with heamophilia on regular prophylaxis before and after injection of their regular FVIII concentrate. Patients had not taken their prophylaxis later than 72 h before blood sampling. TGA was measured according to the method of Hemker on fresh and frozen citrated PPP and PRP samples after activation with tissue factor at 1 pM. ROTEM was performed on citrated whole blood after activation by diluted kaolin or innovin.

Results: In concordance with previous studies thrombin generation correlated poorly to FVIII levels at low FVIII concentrations (1–5%). However a strong correlation between TGA parameters in PRP and ROTEM parameters was seen in patients before injection of factor concentrate. Surprisingly the same parameters did not correlate after replacement therapy was given. This was explained by TGA being much more sensitive to the effect of FVIII compared to ROTEM parameters. When comparing the effect of freezing PPP and PRP, thrombin generation was significantly lower in frozen PPP than in fresh samples, whereas no significant difference was seen in frozen PRP compared to fresh at low FVIII levels.

Conclusions: Both Thrombin generation and Thrombelastometry have a potential to more accurately reflect the hemostatic potential in patients with haemophilia at low levels of FVIII. Thrombin generation in PRP is more sensitive than ROTEM in measuring the effect of replacement therapy. Thrombin generation can be measured on frozen samples of PRP, facilitating the use of this method in multicenter studies.

PB 3.51-4

Comparison of hemoclot thrombin inhibitor® and activated partial thromboplastin time with a reference UPLC-MS/MS method to monitor patients receiving dabigatran etexilate

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Background: The pharmacokinetic properties of dabigatran exetilate (DE) with low bioavailability and a dependence on renal function for its excretion result in considerable variation in plasma drug concentrations. Even if monitoring of dabigatran levels/effects are not needed in the majority of patients, a non-negligible proportion of patients will obtain either insufficient or supra-therapeutic levels of the drug when given a fixed dose. Therefore, possibilities to monitor DE therapy would be useful, e.g., in patients with renal impairment or possible drug interactions, and in cases of severe bleeding or recurrence of thrombosis, in anticoagulation bridging or before urgent or elective surgery. Monitoring may also be useful in infants, pregnant women or in patients with extreme body weights. Assays such as aPTT or the Hemoclot Thrombin Inhibitor® (HTI) have been proposed to evaluate drug concentrations but previous findings are based on *in vitro* analysis and results must be confirmed in clinical samples. The gold standard for therapeutic drug monitoring is mass spectrometry.

Aim: To compare aPTT and HTI measurements with the reference method, Ultra Performance Liquid Chromatography coupled with tandem mass spectrometry (UPLC-MS/MS), in plasma samples from DE treated patients.

Methods: Seventy-one plasma samples were included. aPTT was performed using CKPrest® and Synthasil®. HTI was performed according to instructions from the manufacturer using a STA-R Evolution®.

For the UPLC-MS/MS, after sample preparation, the analytes were separated on an Acquity BEH® column shield RP18 and detected by a Micromass Quattro Premier XE® mass spectrometer operating in positive electrospray ionization mode.

Results: The plasma concentration range was 0–645 ng/mL as measured by UPLC-MS/MS. The HTI and UPLC-MS/MS analyses correlated well with a Pearson correlation coefficient (r^2) of 0.97 (95% CI: 0.95–0.98; $P < 0.0001$). The Bland-Altman analysis showed a mean difference of 9 ng/mL (standard deviation (SD): 21 ng/mL; 95% limits of agreement: −32 to 50 ng/mL). However, the HTI method could not detect low, probably therapeutic levels of DE (<20 ng/mL) whereas the detection limit of UPLC-MS/MS was 2 ng/mL.

The aPTT and UPLC-MS/MS methods were not closely correlated (r^2 values of 0.59 and 0.50 for Synthasil® and CKPrest®, respectively). The Bland-Altman analysis showed a mean difference of −0.8 s between the two reagents (SD: 8.7 s; 95% limits of agreement: −17.9 to 16.3 s).

Conclusions: There is poor sensitivity and an important inter-individual variability with the aPTT and this test cannot be recommended to estimate plasma dabigatran concentrations. A normal aPTT could not exclude therapeutic levels of dabigatran and, conversely, therapeutic levels of dabigatran do not always result in abnormal aPTT. Thus, anticoagulant effects of DE are only detected by aPTT if they are pronounced. The HTI assay, on the other hand, showed little inter-individual variability and good agreement with UPLC-MS/MS measurements in the intermediate – high plasma concentration range for DE. Detection of low levels for, e.g., compliance monitoring or to rule out significant anticoagulant effects in DE treated patients requires the more sensitive UPLC-MS/MS method. HTI measurements and/or drug levels should be studied in relation to clinical outcomes.

PB 3.51-5
Clot waveform analysis in patients with bleeding disorders

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Background: Clot waveform analysis was introduced several years ago to achieve a better discrimination of factor VIII activity in hemophilia A by an extended interpretation of the clot formation process in an aPTT assay. Considering basic physico-chemical laws like Lambert-Beer law and the Michaelis-Menten kinetics, the method may provide more information than just factor FVIII concentration. By using an optical detection system, alteration of absorbance during clot formation is mathematically processed to obtain data regarding fibrin formation (measured curve), thrombin activity (first derivative), prothrombinase activity (second derivative) and tenase activity (third derivative).

Clotting times in aPTT based assays do not provide information about the complexity of the coagulation process, thrombin generation assays are cost- and labor-intensive. Especially, during bleeding episodes a fast, easy and reliable diagnosis is beneficial.

Aim: Disturbances of the coagulation system that lead to a high bleeding tendency are associated with diminished factor Xa or thrombin generation. This study is aimed to show the feasibility of clot waveform analysis in screening and monitoring of patients with bleeding disorders. The results are compared to methods like thrombin generation, PFA-100 and Von Willebrand Factor (VWF)-related diagnostics in different clinical situations.

Methods: Clot waveform analysis is measured using an ACL TOP (Instrumentation Laboratory, Kirchheim, Germany), using a Synthasil-activated aPTT assay (Instrumentation Laboratory). Measured optical data were processed using the Savitzky-Golay algorithm. Coagulation parameters were measured using standard test systems. Thrombin generation was measured as ETP (Siemens Healthcare Diagnostics, Marburg, Germany) or Technothrombin TGA (Technoclone, Vienna, Austria).

Patients were from our center were included after an informed consent was obtained. For all subjects the underlying bleeding disorder was characterized thoroughly.

Results: Patients with bleeding disorders show a significantly altered clot waveform compared to healthy controls. There is a clear correlation between severity of bleeding disorders and clot waveform analysis parameters. In hemophilia thrombin, prothrombinase and tenase have increasing sensitivity for different levels of factor VIII. Clot waveform analysis, especially the calculated course of the tenase complex, correlates with the extent of factor VIII, IX or recombinant FVIIa substitution. Under this conditions thrombin generation shows significantly less sensitivity.

In patients with Von Willebrand disease under DDAVP administration, clot waveform analysis reflects the procoagulant effect in a similar way like conventional test systems like measuring VWF parameters (activity or PFA 100).

Conclusions: Clot waveform analysis is an inexpensive and fast method to screen and monitor patients with bleeding disorders and can be done without any additional measurements beside an aPTT. In cases of replacement therapy the effects on tenase, prothrombinase, thrombin and fibrin formation can be investigated within a short time frame. The method can be easily adapted to other optical detection systems.

PB 3.51-6
Laboratory monitoring of unfractionated heparin therapy

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Background: Unfractionated heparin (UFH) therapy is commonly monitored by activated partial thromboplastin time (aPTT).

Objective: To compare the sensitivity and specificity of aPTT and global tests of haemostasis – Thrombodynamics and thromboelastography (TEG) by adding UFH to blood samples *in vitro* and by monitoring of UFH therapy in patients.

Methods: *In vitro* analyses were performed on 10 blood samples of healthy donors to which a number of UFH concentrations were added. Ten patients with high risks of thrombosis treated by intravenous UFH infusion were enrolled in the study. Blood samples were taken before the UFH infusion beginning, in 3, 6, 12, 24 h ($n = 2$) or once a day during 5 days ($n = 10$). The parameters of TEG (angle), Thrombodynamics (stationary velocity, Vst) and aPTT were determined. M ± SD are shown as a result.

Results: Vst and angle decrease in 2.8 and 2.6 times (25 ± 3 vs. 9 ± 3 μm/min, 26 ± 5 vs. 10 ± 2 deg, respectively) as 0.08 IU/mL UFH were added *in vitro* to blood and in 7.5 and 5.8 times, respectively, as 0.33 IU/mL UFH were added (angle was not determined in 7/10 samples with this dose). APTT increases in 1.6 times (35 ± 2 vs. 56 ± 18 s) and in 2.8 times, respectively. UFH concentration in blood of patients treated by 1000 IU/h is about 0.15 IU/mL.

Vst reveals hypocoagulation in 3 h after the 1000 IU/h UFH infusion beginning (2.2 times decrease) and reaches the lowest constant value in 12 h. APTT reveals hypocoagulation only in 24 h (2.3 times increase). TEG parameters cannot be determined after 12 h.

Statistical comparison of the assays parameters in the blood samples taken before the UFH infusion beginning (group 1) and from patients receiving UFH 300–650 IU/h (group 2) or 800–1300 IU/h (group 3) revealed that Vst is significantly different ($P < 0.05$) between all groups (29 ± 6, 13 ± 7, 5 ± 2 μm/min, respectively). APTT differs only in patients receiving high UFH doses (29 ± 4 s, 38 ± 12 s, 61 ± 20 s, respectively). Angle differs in all groups (39 ± 16, 20 ± 15, 5 ± 5 deg, respectively), but it was not determined ($R > 120$ min) in 13 of 24 blood samples from patients treated by high UFH doses.

Four cases of thromboses in UFH treated patients were observed. Thrombodynamics revealed hypercoagulation in three of them (excluding 1 catheter-related thrombosis), TEG and aPTT only in 1.
Conclusions: Thrombodynamics can be more effective in monitoring UFH therapy than aPTT and TEG.

PB3.52 – Blood coagulation tests – XII

PB 3.52-1

Platelet aggregometer according to the Born method is not suitable to control biological effectiveness of Minirin in the DDAVP test

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Background: Desmopressin (DDAVP, Minirin) is used successfully to normalize hemostasis in patients with thrombocytopathy (TCP), von-Willebrand-Syndrome Type 1 (vWS1) and mild hemophilia A. Unfortunately, not every patient responds adequately to Minirin. To approve the biological effectiveness of Minirin, the PFA-100 is very often used.
Aims: Due to the limitations of the PFA-100 an alternative was wanted and initially seen in the APACT 4004. Suitability for the DDAVP test of both systems was evaluated in the present comparative study.
Methods: Coagulability (platelet aggregation) of the blood from patients with TCP ($n = 7$) or vWS1 ($n = 7$) was tested before and after Minirin infusion (0.3 µg Minirin per kg body weight) in the APACT 4004 and PFA-100. Additionally, thrombocytes were counted and plasma levels of factor VIII activity (FVIII), von-Willebrand factor antigen (vWF) and ristocetin cofactor (Risto) were measured.
Results: Normalization of coagulability by Minirin resulted in the PFA-100 collagen/ADP system in a significant ($P < 0.0001$) reduction of the occlusion time from 131.0 ± 31.1 s before to 54.6 ± 7.9 s after infusion of Minirin in patients with vWS1 and from 95.1 ± 19.1 s to 54.3 ± 9.6 s ($P = 0.0003$) in patients with TCP. FVIII, vWF and Risto normalized from 77.7 ± 25.9%, 60.6 ± 19.7%, and 47.0 ± 26.7% to 141.0 ± 18.7% ($P = 0.0002$), 137.3 ± 33.6% ($P = 0.0002$), and 140.1 ± 26.1% ($P < 0.0001$) by Minirin. Comparable results were obtained with PFA-100 collagen/epinephrine. In contrast, the normalization of coagulation parameters by Minirin could not be demonstrated with the APACT 4004. Maximal aggregation parameters for the initiation of coagulation with ADP, collagen, epinephrine and ristocetin in vWS1 blood were 60.1 ± 9.8%, 50.5 ± 24.9%, 56.4 ± 15.5%, 62.2 ± 10.6% before and 50.1 ± 20.1% ($P = 0.2597$), 37.2 ± 19.8% ($P = 0.2904$), 46.6 ± 21.8% ($P = 0.3515$), 49.8 ± 22.2% ($P = 0.2071$) after Minirin application. Results of patients with TCP were comparable.
Summary/Conclusion: Feasibility of PFA-100 for conformational testing of the effectiveness of Minirin in patients with vWS1 or TCP was verified. However, in contrast the APACT 4004 was not sensitive to the Minirin induced changes of coagulation parameters. Therefore, the APACT 4004 cannot be used to test the biological effectiveness of Minirin.

PB 3.52-2

Effect of eculizumab administrations on the haemostatic changes in patients with paroxysmal nocturnal hemoglobinuria

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Background: Eculizumab is approved to reduce the intensity of intravascular hemolysis and thrombotic risk in patients with paroxysmal nocturnal hemoglobinuria (PNH)[1].
Aim: The aim of this study was to investigate the coagulation state in PNH patients under eculizumab treatment.
Methods: Three patients with PNH in state of permanent hemolysis, requiring constant red cell transfusions, were enrolled in this study. Eculizumab administration was 1 time per week; the tests were performed 1 day before and 1 day after administration for 4 weeks. Further eculizumab administrations last for 2 months, the tests were performed in the day of administration before the infusion. Thrombodynamics (a new method based on a spatial fibrin clot growth registration), thromboelastography (TEG), activated partial thromboplastin time (aPTT), prothrombin index (PI), D-dimer assay, the concentration of lactate dehydrogenase (LDH), and the concentration of hemoglobin (Hb) were performed. In thrombodynamics assay the initial clot growth velocity (Vi) was determined.
Results: TEG parameters, aPTT and PI were in normal or in hypocoagulation area before treatment. Vi was increased (58 ± 7 µm/min with normal range 36–56 µm/min, $P < 0.001$). Enlarged D-dimer levels in two patients (0.8 mg/L, 2.1 mg/L with normal range 0–0.5 mg/L) confirmed the well-known tendency to hypercoagulability during haemolytic crises. D-dimers were in norm for one patient (0.3 mg/L). Coagulation tests data wasn't significantly ($P > 0.5$) changed the day after eculizumab administration: Vi was 60 ± 8 µm/min, the parameters of the TEG, aPTT, PI were in normal or in hypocoagulation area. D-dimers remained in norm in one patient under eculizumab treatment. Other two patients had the decrease of D-dimer level with some incidents of D-dimer's increase during the treatment. LDH level progressively decreased and Hb level increased in all patients indicating the reduction of hemolysis. Physical health was improved. During further eculizumab administrations for 2 months all tests data was not significantly ($P > 0.5$) changed.
Conclusion: Eculizumab administrations did not reduce hypercoagulability in PNH patients, they are still need the prescription of anticoagulant therapy. Thrombodynamics was sensitive to the state associated with the increased risk of thrombotic events in PNH patients.
1. Hillmen et al, Blood, 2007.

PB 3.52-3

Evaluation of assay performance monitoring direct thrombin inhibitors with TECHNOCLOT® DTI in plasma samples contaminated with indirect thrombin inhibitors

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Background: The increasing use of the new oral direct thrombin inhibitors (DTI) dabigatran creates the need of the measurement in clinical routine. Plasma samples obtained from blood drawn through heparinized tunneled venous access devices may contain heparin in unknown concentration. Technoclot® DTI is a modified thrombin time based assay which can be used for measurement of DTIs in the presence of indirect thrombin inhibitors.

Aim: The aim of this study was to evaluate the performance of the new TECHNOCLOT® DTI assay with the DTI dabigatran in plasma samples containing indirect thrombin inhibitors.
Method: Technoclot® DTI is a clotting assay based on the inhibition of a constant and defined concentration of thrombin. Clotting times measured are directly related to DTI concentrations.
Calibration of the assay was performed with Technoview dabigatran. Technoview Dabigatran Controls were used for assay control. The assay was performed automated using the Coasys Plus C coagulation analyzer.
Normal plasma with a defined concentration of dabigatran was spiked with different concentrations of unfractionated Heparin and low molecular weight Heparin.
Results: Dabigatran was calibrated in the range of 0–500 ng/mL and assay performance proved by dabigatran control sample measurements.
The recovery of dabigatran value was below ±10% of target value for all samples spiked with different concentrations of UFH up to 1.2 IU/mL and LMWH up to 1.2 IU/mL. Using the diluted thrombin time the overestimation of dabigatran concentration in samples with 0.8 IU/mL UFH or LMWH was 16% and 13% respective, rising to 37% and 21% for samples with 1 IU/mL UFH or LMWH.
Conclusion: Our data demonstrate that TechnocLOT® DTI is suitable for monitoring the direct thrombin inhibitor dabigatran with good precision in samples containing up to 1.2 IU indirect thrombin inhibitors.

PB 3.52-4
Application of an automated genotyping system (Verigene®) in a thromboembolic disease unit

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Background: The path to an accurate and personalized medicine goes through a genotyping implementation in daily clinical practice with devices able to obtain a fast and simple result. However, several factors such as an appropriate technical training, the availability of specialized equipments or an expert laboratory and consuming time make it difficult.
Aims: To evaluate an automated genotyping system, using whole blood to measure the polymorphisms most frequently associated with acenocoumarol dose requirements as well as frequent molecular variants of thrombophilia: Factor V Leiden (FVL) and prothrombin 20210A (PT20210A).
Methods: Detections of FVL and PT20210A polymorphisms (inherited thrombophilia markers) as well as *VKORC1* and *CYP2C9* polymorphisms (rs2323991 and CYP2C9*2 and *3, respectively) were performed in parallel using a routinely real time PCR genotyping and an automated genotyping system: – Genotyping technology provided by Verigene ® (Nanosphere, Grifols) consists in a low density matrix which does not require previous PCR amplification of DNA. This system uses whole blood (up to 7 days from extraction) and allows the study of one sample per process, with an average duration of 3 h per sample and requiring EDTA or citrate indistinctly as anticoagulants. – The routine protocol for genotyping these SNPs in our laboratory involves an automatic extraction of DNA (QIAmp DNA Blood Kit) followed by real time PCR genotyping (using Taqman commercially available probes; Applied Biosystem). We analyzed blood samples from 177 consecutive unselected patients: 97 were tested for thrombophilia markers and 80 for VKORC1/CYP2C9 genotypes.
Results: In the thrombophilia study, concordance between both genotyping systems was 100%. However 15 samples (15%) were missed in Verigene® system: 11 because of reading problem and 4 because of sample deterioration. Four discrepancies between the two genotyping systems were found when we tested VKORC1/CYP2C9 genotypes (95% concordance). In this study, 10 samples were missed in Verigene® system (10%): eight because of reading problem and two because of sample deterioration.
Conclusions: The Verigene® system is an automated genotyping system whose main advantages are reliability and rapidity. It uses whole blood and does not require a highly specialized training. By contrast, the mean failure rate of automated system (12.5%) in comparison with the routine genotyping (5% for FVL and 1% for the rest of genotypes) could be a point in favor of routine methods. Finally, the high degree of concordance between both procedures (98%) makes of Verigene® system a procedure to take into account to introduce pharmacogenetic testing in any laboratory.

PB 3.52-5
Evaluation of the Grifols Q analyzer for routine and special hemostasis tests

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Background: Robust and standardized validation protocols are needed to assess the performance of new coagulometers. Grifols Q Analyzer is a fully automated coagulometer that analyzes multiple parameters of hemostasis, and can be used for both screening and more specialized coagulation tests, including coagulometric, chromogenic and immunologic assays.
Aim: To evaluate the performance of Q Analyzer (performance characteristics and throughput calculation) and to provide a comparison with an established method in a reference hemostasis laboratory.
Methods: The validation process was established according to the Clinical Laboratory Standard Institute (CLSI Linearity-EP-6-, CLSI full precision-EP-5A, comparison method, CLSI EP-9). We evaluated PT, APTT, FV:C, FVIII:C (three different patient groups: hemophilic, Von Willebrand and thrombotic) and VWF:Ag (three different methods ELISA as reference method and IL and Grifols as two new automated methods), using coagulometric and chromogenic methods. Six parameters were evaluated for each test, by the following strategy: (i) Linearity, by using calibration curves performed consecutively with standard reference material; (ii) preliminary precision (within-run), by repeated testing of a normal and abnormal frozen sample 20 times on the same run; (iii) total precision, by performing 2 runs on 20 separate days, each run consisting of two replicates of the two plasma samples (normal and abnormal); (iv) carryover, by alternating samples spiked with heparin (>1.0 IU/mL UFH) and normal plasma in the APTT determination; (v) throughput, (calculated by subtracting the time to obtain the first completed test panel to that of the last completed panel), by loading the equipment with 30 samples and using different test panels; and (vi) method correlation, for coagulation, chromogenic and immunological assays against the reference method. For the statistical analysis the data was assessed for linear regression r^2, method comparison (Passing & Bablok) and agreement (Bland-Altman).
Results: 140 samples were evaluated for PT and APTT (77/PT and 63/APTT) including 29 patients under warfarin treatment, 15 patients with chronic liver disease, 10 patients treated with unfractionated heparin, 10 patients with hemophilia, and 10 patients with rare coagulation deficiencies. High correlation was obtained between the two methods (r^2 = 0.96 for the PT and r^2 = 0.89 for the APTT). However, differences in reagent performance were observed in selected patient groups when synthetic reagents were compared with reagent obtained from animal sources. The VWF:Ag was evaluated in 30 VWD patients and 33 healthy subjects, with all blood groups equally represented. The automated methods showed high correlation with the classical

ELISA, yielding a linear regression $r^2 = 0.89$. Median results with IL and methods were 46.13 UI/dL (range 0.64–105.5) and 40.70 UI/dL (range 1.3–114.6), respectively, with no statistical significant differences. We also observed satisfactory performance of Q Analyzer for intra, inter and total precision as evidenced by the low coefficient of variations (CV%): 3.6% in normal and abnormal PT, and 4.9% and 3.2% in normal and abnormal levels APTT, respectively. No carryover was detected. The effective throughput was 104 tests per hour for the PT and 64 tests per hour for the APTT.

Conclusion: Using a comprehensive validation protocol, the Q Analyzer showed satisfactory results.

PB 3.52-6

Apixaban: safety, usefulness and practical details of laboratory monitoring

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Background: Apixaban is direct factor-Xa inhibitor that reached the market for the prevention of venous thromboembolism in patients undergoing major orthopaedic surgery and in stroke prevention in patients with non-valvular atrial fibrillation.

Thanks to its predictable pharmacokinetic profile, biological monitoring is not required. Nevertheless, evaluation of plasma drug concentration may be valuable in specific situations such as recurrent thrombosis, bleedings, before urgent surgery, in case of bridging and in case of at least two risk factors among the following ones: drug interactions with caution, moderate renal impairment and moderate hepatic impairment. Monitoring may also be useful in infants, pregnant women or in extreme body weights, although no relevant data on drug levels associated with approximate therapeutic and harmful ranges are currently available.

Aims: The aim of this study is to assess and provide good laboratory practise for the accurate estimation of apixaban plasma concentration using routine or more specific coagulation assays.

Methods: Apixaban was spiked at increasing concentrations (0–500 ng/mL) in pooled citrated normal human platelet poor plasma (PPP) to measure Prothrombin Time (PT), dilute PT and activated Partial Thromboplastin Time (aPTT) with different reagents, dilute Russell Viper Venom Time (screen and confirm), Thrombin Generation Assay (TGA) with different inducers and different anti-Xa chromogenic assays.

Results and Discussion: As mentioned in previous studies, PT is not sensitive enough to allow accurate quantitative measurement of the plasma drug concentration (concentration needed to double the clotting time [2xCT] from 154 to 1367 ng/mL). Indeed, the 2xCT is 154 ng/mL with the most sensitive reagent while the mean C_{max} obtained in a short PK study after one oral intake of 5 mg apixaban (dose given in atrial fibrillation) is 96 ng/mL. This can lead to major concern when urgent or elective surgery is required and in definitive, a normal PT cannot exclude the presence of apixaban at therapeutic concentration. Activated Partial Thromboplastin Time presents a better sensitivity but shows a plateau after 100 ng/mL reflecting the uselessness of this test to estimate the impact of apixaban on the secondary haemostasis. Dilute Russell Viper Venom time shows a sensitivity of 205 ng/mL (screen), which is not enough sensitive.

On the opposite, chromogenic anti-Xa assays seem to be very sensitive (concentration requires to halve the OD/min: 2–20 ng/mL). Nevertheless, the relation is not always linear and some methodologies need to be adapted to ensure a broader range of application.

TGA may be useful to assess the pharmacodynamics effects of apixaban on the coagulation process. Nevertheless, the turn around time and the lack of standardisation are currently limitations that restrict the use of this method.

Conclusion: Prothrombin time could not be used as screening test to assess the risk of bleedings as it was proposed for rivaroxaban another direct FXa inhibitor. A more specific and sensitive assay such as chromogenic anti-Xa assays using calibrators should be used to correctly estimate the concentration of apixaban. Finally, standardization of the time between the last intake of apixaban and the sampling is mandatory.

PB3.53 – Blood coagulation tests – XIII

PB 3.53-1

Haemostatic abnormalities in patients with Noonan syndrome

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Background: Noonan syndrome is a congenital disorder whose main clinical features are short stature, dysmorphic facial features, congenital heart defects and genital defects. Several patients require surgery at a young age. A bleeding diathesis and various coagulation abnormalities have been anedoctically reported.

Patients and methods: A cohort of patients with Noonan syndrome was investigated with coagulation and platelet function tests. If ratios of prothrombin time (PT) and/or activated partial thromboplastin time (aPTT) were prolonged, the activity of clotting factors was measured. Children without history of bleeding admitted for urologic surgery formed the control group. Bleeding score was calculated in all patients and controls.

Results: The study population included 39 patients with Noonan syndrome (median age 10 years, range 1–28) and 28 controls of similar age. PT and aPTT were normal in 21 patients and all controls, while they were prolonged in 18 patients (46%). Clotting factor assays revealed partial deficiency of FVII in 10 (single or combined with deficiency of other vitamin K-dependent factors), of FXII in 3, and of FVIII, FIX or FXI in three other patients. One patient had combined deficiency of FX, FIX and FV and another had lupus anticoagulant. Oral vitamin K was given to three patients with FVII deficiency with normalization of PT. Platelet aggregation and secretion to ADP 4 μM and collagen 2 μg was significantly reduced in patients than in controls. Bleeding score was above 5 in 4 patients, but no correlation with coagulation or platelet abnormalities was observed.

Conclusion: Nearly 50% of patients with Noonan syndrome shows an impairment of platelet function or mild coagulation abnormalities likely related to vitamin K deficiency. This should be considered before performing surgery in these patients.

PB 3.53-2

The impact of blood sampling and major surgery on plasma levels of thrombin and activated protein C

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Background: Due to their high stability, affinity and specific binding-patterns, DNA-based aptamers, in combination with a stabilizing blood sampling strategy, proved to be valuable tools for the measurement of enzymatically active, circulating coagulation factors (Angew Chem Int Ed Engl. 2011; 50: 6075–8; J Thromb Haemost. 2012; 10: 390–8). To date, however, only limited data on preanalytical requirements and the impact of different procoagulant states on plasma enzyme levels are available.

Aims: The aim of this study was to investigate the influence of the blood collection technique on assay results and to assess the impact of major surgeries on circulating enzyme levels.
Methods: Serial blood samples were taken from three healthy donors. An antecubital vein was punctured with 18- or 21-gauge (G) butterfly catheters. In addition, blood samples for determination of reference values were taken from 20 healthy donors (21G). Furthermore, samples were taken during the course of knee- and hip-replacement surgeries from an indwelling venous cannula (18G). All corresponding plasma samples were analyzed for active thrombin, APC, as well as indirect markers of coagulation.
Results: Serial blood sampling showed that slow downed blood flow leads to an artificial increase of direct and indirect markers of thrombin activity. In contrast to thrombin, APC-levels were not influenced by sluggish blood flow. In 19 of 20 tested healthy individuals, plasma levels of both, active thrombin and APC were found to be below the LLOQ of the corresponding assays (1 and 2 pM, resp.). For one donor, blood sampling was difficult, leading to a massive increase of all thrombin markers. During the course of hip- and knee replacement surgery, plasma levels of both, active thrombin and APC increased to high picomolar levels but returned to baseline only few hours after. As expected, observed kinetics of *in vivo* thrombin and APC generation as well as corresponding peak levels were different between these two types of surgery.
Summary/Conclusions: Plasma levels of free thrombin and APC are novel markers that reflect the current state activity of blood coagulation and its regulation. The blood sampling technique used may lead to an artificial increase of plasma thrombin levels. Overall, the found results pave the way for future studies that will lead to a deeper understanding of the *in vivo* kinetics of the plasmatic coagulation cascade.

PB 3.53-3
Von Willebrand Factor and factor VIII inhibitor testing

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Background: The development of inhibitors is a major side effect of hemophilia A treatment. Moreover autoantibodies against factor VIII (FVIII) may emerge in healthy patients. In both cases treatment is focused on the prevention of bleedings and the eradication of the inhibitors by means of excessive administration of FVIII (immune tolerance induction, ITI).
Several assays were designed to monitor inhibitor development or eradication in patients with inherited or acquired hemophilia. Most assays base on the reduction of FVIII activity by mixing a FVIII source with dilutions of an inhibitor-containing sample. Inhibitor assays show a large inter-laboratory variability, varying sensitivity and specificity. In ITI discrepancies between inhibitor strengths, FVIII half-life and recovery are observed regularly; usually the inhibitor assays show no FVIII inhibition despite shortened and diminished FVIII recovery.
However, several publications suggest the usability of inhibitor assays to show a superiority of von Willebrand factor (VWF) containing preparations in prevention and therapy of FVIII inhibitors, based on the competition between VWF and some inhibitors for binding FVIII.
Aim: This study is aimed to reveal the influence of VWF on the results from inhibitor assays. Using basic laws of molecule interaction, it is intended to demonstrate how design and execution influence the assay result.
Method: Effect of VWF on inhibitor assays was simulated by applying the Law of Mass Action and reaction kinetics in computer models. The simulations were compared to inhibitor measurements using classical inhibitor assays or various designs of ELISA-based assays.
Result: If VWF competes with an inhibitor for FVIII binding, VWF influences inhibitor assays in a dose-dependent manner. VWF presence suggests an apparent lower concentration of antibodies. As VWF is found in the patient's sample, in the FVIII source and possibly in dilution media, assay design, sample dilution and reagents interfere with the determination of the true inhibitor concentration. Furthermore, FVIII concentration alters the determined antibody concentration.
The binding kinetics of inhibitors to FVIII is slowed by VWF; therefore, incubation times may alter the result.
Conclusion: As present-day inhibitor assay design distorts the balance between VWF, FVIII and inhibitor compared to the in-vivo situation, the beneficial effect of VWF is overestimated. If an antibody competes with VWF for FVIII binding, its concentration might be underestimated up to factor 30. Inhibitors with low concentration or affinity are easily overlooked; high responders might be classified as low responders. This explains discrepancies between inhibitor strengths, FVIII half-life and recovery in ITI or discrepancies between FVIII activity and ELISA-based assays. An influence of VWF on inhibitor development and inhibitor strength can not be derived from FVIII activity-based assays. Nevertheless, preformed FVIII-VWF-complex might be beneficial in prevention or therapy of acute bleedings in patients with a certain antibody profile.
Based on Law of Mass Action and reaction kinetics, the source of high inter-laboratory variability is more a side effect of the inhibitor assay design than the assay execution itself.
Interestingly, the simulations revealed that FVIII inhibitors that compete with VWF for FVIII show type II-like kinetics.

PB 3.53-4
Application of turbidity monitoring by combined activated partial thromboplastin time and waveform analysis to characterize heterogeneity of hemophilia plasma response to recombinant factor VIII

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Background: Heterogeneity of hemophilia A (HemA) response to therapy is well documented. Response to therapy is often monitored by the one-stage activated partial thromboplastin time (aPTT), which detects clot formation as increased turbidity or viscoelasticity upon fibrin polymerization. Recently, we have applied turbidity monitoring via combined aPTT and waveform analysis to facilitate coagulation testing of HemA plasma (Leong et al., www.multiwebcast.com/isth/2012/ssc/20995/).
Aims: In this study, we determined whether turbidity monitoring can describe clot characteristics and whether differences in clot characteristics might account for the heterogeneity of hemophilia response to recombinant factor VIII (rFVIII).
Methods: Individual HemA plasma samples ($N = 17$) were spiked with 1%, 5%, or 25% rFVIII, and aPTT was performed. Once clot time (CT) values were obtained, raw turbidity data were exported and further analyzed to obtain turbidity parameters.
Results: Turbidity characteristics were correlated with individual plasma aPTT CT values. The HemA plasma samples tested were roughly categorized into two groups following correlation of their maximal turbidity values attained using a 25% rFVIII spike with their CT values. Group 1 (10 of 17) plasma samples showed a linear correlation between turbidity maxima and CT, with the turbidity maxima exceeding that of normal plasma (>297 arbitrary units). The high turbidity maxima of group 1 plasma samples suggests that FVIII deficiency and consequent low thrombin generation would give rise to the thick, porous fibrin meshwork described in the presence of low thrombin (Weisel and Nagaswami, *Biophys. J.* [1992];63:111–128). In the group 2 plasma samples, the CT values did not correlate with the turbidity maxima values obtained, as the turbidity maxima were lower than expected. Correlation of plasma fibrinogen levels with turbidity maxima or maximal rate of turbidity development indicated a direct linear relationship between these parameters, suggesting that the

reduced turbidity maxima of group 2 plasma is a consequence of reduced (but within normal range) fibrinogen levels (<256 mg/dL for normal). This reduced fibrinogen level can potentially impact clot properties, because fibrinogen concentration is a determinant of fibrin structure and viscoelasticity *in vitro* (Ryan et al. *Biophys. J.* [1999];77:111–128)

Conclusions: The application of combined aPTT and waveform analysis indicated that part of the heterogeneity of hemophilia plasma response to therapy may be attributable to subtly reduced (but still within normal range) plasma fibrinogen levels, which can impact clot structure. Future studies correlating clot characteristics (eg, turbidity) with clinical endpoints could elucidate whether differences in clot physical properties can account for the heterogeneity of patient response in hemophilia.

PB 3.53-5

Six SIGMA metric seems not to be applicable as a good quality tool for most laboratory tests of haemostasis

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Six Sigma Metric analyses have been greatly expanded as a tool for clinical laboratories, to measure their procedures performance as a whole. For the calculation of SIGMA the BIAS%, CV%, and allowed Total Error (TEA) of the test are used. In Haemostasis different TEA, according to different requirements are used: CLIA, CAP and biological Variation. The aim of the present study was to calculate Sigma for several tests of the Haemostasis Laboratory, using CLIA, CAP and BV TEA requirements, and comparing the performance obtained. We calculate the BIAS as the mean BIAS obtained in the last four EQC surveys in which our laboratory participated. In all of these surveys the performance of our laboratory was successful. We calculated the cv% from the Internal Quality Control at two levels normal and low of Prothrombin time (PT), activated partial thromboplastin time (APTT) and fibrinogen (FIB), and we also calculated SIGMA for every level of results using CLIA, CAP or BV requirements as TEA. When using BV, none of the tests in any level of control reach an acceptable level of SIGMA (>3). For PT using CLIA requirement as TEA (15%) only 17% of month an acceptable SIGMA was obtained for normal control level, and 25% for low abnormal control. When using CAP requirement of 20% as TEA in 58% of months the SIGMA were acceptable (between 3 and 4), in 25%were good (between 4 and 5) and 8% were excellent (>5) for normal level of control, and in 25% of months the SIGMA were acceptable (between 3 and 4), in 33%were good (between 4 and 5) and 17% were excellent (>5) for low abnormal level of control.

Considering APTT, when CLIA or CAP requirements (both consider 15% as TEA) were used, 8% of months SIGMA calculated were good (4–5), and 92% were excellent for normal level of control, and 8% acceptable, 50% good and 42% excellent for low abnormal control.

Finally, analysing fibrinogen when CLIA or CAP requirements (both consider 20% as TEA) were used only in 33% of months SIGMA reached acceptable values (3–4) and 8% good value for normal control level, and 75% acceptable SIGMA for low abnormal control level.

For factor VII, VIII, Antithrombin (AT) and von Willebrand factor (vWFAg) only BV TEA are published and any SIGMA calculated reached an acceptable value.

For free Protein S and Protein C, although BV requirements were used, results obtained were better. The 83% and 75% of SIGMA calculated were >3.0 for normal and low abnormal control levels of Protein C, respectively. Additionally, 58% of SIGMA calculated were >3.0 in the two levels of control in free protein S.

In conclusion, SIGMA metric seems not to be applicable for most of the tests in the haemostasis laboratory. It is particularly true when the BV is used as TEA, because only free protein S and Protein C reached some acceptable SIGMA values, but no coagulation test, nor chromogenic AT or immunoturbidemetric vWF Ag did with these requirements.

PB 3.53-6

Whole blood thromboelatometry and aggregometry in obese patients

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Background: It was found that obesity is associated with a hypercoagulable state due to either an increased level of clotting factors and/or the inhibition of the fibrinolytic pathway. Furthermore, several authors reported that platelets from obese individuals seem to be more reactive both at baseline and after aspirin, suggesting an innate hyperaggregable state. Traditional coagulation tests cannot evaluate the whole coagulation system. The thromboelastogram record the viscoelastic changes that occur during coagulation, providing a graphical representation of the fibrin polymerization process. Thus, thromboelatogram enables a complete evaluation of the process of clot initiation, formation and stability, using whole blood. The Multiplate® analyzer is a semi-automated point-of-care device that performs multiple electrode impedance aggregometry in whole blood with five different activators.

Aim: We hypothesized that the point-of-care parameters might serve as early biomarkers to identify the prothrombotic state observed in patients with obesity.

Methods: Eighty patients consecutive referred to our Department, of whom 20 were overweight [BMI = 25–29.9 kg/m^2], 20 with I degree obesity [BMI = 30–34.9 kg/m^2], 20 with II degree obesity [BMI = 35–39.9 kg/m^2] and 20 with III degree [BMI>40 kg/m^2]) were enrolled. Eighty age (±3 years) and gender-matched normal weight healthy individuals acted as controls. Viscoelastic clotting measure was performed by thromboelastogram test. The recorded parameters were both in EXTEM and in INTEM tests: clotting time (CT, sec), clot formation time (CFT, sec), maximal clot firmness (MCF, mm), maximum velocity (MaxVel, mm/min), and the area under curve (AUC, mm x100). In FIBTEM test: MCF was considered. Aggregometric impedance measure in whole blood was performed on the Multiplate® function analyser. Platelets were stimulated in different ways: (i) via arachidonic acid pathway (ASPI test) (ii) via the ADP receptor (ADP test) and finally (iii) via the thrombin receptor (TRAP test).

Results: INTEM-test CFT was significantly prolonged ($P = 0.014$) and MCF ($P < 0.001$), VelMax ($P = 0.013$) and AUC ($P < 0.001$) were significantly increased in 3rd degree obese compared with controls. In EXTEM-test MCF ($P < 0.001$), VelMax ($P = 0.034$) and AUC ($P < 0.001$) were significantly increased in patients suffering from 3rd degree obesity compared with controls. MCF in FIBTEM was significantly higher in 1st, 2nd and 3rd degree obesity than controls ($P = 0.027$, 0.002 and <0.001, respectively). A significant difference in platelet aggregation was found between 3rd degree obese subjects and normal controls in each of the tests considered (TRAPtest, $P = 0.011$; ADPtest, $P = 0.005$; ASPItest, $P = 0.02$). A significant correlation between FIBTEM-MCF with BMI (r 0.24, P 0.019) and waist circumference (r 0.21, P 0.04) was observed. Moreover, a correlation between a) BMI with ADP and ASPI test (r 0.49, $P < 0.0001$ and r 0.50, $P < 0.0001$, respectively); b) waist circumference with ADP and ASPI (r 0.62, $P < 0.0001$ and r 0.57, $P < 0.0001$, respectively) was found.

Conclusions: The use of points-of-care is a quick and efficient method for measuring the degree of hypercoagulability and hyperaggregability in whole blood from obese patients, confirming an increase in the prothrombotic state with the increase in fat mass. The possible role of point-of-care devices in leading the anticoagulant prophilaxis/therapy in subjects with obesity is under evaluation.

PB3.54 – Factor II/Prothrombin – II

PB 3.54-1

Des-gamma-carboxyprothrombin (PIVKA-II) in fetus and newborns

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Background: Vitamin K deficiency bleeding (VKDB) is rare and life-threatening condition. In Japan, thrice oral administration of vitamin K (VK2: 2 mg) is recommended for prevention of VKDB. But, there is minimal or no data for vitamin K (VK) status in antenatal and postnatal period during the 1st month of age.
Aims: The purpose of present study was to investigate des-gamma-carboxyprothrombin (PIVKA-II: protein induced by VK absence) level in fetus and healthy newborns who received prophylactic VK.
Method: The present study was performed in 1997–1998. Cord blood samples were obtained from 22 preterm and 51 full term infants, and heel-cut blood samples (500 μL) from 83 full term newborns just before VK administration. The latter was performed together with examination of Hepaplastin test. In the present study subjects suffering from any obstetric complications were excluded. PIVKA-II was measured using the highly sensitive method, ECLIA (electro-chemiluminence immunoassay). Before collecting the samples, informed consent was obtained from the parents in accordance with the regulations of our institute.
Results: (1) PIVKA-II level (mAU/mL) during antenatal period was median 33 (range 19–235) during 14 to 28th gestational week (*n*:6), 28 (12–63) during 28 to 36th week (*n*:16), 42 (12–1557) during 37 to 38th week (*n*:18), and 50 (14–2834) after 39th week (*n*:31). There was a significant increase after 37 th week of gestation ($P < 0.01$, Man-Whitney's U test). 2) PIVKA-II in newborns who received prophylactic VK was 292 (20–9325) on 0–1 day of life just before the first administration of VK (*n*:21), 38 (11–1657) on the 4–6 day just before second administration of VK (*n*:16) with significant decrease ($P < 0.01$), and 27 (8–76) on 1 month of life just before third administration of VK (*n*:46) with significant decrease ($P < 0.1$). All of 83 newborns had no occurrence of VKDB.
Summary: Present transectional study shows physiological changes of vitamin K status in antenatal period. Namely, PIVKA-II level is low during preterm period and remarkably increases in term period. And present study shows a dramatic increase of PIVKA II on 0–1 day of life, reaching to near 10,000 mAU/mL and a remarkable decrease after VK administration. This suggests an effectiveness of thrice VK administration for prophylaxis of VKDB.

PB 3.54-2

The effect of the variant F2C20209T on the detection of the mutation F2G20210A

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Background: Genotyping of the *F2G20210A* mutation in the *F2* gene is performed routinely for diagnosis in thrombophilic disorders. We have developed two automate methods using Real Time PCR to analyze this mutation. It has been reported that the variant *F2C20209T* in the F2 gene may complicate the ability to accurately to analyze the mutation *F2G20210A*. Here we present an example of such a complication that we encountered while screening for this mutation in a patient sent to our center.
Methods: DNA was isolated from peripheral blood leukocytes by Autopure (Qiagen, Izasa). The analysis of *F2G20210A* was performed using two methods: that discriminate various alleles. One method is based on the use of allele-specific fluorescent hybridization probes with the *LightCycler LC480* system (Roche). The other method involved the use of the *TaqMan* system with the *ABI7500PCR* system (Applied Biosystem). Direct sequencing was performed with *Analyzer Genetic* 3500 (*Applied Biosystems*).
Results: The patient was referred to our center for the analysis of the *F2G20120A* mutation. Using the *LightCycler* system for a routine analysis the patient showed atypical values for the melting curves: melting temperatures (Tm) 64 °C (vs. 65 °C) for allele *G* and 55 °C (vs. 58 °C) for allele *A*. In contrast, using the *ABI7500 PCR* system with a specific probe for the *G* allele (probe labelled with FAM) the patient showed an atypical pattern. The direct sequencing data revealed that the patient was heterozygous for the *F2C20209T* variant. During the last 4 years, we have analyzed 6000 samples for the *F2G20210A* mutation using the *Light Cycler* system. Also during the last 6 months, we have analysed 400 samples for the *F2G20210A* mutation using the *ABI7500PCR* system. We have detected six homozygotes for *F2G20210A* and in all cases the result have been confirmed by direct sequencing to rule out the presence of heterozygosity for both *F2G20210A* and *F2C20209T*. So far, we have identified a single patient with the *F2C20209T*, which confirm that this variant is rare in the Spanish population.
Conclusions: The prevalence of the *F2C20209T* variant is very low in the Caucasian population and its position adjacent to the *F2G20210A* mutation makes its identification very difficult. Automated methods using Real Time PCR can detect heterozygous *F2C20209T* variant. With the homozygous for *F2G20210A*, direct sequencing is required to distinguish between individuals who are homozygous for the *F2G20210A* mutation and individuals who are heterozygous for *F2G20210A* and *F2C20209T*.
Spanish grant RD12/0042/0032. AGAUR 2009 SGR1147.

PB 3.54-3

Differentiation of newer oral anti-Xa and Anti-IIa agents with a reference to the regulatory function of thrombin

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Introduction: The newer anticoagualnts represent a group of synthetic antithrombin (Dabigatran: Boehringer Ingelheim) and anti-Xa agents (Abixaban;Bistol Meyer Squib/Pfizer; Rivaroxaban: Bayer/Jenssen; E-doxaban; Diachi Sankyo; Bietrixaban: Portola). All agents are low molecular weiht synthetic organomimetic drugs (MW <500 Da) with targeted antithrombin and anti-Xa activities. Their biochemical and pharmacologic properties differ. Although effective in their specific dosage for given indications these agents differ in their safety profiles, bleeding, risk of myocardial infarction and gastrointestinal disturbances have been reported. The purpose of this study is to differentiate these agents in various biochemical and pharmacologic systems to explain the observed differences in their safety and efficacy profiles.
Methods: Dabigatran and rivaroxaban were obtained from commercial sources and apixaban was of a synthetic origin. Each of these agents were profiled in clot based whole blood and plasma assays. Thrombin generation studies were performed using a flourogenic and chromogenic substrate assay. In addition the effect of these agents on clot stability was also measured. The effect of these agents on platelet aggregation was measured using the aggregometry and flow cyotmetry assays.
Results: All agents produced a concentration dependent anticoagulant effect in the global clotting assays in both the whole blood and plasma samples. Assay based variations were observed. The rank order was Dabigatran>Rivaroxaban>Apixaban. In comparison to the anti-Xa inhibitors, dabigatran was found to be relatively weaker inhibition of thrombin and Xa generation in various systems. Dabigatran also pro-

duced a strong inhibition of thrombin-thrombomodulin mediated activation of protein C, thrombin activatible fibrinolysis inhibitor (TAFI) and factor XIII.
Conclusions: While each of the newer oral anticoagulants are potent inhibitors of thrombin and Xa, the anti-Xa agents are relatively potent inhibitors of thrombin generation, however, do not compromise its regulatory function. In contrast, Dabigatran and related antithrombin agents may compromise thrombin's regulatory functions such as activation of protein C, TAFI and Factor XIII.

PB 3.54-4

Protein-peptide docking of human thrombin: towards improved imaging in cardiovascular disease

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Background: Thrombin plays a central role in thrombosis and haemostasis which is why it remains an important target for anticoagulant therapy. Likewise non-invasive *in vivo* thrombus imaging, detection of thrombin provides important information on thrombus size, location and state of progression. Radioactively labeled peptides, that mimic the natural thrombin (peptidic) substrates could be applied in the thrombus imaging e.g. by Positron Emission Tomography (PET). However, we chose to evaluate the potential use of cyclic peptides, which are known to stay in the blood circulation significantly longer than linear peptides which would give the clinician more time and precision for PET imaging.
Objective: Our aim was to rationally design new cyclic peptides leads for thrombin detection, which could be applied in PET imaging.
Methods/Results: Investigation of new cyclic peptides was based on the structure activity relationship (SAR) and structure based virtual screening (SBVLS) which involved protein-peptide docking. We used information from known peptidic thrombin inhibitors in order to probe the S1–S3 subpockets of the thrombin for cyclic pentapeptides. Rationally designed cyclic pentapeptides were synthesized via solid phase peptide synthesis using Fmoc chemistry. The activity of the cyclic pentapeptides was quantitated by *in vitro* kinetic assay through competition with chromogenic or fluorogenic substrates. We evaluated the activity of the best two cyclic pentapeptides in clotting-based assays. The best cyclic pentapeptide exhibits activity characterized by IC50 = 100 μM and it prolonged the APTT-based clotting time by 30% at concentration 100 μM.
Conclusion: We have characterized and synthetized cyclic pentapetides targeted at thrombin using structure activity relationships. Best peptides so far showed inhibitory activities in range of IC50 = 100 μM. Further optimization of the cyclic peptides for *in vivo* experiments is required.
Disclosure of Interest: The author declares no conflict of interest.

PB 3.54-5

Thrombin generation assay in a patient with acquired factor V deficiency

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Background: Development of acquired inhibitors against factor V (FV) is a rare coagulation disorder. The clinical manifestations range from asymptomatic to severe bleeding episodes. FV inhibitors are associated with various diseases and conditions, most commonly major surgery, exposure to bovine thrombin or transfusion of blood components. The inhibitor titer does not correlate with the level of FV deficiency or with the bleeding risk. Since about 20% of circulating FV is stored in platelets, it has been suggested that platelet FV might be inaccessible to inhibitors and be responsible for the usually benign clinical course seen in these patients.
Materials and Methods: An 18-year-old patient suffering from nephrotic syndrome presented a prolongation of the PT and aPTT, but no bleeding complications. Plasma and platelet FV antigen (FV:Ag) and activity (FV:C) were measured by ELISA and a clotting assay, respectively. Thrombin generation (TG) triggered with 5 pM tissue factor was measured in platelet-poor plasma (PPP) and in platelet-rich plasma (PRP) before and after treatment with intravenous IgG (IVIG) and corticosteroid therapy.
Results: The patient had prolonged PT and aPTT. In mixing studies his plasma prolonged the PT and aPTT of a normal plasma. FV:Ag was low (42%) and FV:C was undetectable (<1%). Platelet FV:Ag was normal (110%). Accordingly, virtually no TG could be detected in the patient's PPP, whereas PRP showed a normal TG. After therapy plasma levels of FV:Ag and FV:C increased (60–106%), whereas platelet FV:Ag decreased (20–50%). TG in PPP progressively increased during the follow-up and eventually normalized.
Conclusions: The TG assay could be an alternative approach for the diagnosis and monitoring of haemostatic changes in patients with FV deficiency due to a coagulation inhibitor. Platelet FV rather than plasma FV plays a significant role in preventing bleeding. FV stored inside platelets is protected from the inhibitor and is probably sufficient to overwhelm the inhibitor when released at the site of injury.

PB 3.54-6

Effects of camel milk on platelet function and coagulation parameters in streptozotocin diabetic rats

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Background: Diabetes mellitus remains a major cause of atherothrombotic disorders, due in part to the associated prothrombotic abnormalities. Other than the conventional anti-diabetic management strategies of diet, hypoglycemic drugs and exercise, camel milk was shown to be effective in the control of the hyperglycemia, shows hypoglycemia and also decreases insulin requirements, in both human diabetics diabetic patients and experimental animals.
Aim: The current study aimed to investigate the effect of camel milk on the blood coagulation and platelet function in streptozotocin (STZ) diabetic rats.
Material and Methods: The study was undertaken in three groups of Wister rats,. Group I: controls (n = 20). Experimental diabetes was induced by single intraperitoneal (i.p) injection of STZ (65 mg/kg) in 40 rats: 20 rats (diabetic camel milk-treated, Group II) received camel milk (50 mL/day/rat) orally; the rest were (n = 20; Diabetic untreated, Group III). After 8 weeks of camel milk treatment blood was collected from retro-orbital venous plexus of anesthetized rats the following tests were undertaken: Coagulation tests: PT, APTT, TT and plasma fibrinogen. Platelets aggregation was undertaken tested in platelet rich plasma (PRP) in response to ADP and arachidonic acid (AA). In addition, plasma activities of aspartate aminotranferase (AST) and alanine aminotransferase (ALT), total bilirubin and protein levels, fasting insulin and glucose levels were also measured.
Results: The untreated diabetic animals showed significant weight loss, marked hyperglycemia, decreased insulin level and increased liver transaminases and bilirubin levels. Additionally, there was significant inhibition of the platelet aggregation response to ADP and AA, decreased plasma fibrinogen (>50%) and shortening of APTT while the PT and TT showed less remarkable changes. Treatment with camel

milk decreased the extent of the weight loss and improved the hyperglycemia, reversed fibrinogen consumption and restored platelet aggregation responses.
Conclusion: Other than its hypoglycemic (anti-diabetic) action, camel milk has reversed the haemostatic activation and the consumption coagulopathy induced by STZ, indicating an anticoagulant/antithrombtic action of camel milk. This would also offer yet additional beneficial action of camel milk as anti-thrombotic agent, in addition further to its already well documented anti-diabetic action.

PB3.55 – Coagulation factor VIII – V

PB 3.55-1

Elevated plasma factor VIII enhances venous thrombus formation and propagation in rabbits: Contribution of thrombin, factor XI, von Willebrand factor and tissue factor

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Background: Elevated plasma levels of factor VIII (FVIII) are associated with increased risk of deep venous thrombosis.
Aims: To elucidate how elevated FVIII levels affect venous thrombus formation and propagation *in vivo*.
Methods: We examined rabbit plasma FVIII activity, plasma thrombin generation, whole blood coagulation, platelet aggregation and venous wall thrombogenicity before and 1 h after an intravenous infusion of recombinant human FVIII (rFVIII). Venous thrombus induced by the endothelial denudation of rabbit jugular veins was histologically assessed. Thrombus propagation was evaluated as indocyanine green fluorescence intensity. Argatroban, a thrombin inhibitor, and neutralized antibodies for tissue factor (TF), factor XI (FXI), and von Willebrand factor (VWF) were infused before or after thrombus induction to investigate their effects on venous thrombus formation or propagation.
Results: The rFVIII (100 IU/kg) increased rabbit plasma FVIII activity two-fold and significantly enhanced whole blood coagulation and total plasma thrombin generation, but did not affect initial thrombin generation time, platelet aggregation and venous wall thrombogenicity. The rFVIII infusion also increased the size of venous thrombus 1 h after thrombus induction. Argatroban and the antibodies for TF, FXI or VWF obviously inhibited such enhanced thrombus formation and all except TF suppressed thrombus propagation.
Conclusions: Elevated plasma FVIII levels enhance venous thrombus formation and propagation. Excess thrombin generation by FXI and VWF-mediated FVIII recruitment appear to contribute to the growth of FVIII-driven venous thrombus.

PB 3.55-2

Risk of venous thrombosis associated with coagulation factor VIII levels and the interrelation of other procoagulant and environmental risk factors

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Background: High levels of various procoagulant factors affect risk of venous thrombosis (VT). However, levels often vary together while the effect of high levels of some factors on the risk of VT is more pronounced than that of others, particularly at the extremes of the distribution (very high levels).
Aim: We aimed to determine (i) the relation between very high coagulation factor levels and thrombotic risk (>99th percentile); (ii) the magnitude of the effect of high levels of various coagulation factors on the risk of venous thrombosis; (iii) if coagulation factor levels have predictive ability in *a priori* high-risk groups.
Methods: To study the relation between VT and high coagulation levels we used data of 2377 patients and 2940 controls from a population-based case-control study (MEGA study) into risk factors for thrombosis. We measured fibrinogen, FII, FVII, FVII, FIX, FX, FXI and VWF. Odds ratios (ORs) were calculated using logistic regression and adjusted for age and sex, unless stated otherwise. We contrasted the levels >99th percentile to the lowest quartile (reference group). Consent and ethical approval were obtained for this study.
Results: The ORs for the various coagulation factor levels (P99 vs.)
We used the factors of which high levels had an effect on risk in multivariable analysis (i.e. FVIII, FXI and FIX) to study their ability to predict thrombosis in groups with *a priori* high-risk (surgery, trauma, plaster cast, hospitalized, bedridden, cancer, oral contraceptive use, pregnancy and obesity). Since the groups were reduced in size a lower cut-off was chosen to classify high levels (P90). In each of the separate high-risk groups factor FXI and FIX levels had lower predictive ability than FVIII levels, which had predictive value in all high-risk groups, up to a most pronounced OR of 16.7 (95% CI 8.6–32.6) for obesity.
Conclusion/summary: Very high levels of procoagulant factors increase the risk of venous thrombosis. In multivariable models, only high levels of FVIII/VWF, FIX and FXI remain associated with risk. This effect was most pronounced for high FVIII and VWF levels, of which at least FVIII levels also had predictive ability in *a priori* high-risk groups.

PB 3.55-3

Characterization of a panel of anti-FVIII antibodies by domain specificity assignment, pairwise epitope overlap analysis, and determination of affinities for FVIII and rFVIIIFc

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Background: Prophylactic therapy for Hemophilia A requires frequent intravenous administration of factor VIII (FVIII) due to the relatively short half-life of FVIII. A recombinant FVIIIFc fusion protein (rFVIIIFc) has been generated to increase the half-life of FVIII. Anti-FVIII monoclonal antibodies are valuable reagents for immunoassay development and for cellular uptake, immunogenicity, and structural studies, as well as for use as probes to assess conformational comparability between FVIII and modified variants such as rFVIIIFc. The utility of these antibodies, however, is often limited by a lack of information regarding their epitope specificities and affinities for FVIII.
Aims: This study was undertaken to characterize members of a panel of both commercially available and internally generated anti-FVIII antibodies with respect to chain and domain specificity, epitope overlap, competition with von Willebrand factor (VWF) for FVIII binding, and affinity for FVIII and rFVIIIFc.
Methods: We first characterized 38 anti-FVIII antibodies to either confirm or determine *ab initio* their chain and domain specificity by using biolayer interferometry (ForteBio Octet). The assay format comprised three sequential steps: (i) binding of a first test antibody to a biosensor probe, (ii) FVIII capture, and (iii) exposure of the FVIII-bound probe to a second test antibody. An increase in the spectral interference signal upon exposure of the probe to the second antibody indicates non-competition between the two antibodies, whereas a lack of change in the signal indicates competition. For determination of chain specificity, B domain-deleted FVIII that had been dissociated into separate heavy and light chains was used. For assignment of domain specificity,

a thrombin exposure step was included after step 2 to enable discrimination between the A domains in the heavy chain, as well as between the a3 acidic peptide and the remainder of the light chain. In a further variation of this approach, overlap between antibody epitopes and the VWF binding site on FVIII was evaluated by substituting a VWF for the test antibody in step 1. The affinities of each antibody for FVIII and rFVIIIc (expressed as K_D), were determined on a surface plasmon resonance (SPR) array instrument (ProteOn XPR36).

Results: Thirty eight anti-FVIII antibodies were characterized with respect to their chain and domain specificity, and antibodies that mapped to the same domain were subjected to pairwise epitope overlap analysis. Collectively, this information was used to generate a FVIII interaction map that included K_D values reflecting affinities for both FVIII and rFVIIIFc. Each antibody exhibited similar affinity for both FVIII and rFVIIIFc, with K_D values spanning the picomolar to nanomolar range. In addition, seven antibodies that mapped to the FVIII C domains competed with VWF for binding to FVIII.

Conclusions: The FVIII antibody interaction map is a valuable resource for assay development and investigation of the function and structure of FVIII. Notably, the observation that members of this panel of antibodies exhibit the similar affinities for both FVIII and rFVIIIFc supports the conclusion that fusion of the Fc domain does not distort the structure of the FVIII molecule.

PB 3.55-4

Preclinical safety of a longer acting recombinant factor VIII (BAX 855)

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Baxter and Nektar have developed BAX 855, a PEGylated form of Baxter's licensed recombinant (r) FVIII based on the same manufacturing process. BAX 855 is derived from a CHO cell line using a plasma-protein-free method and a virus inactivation step. The preclinical program evaluated the safety and immunogenicity of BAX 855 in different species. Two lots of BAX 855 were used to test batch consistency; the licensed rFVIII served as reference item.

Systemic toxicity was tested in rats and macaques. Rats received BAX 855 intravenously at 350 or 700 U/kg every other day for 28 days: macaques were administered 150, 350 or 700 U/kg every 5 days for 28 days. Toxicokinetics and formation of anti-product antibodies were assessed. The thrombogenic potential was assessed in Wessler's rabbit stasis model; general safety pharmacology was evaluated in telemetered macaques. Comparative immunogenicity assessment of BAX 855 and the licensed rFVIII included their potential modulation of both the innate (human cytokine release assay; human complement activation assay) and adaptive immune system (macaques; three different hemophilic mouse models).

No thrombogenicity was observed in rabbits treated intravenously with BAX 855 at 900 U/kg. BAX 855 also did not cause any adverse clinical, respiratory or cardiovascular effects in macaques and was well tolerated at all dose levels tested.

No drug-related changes were observed in rats. Lower levels of FVIII activity and BAX 855 (measured through its bound PEG) in plasma correlated with the presence of neutralising antibodies at study end. No signs of toxicity were observed at any dose level in macaques. Minor findings, such as prolonged APTT in all animals, were noted in the last week of dosing, probably due to development of cross-reactive neutralising anti-FVIII antibodies against endogenous FVIII. The formation of binding and neutralising antibodies was also reflected in a statistically significant decrease in exposure during toxicokinetic analysis at study end. Antibody formation is an expected immune reaction after repeated application of heterologous human proteins to animals, and has been observed with non-PEGylated FVIII products.

BAX 855 and the licensed rFVIII induced similar low levels of cytokine release and complement activation *in vitro* that were not different from the buffer control group. BAX 855 and the licensed rFVIII induced similar levels and incidences of antibodies against human FVIII in all animal models. Importantly, immune tolerance to human FVIII was maintained by both BAX 855 and the licensed rFVIII in hemophilic mice that are immune tolerant to human FVIII.

In conclusion, BAX 855 was well tolerated in rabbits and macaques at all dose levels; no thrombogenic events and no adverse clinical, respiratory or cardiovascular effects occurred. In addition, no adverse systemic effects were observed in either species after intravenous administration of BAX 855 for 1 month. Thus, 700 U/kg was considered the no observed adverse effect level (NOAEL) in these studies. Results obtained in comparative immunogenicity studies demonstrate that BAX 855 and the licensed rFVIII have a similar immunogenicity profile. The favorable preclinical safety profile of BAX 855 provides the basis for proceeding with human trials.

PB 3.55-5

Comparing projected prophylactic consumption and effects of recombinant factor VIII Fc Fusion (rFVIIIFc) and shorter half-life FVIII products in haemophilia

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Background: The mainstay of treatment for individuals with severe haemophilia A is replacement therapy with FVIII. While the treatment of choice is (primary) prophylaxis, the limitation is that it requires people to inject a considerable amount of FVIII. FVIIIs with a longer half-life, such as rFVIIIFc, are currently being studied. Their proposed advantage is less clotting factor utilization and less frequent injections required to receive similar haemostatic effects compared with traditional shorter half-life factor FVIII products. Alternatively, higher trough clotting factor levels could be achieved using identical amounts of rFVIIIFc.

Aims: To develop a decision-analytic model to compare projected prophylactic FVIII consumption and effects (in terms of breakthrough bleeding episodes) of longer-lasting products, such as rFVIIIFc and short-acting FVIII products.

Methods: Factor VIII consumption and bleeding frequency were estimated by adapting PK models of short-acting FVIII developed by Collins et al (2009 and 2010). The first study demonstrated the relationship between clotting factor dose, frequency of infusion, FVIII half-life, and the time individuals are predicted to be below a 1 IU/dL (<1%) FVIII level during the course of a week. The second study estimated the relationship between time under this trough level and bleeding frequency. The developed decision-analytic model links the results from these two studies and also predicts breakthrough bleeding and factor consumption adjusted accordingly for assumptions related to real-world adherence. Half-life data was based on information from the A-LONG phase three clinical trial data (19 h for rFVIIIFc compared with 12.4 for short-acting FVIII). In the base case analysis, results were projected over 1 year for a 30 year old, assuming that adherence was 70% of scheduled prophylactic doses but that missed doses still represented an incurred cost. It was also assumed that individuals receiving short-acting FVIII were infused three times per week with 37.5 IU/kg whereas individuals receiving rFVIIIFc were infused with 37.5 IU/kg twice a week.

Results: The model-generated base case results over 1 year suggest that rFVIIIFc resulted in a similar number of bleeds per year compared with short-acting FVIII (2.03 vs. 2.08 bleeds, respectively) but required 0.16 million fewer IU's of clotting factor (0.31 vs. 0.47 million IU's) compared with a shorter half-life rFVIII. The difference in clotting factor use was considerably higher when a longer time horizon was used: approximately 6.1 million fewer IUs of longer-acting factor were predicted to be used over a 40-year time horizon.

Summary/Conclusions: This decision-analytic model demonstrates the use of a FVIII with a longer half-life such as rFVIIIFc has the potential to reduce the number of injections individuals require and overall factor consumption, without compromising haemostatic efficacy. Indeed, these results suggest that rFVIIIFc, could reduce time under <1 IU/dL and therefore reduce bleeding if similar or improved levels of adherence can be achieved.

PB 3.55-6

PEGylated biopharmaceuticals and safety evaluation of polyethylene glycol (PEG) with focus on PEG-rFVIII

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Biopharmaceuticals are an emerging branch of therapeutic agents. The applicability of such drugs, however, may be impaired by their short half-life, rapid elimination and ability to induce a specific immune response. These disadvantages may be overcome by chemical modification with polyethylene glycol (PEG), which has been shown to enhance the PK and safety profile of several marketed proteins over the last decades.

Baxter and Nektar are currently developing BAX 855, a PEGylated full-length rFVIII based on the FVIII molecule used for Baxter's licensed rFVIII. PEGylation of BAX 855 uses a metabolically stable PEG polymer of 20 kDa and was optimized to retain functionality of the FVIII molecule and improve its pharmacokinetic properties. The protein portion of the PEG- FVIII conjugate is degraded by proteolysis over time, leaving a PEG portion which is rapidly eliminated. An ADME study in rats with radiolabeled BAX 855 showed that the PEG is quantitatively cleared mainly via the kidneys and excreted into urine or via the liver and excreted into feces even at high doses of 2088 and 4176 U/kg. Like other non-degradable entities, physiological clearance mechanisms of PEG may include liver macrophage uptake. Clearance by macrophages in mammals has been reported to cause vacuolization at high cumulative doses of PEG. Generally, vacuoles were shown to consistently resolve over time, with no cellular damage, inflammation at the vacuolation site or functional deficits of affected tissues, and are therefore regarded as non-adverse. Due to the high potency of FVIII, the absolute amount of conjugated PEG applied with PEG-FVIII is within the range of μg per kg body weight and week, which is considerable below the observed threshold for vacuolation in animal studies. As expected for such low PEG doses, no PEG related vacuolations occurred in the preclinical repeated dose toxicity studies conducted where rats received an intravenous dose of 350 or 700 U/kg BAX 855 every other day, and macaques were administered 150, 350 or 700 U/kg every 5 days both within approximately a month. The No Observed Adverse Effect Level (NOAEL) was 700 U/kg for both species.

In summary, no adverse effects were observed in the preclinical studies with BAX 855 and the PEG load was well below the threshold for vacuolation. This favorable safety profile provides the basis for proceeding with human trials.

PB3.56 – Tissue factor – III

PB 3.56-1

The retention and release of tissue factor by endothelial cells results in the differential activation of p38-MAPK and influences the fate of the cells

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Background: We have previously shown that the release of tissue factor (TF) into cell-derived microparticles is mediated by the phosphorylation of serine-253. Moreover, activation of protease-activated receptor 2 (PAR2) in endothelial cells results in the prolonged activation of p38-MAPK in cells expressing TF but only a short-term activation in the absence of TF. The prolonged activation of p38-MAPK is often associated with cellular responses including apoptosis.

Aims: In this study we have explored the mechanisms involved in the activation of p38-MAPK in the presence and absence of TF, and examined the outcome on the mediators of cell apoptosis.

Methods: Endothelial cells were transfected to overexpress wild-type TF and activated by incubation with PAR2-agonist peptide or factor Xa. Furthermore, to mutant forms of TF with alanine (TFAla253) or aspartate (TFAsp253) substitutions at serine-253 were used to suppress or promote the release of TF and treated similarly. The activation of p38-MAPK, Rac1 and Src1 was monitored over 180 min by western blot analysis. In addition, the outcome on the mediators of cellular proliferation and apoptosis (cyclin D, p53 and bax) was examined over 24 h by RT-PCR and western blot analysis. Finally, the outcome on cell proliferation and apoptosis was recorded by cell count and TUNEL assay respectively.

Results: In agreement with our previous work, the activation of PAR2 resulted in two separate peaks of p38-MAPK phosphorylation at 40 and 120 min, in cells overexpressing wild-type TF and was augmented in cells overexpressing the TFAla253-mutant form. In contrast, only the initial peak was present in cells without TF, or those expressing TFAsp253. Rac1 phosphorylation was observed at 40 min in cells expressing wild-type TF and the untransfected cells and at 40 and 120 min in cells expressing TFAla253 mutant. In contrast, Src1 phosphorylation was only observed at 120 min post-activation of PAR2 in the cells expressing the wild-type TF and was significantly enhanced in the cells expressing TFAla253. In all cell samples, the level of cyclin D rose at 8 h following the activation of PAR2 and was higher in cells expressing TFAsp253 and wild-type TF than the untransfected cells. Moreover, the levels of cellular p53 and bax were higher in cells expressing TFAla253 than those in the wild-type TF, which were in turn higher than those in TFAsp253 or the untransfected cells. Finally, an increase in the rate of cell apoptosis was observed at 24 h, in the cells expressing TFAla253.

Conclusions: Our data suggest that the retention of TF following the activation of endothelial cells can induce TF-mediated signalling mechanisms that prolong p38-MAPK activation, culminating in the up-regulation of pro-apoptotic mediators and leading to cell death. Endothelial cells do not normally express TF but may do so in response to pro-inflammatory cytokines. In addition, endothelial cells accumulate TF released as microparticles, from the circulation in particular, during disease conditions. Therefore overwhelming amounts of cellular TF, or the disruption of the release of TF upon activation, may result in cellular apoptosis which can lead to endothelial dysfunction in disease conditions.

PB 3.56-2

Human neutrophils express tissue factor in peritonitis

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Background: The expression of Tissue factor (TF) by human polymorphonuclear neutrophils (PMN) remains controversial. In animal models, PMN recruitment and TF-expressed PMNs have been shown to initiate thrombus formation at the site of injured endothelial wall. Direct evidence of a TF expression by human PMNs is limited. Septic peritonitis is a good model to study chemotaxis, migration and activation of PMNs.
Aims: To study TF expression by human PMNs obtained from patients presenting with peritonitis. To develop an *in vitro* model to mimic inflammation in human PMNs and examine TF expression.
Methods: PMNs were obtained from patients with peritonitis secondary to peritoneal dialysis. PMNs were isolated by immunomagnetic sorting. TF expression was examined by RT-PCR, Western Blot, immunoprecipitation. TF was visualized by indirect immunofluorescence. TF activity was measured by a Xa generation assay and calibrated automated thrombinography. For the *in vitro* study, PMNs from healthy volunteers were purified by density gradient and stimulated by TNF-α and IL-8. The influence of adhesion was studied by the effect of a potent inhibitor of CD11/CD18 (MAb IB4, Calbiochem). We investigated the role of MAPK kinase (MEK) signaling pathway using U0126 (Sigma).
Results: PMNs obtained *ex vivo* from peritonitis strongly express TF (mRNA and protein). Functional assays (Xa generation, thrombinography) indicate that TF is strongly procoagulant. *In vitro*, PMNs expressed TF mRNA in response to TNF. This TF mRNA expression in response to TNF was increased when PMNs were primed with IL-8. On the contrary IL-8 alone weakly induced TF mRNA expression. In contrast, IL-8 alone induced a strong TF protein expression indicating that this chemokine up regulates TF at the translation level. TNF-α induced TF mRNA expression was inhibited by U0126 indicating the involvement of MAPK kinase pathway. When PMN adhesion was inhibited by MAb IB4, we observed a decrease of TNF-α TF expression.
Summary/Conclusions: PMNs from peritonitis allow to study TF expression in inflammatory conditions. Ours results brings direct evidence of a strong TF expression by PMNs in a septic extravascular situation. It iindicates a *de novo* TF synthesis by PMNs. The *in vitro* study suggests that TF expression by PMNs is the result of chemotaxis, adhesion and inflammatory condition.

PB 3.56-3

Plasma levels of bone morphogenetic proteins and circulating monocyte tissue factor in individuals with echolucent and echogenic carotid atherosclerosis

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Background: Tissue factor (TF) potently activates blood clotting via interaction with factor VII (FVII) and FX. Circulating monocytes is a major source of blood borne TF and are sensitive to changes in pro- and anti-inflammatory milieu in blood and vasculature. Lipid-rich and calcified atherosclerotic plaques present as echolucent and echogenic ultrasonic lesions, respectively, bare different risk for thrombus formation on their surface. Bone morphogenetic proteins (BMP)-2 and -7 are potent regulators of vascular calcification and could also modulate TF expression in monocytes. So far no studies have investigated the relationship between plasma levels of BMPs and TF expression on circulating monocytes.
Aims: To assess plasma levels of BMP-2 and BMP-7 and expression of TF on circulating monocyte in individuals with echogenic and echolucent carotid atherosclerosis.
Methods: The study has been approved by the institutional committee on the research ethics. We enrolled subjects with echolucent ($n = 20$) or echogenic ($n = 20$) carotid plaques, as well as controls without carotid atherosclerosis ($n = 20$), judged by ultrasonographic examination. Levels of TF and mRNA expression as well as plasma levels of BMP-2 and BMP-7 were measured using flow cytometry, qPCR, and ELISA (R&D Systems, USA), respectively. Effects of BMPs on TF expression in monocytes were tested *in vitro*. Regression analysis and *t*-test has been chosen to test relationships between variables and differences in mean group values. Two-sided $P < 0.05$ was considered significant.
Results: Plasma from individuals with echolucent carotid atherosclerosis contained significantly higher concentrations of BMP-7 than plasma from individuals with echogenic atherosclerotic plaques ($P > 0.05$) and similar to BMP-7 levels in plasma from healthy individuals. Levels of BMP-2 did not statistically differ between the groups. Relative to echogenic group, higher concentrations of BMP-7 in individuals with echolucent atherosclerosis coincided with stronger TF presentation and higher TF mRNA expression in monocytes. *In vitro*, BMP-7 increased the expression TF mRNA and protein at low μmolar concentrations, whereas BMP-2 had no effect on TF expression in resting monocytes.
Conclusions: Plasma levels of BMP-7 are higher in individuals with echolucent atherosclerotic plaques than in individuals with echogenic lesions. This could lead to increased prothrombotic properties of blood of individuals with soft lipid-rich plaques through BMP-7-mediated stimulation of TF synthesis and presentation in circulating monocytes.

PB 3.56-4

Tissue Factor is expressed by osteosarcoma and regulates IL-8 expression

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Background: Osteosarcoma (OS) is a primary bone tumor mostly affecting adolescents. In spite of aggressive treatment, 30–40% of OS patients develop metastatic disease within 5 years and die. Expression of Tissue Factor (TF), the primary initiator of the coagulation cascade, has been observed in various types of cancer, and expression associates with poor clinical outcome. Apart from its role in coagulation, TF/FVIIa-dependent signaling via PAR2 promotes angiogenesis and tumor progression in various cancer types, through enhanced expression of genes such as IL-8 and CXCL-1. Whether TF is expressed in OS and influences OS cell behaviour has never been documented.
Aims: To investigate whether TF is detected in OS specimens, associates with tumor progression and contributes to OS cellular signaling.
Methods: TF protein expression in 19 high-grade conventional OS biopsies and 14 lung metastases was determined by standard IHC using a specific TF antibody. Scoring was performed semi-quantitatively according to staining intensity on a scale as follows: 0 = total negative slide, 1 = weak, 2 = moderate, and 3 = strong intensity. Semi-quantitative TF mRNA expression in the SAOS-2, U2OS, MNNG-HOS and 143B cell lines was determined by PCR using specific TF primers. TF was downregulated in 143B using an shRNA approach. TF, IL-8 and CXCL-1 levels were determined using qPCR.
Results: All OS biopsies and metastases showed immunoreactivity for TF, but intensity varied between specimens. TF-positivity was significantly increased in metastases compared to primary tumors (Chi-square: $P = 0.019$). The OS cell lines SAOS-2, U2OS, MNNG-HOS and 143B were shown to express TF by semi-quantitative PCR. 143B cells were selected because of its pro-angiogenic and metastatic behaviour in murine xenograft models and 143B cells transduced with TF-specific shRNA showed a 0.6 fold decrease in TF levels by qPCR

($P = 0.02$). IL-8 mRNA expression in TF shRNA transduced cells was reduced by 0.48 fold ($P = 0.013$), while CXCL-1 levels were not significantly reduced.

Summary/Conclusion: Here we show that flTF is widely expressed in clinical OS specimens as well as established OS cells lines. We conclude that targeting TF might be a viable option to inhibit osteosarcoma progression in patients, potentially by inhibiting angiogenesis.

PB 3.56-5

Detection of tissue factor bearing microparticles and the study of the clinical significance in the haemostatic dysfunction of acute myeloid leukemia

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Background: Tissue factor bearing microparticles(MP-TF)has recently been shown to play a critical role in the cancer-associated thrombus formation. The latest research has found that its role can not be ignored in hematologic malignancies. Acute myeloid leukemia (AML) especially acute promyelocytic leukemia(APL) often exists hemostatic dysfunction such as disseminated intravascular coagulation (DIC) and threaten the lives of patients seriously. The study of the MP-TF in the haemostatic dysfunction of AML is very limited.

Aims: To analyze the clinical significance of the MP-TF in the haemostatic dysfunctionin of AML patients.

Methods: MP-TF and TF were detected by FCM (antibody: TF-PE and Annexin-V-FITC) and ELISA respsctively in 64 newly diagnosed AML patients including 22 cases of APL.

Results: MP-TF and TF of AML and APL patients are both higher than those in the healthy adults($P < 0.05$), MP-TF especially presents high expression($P < 0.001$). The MP-TF in the patients whose prothrombin time (PT) is prolonged more than 3s is higher than those with normal PT value($P < 0.05$). MP-TF increases along with prolonged PT, TF is not different between the two groups. The MP-TF of APL patients and TF in AML patients with DIC before chemotherapy are both higher than after chemotherapy($P < 0.05$, $P < 0.001$). The level of TF in the patients with abnormal FDP value is increased ($P < 0.05$), but there is no relation between MP-TF and FDP. There was no statistically significant difference of MP-TF and TF between the AML and APL($P > 0.05$), AML with DIC and without DIC ($P > 0.05$)

Conclusions: MP-TF and TF associated with the extrinsic coagulation pathway participate in the haemostatic dysfunction in the AML.

PB 3.56.6

Oral anti-factor Xa and factor IIa agent mediated inhibition of tissue-factor mediated generation of thrombin in prothrombin complex concentrates

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Introduction: Several oral anti-factor IIa and factor Xa agents have recently been developed. These include the thrombin inhibitors Ximelagatran/Melagatran (M) and Dabigatran Etexilate/Dabigatran (D), which require endogenous conversion to the active agents D and M. The factor Xa inhibitors, Rivaroxaban (R) and Apixaban (A), are anti-Xa agents that do not require any endogenous activation. Ximelagatran was withdrawn from the market due to adverse reactions. Dabigatran, Rivaroxaban, and Apixaban are approved for various clinical indications. Antagonism of the anticoagulant effect may be required in bleeding complications. Contradictory results were reported for the efficacy of various prothrombin complex concentrates (PCCs) with these new oral anticoagulants (NOACs). The purpose of this study was to determine the differences in the thrombin generation inhibitory profiles of the newer oral anticoagulant agents.

Methods: Commercially available PCCs namely Octaplex and Beriplex, were used as a source of Factors II, VII, IX and X. To investigate the effect of each of these agents, a working solution of 1 U/mL of both PCCs were supplemented in a graded concentration of 0–1250 ng/mL with M, D, R and A. Thrombin generation studies were carried out using a thromboplastin activator (RC High, Technoclone Vienna, Austria). Total thrombin generated was measured in terms of nM's. The IC-50 for each agent was calculated individually. The time course of thrombin generation was also measured following the kinetic profiles and AUC.

Results: Dabigatran and Melagatran produced relatively weaker inhibition of thrombin generation with the IC-50 values ranging from 410 to 110 ng/mL in Beriplex and 350–1120 ng/mL in Octaplex. Both Rivaroxaban and Apixaban produced strong inhibition of thrombin generation, with the IC-50 ranging from 58 to 62 ng/mL in Octaplex; whereas, in Beriplex these values ranged from 48 to 50 ng/mL. The onset time for thrombin generation and total thrombin formation was concentration dependent. The kinetics of thrombin generation with A and R were distinct from D and M. At concentrations below 310 ng/mL the total amount of thrombin generated was comparable to the control; however, its formation was delayed. In both systems, D exhibited the weakest thrombin generation inhibitory potential. While the onset time of thrombin generation was delayed at concentrations below 310 ng/mL the levels were comparable to or higher than the control.

Discussion: This data suggests that PCC's such as Octaplex and Beriplex can be used to generate thrombin and it's inhibition by new oral anticoagulant drugs. Octaplex generates much higher amount of thrombin than Beriplex at equivalent units. These results also show that in comparison to the oral anti-Xa agents, the oral anti-IIa agents are relatively weaker inhibitors of thrombin generation. These studies also suggest that the differential inhibition of the generation of thrombin through tissue factor by the anti-Xa and IIa agents may contribute to the potential neutralization profile of PCC's for these drugs.

PB3.57 – Fibrinogen/Fibrin – IV

PB 3.57-1

Fibrinogen Geisinger?W335C: impaired self-assembly, diminished clot stiffness, accelerated clot lysis, and association with thrombophilia

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The poorly understood association of thrombosis with many congenital dysfibrinogens led to the present investigations. A 58 year old woman experienced numerous venous thromboembolic events since age 20 years, as did her father since age 60. A nephew incurred venous sinus thrombosis at age 15. Except for past use of contraceptives and one episode occurring during her only full term pregnancy, exhaustive hypercoagulability testing disclosed no other risks. Fibrinogen levels were 111 mg/dL and 123 mg/dL by functional and antigen assays, respectively. Similarly decreased by both assays were corresponding levels of her father and nephew. PCR and DNA sequencing disclosed a heterozygous missense mutation encoding for fibrinogen γW335C. Her isolated fibrinogen was >96% coagulable and its thrombin time (1 mg/mL, 2 mM $CaCl_2$, pH 7.4) was 28.1 s, control 19.2 s ($n = 2$). Unreduced and reduced SDS-PAGE bands, including those of clots cross-linked by Factor XIIIa, were identical to those of normal controls. Turbidity maxima (350 nm) of single batroxobin- and throm-

bin-induced clots were 41% and 34% of respective controls, and its thrombin-induced polymerization rate (absorbance units/minute $\times 10^{-3}$) was 33, control 108. By thromboelastography (TEG), duplicate clots formed in the presence of gel-sieved platelets (190×10^3/μL, 4 mM $CaCl_2$, pH 7.4, 15% afibrinogenemic plasma, fibrinogen 2 μM, thrombin 0.5 U/mL) yielded maximum signal amplitude (reflecting shear modulus and clot stiffness) 13% and 15% of controls. Thrombin (0.2 U/mL) induced duplicate clots of 3 μM fibrinogen, containing 125 nM recombinant tissue plasminogen activator (rtPA) and 15% afibrinogenemic plasma, yielded lysis times of 91 and 93 min, controls >300 min. In chromogenic measurements, the fibrin (50 μM) enhanced glu-plasminogen activation rate by rtPA (absorbance, 405 nm, increments in units $\times 10^{-3}$/min) was similar to that of controls, $n = 3$. Scanning electron microscopy revealed that 19% of fibrin fibers displayed one or more unevenly spaced bead-like dense bulges, an occasional fiber end, and mean fiber width 81% ($n = 72$) that of controls. The known proximity of γ335 to the 'a'pocket (γ337–379) led to examination of fibrinogen networks, known to consist of fibers whose periodicity and stiffness are virtually identical to those of fibrin counterparts (Koo et al, J Thromb Haemost, 2010; 8: 2727). Fibrinogen, depleted of its soluble fibrin, was incubated (4 mg/mL, 50 U/mL aprotinin, 20 U/mL hirudin, 0.5 mM EDTA, pH 7.4, 4 h) on polystyrene-coated (1 × 1 cm, 100 μm thick) silica plates. Light microscopy disclosed randomly distributed insoluble patches, which by atomic force microscopy (topographic scanning) consisted of three-dimensional fiber networks (≤1 μm height). Fibers were typically fine (diameter 100 ± 40 nm, control 154 ± 27 nm, $n = 7$), short, and branched. Most fibers displayed multiple bead-like bulges, and many were laterally coalesced into loosely packed coarser fibers (diameter 405 ± 100 nm, control 349 ± 63 nm, $n = 7$). Control network fibers were long, smooth, with occasional overlaps and branching. Abnormal fibrinogen and fibrin networks suggest impaired polymerization contact(s) at site(s) other than that enabling 'A knob': 'a'pocket interaction. Accelerated clot lysis is attributable to decreased plasmin resistance, and its co-presence with hypofibrinogenemia suggests a possible decrease of thrombin sequestration by fibrin, enabling pathologic thrombus extension.

PB 3.57-2

The spectrum of mutations associated with hereditary fibrinogen disorders in the UK

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Hereditary fibrinogen disorders may be quantitative (afibrinogenemia/hypofibrinogenemia) and/or qualitative (dysfibrinogenemia). The clinical significance of fibrinogen disorders are variable and difficult to predict, particularly with dysfibrinogenemia, which although frequently asymptomatic, may also be associated with an increased risk of bleeding and/or thrombosis

The aims of this study were to investigate the spectrum of mutations associated with fibrinogen disorders in the UK and evaluate the role of genetic analysis in assisting clinical decisions.

Inherited fibrinogen disorders are associated with a range of different mutations found throughout the three genes that encode fibrinogen (FGA, FGB and FGG). Genetic analysis was carried out by nucleotide sequencing of the coding regions of these genes. 161 patients from 120 families originating from throughout theUKwere studied, and this approach identified a causative mutation in 154 patients.

123 patients (84 families) had dysfibrinogenemia as defined by discrepant fibrinogen antigen and Clauss activity. All cases were found to be associated with heterozygous missense mutations.

74 patients (61%) from 56 families were heterozygous for FGA c.104G>A (Aα p.Arg35His). Patients with this variant consistently had a clear antigen:activity discrepancy (mean FibAg 3.14 g/L, Fib Clauss 0.6 g/L). This variant was associated with a small increased risk of bleeding but no increased risk in thrombotic or pregnancy complications. Other recurrent mutations included FGG c.901C>T (γ p.Arg301Cys) which was seen in 17 patients (14%) from 13 families. Eleven other missense mutations were detected including three novel variants; Bβ p.Val169Asp and Aα Pro222Thr, in association with hypodysfibrinogenemia and Bβ p.Ile235Thr in association with dysfibrinogenemia.

Genetic variants were detected in 35 patients with quantitative disorders (27 families). Most cases were heterozygous, while severe deficiency was associated with homozygosity/compound heterozygosity. The nonsense mutation Bβ Arg47* was detected in 10 patients (28%) from six families. Seventeen other genetic variants were detected, none recurrently. These included four splice site variants, four small insertion or deletions, one nonsense mutation and eight missense changes. Novel variants include heterozygosity for Aα p.Ser294 fs, FGG c.124-1G>C, γ p.Lys222Asn and γ p.Ala353Asp in association with hypofibrinogenemia, and homozygosity for splicing variant Bβ Gly320Ser in a patient with afibrinogenemia.

This study has given insight into the spectrum of mutations associated with fibrinogen disorders in theUK, which differs from previously reported incidences, and targeted analysis for recurrent variants may offer a more cost-effective strategy for genetic testing.

Although it is important to consider additional genetic and environmental factors, the increasing availability and critical analysis of clinical data in relation to recurrent mutations – particularly Aα p.Arg35His, may support more confident predictions of bleeding/thrombotic risk in dysfibrinogenemia. This study has also allowed the identification and characterisation of novel variants which may further our understanding of fibrinogen structure and function.

PB 3.57-3

Progression in D-Dimer and biomarkers of fibrin formation correlate with outcome in sepsis

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Background: Sepsis is associated with an increase in intravascular fibrin formation and its detection is though fibrin-related biomarkers (FRM). Although D-Dimer (DDI) is the most commonly used, fibrin degradation products (FDP) and fibrin monomer (FM) may also have clinical utility in meeting the unmet challenges of early diagnosis and prognosis of sepsis.

Aim: To examine the hypothesis that increased FRM levels are useful in the diagnosis and prognosis of sepsis.

Methods: FDP, DDI & FM assays were measured on 130 plasma samples from patients on the intensive care unit (ICU) with and without sepsis and healthy individuals. Levels were correlated with each other (Pearson correlation coefficient) and also with fibrinogen (FIB), prothrombin time (PT) and platelets (PLT) as global indicators of clot activation as well as antithrombin (AT). Clinical correlation was also determined for outcome (survival/death) from FRM levels on admission and from serial time-dependent changes (ANOVA).

Results: There was strong correlation between all three FRM ($r = 0.38$–0.93, $P < 0.0001$) but only FM correlated significantly with PT, FIB, PLT and AT. However, FDP & DDI levels could significantly discriminate between patients with sepsis, non-sepsis & healthy individuals [Mean FDP: 28.74, 7.27, 1.42 (μg/mL) respectively] [Mean DDI: 7.17, 2.23, 0.29 (μg/mL) respectively] ($P < 0.05$). A single admission level did not predict outcome in all three FRM but longitudinal time-dependent analyses showed serial trends in FDP & DDI levels correlating to outcome at day 28. Statistical significance was only achieved by serial FDP levels [Mean FDP (μg/mL) from 35.36 (first day) to 21.37 (last day), $P < 0.005$].

Conclusion: For early identification of sepsis via identification of coagulation activation, FM is the most clinically useful. For monitoring the patient with sepsis, FDP and DDI levels can identify sepsis on ICU admission with FDP preferable for monitoring due to its statistically significant time-dependent changes in predicting outcome.

PB 3.57-4

Impairment of fibrin properties resulting from coagulopathy at haemodilution and its reverse by a fibrinogen concentrate Haemocomplettan® (Riastap®)

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Background: Resuscitation of intravascular volume with crystalloid infusion post trauma leads to plasma dilution and a coagulopathy. In haemodilution, fibrinogen is the first factor to become critically low, giving rise to increase of bleeding risk. Although the plasma levels of other coagulants decline in parallel, usually no decrease of thrombin generation (TG) is found in such patients. This may be explained by greater response of TG for dilution of coagulant inhibitors than for dilution of coagulants (De Smedt, 2009).

Aim: To assess whether at haemodilution, i) the decrease in amounts of formed fibrin is more profound than that in TG, ii) the decrease of fibrin quantity is more important than of the network porosity for down-regulating clot stability against fibrinolysis, a natural sequel to haemostasis, and ii) Haemocomplettan® (a fibrinogen concentrate) can restore the impairment of proteolytic resistance of fibrin.

Methods: *In vitro*, a haemodilution model was examined by determining Calibrated Automated Thrombogram, quantity of clotted fibrinogen i.e., fibrin (He, 2013), fibrin network permeability, images of Scanning Electron Microscope (SEM) and Rotational Thrombelastogram (ROTEM). This model was created with a normal plasma pool (NPP) diluted from 100 to 75, 50 and 25%, and then spiked with Haemocomplettan®. Coagulation and fibrinolysis were triggered by tissue factor and t-PA respectively.

Results: At haemodilution, the global marker of TG i.e., Endogenous Thrombin Potential was not changed due to delay of thrombin inactivation. In parallel with dilution, less fibrin was generated and a network with lower permeability (Ks) was formed. Haemocomplettan® added to NPP25% made the fibrin amounts rise but the Ks further decline. According to SEM images, the addition of Haemocomplettan® giving total fibrinogen 2 g/L tightened the network significantly. Noteworthy, an additional network was formed between the major fibrin fibres, comprising thinner fibres and smaller pores than those in the major network. Haemocomplettan® at a higher concentration (total fibrinogen 4 g/L) further modified the appearance of clot: the additional network still existed but the major fibres tended to lateral association forming thick 'fibrin fibre bundles'. In ROTEM assays, haemodilution did reduce the maximum visco-elasticity i.e., Maximum Clot Firmness (MCF) and did shorten the fibrinolysis rate i.e., Clot Lysis time (CLT). When the total fibrinogen rose to 2 g/L after addition of Haemocomplettan®, normalized levels were found in these ROTEM parameters. Greater concentrations of Haemocomplettan® added (total fibrinogen 4–6 g/L) brought parallel enhancements in MCF, but no further prolongation of CLT was induced by the enhancement of fibrin amounts.

Summary: Due to the unequal effect of haemodilution on TG and fibrin formation, clots derived from diluted plasma with insufficiency of fibrinogen are less stable to plasmic digestion, notwithstanding the tightened network structure. This study *in vitro* suggested that normalization of clot strength against fibrinolysis which may have a positive influence on haemostasis needs fibrinogen concentrations no less 2 g/L (after administration of Haemocomplettan®); there may be no more advantage at using higher doses of this concentrate, since 'fibrin fibre bundles' probably formed as seen *in vitro* favor fibrinolysis by exposing more binding-sites to plasminogen (Veklich, 1998)

PB 3.57-5

Global development plan for a double virus inactivated fibrinogen concentrate for the treatment of congenital fibrinogen deficiency

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Introduction: Congenital afibrinogenaemia and hypofibrinogenaemia are rare inherited disorders occurring in homozygotic patients with an estimated incidence of 1 in 10^6. Patients present with frequent severe bleeding episodes since birth or early childhood. Bleeding may occur after a minor trauma or a small surgical intervention, into the skin, mucosa, muscles, gastrointestinal tract, or the brain. Therapeutic substitution with human fibrinogen concentrate can correct the haemostatic defect and arrest the bleeding in patients with these fibrinogen deficiencies. Octafibrin is a highly purified, lyophilized, human plasma fibrinogen concentrate, without added albumin. Octafibrin is double virus inactivated using two dedicated virus inactivation/removal steps, solvent/detergent treatment, and nanofiltration.

Aim: This poster presents a plan for the global development of Octafibrin, taking into account discussions with European Regulators and the FDA. This plan also involves discussions with the European pediatric committee (PDCO) which oversees the inclusion of pediatric subjects into drug development under the new EMA guidelines.

Methods: The development plan calls for a prospective, randomized, open label, multinational, pivotal PK comparison of Octafibrin to an existing marketed product in 18 adult and adolescent patients, including comparison of a surrogate efficacy endpoint measured by TEG. In a second study the efficacy and safety of the product in bleeding and invasive procedures will be assessed in 24 adult and adolescent patients. Finally a pediatric PK, efficacy and safety study in patients below 6 years will be performed but because of the rarity of these patients this study will not need to be completed before review and approval by the regulatory agencies. Clinical studies are planned to be started end of 2012.

Results: A double virus inactivated, plasma derived fibrinogen concentrate (Octafibrin) will be globally developed in an ultra rare congenital disease after harmonized discussions with EU and US regulators. Pivotal comparative PK data and interim efficacy and safety data will be available at time of regulatory submissions while the finalization of the pediatric study is deferred.

PB 3.57-6

Acquired hypofibrinogenemia assessed by whole blood thromboelastometry profiles in children with acute lymphoblastic leukemia treated with l-asparaginase

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Background: Treatment with L-Asparaginase (L-Asp) or PEG-L-Asparaginase (PEG-L-Asp) in patients with acute lymphoblastic leukemia (ALL) is associated with coagulation disturbances, being deep venous thrombosis the most common clinical consequence. Coagulation alterations most frequently reported were hypofibrinogenemia, low plasminogen and antithrombin (AT) plasma levels, protein C and S deficiencies. Whole blood rotation thromboelastometry (ROTEM®),

using FIBTEM test, has been reported to be a good tool to identify hypofibrinogenemia.
Aim: To evaluate whole blood rotation thromboelastometry profiles in children with ALL treated with PEG-L-Asp in order to identify strategies for monitoring and treating complications in LLA children.
Methods: Children treated with PEG-L-Asp for ALL consecutively referred to the Pediatric Hemato-Oncology Clinic of the Padua University Hospital, from June to December 2012, were enrolled. After informed consent, samples were obtained at predefined time points during the induction and reinduction phases of chemotherapy. Maximum Clot Firmness (MCF, normal value 9–25 mm), i.e. the maximum amplitude in millimeters reached in FIBTEM thromboelastogram, which is used to assess the specific role of fibrinogen in whole blood clot formation following inhibition of the platelets by Cytocalasin D, was performed. Moreover, AT and fibrinogen plasma levels were measured on the same samples.
Results: One hundred seventy two sets of thromboelastometry measurements were collected from 26 ALL patients (age range 20 months–17 years; M/F 13/13). Seventy-five MCF measurements (40%) resulted below the lower limit of MCF normal value. A significant linear correlation was found between MCF values in FIBTEM and fibrinogen plasma levels measured with Clauss method ($P < 0.001$). No influence of reduced AT plasma levels on thromboelastometry determination was observed. Replacement with i.v. fibrinogen concentrates was suggested for MCF values <2 mm. No hemorrhagic complication was reported in our cohort. One 6-year old boy presented cerebral venous thrombosis not related to supportive (i.e. fibrinogen, plasma and/or platelets) therapy.
Conclusions: The MCF value in FIBTEM may be used as an alternative test to determine the clotting efficacy of fibrinogen respect to the Clauss assay. The determination of fibrinogen function by means of ROTEM® can be considered a complementary test to the conventional clotting profile used for the management of asparaginase-induced coagulopathy in children with ALL. Larger studies are needed to evaluate the role of thromboelastometry to guide fibrinogen replacement therapy in acquired hypofibrinogenemia due to asparaginase treatment.

PB3.58 – Other coagulation factors – III

PB 3.58-1

Platelet uptake of recombinant factor VIIa takes place *in vivo* but does not prolong its circulating half-life

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Background: The half-life of Factor VIIa (FVIIa) in the circulation in humans is around 2–3 h. However, a once-daily administration of recombinant Factor VIIa (rFVIIa) has been reported to prevent bleeding in patients with hemophilia A, suggesting that the product has a longer therapeutic effect than suggested by its half-life. Previously, we and others have shown that platelets internalize rFVIIa *in vitro*.
Aims: The aim of this study was to investigate if uptake of rFVIIa by platelets also takes place *in vivo* and to evaluate whether this process influences the half-life in the circulation.
Methods: We administered a single bolus dose of 90 μg/kg rFVIIa to six pigs, and collected blood samples before and 12 time-points after administration. The concentration of FVIIa within platelets was assessed in lysates of isolated, washed platelet preparations. We determined VIIa concentrations in these lysates by a functional assay in which we determined the prothrombin time (PT) in Factor VII deficient plasma to which lysates were added. In addition, we assessed FVIIa antigen levels in platelet lysates using a novel ELISA that recognizes activated but not zymogen factor VII.
Results: We detected FVIIa in platelet lysates using both the functional assay and the ELISA. The highest amount of FVIIa detected in platelet lysates was present in the sample taken 1 h after administration of rFVIIa. Platelet FVIIa levels remained elevated up to 12–16 h after infusion. The half-life of platelet FVIIa was similar to that of the half-life of FVIIa in plasma as shown by an unchanged ratio of FVIIa in plasma over FVIIa in platelets in samples taken 1 h or more after injection.
Conclusions: Here we have demonstrated that rFVIIa is taken up by platelets *in vivo* using a model of non-bleeding pigs. The half-life of rFVIIa in platelets is similar to the half-life in plasma, suggesting a continuous exchange of rFVIIa between platelets and plasma. Our hypothesis that platelet uptake protects rFVIIa against clearance is thus not supported by the present experiments.

PB 3.58-2

A fusion of thrombin-activatable FVII and soluble tissue factor displays improved activity and pharmacokinetic properties compared to activated FVII

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Background: Improvements in the pharmacokinetics of recombinant activated factor VII (rFVIIa) are limited by its susceptibility to inactivation by protease inhibitors such as antithrombin III (ATIII), αII-macroglobulin, and tissue factor pathway inhibitor. Furthermore, this susceptibility can be enhanced by mutations aimed at improving the enzymatic activity of rFVIIa. We have previously shown that a thrombin-activatable rFVIIFc variant is resistant to ATIII inhibition, resulting in enhanced pharmacokinetic properties relative to rFVIIa and rFVIIaFc. However, such variant showed poor activity in *in vitro* coagulation assays. We have generated a thrombin-activatable rFVII recombinantly fused to the soluble form of tissue factor (sTF, residues 1–219). We reasoned that such variant would remain in the zymogen form in circulation and resistant to protease inhibitors, but activated at the site of an injury by thrombin. Upon activation, the sTF protein would act as a cofactor to enhance the activity of thrombin-activatable rFVIIa significantly.
Aims: To develop a FVII variant with improved efficacy and pharmacokinetic properties relative to rFVIIa.
Methods: We have generated a thrombin-activatable rFVII variant as an Fc fusion heterodimer, where one Fc moiety is recombinantly fused to the C-terminus of thrombin-activatable rFVII, while the second Fc moiety is fused to the C-terminus of a soluble form of tissue factor. To assess potential unintended effects of the sTF protein on endogenous FVIIa, we generated a negative control heterodimer variant where an arginine essential for thrombin cleavage was mutated to an alanine. The clotting activity of rFVIIa variants was measured by the soluble tissue factor dependent prothrombin time assays (sTF-PT) and rotational thromboelastometry assays (ROTEM). The Factor Xa (FXa) generation activity was determined by monitoring FX activation using a chromogenic substrate specific for FXa. The pharmacokinetics of rFVII variants were determined in hemophilia B mice by ELISA, and ATIII inhibition was assessed by SDS PAGE electrophoresis, sTF-PT and ELISA of the rFVII-ATIII complex.
Results: Both the thrombin-activatable FVII and negative control heterodimers displayed no activity in sTF-PT assays and, consequently, were not susceptible to ATIII inhibition. Furthermore, the thrombin-activatable heterodimer showed enhanced FXa generation activity *in vitro* vs. equal molar levels of rFVIIa following activation by thrombin. When we evaluated activity by ROTEM using blood from hemophilia A donors, the thrombin activatable variant showed an enhancement of activity >10-fold relative to rFVIIa. In contrast, the activity of the negative control variant by ROTEM was negligible, demonstrating that the activity associated with the thrombin-activatable heterodimer is mediated by the FVIIa/sTF complex, not sTF alone. Furthermore, the thrombin-activatable heterodimer displayed

improved pharmacokinetics and prolonged efficacy in HemB mice compared to rFVIIa as determined by ELISA and ex vivo ROTEM.
Summary/Conclusions: The thrombin-activatable rFVII/sTF heterodimer shows high activity following thrombin activation and displays a longer half-life not impacted by ATIII, resulting in a prolonged efficacy relative to rFVIIa. Taken together, these results set the basis for a potential long-lasting recombinant bypass treatment option for hemophilia patients with inhibitors.

PB 3.58-3
Hemostatic effect of an anti-TFPI peptide in a murine model of hemophilic joint bleeding

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Background: Tissue factor (TF) is part of the extrinsic pathway of the coagulation system, triggering the coagulation cascade after tissue damage. Tissue factor pathway inhibitor (TFPI) inhibits factor X activation by TF/factor VIIa and thus down regulates coagulation. Anti-TFPI is hypothesized to amplify the effect of TF and therefore boost blood coagulation. Anti-TFPI could therefore be used as a therapeutic agent in hemophilia patients.
Attachment of polyethylene glycol (PEGylation) is known to prolong circulation time of therapeutic agents. A PEGylated, linear anti-TFPI peptide (JBTA8) was developed with improved pharmacokinetic (PK) characteristics. JBTA8 interacts with murine TFPI and is therefore suitable for testing in a murine model of hemophilia.
Aims: The goal of this study was to evaluate the effect of a PEGylated anti-TFPI peptide on puncture induced hemarthrosis in a murine model of hemophilia A and compare its effect with recombinant factor (F) VIII (Advate).
Methods: Mice deficient in FVIII activity (E16 FVIII B6;129S4-F8^{tm1kaz}/J) with severe hemophilia A were treated with JBTA8 (1 mg/kg) or various doses Advate (10, 50 and 100 IU/kg) by intravenous tail vein injection. At specified times after each product administration, the right knee joint capsule was punctured with a 30 gauge needle to induce a onetime hemorrhage. Animals were sacrificed 3 days following joint injury and assessed for the presence of bleeding using gross and histological methods. Total bleeding scores (TBS), which includes gross and histological bleeding scores, were assigned to each joint. The data show that JBTA8 prior to injury significantly reduced joint bleeding. The protective effect of 1 mg/kg JBTA8 on puncture induced hemarthrosis was significantly greater than the effect of 10 IU/kg but <100 IU/kg of Advate assessed by TBS. There was no statistically significant difference in TBS after pretreatment with 1 mg/kg JBTA8 compared to 50 IU/kg Advate.
Summary: These preliminary results suggest that anti-TFPI peptide may provide a protective effect against experimental joint bleeding. The effect of investigated anti-TFPI peptide dose on hemarthrosis was comparable to that of Advate and can contribute to blood coagulation in hemophilia.

PB 3.58-4
Structure and function of chicken protein C inhibitor

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Background: Protein C inhibitor (PCI), a member of the plasma serine protease inhibitor (SERPIN) family, is a physiological inhibitor of activated protein C (APC) and thrombin- thrombomodulin complex; they are the main proteases of the anticoagulant protein C pathway. Human PCI also inhibits other serine proteases of the hemostatic system, such as thrombin, factor Xa, factor XIa, factor VIIa (complexed with tissue factor), plasma kallikrein, and urokinase-type plasminogen activator, as well as proteases of the reproductive system such as prostate-specific antigen and acrosin of the sperm. We showed that PCI is expressed in many organs including liver, kidneys, lungs, and the reproductive organs, such as testes, seminal vesicles and ovary in primates, but only in the reproductive organs in rodents. However, it is still unknown whether PCI is present in birds.
Aim: In this study, we cloned a full-length chicken PCI cDNA, and determined its mRNA distribution in chicken tissues, and functional characteristics using recombinant chicken PCI.
Methods: Full-length chicken PCI cDNA was isolated by screening of the Lambda ZAP chicken liver cDNA library using chicken partial PCI cDNA fragment as a probe. DNA sequence determination and Northern blot analysis were performed using standard methods. Recombinant chicken PCI was prepared using Baculovirus expression system. Inhibitory activity of the recombinant chicken PCI for APC prepared from chicken plasma was determined in the presence or absence of heparin.
Results: Chicken PCI cDNA encoded a putative 19-residue signal peptide and a 413-residue mature protein; this showed 46.2%, 46.3%, 42.3%, 45.3% and 44.7% amino acid sequence homology with the human, rhesus monkey, bovine, rat and mouse PCIs, respectively. Chicken PCI had a putative reactive site peptide bond, Arg-Ser, that is the same as the reactive site sequence (Arg-Ser) of other species' PCI, except for bovine PCI. Chicken PCI mRNA (2.1 kb in size) was expressed strongly only in the liver. Recombinant chicken PCI inhibited chicken APC, and this inhibition was dependent on the heparin concentration. The three dimensional structure of chicken PCI, estimated on the basis of the amino acid sequence of chicken PCI, was similar to that of human PCI, which inhibits human APC dependent on heparin.
Conclusion: The data suggest that chicken may have the anticoagulant protein C pathway in plasma, in which PCI regulates APC in heparin-dependent fashion, as in human plasma.

PB 3.58-5
Fibrinogen protects activated factor XIII from early inactivation in human plasma

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Background: Activated factor XIII (FXIIIa) is a transglutamase that plays a central role in the regulation of thrombus stability, thrombus regulation, cell-matrix interactions and wound healing. While activation kinetics of FXIII have been extensively analyzed, little is known about the mechanisms involved in the regulation of the catalytic activity of FXIIIa.
Aims: To study the catalytic half-life of FXIIIa in human plasma and to study if fibrinogen plasma levels interfere with the catalytic half-life of FXIIIa.
Methods: To measure time-related changes in FXIIIa activity, recombinant human FXIIIa was added to human plasma and incubated at 37 °C. The loss of FXIIIa activity was measured using a fluorometric FXIIIa assay. This assay was sensitive to a lower limit of quantification of 0.01 IU/mL. To test the interindividual variability, FXIIIa inactivation kinetics were measured in plasma samples obtained from five healthy blood donors. All blood donors gave written informed consent.
Results: In pooled normal plasma the catalytic activity of FXIIIa declined to 74%, 45% and 33% of initial after 5, 30, and 60 min of incubation. The half-life of FXIIIa was approximately 20 min showing a low interindividual variability (CV <10%). In fibrinogen-deficient plasma, the half-life was reduced to 5 min with residual FXIIIa-activi-

ties of 24% and 20% measured after 30 and 60 min of incubation. The catalytic half-life returned to normal after normalization of the fibrinogen level by addition of purified fibrinogen.
Summary/Conclusion: Our data indicate that fibrinogen protects FXIIIa from early inactivation.

PB 3.58-6

Novel mutation of factor XII gene in five Taiwanese families of congenital factor XII deficiency

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Background: Factor XII deficiency was first described by Ratnoff and Colopy in 1955, it was not associated with abnormal bleeding despite causing a marked prolongation of aPTT. Most patients are diagnosed by incidentally found prolonged PTT and normal PT. Factor XII gene is 12 Kb long with 14 exons and is located on chromosome 5q33-qter. More than 200 cases, with more than 20 mutations in the factor XII gene have been reported previously. However, there were no cases reported fromTaiwan.
Aim: We have seen five unrelated families of congenital factor XII deficiency since 1995. Their clinical, laboratory and genetic studies will be presented.
Methods: Factor XII coagulant activity (FXII:C) was measured by one-stage method. All exons and their junctions of factor XII gene were amplified and sequenced. Informed consent was obtained from patients and family members.
Results: Age (year)/sex of the five index patients were 55/Female, 71/Male, 44/F, 59/F and 48/M, respectively, all had no personal or family history of abnormal bleeding. There were no consanguinity in all families. PT/PTT (sec) were 12/257, 11.4/150.7, 10.7/81.6, 10.7/>240 and 10/108, respectively (reference ranges: PT 9.9–12.0, PTT: 28.6–38.6). These were detected during preoperative screens in all index patients, except patient I who was also found to have prolonged PTT and normal PT before she received liver biopsy for evaluation of HBV hepatitis. FXII:C levels (%) were 0.2, 2.2, 2.6, 1.2 and 1.3, respectively (reference range:39–154). Direct DNA sequencing in index patient I identified a homozygous 11908 Gto A transversion resulting in FXII Glu502Lys substitution in exon 13 of the FXII gene. A similar heterozygous mutation was detected in both her parents, sister and daughter. In index patients III, IV and V, a homozygous single nucleotide transversion at 12118 Gto A in exon 14, resulting in FXII Gly542Ser substitution was identified. Two daughters (patient III) and one son each (patients IV and V) had similar heterozygous mutations. In index patient II, a heterozygous 11903 T to A transversion in exon 13 resulting in Leu500Gln substitution and a heterozygous Gly542Ser mutation were identified. The patient's son had similar heterozygous mutation of Leu500Gln. All families showed 46CC at residue 46 of FXII gene. Glu502Lys and Gly542Ser mutations of FXII gene have been reported fromChina. Leu500Gln is a novel mutation which is located within the catalytic domain of FXII gene, and the hydrophobic Leucine substituted to hydrophilic Glutamine may suggest to cause unstable structure of the catalytic triad His393-Asp442-Ser544, resulting in impaired secretion or decrease of the enzymatic activity.
Conclusion: One novel mutation of Leu500Gln in FXII gene was identified among five Taiwanese families of congenital factor XII deficiency. This mutation may suggest to cause reduced secretion of factor XII protein or decrease of the functional activity.

PB3.59 – Regulation of coagulation and fibrinolysis – III

PB 3.59-1

An additional pathway for hypofibrinolysis in diabetes: the role of complement C3

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Background: Plasminogen activator inhibitor (PAI)-1 has been regarded as the main antifibrinolytic protein in diabetes but recent work indicates that complement C3, an inflammatory protein, compromises fibrinolysis in individuals with type 1 diabetes (T1DM). However, it is currently unknown whether this holds true in patients with type 2 diabetes.
Aim: To investigate the association between plasma complement C3 levels and fibrinolysis in individuals with T2DM.
Methods: Fibrin clot lysis was determined in 838 patients enrolled on the Edinburgh type 2 diabetes study using a turbidimetric assay. Plasma levels of complement C3, C-reactive protein (CRP), PAI-1 and fibrinogen were analyzed by ELISA. Correlations were analysed using Pearson's test after log transforming non-normally distributed data. The R environment for statistical computing was employed to fit regression models and determine independent predictors of clot lysis time and complement C3 levels.
Results: Clot lysis time showed a highly significant correlation with C3 and PAI-1 plasma levels ($r = 0.25$, $P < 0.0001$ and $r = 0.15$, $P < 0.0001$; respectively). In contrast, a relatively weak correlation was detected with CRP ($r = 0.08$, $P = 0.02$) and fibrinogen ($r = 0.08$, $P = 0.01$). In a regression model involving PAI-1, C3, CRP and fibrinogen, C3 was a predictor of lysis time (predictive value for 0.1 mg/mL change in plasma levels 14.4 [95% CI 7.9, 21.0] $P < 0.001$), as was PAI-1 (predictive value for 0.1 ng/mL change in plasma levels 8.2 [4.2, 12.1; $P < 0.001$]) with a smaller effect shown for 0.1 mg/mL change in fibrinogen levels (5.3 [0.01, 10.5]; $P = 0.049$) and none detected for CRP (−2.5 [−9.7, 4.8]; $P = 0.50$). No correlation was demonstrated between C3 and PAI-1 plasma levels, consistent with previous observations of the two proteins affecting different pathways in fibrin clot lysis ($r = 0.04$, $P = 0.37$). Although women showed higher plasma levels of CRP compared with men (4.49 ± 0.33 and 2.95 ± 0.22 mg/L, respectively; $P < 0.01$) no such difference was found in complement C3 plasma levels (1.336 ± 0.02 and 1.344 ± 0.03 mg/mL, respectively; $P = 0.83$). Plasma levels of C3, CRP, fibrinogen and PAI-1 did not differ in the presence of previous history of myocardial infarction or cerebrovascular disease. Plasma levels of all four proteins correlated with body mass index, but only fibrinogen showed an interaction with age and duration of diabetes. To investigate interactions between treatment and plasma levels of proteins studied, a regression model including all various therapies was constructed. CRP plasma levels were positively predicted by long lasting insulin and nitrate therapy (1.78 [0.86, 2.07] and 1.68 [0.66, 2.53]; $P < 0.05$ for both). In contrast, none of the medication predicted complement C3 plasma levels although a trend was observed in men towards a positive and a negative association with sulphonylurea and antiplatelet therapy, respectively (0.08 [0.05, 1.17] $P = 0.08$ and −0.09 [0.04, −1.83] $P = 0.06$).
Conclusions: C3 is at least as strong as PAI-1 at predicting fibrin clot lysis in subjects with T2DM. Gender and medications are not associated with changes in C3 plasma levels in the population studied. Therefore, future studies investigating fibrinolysis potential should

also analyze C3 plasma levels as a surrogate marker of fibrin clot lysis in individuals with T2DM.

PB 3.59-2
Specific detection of polyphosphate in cells, tissues, and thrombi

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Background: Inorganic polyphosphate (polyP) is widely present in biology. The recent discoveries that polyP is secreted from activated human platelets and that it potently modulates coagulation and inflammation have led to a burst of interest in this ancient molecule regarding its roles in human pathophysiology.
Aims: Our goal was to localize polyP in animal thrombosis models, using the isolated polyP-binding domain of *E. coli* exopolyphosphatase (PPXbd) as the candidate detection agent. PPXbd binds polyP with high affinity and has been used to detect polyP in cells, but we felt it essential to determine if PPXbd also binds to anionic glycosaminoglycans like heparin. If PPXbd is specific for polyP, it could be used to reliably detect polyP *in vitro* and *in vivo*, and could function as a novel antithrombotic agent, akin to a blocking antibody.
Methods: We evaluated the ability of PPXbd to specifically identify polyP in the mast-cell like cell line, RBL-2H3, known to have distinct classes of secretory granules containing polyP and/or heparin. PolyP was detected in fixed, permeabilized cells using recombinant PPXbd fused to maltose-binding protein (MBP), followed by rabbit anti-MBP and fluorescent donkey anti-rabbit IgG. To visualize heparin, permeabilized RBL-2H3 cells were treated with human antithrombin, followed by a rabbit antibody to antithrombin and fluorescent donkey anti-rabbit IgG. Control cells were pretreated with exopolyphosphatase or heparinase to destroy endogenous polyP or heparin, respectively. To visualize polyP in thrombi formed *in vivo*, clotted sections of mouse common carotid arteries treated with 5% $FeCl_3$ were removed, formalin-fixed, and paraffin-embedded. Serial 5 μm sections were stained with purified PPXbd-MPB fusion protein followed by peroxidase-conjugated mouse anti-MBP and detection with peroxidase substrate (DAB).
Results: Binding of PPXbd and antithrombin to permeabilized RBL-2H3 cells was readily detectable by immunohistochemistry, displaying punctate staining typical of mast cell secretory granules. Pretreating cells with exopolyphosphatase significantly ($P < 0.001$) reduced the fluorescent signal from PPXbd but not from antithrombin. Conversely, pretreating cells with heparinase significantly decreased the fluorescent signal from antithrombin but not from PPXbd. PolyP was readily detectable in sections of RBL-2H3 cells after formalin fixation and paraffin embedding. We are currently employing this method to detect polyP in sections of thrombi from mouse models of carotid artery thrombosis.
Summary/Conclusion: Immunodetection of polyP via PPXbd binding is robust, even in paraffin sections of cells and tissues, and appears to be highly specific for polyP. This approach can therefore provide important information about the localization of polyP in thrombi and tissues. Our findings also suggest that PPXbd can specifically inhibit the procoagulant activity of polyP *in vivo*, allowing us to test the concept of polyP as a novel antithrombotic target.

PB 3.59-3
Serpins interfering with procoagulant activity and pressure regulation in a basal vertebrate

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Background: Blood coagulation of gnathostomes evolved from a simple cascade of procoagulant proteases at the dawn of vertebrates. Previous analyses showed that lampreys, an extant group of primordial vertebrates, encode many genes pivotal for coagulation, including those coding for prothrombin, factor X, and fibrinogen. However, how control of procoagulant enzymes emerged is largely unknown.
Aims and Methods: In order to reconstruct the ancestry of anticoagulant proteins, we have isolated lamprey cDNAs coding for serpins and characterized the activities of the recombinant proteins towards clotting proteases.
Results: Three serpins were identified that exhibit protease-inhibiting activity directed against central coagulation-promoting enzymes. The lamprey heparin cofactor II (HCII) gene, clearly identified by its syntenic position conserved in all vertebrate genomes, appears to fulfil a role comparable to its counterpart in gnathostomes. Uniquely in vertebrates, lamprey angiotensinogen serves as protease inhibitor that is not only a potent, but also a highly selective thrombin inactivator, as all other serine proteases investigated did not interact with the angiotensin precursor protein. Moreover, heparan sulphate, a widely occurring glycosaminoglycan, strongly stimulates thrombin inhibition, similar to heparin. We also isolated a lamprey serpin depicting inhibitory activity towards human factor Xa, though the serpin was proteolytically cleaved to some extent.
Summary: Together, these three serpins appear to be major regulators of the coagulation cascade in lampreys that are believed to mirror many essential aspects of the early coagulation system. Antithrombin, seemingly not present in lampreys, may have adopted some of physiological roles of these ancestral inhibitors during the advent of 'crown' vertebrates. A scenario now emerges that provides insight into major aspects of the basal interaction network enabling and controlling fibrin formation.

PB 3.59-4
Glycan associated functional changes of desialylated, deglycosylated and recombinant acutobins

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Acutobin is a specific α-fibrinogenase isolated from the venom of *Deinagkistrodon acutus* (hundred- pace pitviper), and this serine protease has been used on patients to treat or prevent strokes via intravenous injection. We previously showed that acutobin (40 kDa) contains unique disialyl-rich complex type glycans at four Asn positions. However, whether the glycan's structural variations would affect the enzymatic properties and the pharmacokinetics of acutobin remain to be clarified. Sialidase treated (DS-acutobin) and PNGase treated acutobin (PNG-acutobin) retained the kinetic specificity for fibrinogen Aα and released fibrinopeptide A as effectively as the native acutobin, as shown by SDS-PAGE and HPLC analyses. The sugar-modified acutobins showed slightly increased fibrinogenolytic activity, but induced plasma coagulation as fast as the native acutobin *in vitro*. Moreover, although similar amounts of fibrinogen/fibrin degradation products (FDP) were generated after intravenous injection of either native acutobin or the sugar-modified acutobin in mice, the generation of FDP after intraperitoneal injection of the sugar-modified acutobin was significantly less than that after native acutobin injection. These results suggest the contribution of acutobin's sugars for vascular localization of the protease. Recombinant acutobins (rATBs) were expressed and purified from HEK293T and SW1353 cells, and their N-glycans were profiled by mass spectrometry. The glycans of rATB from HEK293T were found to be smaller than that of native acutobin, while the glycans of rATB from SW1353 were found to be larger. Both rATBs, PNG-acutobin and DS-acutobin showed similar reactivities and kinetic specificities toward the chromogenic substrate N-Tosyl-Gly-Pro-Arg-*p*-nitroanilide. Compared with acutobin, SW1353-rATB showed lower α-fibrinogenase activity, and HEK293T-rATB was no longer specific for the Aα chain. We also found that the N-glycans could protect the stability of acutobin and rATBs at 55 °C but were less protective at higher temperature (≥65 °C). Taken together, our

data suggest that the glycans of acutobin are not necessarily required for substrate binding and catalysis, but are essential for increasing the stability, specificity and pharmacokinetics of the enzyme. The different glycans found in various pitviper thrombin like proteases are probably resulted from co-evolution of the protein sequences and the venom gland N-glycosylation machinery. (The mouse experiments were carried out under the guideline of the regulation of Academia Sinica, Taiwan; grant support was from the National Science Council and Academia Sinica, Taiwan)

PB 3.59-5

An ex vivo evaluation of the hemostatic effects of plasma-derived C1 inhibitor

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Background: C1 inhibitor (C1-INH) is used to treat or prevent angioedema attacks in patients with hereditary angioedema. Because C1-INH inhibits bradykinin and has the potential to interact with the coagulation system, we evaluated the hemostatic effects of increasing concentrations of C1-INH on human blood using thromboelastometry and thrombin generation.

Methods: Following institutional approval, blood was collected from 10 healthy volunteers who provided informed consent. Samples were processed immediately for thromboelastometry or were centrifuged to obtain platelet poor plasma that was subsequently used to assess thrombin generation. Samples were supplemented with C1-INH (ViroPharma Biologics, Inc., Exton, United States) at concentrations of 0.14, 0.7, 1.4, 2.8, and 7.0 IU/mL. Controls included samples without C1-INH supplementation, samples with the procoagulant recombinant factor VIIa (rFVIIa) (1.5 μg/mL) or prothrombin complex concentrate (PCC) (0.6 U/mL) added, and samples with the anticoagulants antithrombin III (AT-III) (1.2 IU/mL) or desirudin (10 nM/0.5 μg/mL) added. The viscoelastic effects of C1-INH were assessed using the ROTEM® device, while thrombin generation was evaluated using a calibrated automated thrombin generation system. Data are expressed as mean ± SD. Kolmogorov-Smirnov statistics were used to test for normal distributions. Increasing C1-INH concentrations, as well as pro-and anticoagulants vs. control were evaluated by ANOVA for repeated measurements, followed by post hoc analysis using Bonferroni correction. $P < 0.05$ was considered significant.

Results: In the studies testing viscoelastic effects, increasing concentrations of C1-INH did not have a procoagulant effect based on ROTEM measurements when compared to the control data regardless of the activator ($P > 0.05$). At concentrations of 2.8 and 7.0 IU/mL, C1-INH had a mild but statistically significant anticoagulant effect based on maximum clot firmness ($P < 0.05$). rFVIIa significantly decreased clotting time, and desirudin increased clotting time from 59 ± 11 to 103 ± 44 s when the EXTEM reagent was used as an activator ($P < 0.001$) and from 163 ± 8 to 269 ± 42 s with the INTEM reagent ($P < 0.001$). In the thrombin generation studies, there was no difference in the lag time of thrombin generation between C1-INH-supplemented samples vs. control samples regardless of the activator used ($P > 0.05$). Addition of 30 nM rFVIIa shortened tissue factor-activated thrombin generation lag time by ~48%, and by ~ 30% with dilute Actin. Desirudin, a direct thrombin inhibitor, significantly delayed the thrombin generation lag time. Increasing concentrations of C1-INH had no effect on peak thrombin generation as compared with control samples. AT-III decreased peak thrombin generation by >40%, while PCC increased peak thrombin generation by >60%.

Summary/Conclusions: Increasing C1-INH concentrations up to 7.0 IU/mL did not have a procoagulant effect based on viscoelastometry parameters or thrombin generation in blood obtained from healthy volunteers. C1-INH demonstrated a weak anticoagulant effect based on maximum clot firmness at the two highest concentrations studied. There was no evidence of an *ex vivo* procoagulant effect of plasma-derived C1-INH when studied at concentrations up to 10-fold higher than those achieved with clinical dosing in patients with hereditary angioedema.

PB 3.59-6

How aspirin inhibits human cyclooxygenase-1? Hybrid quantum mechanical/molecular mechanical (QM/MM) calculations on the mechanism

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Background: Aspirin (acetylsalicylic acid; ASA) suppresses the generation of prostaglandin H2, the precursor of thromboxane A2. Aspirin acts as an acetylating agent; its acetyl group is covalently attached to a serine residue (S529) of the substrate-binding cavity of the cyclooxygenase-1 (COX-1) enzyme. The exact reaction mechanism has not been revealed by experimental methods.

Aim: The goal of this study was to characterize the reaction mechanism of the irreversible inhibition of COX-1 by aspirin using static ONIOM (Our N-layer Integrated molecular Orbital molecular Mechanics) type hybrid QM/MM methods. We intended to decide whether the above methods predicted a single-step elementary reaction or multiple-step one(s), leading from the reversible ASA-COX-1 complex to the salicylic acid and acetylated COX-1 products. A further aim was to determine the 'exact' transition state(s) and local minima on the potential energy surface and prove their existence by vibration analyses.

Methods: We used 'high level' *ab initio* and density functional theory (DFT) calculations on a model system; the steric and electrostatic effect of the neighboring amino acids, in a relatively large surrounding region, was also taken into account. The latter was achieved by using the electronic embedding method in ONIOM, which allows polarizing the wave function by the surrounding partial charges. Series of calculations were carried out to assess the dependency of the results on the level of theories applied in quantum chemical calculations.

Results: As there was no available X-ray structure for human COX-1, we derived a model for the respective human protein by homology modeling, using the known structure of sheep COX-1 as a template. Calculations were carried out on this model. ASA was docked into the substrate-binding cavity of the enzyme. The most stable and most populated (~70%) pose obtained by docking resulted in a complex suitable for the next step of the reaction. The potential energy surfaces as a function of the S529 Oγ – C (ASA acetyl carbonyl) and the S529 Hγ – S529 Oγ distances suggested only a single saddle point (i.e. one elementary reaction step) for all the ONIOM QM/MM calculations. The 'exact' transition states can be featured by an almost completed proton transfer, i.e. the reaction has asynchronous character. All our failed efforts to find the 'tetrahedral intermediate' and also the normal mode corresponding to the imaginary frequency in the transition states confirmed a single elementary reaction step for the ASA-COX-1 trans-esterification.

Conclusion: A model for the human COX-1 was constructed by homology modeling. ONIOM type QM/MM methods suggested a plausible single elementary step mechanism for the aspirin – human COX-1 trans-esterification reaction.

PB3.60 – Cancer and Thrombosis – VII

PB 3.60-1

Coagulation factors V and X gene polymorphisms are associated with breast cancer risk and correlate with phenotype

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Background: The bidirectional relationship between cancer and thrombosis is now recognized. Tumor cells express procoagulants and may stimulate cancer progression by both coagulation independent and dependent signaling. Tissue factor (TF), FXa and thrombin have been shown to promote migration, angiogenesis, adhesion and invasion. Blood clots may provide a structural platform for settlement and growth of cancer cells.

Aim: We aimed to yield a better understanding of the molecular mechanisms underlying the link between cancer and the hemostatic system. To address this, levels of hemostatic markers in blood of breast cancer patients were compared to controls, and association to single nucleotide polymorphisms (SNPs) in hemostatic marker genes were explored. Genotype-phenotype correlations were conducted between SNPs and mRNA- and protein expression.

Methods: Blood from 390 breast cancer patients* and 350 controls*, and tumor tissue from 150 of the patients have been analyzed. Blood samples were taken immediately before surgery (no prior treatment).

Plasma levels of TF, FVIII, FIX, FX, fibrinogen, VWF, D-dimer, TF pathway inhibitor (TFPI), antithrombin, protein C (PC), protein S, endogenous thrombin potential (ETP) and activated PC resistance (APCR) were determined.

Selected SNPs in hemostatic genes, including F5 rs6025 (factor V Leiden) and F2 rs1799963 (prothrombin G20120A variant) were genotyped by multiplexing (Sequenom). mRNA expression in tumor was measured by Agilent microarray (G4851A).

*All attendants signed a written consent. The study was approved by the Regional Ethics Committee.

Results: A procoagulant state in breast cancer patients was observed as demonstrated by increased APCR and D-dimer levels, as well as higher maximum thrombin generation. The increase in global coagulation may result from elevated levels of FVIII, FIX, VWF and decreased levels of antithrombin detected in patients.

Genotyping of the two most common genetic thrombotic risk factors revealed that F5 rs6025 was equally distributed among patients and controls, whilst the appearance of F2 rs1799963 was twice as common in patients. However, this was not significant due to the low minor allele frequency.

Preliminary results demonstrate novel associations of F10 and F5 SNPs to breast cancer risk. The rare allele of F10 rs3093261 was more frequent among patients (OR = 1.60, [95% CI, 1.17–2.17], $P = 0.003$). The rare allele of F5 rs12120605 was also more frequent in the patients (OR = 1.52, [95% CI, 1.07–2.17], $P = 0.02$), and showed association with increased TF mRNA levels in tumor, implying a possible indirect regulation of pro-coagulant expression in the tumor. In contrast, the rare allele of F5 rs6427199 was more frequent in controls (OR = 0.71, [95% CI, 0.52–0.96], $P = 0.03$), and correlated with antithrombin. This might explain the higher antithrombin levels observed in controls. Furthermore, the same SNP also correlated with lowered APCR, explaining parts of the variation in APCR observed between cases and controls, which could not be explained by FV Leiden.

Conclusion: This study displays activated coagulation in breast cancer patients, and demonstrates new genetic polymorphisms in the F5 and F10 genes that associate to breast cancer risk, and correlate with phenotype. The study provides novel findings, which extend the knowledge on the molecular mechanisms underlying how the hemostatic system relates to breast cancer.

PB 3.60-2

Biomarkers predictive of venous thromboembolism in patients with high grade gliomas

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Background: High grade gliomas (HGG) are among the most prothrombotic malignancies. Thromboprophylaxis in patients with HGG is challenging because of the high risk of intracranial hemorrhage. Biomarkers predictive of venous thromboembolism (VTE) might help to identify HGG patients who are likely to benefit from primary thromboprophylaxis.

Aims: To investigate the predictive role of blood biomarkers for occurrence of VTE in patients with HGG. We also examined, whether the development of a specific risk assessment model (RAM) for patients with HGG further improves risk stratification for VTE in these patients.

Methods: We investigated 11 potential biomarkers of future VTE in HGG patients who were included in the Vienna Cancer and Thrombosis Study, which is a prospective and observational cohort study approved by the institutional ethics committee. At study inclusion detailed data on patients' characteristics and information on the tumour histology were recorded.

A blood sample was drawn at study inclusion and each patient was followed for a period of 2 years, until occurrence of VTE and/or death or loss of follow-up.

Results: We included 169 patients with HGG in this study. Hundredtwelve patients had glioblastomas, 37 anaplastic astrocytomas, 11 anaplastic oligoastrocytomas and nine anaplastic oligodendrogliomas. Twenty-six (15.7%) patients developed VTE during follow-up. In multivariable analysis (including age and type of HGG) a significant association ($P < 0.05$) was found between risk of future VTE and the following parameters: leukocyte count (hazard ratio [HR] per doubling: 1.88 [95% CI: 1.02–3.39]), thrombocyte count (HR per 50 increase: 0.71 [95% CI: 0.53–0.92]), sP-selectin (HR per doubling: 2.73 [95% CI: 1.36–6.70]), prothrombin fragment 1 + 2 (HR per doubling: 1.55 [95% CI:1.00–2.34]), FVIII activity (HR per 20% increase: 1.13 [95% CI: 1.03–1.23]) and D-dimer (HR per doubling: 1.50 95% CI: 1.15–1.94]). No association was found with hemoglobin-, C-reactive protein- and fibrinogen levels or peak-thrombin generation.

Using a stepwise forward selection process three dichotomized biomarkers were chosen for an exploratory VTE RAM. This RAM comprised platelet count (cut-off: £25th percentile), FVIII activity (cut-off: ³75th percentile) and sP-selectin (cut-off: ³75th percentile). One point was assigned for each parameter according to the respective cut-off. The cumulative probability of developing VTE after 12 months was 5.9% (2.2–14.9%) in patients with score 0 ($n = 75$), 18.6% (10.4–32.0%) in patients with score 1 ($n = 60$) and 32.3% (17.3–55.1%) in patients with score 2 ($n = 28$). Only three patients had a score of 3 and two of them developed VTE.

Summary/Conclusions: : We identified six biomarkers that indicated an increased risk of developing VTE in HGG patients. Surprisingly, in contrast to previous results obtained in solid tumours, we detected an inverse association between platelet count and risk of VTE, indicating that HGG patients with low platelet count are at particularly high risk of developing VTE. We also developed an exploratory RAM for

prediction of VTE that is able to reliably stratify patients according to their risk of VTE.
The present study provides data for assessing the risk of VTE in HGG patients and identifying those patients at particularly high risk of developing VTE who might benefit from primary thromboprophylaxis.

PB 3.60-3
Pre-existing pulmonary thrombi in cancer patients diagnosed with an unsuspected pulmonary embolism

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Background: Pulmonary embolism (PE) is increasingly identified in cancer patients who undergo CT imaging in the absence of clinical suspicion. The morbidity potential of such findings is significant, hence prompt identification and treatment with anticoagulants is warranted. Nevertheless some pulmonary thrombi may remain inconsequential or just clinically unsuspected, at least for a period of time.
Aims: To investigate the prevalence and duration of persistence of pre-existing thrombi in the pulmonary vasculature in patients diagnosed with a clinically unsuspected pulmonary embolism (UPE).
Methods: From March 2010 all cancer patients with a PE as incidental finding are identified and managed under a specialized pathway which involves specifically trained radiographers, radiologists and clinical nurse specialists (previously published). We retrospectively reviewed previous CT imaging of patients with UPE managed under this pathway for pre-existing thrombi in the pulmonary vasculature. All reference CT scans were performed with IV contrast and slice thickness of 1 mm as per institutional standards, but not with pulmonary angiography (CTPA) protocols. Digital films were reviewed by two of the authors (GA and RB) independently. Differences between their assessments were reconciled by mutual consent.
Results: Between March 2010 and March 2012, 108 patients with UPE were included in the pathway. Ninety-one of these patients had assessable previous CT imaging (median time since last imaging: 105 days, range: 47–707). Post reconciliation, the prevalence of pre-existing pulmonary thrombi was 13% ($n = 12$). In two (17%) of these patients pre-existing thrombi were located in the lobar arteries and in 10 (83%) in segmental and sub-segmental branches. In one of the patients, thrombosis was suspected but findings were attributed to tumour infiltration in previous imaging and no further action was taken at the time. Median interval between a current and a previous positive CT chest was 105 days (range: 51–547). Comparison of the distribution of PE in baseline and previous imaging showed that in six (50%) cases it had remained similar for a median of 92 days (range: 63–253), in three (25%) there was progressive extent of thrombosis (scan intervals: 84, 174, 193 days respectively) and in three (25%) distributions of thrombi were different (scan intervals: 51,232, 547 days respectively).
Conclusions: Clinically unsuspected pulmonary thrombi are often missed in non CTPA imaging but in some cases they may not progress for a period of time even without anticoagulation. The sequence of imaging appearances in our cohort of UPE patients might also support the notion that for some patients pulmonary thrombosis may be a local phenomenon rather than a thromboembolic event from a distant site of DVT.

PB 3.60-4
Different patterns of death among cancer patients and venous thromboembolism

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Background: Venous thromboembolism (VTE) is a significant cause of morbidity and mortality in patients with cancer. VTE is also associated with early death in subjects with hidden cancer. The timing and evolution to death could be different according to the time of VTE and cancer diagnosis.
Objective: To determine the mortality among patients with cancer according to the time of diagnosis of VTE.
Methods: In a retrospective study, the survival of cancer patients who developed a VTE during 2011 was analyzed. All patients were followed until death or the date of the end of study on 31st December 2012. Subjects were categorized into three groups according the time of VTE diagnosis: (i) Before Cancer, (ii) At the time of cancer diagnosis, (iii) After cancer. The survival time was calculated. The cause of death was also included.
Results: Seventy-two patients were included in the study. Of them, 43 (60%) were male and median (Q1-Q3) was 72 (58–79) years. Median time (Q1-Q3) was 4.4 (1.4–11.2) months. At the time of VTE diagnosis, 34 (47%) and 32 (44%) were under oncology treatment and presence of metastasis. Forty-three (60%) died during the follow-up. The causes of death were: 8 (19%) due to VTE and 35 (81%) secondary to advanced cancer. Most frequent primary cancer included lung, gastrointestinal and pancreas in 14%, 24%, 14%, respectively. According to the time of VTE and cancer diagnosis, 5 (71%), 12 (75%), 26 (53%) subjects died in groups 1, 2 and 3, respectively ($P = 0.07$). Survival time al 1, 3 and 6 months were: i) 76%, 47% and 29% in VTE patients with hidden cancer or at the time of cancer, and, ii) 84%, 64% and 38% in patients who developed VTE after cancer ($P = 0.08$).
Conclusions: VTE patients with hidden cancer or diagnosis at the time of cancer have a worse prognosis than those diagnosed after cancer.

PB 3.60-5
TF, TFPI and TAT complexes in myeloproliferative neoplasms

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Background: Myeloproliferative neoplasms (MPNs) are caused by the neoplastic transformation of multipotent stem cell. In patients with MPNs are observed frequent hemostatic disturbances such as thrombotic disease and bleeding disorder. It is known that the most clinically important coagulation pathway is the tissue factor (TF) -mediated coagulation pathway. In the literature there are no data on the role of TF in the pathogenesis of thrombotic complications in MPNs.
Aims: The aim of this study was to evaluate the blood coagulation mediated by TF in patients with MPNs.
Methods: The study was carried out in a group of 32 patients with MPNs aged 21–86 (mean age 57), treated in the Clinic of Haematology, Dr Jan Biziel University Hospital No. 2 in Bydgoszcz, Poland. These patients were enrolled in the study at the time of the diagnosis of MPNs and prior to the implementation of appropriate treatment. The study group included: 16 patients with essential thrombocythemia (ET), eight with polycythemia vera (PV), five with chronic myelogenous leukemia (CML) and three patients with primary myelofibrosis (PMF). The control group consisted of 30 healthy volunteers, age and sex matched. In blood samples were determined TF, tisssue factor pathway inhibitor (TFPI) and thrombin-antithrombin complexes (TAT) concentration using ELISA tests (Imubind ELISA kits,

American Diagnostica Inc.). The study was approved by the Bioethics Committee of Collegium Medicum in Bydgoszcz, the Nicolaus Copernicus University in Torun, Poland (no. KB/396/2010).
Results: The study showed that patients with ET (Me = 378.36 pg/mL), PV (Me = 319.70 pg/mL), CML (Me = 466.08 pg/mL) and PMF (Me = 378.25 pg/mL) had significantly higher TF concentration than the control group (Me = 130.13 pg/mL). Moreover, patients with ET (Me = 47.98 ng/mL) and PV (Me = 41.98 ng/mL) had also significantly lower concentration of TFPI than controls (Me = 72.50 ng/mL) ($P = 0.010820$ and $P = 0.030290$, respectively). Furthermore, it was observed that in patients with CML was significantly higher level of TAT complexes in comparison to the control group (19.14 ng/mL vs. 2.24 ng/mL, $P = 0.006710$).
Conclusions: TF-dependent activation of coagulation process is reported in the blood of patients with MPNs. In patients with ET and PV is increased tendency to hypercoagulability due to the significantly lower concentration of TFPI.

PB 3.60-6
Prolonged LMWH treatment of venous thromboembolism in patients with cancer-A restrospective observational study

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Background: Patients with cancer have an increased risk of venous thromboembolism (VTE) compared with patients without cancer. The treatment of choice for acute episodes of VTE is 200 UI/kg B.W. of low molecular weight heparin(LMWH) for the first month and 150 UI/kg B.W. for the following 5 months.
Aims: In this retrospective observational study we evaluated if patients with cancer and VTE had been treated according to the current guidelines. We also evaluated the efficacy and safety of the prolonged treatment.
Methods: We considered adherent to the recommended therapy patients who received LMWH at a dose of 200 IU/kg BW ±20% for the first month and 150 IU/kg BW ±20% for the following 5–11 months. LMWH' s dosages were adjusted to fulfill the therapeutic intent, according to renal function and transient chemotherapy induced thrombocytopenia. New VTE episodes were confirmed by objectives tests. The main safety outcome was a clinically relevant bleeding.
Results: We enrolled 204 patients with cancer-related VTE. Mean age of the patients was 66.3 years (36–89). We lost on follow up 55 and therefore 149 remained eligible for data analysis: 70 had pulmonary embolism (PE), 27 had PE and Deep Vein Thrombosis (DVT) of the legs, 52 had lower limbs DVT only.
During the first month of antithrombotic approach,125 of 149 (84%) patients were treated with LMWH, 61% of which with recommended LMWH dosages and 14 patients received from the onset vitamin K antagonist(VKA) therapy through an initial LMWH bridging. Mean LMWH dose during the first month of treatment was 164 IU/kg (46–230).
From the 2nd to the 6th month, 102/149 (68%) patients received LMWH, 50% of which at the recommended dosage. Seventeen switched to oral anticoagulant therapy (OAT) with vit k antagonists (VKA) at variable times .
Forty-four patients received LMWH for 12 months, 27 of them (61%) at 150 IU/Kg BW.
The incidence of a recurrent VTE event while on LMWH at recommended dosages, LMWH at lower dosages and VKA treatment resulted respectively of 0.014, 0.016 and 0.016 event/patient-month. The incidence of major bleeding on LMWH at recommended dosages, LMWH at lower dosages and VKA treatment were respectively 0.0042, 0.0033 and 0.015 event/patient-month with a relative risk of 0.28 for recommended LMWH dosages compared to VKA therapy.
Ten patients were treated with fondaparinux from the onset. From the 2nd to the 6th month 6 switched from LMWH to Fondaparinux. Seven received Fondaparinux continuatively for 6 months. Four patients received fondaparinux for 12 months. In Fondaparinux group we registered two bleeding episodes(0.013 event/patient-month) and one VTE recurrence(0.006 event/patient-month)
Conclusions: LMWH treatment is well tolerated and well accepted in patients with cancer and VTE, in a significant part of which up to the 12th month. Our study seems to confirm that patients on LMWH are submitted to a similar rate of thromboembolic complications as compared with those on OAT, while they seem to be exposed to a lower rate of bleeding complications.

PB3.61 – Cancer and Thrombosis – VIII

PB 3.61-1
Cerebral venous thrombosis and myeloproliferative neoplasms: results from a combined analysis of 706 patients with cerebral vein thrombosis and 2267 patients with myeloproliferative neoplasms

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Background: Philadelphia-negative (Ph−) myeloproliferative neoplasms (MPNs) include polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF) with about 70% of patients carrying *JAK2*(V617F) mutation. In MPNs venous and arterial thrombosis are the most common clinical manifestations accounting for a prevalence that ranges from 6 to 12%. In the past, we conducted a meta-analysis finding that the mean prevalence of *JAK2* (V617F) mutation was 32.7% in patients with splanchnic vein thromboses (SVTs) and very low (range, 0.88–2.57%) in other venous thromboembolic events indicating that MPN should be investigated in patients with SVT. Concerning cerebral venous thrombosis (CVT), the scenario of prevalence in MPNs is still unknown.
Aim of the study: To asses if an association between CVT and MPN exists to finally justify a deeper MPN-oriented evaluation in patients with CVT or viceversa.
Methods: We conducted a retrospective study including two large cohorts of patients. The CErebral VEin Thrombosis International (CEVETIS) database was used to collect MPN history and MPN evolution in 706 patients with a well-defined diagnosis of CVT. The Pavia-MPN-database was used to collect CVT history and CVT occurrence after MPN diagnosis in 2267 patients with a well-defined MPN diagnosis.
Results: The CEVETIS database includes 304 patients (43.0%) with idiopathic CVT and 402 patients (57.0%) with secondary CVT (at least one risk factor identified) followed for a median time of 40 months (range 6–297 months). Mean age at CVT diagnosis was 40 years (standard deviation + 16.3 years) and 73.7% were females. In nine patients (1.3%) MPN was diagnosed before CVT, in four patients (0.57%) CVT and MPN were synchronous and in 14 patients (1.98%) MPN occurred after CVT in a median time of 21 months (range 11–120 months). Among these, seven (50%) had idiopathic CVT. Overall, demographics did not reveal any statistically significant differences between patients who had MPN or did not. The Pavia-MPN-database includes 735 patients with PV, 964 with ET and 444 with PMF. Median age was 55.3 years (range 13–91 years) and 51% were females. Nine CVTs were collected: three occurred before MPN diagnosis (at 9, 5.4 and 5 years before MPN), three at MPN diagnosis

and three during MPN follow up (at 0.7, 2.5, and at 3.5 years). To note that seven out of nine CVTs occurred in female and the median age was slightly younger (48.6 years) than the overall MPN population (55.3 years)

Conclusions: This study demonstrates that CVT is not strongly associated to MPN phenotype, indicating that a deep MPN-screening is not needed at least in asymptomatic patients with CVT.

PB 3.61-2

Low molecular weight heparin in hospitalized cancer patients: a systematic review and pooled analysis of placebo-controlled randomized trials

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Background: Cancer patients are at high risk of venous thromboembolism (VTE). Current Clinical Practice guidelines (ASCO, ACCP and ESMO) recommend the use of pharmacological parenteral thromboprophylaxis for cancer patients requiring hospitalization. These recommendations are based on extrapolation from large placebo-controlled trials assessing the efficacy and safety of thromboprophylaxis in medically-ill hospitalized patients. However, the risk benefit ratio of thromboprophylaxis in hospitalized cancer patients has never been formally assessed.

Aim: To summarize the rates of VTE and major bleeding episodes among medically-ill cancer patients receiving low molecular weight heparins (LMWH) or placebo.

Method: A systematic literature search strategy was conducted using MEDLINE, EMBASE, the Cochrane Register of Controlled Trials and all EBM Reviews to identify all randomized controlled trials comparing a LMWH to placebo in hospitalized medically ill patients. Two reviewers independently extracted data onto standardized forms. The primary endpoint was VTE. Venous thromboembolism was defined as a composite outcome of: (i) deep vein thrombosis (DVT) by protocol scheduled screening studies; (ii) symptomatic DVT (distal or proximal) or pulmonary embolism (PE); (iii) fatal PE; or (iv) sudden death without another plausible cause. Secondary endpoints included major bleeding episodes and symptomatic VTE (lower limb DVT and PE). Corresponding author of manuscript were contacted if primary or secondary outcomes could not be extracted from the original manuscript. Relative risk (RR) using a random effect model was used as the primary measurement with 95% confidence intervals (CIs).

Results: The search strategy identified 242 potentially eligible papers. Four placebo-controlled randomized trials reported the primary outcome measure. Data could be extracted for 96% of cancer patients enrolled into these trials. The three major trials ($n = 278$ cancer patients) compared thromboprophylaxis (enoxaparin 40 mg, dalteparin 5000 IU or fondaparinux 2.5 mg daily) to placebo and were included within the pooled analysis. The pooled RR of VTE was 0.91 (95% CI: 0.21 to 4.0; I^2: 68%) among hospitalized cancer patients receiving thromboprophylaxis compared to placebo. Extreme case modeling of the remaining 4% of the missing data assuming an event rate of 40% in the placebo group did not significantly alter the results (RR: 0.80 (95% CI: 0.23–2.7). An additional three placebo-controlled randomized trial reported ($n = 720$ cancer patients) symptomatic VTE as a secondary endpoint. However, none of the trials reported the symptomatic VTE rates according to cancer status and corresponding authors have yet to provide requested data. Similarly, none of the trials have reported major bleeding rates among cancer patients.

Summary/Conclusion: We observed no significant benefit for LMWH primary thromboprophylaxis in hospitalized patients with cancer. However, only a limited number of cancer patients were enrolled in the three major thromboprophylaxis clinical trials from which current practice guidelines are based. We hope additional data from published trials will become available for analysis to address this important clinical question.

PB 3.61-3

The impact of combined radiation and hormon therapy on the microparticles and tissue factor generation in patients with prostate cancer

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Background: Tissue factor (TF) plays a critical role in tumour growth and metastasis, and its enhanced release into plasma in association with cellular microparticles (MPs) has been recently associated with cancer progression. Accumulating evidence indicates that combining radiotherapy and hormone therapy in patients with prostate cancer has significantly improved men's survival as compared to hormone therapy treatment alone.

Aim: The purpose of the study was to evaluate the influence of combined hormone therapy and radiation on plasma levels of TF, TFPI and MP generation in patients with prostate cancer.

Methods: 40 patients with prostate cancer (mean age of patients was 67.7 years) were divided according to their cancer status and line of management into: group 1- androgen deprivation therapy combined with radiotherapy (22 patients) and group 2- radiotherapy alone (18 patients). For control, plasma samples were collected from 30 age – matched healthy males with no evidence of cancer. In cancer groups blood samples were collected before, during and 30 days after radiation therapy. Procoagulant activity of circulating MPs, concentrations of TF and TFPI antigens were measured by immunoenzymatic method (ELISA, American Diagnostica).

Results: Before radiation the plasma levels of MP, TF and TFPI were similar in group 1 (respectively: Me 9.94 (Q1- 6.14; Q3–13.44 nM), Me 59.42 (Q1- 35.24; Q3- 97.22 pg/mL), Me 117.26 (Q1- 83.08; Q3–138.26 ng/mL) and group 2 of patients with prostate cancer (respectively: Me 9.75 (Q1- 8.74; Q3–14.44 nM), Me 77.46 (Q1- 53.52; Q3-116.01 pg/mL), Me 105.40 (Q1- 83.64; Q3–147.76 ng/mL). However MP were significantly higher as compared to the control group ($P < 0.01$).

Radiotherapy caused a transient increase in MPs generation in group 1 of patients as compared to group 2 (Me 10.36 (Q1- 7.32; Q3- 15.22) vs. Me 15.83 (Q1- 10.83; Q3- 22.70) nM, $P < 0.02$)

Conclusion: Our data suggests a role of circulating MPs in intravascular coagulation activation in patient with prostate cancer, especially those with radiation therapy treatment alone.

PB 3.61-4

The impact of prophylactic heparin on survival in cancer patients: a meta-analysis

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Background: Cancer is associated with alterations in clotting and fibrinolytic systems and increased risk of venous thromboembolism. Previous research suggests that tumors may exploit the coagulation system allowing for immune evasion and angiogenesis, resulting in survival and dissemination of tumor cells. Several studies suggest that heparin and low molecular weight heparin (LMWH) may have an anti-tumor effect in experimental *in vivo* and *in vitro* models. The clinical implica-

tion of this is unclear. Earlier clinical trials have reported conflicting findings and our previous meta-analysis (Lazo-Langner et al 2007) suggested a beneficial albeit modest effect of LMWH on survival in cancer patients.

Aims: We aimed to evaluate the effect of LMWH compared with placebo or no-anticoagulant intervention on the overall survival of patients with cancer.

Methods: We conducted a systematic review and meta-analysis of randomized trials evaluating use of LMWH compared with placebo in cancer patients without acute venous thrombosis. Data sources included: MEDLINE, HealthSTAR, EMBASE, the Cochrane library, thrombosis and hematology conference abstracts and cross-referencing from reference lists. Data was extracted by one reviewer and verified independently by a second reviewer. The meta-analysis was conducted using the random-effects model and data was analyzed using odds-ratio (OR) and relative risk (RR) calculated for 1 year overall survival.

Results: We identified 301 trials involving use of low molecular weight heparin in cancer patients using our search strategy. Sixteen of these studies met our inclusion criteria, although only seven of these reported data on overall survival or number of deaths at 1 year and these were included in the meta-analysis. Five of the seven studies included only patients with locally advanced or metastatic disease; one study included patients with both limited and advanced solid tumors and one study included only patients with malignant glioma. The pooled odds-ratio for death was 0.96 [95% CI 0.86–1.07, $P = 0.5$] in favor of the LMWH group. The pooled relative-risk for death was 0.98 [95% CI 0.93–1.04, $P = 0.51$] in favor of the LMWH group.

Conclusions: In contrast with our previous findings, the results of this updated meta-analysis suggest that the use of LMWH does not appear to improve overall survival in cancer patients. The trials meta-analyzed included primarily patients with advanced and metastatic disease with a low likelihood of long-term survival or cure. The effect of LMWH on overall survival in patients with limited stage disease still is unknown.

PB 3.61-5

Pro-coagulant activity of malignant ascites

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Background: Malignancy accounts for approximately 10% of all ascites cases. Ovarian, pancreatic, stomach and colon cancers being the most common primary malignant sites. Tumour-cell infiltration within the peritoneal cavity presents the opportunity to use malignant ascitic fluid to study therapeutic effects of agents directly on the cancer cell. As a first stage in a study planned to investigate the direct and indirect effects of anticoagulants in cancer patients with ascites, we have compared the cancer related procoagulant activity (PCA) and it's driving factors in ascites and plasma.

Aim: The aim of this study was to analyse tissue factor microparticles (TF + MP) and the related PCA of malignant ascites and compare it with the PCA of patient plasma.

Methods: This study was conducted *in accordance with* the *Helsinki Declaration and ethical approval from the regional medical ethics committee was obtained prior to commence (R1220 - 11/YH/0370). Informed written consent was obtained prior to sample collection.*

We included ovarian ($n = 9$), pancreatic ($n = 3$) and colonic ($n = 3$) cancer patients with clinical and radiological evidence of malignant ascites. Those with a known concurrent coagulopathy or receiving anticoagulant therapy were excluded. One litre of ascites and 5 mL of citrated blood were simultaneously obtained. Ascites samples and platelet poor plasma were assessed for PCA using a pro-thrombin time assay and TF + MP, annexin + MP and TF+/annexin + MP were enumerated by flow cytometry. Ascites was analysed for PCA before and after centrifugation (to remove cells) and filtration through a 0.1 μm filter (to remove large microparticles).

Results: Ascites was found to contain TF + MP in all cases. A logarithmic relationship was observed between TF + MP and PCA of ascites, whereby higher concentrations of TF + MP were associated with lower prothrombin time and hence PCA. No relationship was observed between plasma PCA and MP concentration. Centrifugation of ascites resulted in a consistent, proportional reduction in PCA and filtration through a 0. 1 μm filter removed almost all PCA. No relationship was observed between PCA of plasma and ascites.

Conclusions: PCA of ascites was observed to be TF + MP and cell dependent. A reduction in PCA through centrifugation suggests a proportion of the PCA was cell associated and furthermore filtration removed most PCA suggesting PCA in ascites was MP associated.

PB 3.61-6

Clinical profiles of DIC patients with bone marrow carcinosis, aiming for earlier diagnosis and treatment to improve their prognosis

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Background: The clinical course of disseminated intravascular coagulation (DIC) in patients with bone marrow carcinosis (BMC) due to solid cancer is thought to be aggressive with no effective treatment.

Aim: The clinical profiles of patients with DIC and BMC were studied in order to improve the possibility of making early diagnosis and establishing treatment to improve their prognosis.

Methods: Seventeen patients with BMC were enrolled and their background, clinical symptoms, hemostatic profiles, presence of DIC, treatment and prognosis, complications and other laboratory examinations were analyzed.

Results: The primary cancers originated in stomach (seven), lung (three), prostate (two), colon, breast (mamma), urinary bladder, and unknown origin (one). Most of the gastric cancers were poorly differentiated. All cases of lung cancer were of the small-cell type. Lumbago was the most frequent symptom of BMC. Nine cases, comprising six gastric cancers, one lung cancer, and one each of urinary bladder cancer and unknown origin cancer, were diagnosed as DIC. The values for platelet count, fibrinogen, FDP, XDP, AT, α2PI and plasminogen at diagnosis of DIC were $3.2 \pm 1.8 \times 10^{*}4$, 155 ± 109 mg/dL, 100 ± 79 μg/mL, 54 ± 43 μg/mL, $93 \pm 36\%$, $61 \pm 21\%$ and $70 \pm 17\%$, respectively. The period from diagnosis of DIC until death was 15.7 ± 16.3 days. Four cases were complicated with thrombotic microangiopathies (TMA); they were all poorly differentiated gastric cancer or mucin-producing gastric cancer. All cases were accompanied by anemia, high serum levels of lactate dehydrogenase (LDH) and alkaline phosphatase (ALP). All cases that originated as pre-BMC had high serum ALP levels. CA19-9 levels in DIC cases were higher in gastric cancer than were CEA levels and were better tumor markers.

[Conclusions] Markedly low platelet counts, decreased levels of fibrinogen and hyperfibrinolysis were revealed in patients with DIC due to BMC. Patients with BMC from mucin-producing poorly differentiated gastric cancer died earlier. It is very important to check for lumbago, progression of anemia, sustained elevation of ALP or LDH levels, leukoerythroblastosis and levels of tumor markers. Early bone marrow aspiration or biopsy helps to establish an early diagnosis of BMC. Aggressive introduction of chemotherapy could help to prolong the survival period in lung or breast cancer patients, especially in breast cancer.

PB3.62 – Antiphospholipid- IV

PB 3.62-1

Antiphospholipid syndrome in children

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Background: Antiphospholipid Syndrome (APS) is more frequent in adults than in children. Cohort studies estimate up to 7% of children with venous thromboembolism (VTE) may meet criteria for APS; however, pediatric studies are lacking.
Aims: To describe a large cohort of childhood APS, examining laboratory and clinical characteristics.
Methods: A retrospective cohort from the Hospital for Sick Children (Toronto) included patients <18 years of age diagnosed between 1999 and 2011. Patients were identified from the pediatric thrombosis/rheumatology/stroke databases. Only patients meeting revised Sapporo criteria were included. Demographic, laboratory and clinical data were extracted from medical records. Thrombus resolution for multiple initial thrombi was defined as complete resolution of ≥50% of thrombi. Complete thrombophilia evaluation included factor V Leiden (FVL), prothrombin variant, deficiencies in protein C/S and antithrombin testing. Patients with primary vs. secondary APS were compared using descriptive, parametric and nonparametric statistics as appropriate. Study was approved and consent waived by the local research ethics board.
Results: Over the 12-year study period, 53 patients were identified. Seventy-five percent of the cohort had secondary APS (systemic lupus erythematosus [33%], others [67%]). The overall cohort median age was 11 years (IQR: 1–14; M:F 1.94:1.0). There were 57% venous, 34% arterial and 9% mixed thrombi, distributed as follows: central nervous system (CNS) (38%); non-CNS (49%); and both (13%).
APS laboratory criteria consisted of persistent elevation in anticardiolipin IgG antibodies (75%) and persistent positive lupus anticoagulant (55%). Complete thrombophilia evaluation was done in 44/53 (83%) patients, partial in 6/53 (11%), and missing in three patients. Overall, 36/53 (68%) patients had no concomitant thrombophilia, 9% had incomplete evaluation but showed no defects, and 17% had a positive finding, as follows: (FVL [homozygous (1), heterozygous (5)], prothrombin variant [heterozygous (1)], protein S deficiency (1), none with combined defects). Eighty-nine percent of children received some duration of anticoagulation.
On follow-up imaging 64% had no/incomplete radiological resolution, 34% had complete resolution, and 2% had progression. At least one clinical recurrence occurred in 7/53 (13%) patients.
There was no statistically significant difference in sex, thrombus type/location, thrombophilia status, resolution or recurrence in children with primary vs. secondary APS. There was however a significant difference in proportion of children ≤10 vs. >10 years of age in primary vs. secondary APS groups ($P = 0.005$). There was no statistically significant difference in clinical recurrence based on thrombophilia status nor treatment status; 6/7 recurrences occurred in patients with a negative complete thrombophilia evaluation. At the time of recurrence only 1/7 was therapeutic in their anticoagulation.
Summary/Conclusion: Diagnostically proven APS is rare in children, with secondary APS being seen more frequently. Younger children more frequently had secondary APS, while older children had a more even distribution of primary and secondary cases. Clinical recurrence was low in this cohort, particularly considering the concomitant thrombophilia rate described, and recurrence was not associated with thrombophilia status. Interestingly, most children within this cohort received some duration of anticoagulation and recurrence occurred mostly during subtherapeutic anticoagulation levels. Further prospective work is necessary to explore the ideal anticoagulation length in this population.

PB 3.62-2

RAPS: a prospective randomised controlled phase II/III clinical trial of rivaroxaban vs. warfarin in patients with thrombotic antiphospholipid syndrome, with or without SLE

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Background: The current mainstay of the treatment of thrombotic antiphospholipid syndrome (APS) is therapeutic anticoagulation with vitamin K antagonists (VKA), such as warfarin. However, the limitations of VKA have driven a search for new agents, which include rivaroxaban (Xarelto®; Bayer HealthCare). These drugs have been approved for several clinical indications at present based on phase III prospective randomised clinical trials, but these trials may not be directly applicable to patients with APS where there remains an unmet need.
Aim: To demonstrate that the intensity of anticoagulation achieved with rivaroxaban is not inferior to that of warfarin, by measurement of the dynamics of ex vivo thrombin generation using the thrombin generation test (TGT) with the endogenous thrombin potential (ETP) as the key parameter, in patients with thrombotic APS, with or without SLE. Secondary aims: to compare rates of bleeding and recurrent thrombosis, and compare quality of life in patients on rivaroxaban with those on warfarin.
Hypothesis: The intensity of anticoagulation, assessed using the ETP, achieved in thrombotic APS patients on rivaroxaban is not inferior to that obtained on warfarin. The TGT, as a global measure of anticoagulation, can assess the anticoagulant effects of both rivaroxaban and warfarin. Clinically, our hypothesis is that in patients with thrombotic APS, rivaroxaban could induce more predictable anticoagulation and, therefore, a greater sustained reduction in thrombin generation than would warfarin, with additional patient benefits because there is no requirement for regular anticoagulation monitoring
Methods: 156 eligible patients with thrombotic APS, with or without SLE, who have had either a single episode of VTE whilst not on anticoagulation or recurrent episode(s) which occurred whilst off anticoagulation or on sub-therapeutic anticoagulant therapy, currently taking warfarin, target INR 2.5 (range 2.0–3.0), will be randomised either to continue warfarin (standard of care) or to stop warfarin and start rivaroxaban 20 mg once daily as standard. Each patient will have a 6 month treatment period and a final visit 30 days after the end of trial treatment. The planned recruitment period is 10 months and overall trial duration is 24 months.
Results: The primary outcome measure is the percentage change in ETP from randomisation to day 42. The differences between the two arms for the quality of life, efficacy (venous thromboembolism and other thrombotic events) and safety (serious adverse events and all bleeding events) secondary outcomes will be presented as estimates and 95% confidence intervals.
Conclusion: If the trial demonstrates: i) that the anticoagulant effect of rivaroxaban is not inferior to that of warfarin using the ETP; and ii) absence of any adverse effects that cause concern with regard to the use of rivaroxaban, we believe that this would provide sufficient supporting information to change practice for our patients, i.e. to make rivaroxaban the standard of care for the treatment of patients with thrombotic APS, with or without SLE.

PB 3.62-3

Contribution of immunoregulatory cytokines in the pathogenesis of primary antiphospholipid syndrome

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Background: The primary antiphospholipid syndrome (PAPS) is an autoimmune disease characterized by the presence of antiphospholipid antibodies with associated vascular thrombosis and obstretical complication. Recent studies suggest defective immunoregulatory mechanisms as a potential cause of autoimmunity.
Aims: To investigate the contribution of immunoregulatory and Th17-associated cytokines in the pathogenesis of PAPS.
Methods: We measured serum concentration of TGF-β1, IL-10, IL-23, IL-17A and IL-27 (high sensitivity ELISA kits) in patients with confirmed diagnosis of PAPS ($n = 20$) and matched group of healthy controls ($n = 20$). Additionally, anticardiolipin (aCL) and anti-β2 glycoprotein I (aβ2GPI) antibodies, and CRP were measured in all subjects studied.
Results: Serum levels of both IL-10 and IL-23 were significantly elevated in PAPS patients as compared to controls (median IL-10: 3.7 vs. 1.7 pg/mL, $P < 0.001$; IL-23: 19.9 vs. 11.6 pg/mL, $P < 0.05$). There was a trend ($P = 0.056$) toward lower concentration of TGF-β1 in PAPS group (14.5 vs. 13.3 ng/mL in controls). IL-17A was detected only in one PAPS patient. All other measurements for IL-17A and IL-27 were below the assay threshold. There was a significant negative correlation between concentrations of TGF-β1, and both aCL IgG ($r = -0.65$) and aβ2GPI IgG ($r = -0.61$). We also observed a positive correlation of IL-23 with IgM aCL ($r = 0.7$) and IgM aβ2GPI ($r = 0.6$). PAPS patients with the history of deep venous thrombosis (DVT) were characterized by significantly higher levels of serum IL-10 ($P < 0.001$) and lower concentrations of TGF-β1 ($P < 0.05$). Finally, serum CRP did not differ between the groups, and there was no association between CRP levels and concentration of measured cytokines.
Conclusions: Our data suggest, that PAPS is characterized by low-grade immune activation and imbalance between immunoregulatory and pro-inflammatory cytokines, which may be responsible for production of antiphospholipid antibodies and subsequent thrombotic symptoms.

PB 3.62-4

Pro-inflammatory and pro-thrombotic markers in antiphospholipid antibody positive patients: subgroup analysis from an open-label prospective pilot study

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Background: Antiphospholipid Syndrome (APS) is a multisystemic disease, characterized by recurrent thrombosis and/or pregnancy loss and antiphospholipid (aPL) antibodies. Elevated aPL antibodies can also be found in 'asymptomatic' individuals and in patients with systemic lupus erythematosus (SLE). Pro-inflammatory and pro-thrombotic markers are associated with aPL-mediated pathogenic effects *in vitro* and *in vivo* and are increased in aPL-positive patients.
Aims: We examined whether those markers are differentially upregulated in persistently aPL positive patients with or without autoimmune disease (APS and/or SLE), utilizing data collected from an open-label prospective pilot study.
Methods: Forty-one persistently aPL-positive patients (29F and 12M) and 30 age/sex-matched controls were included. Exclusion criteria included pregnancy, statin use, prednisone <10 mg/day, and immunosuppressive use (except hydroxychloroquine). Interferon (IFN)-α, Interleukin (IL)1β, IL6, IL8, inducible protein (IP)10, tumor necrosis factor (TNF)-α, vascular endothelial growth factor (VEGF), and soluble CD40 ligand (sCD40L) levels were determined by a multiplex assay (Millipore, Billerica, MA) in the sera of patients and controls. Plasma samples were used to detect sTF using a chromogenic assay. aCL (IgG, IgM and IgA), aβ2GPI (IgG, IgM and IgA), soluble intercellular cell adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, and E-selectin (E-sel) were evaluated by ELISA. For the purpose of this study patients were divided into groups: Primary APS (PAPS), APS with SLE (SAPS), SLE with aPL antibodies (SLE/aPL), and 'asymptomatic aPL' (asympt aPL). Mann-Whitney Rank Sum test was used to analyze the data.
Results: Of the 12 biomarkers assessed, nine biomarkers (IL6, IL1β, TNFα, IP10, sCD40L, sTF, sICAM1, sVCAM1 and sEsel) were significantly elevated in PAPS patients, 7 (IL6, IL1β, TNFα, IP10, sCD40L, sTF and sICAM1) were significantly elevated in SLE/APS patients, 4 (IL6, IP10, sCD40L and sTF) were significantly elevated in SLE/aPL patients and 3 (TNFα, sCD40L and sTF) were significantly elevated in patients with asymptomatic aPL when compared to controls. Soluble TF and sCD40L were elevated in all four subgroups and there was no difference in sTF titers among aPL-positive groups ($P = 0.055$). TNFα was elevated in three subgroups – [PAPS, SLE/APS and asymptomatic aPL patients] – while IL-6 levels were elevated in the three subgroups – [PAPS, SLE/APS and SLE/aPL]. IL-8, TNFα and IP-10 concentrations were significantly higher in PAPS, SLE/APS and SLE/aPL patients when compared to patients with asymptomatic aPL. VEGF, sICAM-1, and sVCAM-1 concentrations were significantly higher in PAPS patients when compared to the all other groups. There was no difference in the titres of aCL or antiβ2GPI (all isotypes compared) among the aPL-positive subgroups.
Conclusion: Pro-inflammatory and prothrombotic markers are differentially upregulated in aPL-positive patients. These findings may have implications in the pathophysiology of APS and underscore the importance of detecting biomarkers to help in the identification of subgroups of aPL-positive patients.

PB 3.62-5

Laboratory evaluation of antiphospholipid antibodies: have the recommended guidelines for diagnosing antiphospholipid syndrome been followed

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Background: In addition to clinical indicators, laboratory evaluation of antiphospholipid antibodies (aPLAs) is an integral part of the diagnostic criteria for antiphospholipid syndrome (APS). Based primarily on the method detection, there are three major subgroups of aPLAs: lupus anticoagulant (LA), anticardiolipin antibodies (ACL) and anti-b_2-glycoprotein-1 antibodies (anti-b_2GPI). According to the International Society of Thrombosis and Haemostasis (ISTH) recommended guidelines, the laboratory investigation of aPLAs should always include determination of all three groups of antibodies.
Aim: The aim of this study was to analyze the results of laboratory evaluation of aPLAs in unselected consecutive patients who referred for testing during 1 year period.
Methods: LA antibodies were identified by a panel of commercial coagulation assays (Siemens, Germany) including screening tests (activated partial thromboplastin time (APTT, Actin FSL), APTT mixing test, dilute Russell¢s viper venom time dRVVT screen and dRVVT confirmation test. The laboratory evaluation of ACL and anti-b_2GPI antibodies was performed using enzyme-linked immunosorbent (ELISA) assays (Orgentec, Germany) for immunoglobulin M (IgM) and immunoglobulin G (IgG) isotypes.
Results: The prevalence of positive results for individual aPLAs were: LA = 33/991, 3.3%; ACL = 67/1253, 5.3% (IgG = 22/67, 33%; IgM = 31/67, 46%; IgG and IgM = 14/67, 21%) and anti-b_2GPI = 7/

177, 4.0% (IgG 4/7; IgM 3/7, IgG and IgM 1/7). Only for 11/86 (13%) of all positive patients, laboratory investigation included all three groups of aPLAs. Anti-b_2GPI were ordered only for 16/86 (18.6%) patients. LA and ACL were simultaneously ordered for 48/86 (56%) of positive patients, while LA alone was ordered for 10/86 (12%) patients and ACL alone for 33/86 (38%) patients. Among 44 positive cases for simultaneously ordered LA and ACL, 15/44 (34%) were positive for both LA and ACL, 9/44 (20%) were LA positive and ACL negative and 20/44 (46%) were ACL positive and LA negative. Of the total 86 positive results, LA was ordered for 58 patients (67%) with positivity of 57% (33/58), while ACL antibodies were ordered for 77 (90%) patients with positivity of 87% (67/77). Only in 4/33 (12%) of LA positives, 8/67 (11.9%) of ACL positives and 1/7 of anti-b_2GPI positives, laboratory evaluation was performed on two separate occasions at least 12 weeks apart.

Conclusions: The laboratory investigation of individual aPLAs among unselected consecutive patients had shown the prevalence of 3.3–5.3% for individual subgroups of antibodies, with the highest prevalence of ACL antibodies. Our results indicated that laboratory investigation has not been completely performed in accordance with the recommended guidelines on testing, since only for a small number of patients testing was performed for all three subgroups of antibodies or at least for two (LA and ACL). Additionally, for a minority of positive patients testing was ordered on two separate occasions at least after 12 weeks. It is obvious that laboratory has to take the more substantial role in the investigation process of APS, than just providing doctors with numbers. The corrective actions should primarily include ongoing education of clinicians pointing the importance of determining all three subgroups of aPLAs, since patients may be negative for one subgroup of aPLAs and positive for others.

PB 3.62-6

Taipan snake venom time coupled with ecarin time testing enhances lupus anticoagulant detection in non-anticoagulated patients

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Background: Accurate and timely detection of LAs is crucial to diagnosis and management of antiphospholipid syndrome (APS). It is hampered by the lack of a gold standard assay or reference preparation, exacerbated by antibody heterogeneity, reagent variability and inconsistencies in raw data interpretation. Limiting analysis to just dRVVT and APTT assays has been proposed, at least in part to foster standardisation, yet even this combination of stalwart tests is by no means infallible and will not detect all clinically significant LAs. Numerous alternative assays have been described but few have been subjected to the same degree of scrutiny as the dRVVT/APTT pairing, although this does not necessarily invalidate them as useful diagnostic adjuncts. Taipan snake venom time (TSVT) screening with an ecarin time (ET) confirmatory assay has been shown to be useful for detecting LAs in patients receiving warfarin or rivaroxaban that compromise dRVVT and APTT assays.

Aims: To assess the diagnostic impact of incorporating TSVT/ET analysis into an existing dRVVT and LA-responsive APTT repertoire when testing non-anticoagulated patients for the presence of LAs.

Methods: dRVVT was performed with Life Diagnostics LA Screen and LA Confirm reagents (Life Therapeutics). APTT was performed using PTT-LA (Diagnostica Stago) in the screen and addition of platelet-derived LA confirmation reagent (Bio/Data Corporation) for the confirmatory test. TSVT was performed using Diagen Taipan venom and diluted Diagen Bell and Alton Platelet Substitute (Diagnostic Reagents) and *E. carinatus* venom (Diagnostic Reagents) for the ET. All LA assays were performed on a CS2000i automated analyser (Sysmex). Screen and confirm clotting times were each converted to normalised ratios via the mean clotting time for each reference interval (RI). Test plasmas were defined as being consistent with the presence of a LA if the screening test ratio was greater than the upper limit of the RI and there was ≥10% correction by the confirm ratio after exclusion of other causes of prolonged clotting times. One hundred and fifty two samples from non-anticoagulated patients being investigated for APS were tested for LA by all three assay systems.

Results: Ninety five of 152 samples (62.5%) were negative for LA by all three assays systems. Twenty two (14.5%) were positive by TSVT/ET and one or both of dRVVT and APTT. Twenty five (16.4%) were positive for LA by one or both of dRVVT and APTT but negative by TSVT. Ten (6.6%) were positive for LA by TSVT/ET but negative with both dRVVT and APTT.

Conclusions: Most of the LAs manifested in dRVVT and/or APTT analysis, as might be anticipated from this reagent pairing. The small number of samples positive by TSVT/ET only, as has been previously demonstrated, emphasise that the many variables that impact on LA testing mean that even a well established dRVVT and APTT pairing cannot deliver diagnostic certainty. In view of low frequency and financial/logistic considerations, TSVT/ET analysis is best placed as a second-line assay where clinical suspicion of APS is high and standard assays are negative, and for use in anticoagulated patients.

PB3.63 – Arterial Vascular Disorders – IV

PB 3.63-1

The ABO, non-O blood groups and their association with hematoma growth in acute intracerebral hemorrhage

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Background: ABO blood groups have been associated with various diseases, particulary thrombotic diseases. We tested the hypothesis that the ABO, non-O blood groups are associated with hematoma growth (HG) in patients with acute intracerebral hemorrhage (ICH).

Methods: We studied patients who had a spontaneous ICH within the first 6 h after the onset of symptoms. We included 90 patients (mean age 71 ± 10.8 years), 61% were men and 39% were women. HG was observed in 35 (39%) of the patients. The HG group and the non-HG group were demographically similar. The frequency of intraventricular hemorrhage, and volume of the hematoma at baseline were similar also. HG was defined as an increase of 33% in the volume of hematoma using the computed tomography (CT) data obtained 24–72 h after the onset of symptoms compared to the CT at admission. The volume was calculated using the formula (AxBxC):2. Functional outcome at 3 months was assessed with the Rankin scale score, and it was considered favourable when Rankin score was 0–1 (groups: Rankin 1 0–1; 2nd Rankin ≥2). For genetic analysis, the ABO blood group was determined using the polymerase chain reaction (PCR) with two fragments: one of 252 bp of the exon 6 and other of 843 bp of the exon 7. The fragments were digested with the restriction endonucleases *KpnI* and *MspI*. We used Chi-Square test to compare the frequencies of the two groups: *A2O* and O group (*OO*, *A2O*, *A2A2*) and the non-O group (*A1A1*, *A1B*, *BB*, *A1O*, *BO* and *A2B*).

Results: The frequencies between groups were not significantly different. However, we observed that there was a greater number of patients with HG in the A_2O and O group than in the non-O group (61.3% vs. 38.7%. ns). Also, we observed a higher proportion of non-O group in patients who did not exhibit HG (57.7% vs. 42.3% ns). The Rankin scale at 3 months was not significantly different between the two groups.

Conclusion: Despite the small sample size, we observed that prothrombotic genetic factor, non-O blood group (except *A2*), might play a pro-

tective role in hematoma growth. Further studies with a greater number of subjects need to be done to confirm our results.
Spanish grant RD12/0042/0032. AGAUR 2009 SGR1147.

PB 3.63-2

Pathomechanism of reobstruction after endovascular treatment of the superficial femoral artery: impact of inflammatory cells

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Background: Pathophysiology of reobstruction after arterial intervention is not well understood so far and seems to differ from typical atherosclerotic plaque lesions. Development of a neointima as a response to injury is induced by different cellular components as platelets, vascular smooth muscle cells and immunity cells. Various laboratory parameters reflect the inflammatory state in atherosclerotic lesions. So far it is unknown whether those parameters influence development of restenosis after endovascular treatment of the superficial femoral artery (SFA) as well.
Methods: We performed a retrospective data analysis of 1064 patients with peripheral arterial occlusive disease treated at our institution between 2005 and 2010 by means of endovascular recanalization (percutaneous transluminal angioplasty (PTA) and stent insertion) of the SFA. We evaluated laboratory parameters reflecting inflammatory state and occurrence of symptomatic reobstruction during a mean follow up of 58 months.
Results: In patients developing symptomatic restenosis after PTA median percentage of monocytes was significantly lower than in patients without reobstruction (median 6.0% vs. 7.0% *P*-value 0.02). Reobstruction rate in stent patients was not associated with monocyte-count. We were not able to find statistically significant differences in levels of lymphocytes, neutrophiles, c- reactive protein and fibrinogen.
Conclusion: Low levels of monocytes are associated with a higher risk of restenosis after PTA but not after stent insertion. We hypothesize that monocytes might have a protective effect in vascular lesions and development of restenosis after PTA as they might harbour properties of endothelial cells.

PB 3.63-3

Presence of organized thrombus in coronary aspirated materials is a predictor of in-hospital mortality in patients with acute myocardial infarction

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Background: Thrombus propagation on disrupted atherosclerotic plaque leads to acute myocardial infarction (AMI). Recent studies have shown that the histopathology of coronary thrombus is associated with myocardial reperfusion, ST-segment recovery, distal embolization and long-term mortality in patients with AMI.
Aims: We investigated the histopathological characteristics of material aspirated during percutaneous coronary intervention (PCI) in patients with AMI, and assessed whether histological findings are related to in-hospital mortality.
Methods: In this prospective single-center registry, coronary materials were obtained during PCI from 264 AMI patients within 24 h after the onset of anginal symptoms. The presence of organized thrombus, calcification and plaque components in aspirated materials were morphologically assessed.
Results: In-hospital deaths occurred in 17 (6%) patients. Organized thrombi were found in 91 (34%) of 264 patients, calcification was identified in 44 (17%) and plaque components were aspirated from 117 (44%) patients. Rates of in-hospital all-cause mortality were significantly higher among patients with, than without organized thrombus ($P < 0.05$). However, organized thrombus was not associated with peak creatine kinase ($P = 0.94$). Multivariate analysis also identified organized thrombus as an independent predictor of in-hospital death, as well as age, a history of myocardial infarction and the presence of shock ($P < 0.05$). In contrast, calcification and plaque components were not significantly associated with in-hospital mortality.
Conclusions: Our results suggest that the presence of organized thrombus in coronary aspirated thrombus is an independent predictor of in-hospital mortality in AMI patients.

PB 3.63-4

Correlation between burst of thrombin and microvacular obstruction (no reflow) during ST elevation myocardial infarction treated by primary percutaneous coronary intervention

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Background: The principal challenge in primary angioplasty is not to achieve the culprit vessel reopening (which is regularly obtained) but to prevent the myocardial microvascular obstruction: the no-reflow phenomenon which determines initial and long term prognosis of patients. The main factors of the no-reflow are the micro-thrombotic distal embolizations from the main thrombus and the ischemia reperfusion which includes endothelial reactivity induced by numerous cellular reactions. The thrombotic burden at the acute phase of STEMI, is a consequence of platelet and coagulation activation, for which thrombin generation plays a major role due to its multiple molecular and cellular effect in thrombosis, inflammation micro vascular damage
Objective: During the acute phase of STEMI we evaluated the generation of thrombin as assessed by circulating thrombin-anti thrombin complex. In order to study the consequence, we correlated this thrombin generation with other markers of coagulation activation (fibrin D-Dimers, fibrin monomers), markers of cellular activation (microparticles derived from platelets and endothelial cells), microvascular injury and obstruction and infarct size.
Methods: 36 consecutive patients with STEMI admitted for primary PCI were enrolled. Blood samples were collected and analyzed before angioplasty, at the end of angioplasty and 2, 6, 12 and 24 h after angioplasty. Plasma TAT complexes and D-Dimers were evaluated by ELISA methods, fibrin monomers by immunotubidimetric test. Platelets and endothelial microparticles were determined by flow cytometry analysis. Microvascular obstruction was assessed by the myocardial blush at angiography and index of microvascular resistance. Infarct size was assessed by AUC of plasma troponin and CK-MB, angiographic evaluation of myocardium at risk and by MRI.
Results: a burst of thrombin was observed in 33% of the patients and in these patients the peak occurred during primary percutaneous coronary intervention. The same patients increased their circulating platelets microparticles but the kinetics was delayed 2 h after primary PCI and the levels remained high during 24 h. The endothelial microparticles, D-Dimers and fibrin monomers generation remained stable at the acute phase, without burst. Prolonged ischemic time and significant myocardium at risk were correlated with a higher level of thrombin generation ($P < 0.05$). Patients with burst of thrombin had an alteration of myocardial blush ($P < 0.05$), an increased micro-vascular obstruction ($P < 0.05$) and higher troponin I and CK levels ($P < 0.05$)
Conclusion: This study showed a burst of thrombin at the time of primary PCI during STEMI in one third of the patients. When present this burst was associated with more cellular, myocardial and microvascular damage.

PB 3.63-5

Thrombophilic risk factors predisposing to thrombosis in patients sufferd from ocular arterial occlusions

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Introduction: Retinal vascular occlusion is common causes of blindness and visual morbidity. The role of thrombophilia in the etiology of retinal artery occlusion has not been adequately clarified. Classic risk factors are commonly associated with retinal artery thrombosis, while unclear it is the role of the thrombophilic and coagulation disorders.

Aim: The aim of our study was to establish the prevalence of major and potential inherited and acquired thrombophilic risk factors in 65 patients (39 females, 26 males; age: 56.7 years; range 24–72) with ocular arterial occlusions. The control group consisted of 60 (32 females, 28 males; age: 55.7 years; range 20–72) healthy subjects, without any vascular, eye-related disease.

Methods: In all participants the prevalence of Leiden mutation (FV Leiden), protrombin variant (20210 G/A mutation), Leu 34 polymorphism of the factor (F) XIIIA-subunit, deficiency of protein C, S, antithrombin and antiphospholipid antibodies (aPL) were assessed. Also intima-media thickness (IMT) in carotid arteries were measured using ultrasonography.

Results: Elevated aPL antibody level (IgG>10 GPL; IgM>20MPL) were detected in 15 of 65 patients (23%) and in 4 of 60 controls (6.6%). The incidence of FV Leiden, deficiency of protein C were significantly more common in patients suffered from ocular arterial occlusions ($P < 0.05$) compared to controls. Patients with ocular arterial occlusions had greater IMT than healthy subjects (0.91 mm vs. 0.72 mm; $P < 0.05$). There was no significant difference in the prevalence of deficiency of protein S, 20210 G/A mutation, FXIII Val34Leu polymorphism and antithrombin level between patients and healthy subjects. Among classic risk factors only hypertension, diabetes mellitus, hypercholesterolemia and cigarette smoking were significantly more frequent in patients suffered from ocular arterial occlusions in comparison to controls.

Conclusions: These results show that thrombophilia plays a much important role in the pathogenesis of retinal artery occlusions. According to these data, thrombophilia screening should be consider in preventing and treatment of such events.

PB 3.63-6

TAFI Thr325Ile polymorphism and abdominal aortic aneurysms

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Background: Abdominal Aortic Aneurysm (AAA) is a permanent dilatation of the abdominal aorta, with a natural history of expansion and eventual rupture, accounting for up to 8000 deaths per annum in the UK alone. The presence of intra-luminal thrombus (ILT) is an independent risk factor for expansion and rupture of AAA. Beyond the ILT, the high cardiovascular risk in this group of patients indicates thrombosis may be altered systemically. Recent studies from our laboratory showed that patients with AAA form denser clots with smaller pores, which are more resistant to fibrinolysis.

Thrombin-activatable fibrinolysis inhibitor (TAFI) inhibits fibrinolysis through cleavage of C-terminal lysines from fibrin, impairing the conversion of plasminogen into plasmin and thereby promoting clot stability. A known polymorphism of TAFI, Thr325Ile, has been shown to increase the stability and anti-fibrinolytic activity of TAFI *in vitro*. A recent study indicated a role for TAFI in AAA development, since TAFI deficient mice developed larger AAA upon aortic elastase infusion compared with controls (Schultz G et al, ATVB 2010). Whilst TAFI Thr325Ile has been previously associated with cardiovascular disease, its role in human AAA remains unexplored.

Aim: To investigate the association between the TAFI Thr325Ile polymorphism and AAA, ILT and survival.

Methods: All participants were recruited through the Leeds Aneurysm Development Study (LEADS). A total of 1111 Caucasian subjects ≥55 years (604 patients with AAA ≥3 cm, and 507 age and sex-matched controls, aortic diameter <2.9 cm) were included. DNA was extracted from blood cells, and Thr325Ile genotype was identified using real time PCR, through a commercially available genotyping assay (rs1926447, TaqMan® SNP Genotyping Assay, Life Technologies). Data was analysed using IBM SPSS Statistics version 20.

Results: Genotypes across the study population agreed with the Hardy Weinberg Equilibrium (Controls $x^2 = 0.01$, $P = 0.92$, Aneurysms $x^2 = 0.008$, $P = 0.93$, Total population $x^2 < 0.001$, $P = 0.98$). More patients than controls were both homozygote (9.8% vs. 8.1%) and heterozygote (42.7% vs. 41.0%) for the Ile325 allele; these differences were not statistically significant ($P = 0.429$). The presence of ILT was confirmed on ultrasound scan in 226 of the aneurysm patients (37.4%) but presence or absence ILT was not related to genotype ($P = 0.663$). Although there was a significant difference in survival between aneurysms and controls ($P < 0.001$), survival was not related to TAFI genotype within either group (controls $P = 0.951$, aneurysms $P = 0.549$), or across the population as a whole ($P = 0.234$).

Conclusion: The TAFI Thr325Ile polymorphism did not associate with AAA, presence of ILT, or survival in this group of AAA patients and their controls. These data do not support a major role for this polymorphism in AAA development or intra-luminal thrombus formation.

PB3.64 – Diagnosis of VTE – V

PB 3.64-1

Hestia criteria with the POMPE-C tool identifies patients with cancer and pulmonary embolism at very low risk for short-term complications

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Background: The Hestia criteria and the Prediction of Mortality from Pulmonary Embolism with Cancer (POMPE-C) are well-suited to risk stratify patients with active cancer and PE. We test the hypothesis that the tandem application of Hestia and a POMPE-C score ≤5% in emergency department (ED) patients with active cancer and PE, would be efficient (>25% categorized as eligible for home treatment) and safe (zero mortality at 30 days).

Methods: Patients with confirmed PE were enrolled from the ED of a large academic hospital. Variables required for Hestia and POMPE-C were collected prospectively and 30 day survival was confirmed by direct observation. Active cancer was defined as under treatment or metastatic.

Results: We enrolled 299 patients with PE, including 107 with any history of cancer and complete data. The 30 day mortality rate was 10/107 (9%, 95% CI: 5–17%). Application of Hestia to all 107 with any cancer excluded 29 patients, leaving 79 evaluable by POMPE-C which was ≤5% in 46 patients (43% 34–52%). None of these 46 (0%, 0–8%) died within 30 days. Fifty-eight patients had active cancer and both the Hestia criteria were negative and POMPE-C were ≤5% in 24/58 (41%, 29–55%). None of these 24 patients died within 30 days (0–13%).

Conclusion: The Hestia criteria negative together with POMPE-C ≤5% identified 41% of outpatients with active cancer and PE at very low risk of short-term mortality. Hestia plus POMPE-C offer an efficient and safe method to risk-stratify patients with cancer and PE.

PB 3.64-2

Prevention of venous thromboembolism after major trauma: efficacy of prophylactic measures and associated bleeding complications

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Background: Trauma patients are at a particularly great risk for the development of venous thromboembolism (VTE) due to hypercoagulability, immobilization and venous injury. Without thromboprophylaxis the incidence of deep vein thrombosis (DVT) has been reported to be as high as 63%. The optimal VTE prophylaxis strategy for trauma patients with a contraindication to pharmacological prophylaxis due to the risk of bleeding remains unknown.
Aims: To determine the VTE incidence in major compared to minor trauma patients admitted under a multidisciplinary trauma team as well as assess the efficacy of prophylactic modalities used and associated bleeding complications.
Methods: In total, 1182 patients admitted at Westmead Hospital Level 1 trauma centre were identified from the prospectively maintained Trauma Registry and reviewed (with waiver of consent) over a 6 month period in 2011 and 2012. Patients with an injury severity score (ISS) >12 were categorized as major trauma. Demographic details, mechanism and type of injuries, hospital length of stay (LOS), ISS, prophylactic modalities (mechanical and pharmacological), operative duration, bleeding complications, outcome including in-hospital and 3 month incidence of DVT and pulmonary embolism (PE) were analyzed.
Results: Overall male to female ratio was 2.4:1. 78.4% of patients were minor and 21.6% were major trauma. Mean age was 44.0 years (14.0–100.0) for minor and 46.0 years (16.0–91.0) for major trauma patients with a mean hospital LOS of 4.6 days (1.0–86.0) and 17.2 days (1.0–133.0) respectively ($P < 0.0001$). Mean intensive care LOS was 0.1 days (0.0–7.0) for minor and 2.3 days (0.0–46.0) for major trauma ($P < 0.0001$). No pharmacological prophylaxis was used in 36.6% of minor and 7.7% of major trauma patients. Unfractionated heparin (UH) was used in 56.3% of minor and 88.5% of major trauma patients and low molecular weight heparin (LMWH) in 7.0% and 3.8% respectively. Mechanical prophylaxis was used in 70.4% of minor and 96.2% of major trauma patients. Major bleeding rates for major trauma were 10.7% with no pharmacological prophylaxis and 34.8% with use of UH. For minor trauma patients, the overall in-hospital VTE incidence was 1.0% (0.3% for DVT and 0.7% for PE). For major trauma patients, the overall in-hospital VTE incidence was 7.8% (7.0% for DVT and 0.8% for PE). At 3 months, the VTE incidence for minor trauma patients was 1.2% (0.5% for DVT and 0.7% for PE) and 9.0% (7.4% for DVT and 1.6% for PE) for major trauma patients. Mortality rates were 0.6% for minor and 7.8% for major trauma.
Summary/Conclusion: Our in-hospital incidence of VTE in trauma patients was kept relatively low with use of prophylactic protocols. The 7.8 fold increase in the in-hospital VTE incidence for major trauma together with the two fold rise in PE rates at 3 months reinforces the importance for maximizing optimal thromboprophylaxis relative to risk factors, type and severity of injury, surgical intervention, hospital LOS and bleeding risk. Selective ultrasound screening in patients unable to receive pharmacological prophylaxis due to bleeding risk or inability to use mechanical prophylaxis may be of benefit.

PB 3.64-3

A swine model for pulmonary embolism with autologous clot mimicking sub-massive PE

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Background: Safer treatments are needed for severe, submassive pulmonary embolism (PE). Since rodent models are inadequate, a standardized reliable and reproducible large animal model of submassive PE that reproduces physiological abnormalities observed in humans with severe submassive PE would facilitate new drug development.
Aims: Create a swine model of PE with autologous clot, which would resemble severe, submassive PE in humans
Methods: Domestic pigs ($N = 5$ 30–40 kg body weight) were sedated and placed on isoflurane anesthesia. The femoral vein and artery were cannulated to permit blood sampling and pressure measurements, respectively. For autologous clot formation, 2 mL/kg of blood volume was taken and replaced 3:1 with 0.9% NaCl. Whole blood was incubated for 37 °C for 60' in 12 mm diameter glass tubes. Cylindrical clots were removed, washed with warm saline, and loaded into Tygon silastic tubing (ID 6.35 mm, OD 7.94 mm). A 7F Edwards Scientific thermodilution Swan-Ganz catheter was placed via ultrasound guidance in the right external jugular. A cut-down was performed and left external jugular isolated, silastic tubing was introduced into the vein, and clot was gently introduced. Clot was confirmed to be present in the heart with live echocardiography and fluoroscopy. All measurements were taken prior to and 1 h. after delivery of clot.
Summary/Conclusions: Infusion of ~0.5 g/kg clot produced tachycardia (106 ± 6 beats/min), pulmonary hypertension (sPAP 45 ± 4 mm Hg), and mild hypoxemia (95 ± 1%), and a 20 ± 7% decrease in cardiac output. Increasing the clot burden to ~0.7 g/kg showed immediate changes consistent with marginally compensated shock. Heart rate increased to 121 ± 3 beats/min), a marginally decreased systolic arterial blood pressure (101 ± 6 mm Hg), without hypotension, but with severe right ventricular dysfunction, pulmonary hypertension (sPAP 60 ± 2 mm Hg), and hypoxemia (91 ± 1%). At this level a 30 ± 8% decrease in cardiac output was seen. The mean change from basal control values to +60 min post infusion of clot mass of 0.7 g/kg was as follows: mean arterial pressure (MAP) = 80 ± 5 vs. 64 ± 3 mm Hg; heart rate increased from 81 ± 3 to 119 ± 6 beats/min; oxygen saturations dropped from 98 ± 0 to 91 ± 1%; the systolic pulmonary arterial pressure (mPAP) increased from 10 ± 2 vs. 36 ± 2 mm Hg and cardiac output dropped from 2.2 ± 0.1 to 1.6 L/min, PCWP increased from 8 ± 1 to 18 ± 3 mm Hg, and PVR increased from 72 ± 11 to 742 ± 54 dyne*S/cm5. Cardiac arrest occurred as a result of rapidly injecting clot at 0.9 g/kg.
Conclusions: In anesthetized domestic swine, 0.7 g/kg autologous, cylindrical clots instilled via the external jugular vein elicit the pulmonary vascular, hemodynamic and echocardiographic responses of humans with severe submassive PE. This model will be useful for preclinical testing of new fibrinolytic agents in preparation for phase I human studies.

PB 3.64-4

Clinical outcome and prognostic factor of cerebral venous sinus thrombosis in Siriraj hospital

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Background: Cerebral venous sinus thrombosis (CVST) is an uncommon disease with the potentially serious consequences. The clinical outcome of patients with CVST varied from complete recovery to permanent neurological deficits and death.
Aims: To describe the outcome and the prognostic factor.
Methods: Medical records of patients with CVST in Siriraj hospital between year 2001 and 2009 were reviewed retrospectively. Collected data included clinical features, thrombotic risks, imaging result, treatment, and outcome until the second visit after discharge. Patients without data of clinical status at the time of hospital discharge were excluded. Bleeding complication after discharge was also reviewed. The outcome was categorized into two groups with a simple and practical scale: good outcome (able to walk) and poor outcome (need wheelchair, bedridden, and death).

Results: One hundred and fourteen patients (103 women) had a median age of 34.0 years (range 21–65). Most common thrombotic risk was oral contraceptives (30.9%) whereas congenital deficiency of natural anticoagulants was found in 10/110 (9.1%). Mean length of hospital stay was 16.7 + 4.2 days (range 2–25). On discharge date, 29 patients (25.4%) had a poor outcome (five deaths). In the univariate analysis, factors significantly related to the poor outcome were male sex, older age, weakness, cranial nerve palsy, seizure, coma, blurred vision, papilledema, >2 thrombotic risks, number of sinus involvement, involvement of sagittal or transverse sinus, intracerebral hemorrhage, cerebral infarction, and cerebral edema whereas factors associated with the good outcome were Delta's sign, low-molecular-weight heparin use, and warfarin use. In the multivariate analysis, coma and number of sinus involvement were significantly associated with the poor outcome. Poor outcome rate was 90% (9/10) in patients with coma. Among patients without coma, poor outcome was 2.6% (2/77), 60% (12/20), 100% (6/6) for the number of sinus involvement of 1, 2, >3 sinuses, respectively. Among alive patients with poor outcome at discharge, status at follow-up visit (median follow-up time = 7 weeks; range 4–12) was improved in 7/24 and worse to death in 3/24. Major bleeding complication occurred only in the poor-outcome group (2/24 patients; INR = 9.8 and 10.2).
Conclusions: At a 7-week follow-up after discharge, rate of poor outcome was 21.9% whereas mortality rate was 7%. Coma and multiple venous sinus involvement were poor prognostic factors of cerebral venous sinus thrombosis.

PB 3.64-5

Implementation of a venous thromboembolism prophylaxis program in Brazilian hospitals: VTE Safety Zone Brazil

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Background: The ENDORSE study showed that 46% of medical patients in Brazil are at risk for venous thromboembolism (VTE), but only 59% of these were receiving prophylaxis according to the American College of Chest Physicians (ACCP) guidelines. The implementation of hospital prophylaxis programs based on passive distribution of VTE risk-assessment algorithms and continuous medical education are not sufficient to improve the utilization of prophylaxis.
Aims: (i) To evaluate the impact of a hospital prophylaxis program (VTE Safety Zone) implementation based on the standardized distribution of VTE risk-assessment algorithms, lectures and the participation of a hospital VTE prophylaxis committee (VTEPC) on the adequacy of VTE prophylaxis in medical patients; (ii) To compare the evaluation of VTE risk by the ACCP and the Brazilian Guideline for VTE Prophylaxis, and (iii) To evaluate the impact of VTE Safety Zone with and without a formal VTEPC on the utilization of prophylaxis.
Methods: We performed a cross-sectional survey, during a single day, in medical patients admitted to 11 Brazilian hospitals that participated in ENDORSE and that were randomized to be implemented during 12 months with one of two groups of the VTE prophylaxis program. The more intensive (MI) group included a formal VTEPC supported by the hospital director. The rates of adequacy of prophylaxis were compared between the two hospital groups, between the two guidelines recommendations and also with the 649 historical controls evaluated during ENDORSE.
Results: Data of 725 patients were collected: 400 (55%) patients in the MI group and 325 (45%) in the less intensive (LI). The mean age of patients was 65 ± 14 years-old, 50% were men and had a median of 10 ± 8 days of hospitalization. We found that 15% of patients were obese, 57% had reduced mobility, 96% had at least one risk factor (RF) for VTE; mean of 3 RF/patient, but higher in the MI group (3.6 ± 2 vs. 2.4 ± 1, $P < 0.0001$). The more frequent RF were age ≥55 years (75%), intensive care admission (31%), infection (25%), central venous catheter (21%), chronic pulmonary disease (21%), heart failure (19%), pneumonia (19%) and cancer (16%). At least one RF for bleeding was present in 32%. According to ACCP, 54% of the medical patients were at-risk for VTE and 73% had adequate prophylaxis, which was significantly more than the 59% reported in ENDORSE. According to the Brazilian Guideline, 89% were at-risk and 60% had adequate VTE prophylaxis. The adequacy was significantly higher in the MI group as compared with the LI, according to the Brazilian Guideline (73% vs. 50%, $P < 0.0001$).
Conclusions: There were important improvements in the adequacy of prophylaxis in medical patients admitted to Brazilian hospitals between the ENDORSE and the VTE Safety Zone studies. According to the ACCP criteria, after the implementation of the VTE Safety Zone program, more patients at-risk were identified and consequently more patients were receiving adequate VTE prophylaxis. Including a formal VTEPC into a hospital prophylaxis program seems essential to achieve higher goals of adequacy of VTE prophylaxis in medical patients.

PB 3.64-6

The influence of the thrombotic burden on D-dimer plasma levels in acute symptomatic deep vein thrombosis of the lower limbs

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Background/Aims: Thrombotic burden might influence the concentration of D-dimer in patients with acute symptomatic deep vein thrombosis (DVT) of the lower limbs. Patients with small thrombi may thus present with relatively low concentrations of D-dimer. To establish whether D-dimer levels reflect the extent of acute deep vein thrombosis (DVT) of the lower limbs. Design: cross-sectional, single centre study.
Patients and Methods: We evaluated consecutive patients with suspected DVT of the lower limbs referring to our vascular emergency department from 2002 to 2009. All patients underwent prestest clinical probability (PCP), compression ultrasonography and D-dimer testing (STA, Stago, cut-off: 500 ng/mL).
Results: We investigated 1187 patients (M:477 – 40%, mean age 65, median 68, range:12–97) among whom 233 proximal DVTs (20%) and 164 distal DVTs (14%) were diagnosed. Prestest clinical probability was likely in 194 (83%) of patients with proximal DVT and in 95 (57%) of subjects with distal DVT. D-dimer levels were higher in patients with proximal DVT (mean: 4.38 μg/mL, median: 3.55 μg/mL; range:0.39–23.65 μg/mL) than in patients with distal DVT (mean: 1.23 μg/mL; median:0.71 μg/mL, range:0.01–12 μg/mL) ($P < 0.0001$) and patients without DVT (mean 0.80 μg/mL, median: 0.46 μg/mL, range:0.01–11 μg/mL) ($P < 0.0001$). D-dimer was below the cut-off in 38% of patients with distal DVT and in 2% of patients with proximal DVT. Among patients with unlikely PCP and D-dimer below the cut-off, distal DVT was diagnosed in 16% and proximal DVT in 0.83%. D-dimer levels were not correlated with the extent of proximal DVT.
Conclusions: D-dimer levels are higher in patients with proximal DVT than in patients with distal DVT. Patients with acute small DVT might present with low concentrations of D-dimer, thus affecting the yield of diagnostic algorithms in suspected acute DVT of the lower limb.

PB3.65 – Hormones, pregnancy, women's issues – III

PB 3.65-1

Does systematic risk assessment in pregnancy identify women at risk for venous thromboembolism and so avoid thrombosis? Experience of an 18 month programme based on national guidance

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Background: Pulmonary embolism (PE) remains the leading direct cause of maternal mortality in the UK, whilst pregnancy related DVT may result in significant morbidity. Venous thromboembolism (VTE) has an overall incidence of 1–2/1000 pregnancies. The UK Royal College of Obstetricians and Gynaecologists (RCOG) Green-top guidance 37a (2009) recommends risk assessment (RA) for VTE in early pregnancy and at delivery to guide decisions on thromboprophylaxis. A schema was derived from the RCOG guidance, based on personal and family history of VTE and identified risk factors such as age, multiparity, raised body mass index, thromophilia and the means of delivery. Routine midwife lead RA was introduced at the initial early pregnancy 'booking' visit, with women catagorised as low, intermediate or high risk for VTE by pro-forma. Referral of high risk women to a Joint Haematology Obstetric Clinic enables specialist assessment and consideration for thromboprophylaxis. RA is repeated post delivery, using a hospital prescription chart based schema, or in the event of antenatal admission to hospital.

Aim: To study the effectiveness of a risk assessment schema in identifying at risk women and the prevention of VTE.

Method: Information was collected on patients who developed VTE in pregnancy or the post partum, known to the Joint Haematology Obstetric Clinic. Data from the available pro-forma records was collated for RA catagorisation, or otherwise this was derived from the available clinical and electronic records. Pregnancy outcomes and complications were assessed from the obstetric record.

Results: Over an 18 month period of study, there were a total of 7533 pregnancies at RCHT. Twelve cases of VTE were identified, five PE's and seven DVT's, an incidence of 1.59/1000 pregnancies. Seven cases occurred ante-natally, with five cases in the puerperium. There were no maternal or fetal deaths. Booking RA pro-formas were identified for 10 women and an RA calculated for the two other cases, all subjects being catagorised as low risk. For those five women with VTE in the puerperium, three had a RA recorded post delivery, one assessed as low risk for VTE and two at intermediate risk, while for the two other women their RA. derived from the clinical record, was one at low risk and one at intermediate risk. Only one of three women at intermediate risk for VTE, who by the schema were to be offered heparin thrombophrophylxis, had documentation of this proposed management but she declined therapy.

Conclusion: The observed incidence of 1.59/1000 pregnancies is comparable to the literature. Despite a low RA catagorisation seven women sustained ante-natal VTE, suggesting that this ante-natal schema may not identify risk or avoid VTE. For those post partum VTE cases 60% were classified as intermediate risk, however none received heparin thromboprophylaxis, which may reflect failure to implement new practice and the need for continuing education. Further prospective data should be collected for the schema, with determination of any additional evident risk factors, specifically in antenatal VTE, with comparison to other Units with a similar obstetric practice but with alternative RA schemas.

PB 3.65-2

Incidence of pregnancy outcomes in the conventionally treated purely obstetric antiphospholipid syndrome, pregnancy loss subtype: the NOH-APS observational study

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Background: The incidence of pregnancy outcomes in the purely obstetric form of antiphospholipid syndrome (APS) treated according to guidelines is uncertain.

Aims: We aimed to design a prospective study in women selected on the same clinical criteria, according to the presence of APS, or to the absence of any thrombophilia.

Methods: We performed a 10-year observational study of 1592 non-thrombotic women who had experienced three consecutive spontaneous abortions before the 10th week of gestation or one foetal death at or beyond the 10th week of gestation (*NOH-APS, Blood 2012;119 (11):2624–32*).

We compared the frequencies of pregnancy outcomes among women positive for antiphospholipid antibodies (n = 517; treatment: prophylactic LMWH plus low-dose aspirin LDA), women carrying the *F5* 6025 or *F2* rs1799963 polymorphism (n = 279; recurrent abortions: no treatment; foetal death: prophylactic LMWH), and women with negative thrombophilia screening results (n = 796; no treatment).

We report here the results comparing APS women and thrombophilia negative women.

Results: In control women with negative thrombophilia screening results, initial foetal loss was associated with an increased risk of early spontaneous abortion and of foetal death; of pre-eclampsia (PE), severe PE, PE before 34 weeks and PE with delivery of a small-for gestational age neonate (SGA); of any ischemic placenta disease (IPD); of SGA neonate; of preterm life birth before 37 weeks and before 34 weeks.

In women with recurrent abortions, treated APS women were at increased risk of foetal death; of PE, severePE, PE before 34 weeks and PE with delivery of a SGA neonate; of any IPD; of neonatal death. A trend ($P < 0.15$) for an increased risk of premature birth before 37 weeks and before 34 weeks, for birth of a SGA neonate and for early neonatal mortality was also detected.

In women with foetal death, treated APS women were at lower risk of early abortion and of foetal death. They were at higher risk of PE and of PE before 34 weeks. A trend for an increased risk of premature birth before 37 weeks, of severe PE, of HELLP syndrome, of PE with a SGA neonate and of late neonatal mortality was also detected.

Summary/Conclusions: In women with a purely obstetric APS syndrome attempting a new pregnancy treated according to guidelines, the relative risks of the various pregnancy outcomes depend on the initial clinical presentations, which by themselves impact on the reference prognosis. Treatment does not overcome the risk of pre-eclampsia. Neonatal mortality rates remain increased. New therapeutic options are thus needed.

PB 3.65-3

Prevalence of hereditary thrombophilia is the highest in women who developed pregnancy related thrombosis during first trimester

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Background: Venous thrombosis is one of the leading causes of maternal morbidity and mortality. In women of reproductive age, over half

of all venous thrombotic events are related to pregnancy and puerperium.

Aims: We evaluated the presence of hereditary thrombophilia in women with thromboembolic complications during pregnancy and puerperium.

Methods: We conducted a retrospective analysis of 143 consecutive women that developed thromboembolic complications during pregnancy or puerperium (6 weeks after the delivery), and who were referred to our institution for thrombophilia testing from January 2004 to October 2012. Venous thromboembolism was defined as deep vein thrombosis (DVT) of upper or lower extremities, cerebral vein thrombosis and pulmonary embolus (PE). When deep vein thrombosis of lower extremities was present with PE, the event was accounted as PE. Women with thrombosis of superficial veins were excluded from this study. All episodes of venous thromboembolism were confirmed with objective methods (duplex ultrasonography, CT angiography, perfusion scintigraphy, NMR angiography). In all women following causes of hereditary thrombophilia were tested: factor V Leiden and prothrombin G20210A mutations, antithrombin deficiency, protein C deficiency and protein S deficiency. Blood for thrombophilia testing was obtained at least 3 months after cessation of anticoagulant therapy.

Results: Out of 143 women with pregnancy related thrombosis, 54 (38%) developed thromboembolic complications during pregnancy and 89 (62%) during puerperium. The presence of congenital thrombophilia was detected in 29 (54%) women who developed thrombosis during pregnancy and in 23 (46%) women with thromboembolic complications during puerperium.

Out of 54 women who developed thrombosis during pregnancy, 15 (28%) had thromboembolic event in the first trimester of pregnancy, 11 (20%) in the second and 28 (52%) in the third, respectively. Prevalence of hereditary thrombophilia among women with thrombosis in the first, second and third trimester of pregnancy was 72%, 50% and 53%, respectively.

In women who developed thrombosis during pregnancy distribution of thrombosis according localization was as follows: proximal vein thrombosis of lower extremities in 38 (70%), distal veins in 8 (16%), PE in 5 (9%), cerebral vein thrombosis in 2 (4%) and subclavio-axillaris thrombosis in 1(1%). On the other hand, in women with occurrence of thrombosis during puerperium was: proximal vein thrombosis in 42 (47%), distal vein in 21 (23%), PE in 17 (19%), ccerebral vein thrombosis in 8 (10%), subclavio axillar thrombosis in 1(1%).

Summary/Conclusions: We observed the highest prevalence of hereditary thrombophilia in women who developed thrombosis during first trimester of pregnancy compared to advanced stages of pregnancy or puerperium. This finding may indicate the importance of acquired factors in occurrence of thrombosis in second and third trimester of pregnancy and during puerperium. Recognition and elimination of these factors may play an important role in prevention of pregnancy related thrombosis. Two times higher prevalence of PE in puerperium than during pregnancy may be a consequence of underdiagnosing this complication (reluctance to use CT angiography or perfusion scintigraphy) during pregnancy although we cannot exclude the real difference in prevalence of thrombosis during puerperium and during pregnancy.

PB 3.65-4

Protein Z deficiency and Lipoprotein (a) increase are the most frequent abnormalities in women with recurrent miscarriage

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Introduction: More than 1–2% of women suffer from recurrent miscarriage (RM), vascular complications of pregnancy or implantation failure after assisted reproduction. Guidelines recommend ruling out chromosomal or anatomical abnormalities as well an antiphospholipid syndrome or thyroid dysfunction. In Europa, complete thrombophilia work up is commonly requested. By metaanalysis hereditary thrombophilia (e.g. FV-Leiden-/prothrombinmutation) is questionable in RM. However, low Protein Z (PZ)-levels (<1000 μg/L) (inherited or acquired due to antibodiesare associated with vascular complications in pregnancy (OR 4.17) (Sofi 2010). Lp(a) influences the fibrinolytic system which is important in implantation and placental development and elevated Lp(a) (>30 mg/dL) has been reported in women with vascular complications in pregnancy (e.g. preeclampsia). Elevated FVIII is not associated with poor pregnancy outcome (Middledorp 2004).

Material and Methods: Over 1 year 107 women, median age 32 years, were transferred to our coagulation outpatient clinic to rule out hypercoagulability in women with RM. In all women laboratory work up consists of: TSH, Lp(a), Homocysteine (fasting level), FV- Leiden- and Prothrombinmutation, Antithrombin, Protein C, free Protein S, Protein Z, FXII, Lupusanticoagulant (dRVVT, PTT-LA), Anti-Cardiolipin IgG and IgM, Anti-beta2-GPI IgG, Anti-Prothrombin IgG and Anti-Phosphatidylserine IgG; BMI calculation and medical history, also in complete pregnancy, were documented.

Results: In 32% of our cohort no laboratory abnormality could be detected. 13% had at least two abnormalities. FV-Leiden-/prothrombinmutation were present in 7%, no Antithrombin deficiency; Protein C and S deficiency in 3% and low FXII-levels in 2% and antiphospholipid syndrome in 6%. Hyperhomocysteinemia was detected in 2%. Decreased Protein Z was detected in 20% and Lp(a) >30 mg/dL in 16%. Mean BMI 24.8 (range 17.6–39.1). Abnormal thyroid function was present only in 3%, while some women were already on thyroid medication when investigated.

Discussion: RM is a multifactorial disorder; increased maternal age, BMI, thyroid function as well certain coagulation abnormalities influence pregnancy outcome. The frequency of FV-Leiden-/prothrombinmutation as well as hereditary deficiency of Protein C and S and FXII-deficiency reflects the prevalence found in normal population and therefore cannot be causal in RM, solely. In our cohort low PZ-levels and elevated Lp(a) were the most frequent abnormalities. Such risk factors may have a potent influence on early implantation and placental development. The question remains, which treatment options are feasible: preconceptional ASA and/or LMW Heparin as in women with APS?

PB 3.65-5

Elevated endogenous thrombin potential prior to clinical diagnosis of preeclampsia

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Background: Preeclampsia is a multisystem disorder of unknown cause manifesting in the second half of pregnancy. Endothelial cell dysfunction, enhanced platelet aggregation, activation of coagulation and increased vascular resistance may be involved. Increased thrombin generation was described in patients with preeclampsia and in women with a history of preeclampsia. The measurement of thrombin generation with calibrated automated thrombography is a global function test of the hemostatic system, which allows sensitive detection of prothrombotic states.

Aim: We investigated whether increased thrombin generation is measurable in early pregnancy, before the clinical diagnosis of preeclampsia.

Methods: Blood of 56 pregnant women was drawn repeatedly within the duration of pregnancy. Five women were excluded from the study due to heparin treatment. Eleven women developed a preeclampsia during pregnancy. Blood of six women was drawn before the clinical diagnosis of a preeclampsia. Measurements of thrombin generation were performed in platelet poor plasma using calibrated automated

thrombography. Each sample was measured with PPP reagent (5 pM tissue factor) and MP reagent (no tissue factor). Lag time, time to peak, start tail, peak height and endogenous thrombin potential were calculated from the obtained thrombin generation profiles. In addition prothrombin fragment F1, F2 and thrombin antithrombin complex were measured. All pregnant women were split into groups depending on gestational weeks. Within these groups all parameters were statistically evaluated. Differences between women with an uncomplicated pregnancy and women with a preeclampsia were calculated using *t*-test or Man- Whitney U test.

Results: No differences were found in the dynamic parameters of thrombin generation (lag time, time to peak, start tail) between women with an uncomplicated pregnancy and women with preeclampsia. In the same gestational group, peak height, prothrombin fragment F1, F2 and thrombin antithrombin complex were similar between women prior to diagnosis of preeclampsia and women with a uncomplicated pregnancy. Women who developed preeclampsia exhibited an increased endogenous thrombin potential compared to women with an uncomplicated pregnancy that became statistically significant already at the 16th week of gestation.

Conclusion: Our preliminary results suggest that a high endogenous thrombin potential is associated with the development of preeclampsia. Tthe measurement of thrombin generation with calibrated automated thrombography may have predictive value. A prospective study of larger scale is in preparation.

PB 3.65-6

Effect of prophylaxis with LMWH on implantation in women undergoing assisted reproductive procedures (IVF or ICSI): an interim report of a prospective randomized study

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Several studies have shown that a pharmacological prophylaxis in women subjected to MPA treatment, lead to an increase of the number of taken oocytes as well as higher rates of implantation and pregnancy.

An experimental strategy employed to improve embryo implantation and possibly positive pregnancy outcome was attributed to the use of heparins, particularly low molecular weight heparin (LMWH).

Aims of the study: The aim of the study is to test whether prophylactic dose of LMWH could improve the implantation rate in women with inherited thrombophilia or a history of two or more than two consecutive implantation failures.

Study population: Women consecutively referred to the Medically Assisted Procreation (MAP) Center of Gynecology Clinic of Padua are considered eligible for the study. All women undergoing intrauterine insemination techniques are excluded because of the inability in this first level technique to ensure fertilization.

Inclusion criteria are: heterozygosity of inherited common thrombophilias (FVL, PTm), hetero/homozygosity of MTHFR, two or more than two previous implantation failures, age >18, informed consent

Exclusion criteria are: deficiency of antitrombin, protein C, protein S, antiphospholipid antibodies, homozygosity or double heterozygosity for FVL/PTm, history of venous thromboembolism.

Women enrolled in the study are randomly divided into two groups. One group receives prophylaxis with LMWH (dalteparin 5000 UI/die) (cases), the other does not (controls).

Cases receive prophylaxis with LMWH from the beginning of MPA procedures to the positive/negative outcome (implantation/implantation failure).

Statistical analysis: The implantation rate in procedures of medical assisted reproduction (whether by IVF or through ICSI technique) is 20–25%. The results of an our previous observation evidenced a risk reduction for implantation failure of 50% [rRR 0.5 (95% IC 0.3–0.8)] in women under prophylaxis. Then, about 257 women in both groups (with and without prophylaxis) need to be enrolled in the study: a power of the study of 90% is needed to demonstrate a reduction of implantation failure of 30% with a statistical significance of 5%, by considering a drop-out of 15%. Statistical analysis is performed by SPSS version 17.0.

Preliminary Results: From September 2012, 50 women have given their consent to participate in the study. At present 20 women have completed a course of procedures: 10 in the group of cases (mean age 39 years, two homozygous for MTHFR C677T, five heterozygous for MTHFR C677T, seven with more than two implant failure) and 10 in the control group (mean age 40 years, one homozygous for MTHFR C677T, four heterozygous for MTHFR C677T, seven with more than two implant failures). Four implants occurred in the group of cases and one occurred in the control group. The odds ratio was 6.0 (95% CI 0.54–68).

Conclusions: The results of this ongoing study are clearly preliminary due to the small number of women evaluated so far. This interim report shows a trend toward a potential advantage of anticoagulant prophylaxis in the improvement of the implantation rate in MAP.

PB3.66 – Inflammation: Clinical – II

PB 3.66-1

New insight on the systemic inflammation response syndrome (SIRS) in STEMI patients undergoing primary percutaneous intervention: clinical features, blood markers, and prognostic significance

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Background: Acute myocardial infarction (MI) is recognized as a classical trigger to the development of a systemic inflammation response syndrome (SIRS).

Aims: This study prospectively explored how the traditional SIRS criteria, with or without selected blood markers, could help predict prognosis and orient management in the contemporary era of reperfusion therapy.

Methods: A predictive model for 90-day rates of death and of death or shock was built using clinical variables and blood markers obtained at hospital admission in 337 consecutive patients with ST-segment elevation MI (STEMI) oriented promptly to primary percutaneous intervention.(PPCI).

Results: The traditional SIRS criteria used alone and in combination were found to poorly correlate with clinical outcome and markers. Using ROC curve analyses, a modified SIRS definition that includes ≥3 of 4 of the criteria of age ≥75 years, heart rate >90 beat/min, systolic blood pressure ≤130 mm Hg and white blood cell count of $>10 \times 10^9$/L was strongly predictive of 90-day death (odds ratio, 11.0; 95% CI, 4.2–28.9, $P < 0.001$) and of death/shock (10.8; 4.8–24.0, $P < 0.001$). This model was associated with high levels of blood markers at baseline (IL-6, NT-proBNP and glucose levels $P < 0.001$; C-reactive protein (CRP) $P = 0.004$, and TNFα $P = 0.005$). Incorporating CRP, NTproBNP, and IL-6 in the modified SIRS definition added independent significant prognostic value over SIRS criteria alone (odds for death, 8.0; 95% CI, 2.5–25.9; 9.1, 2.3–35.4; and/or death/shock 5.0, 1.7–15.1).

Summary/Conclusions: A SIRS-like syndrome supported by inflammation markers can be identified at first medical contact and before PPCI in STEMI, and is strongly predictive of death and death or cardiogenic shock during follow-up.

PB 3.66-2

Do therapeutic infliximab concentrations influence TAFI and PAI-1 plasma levels in IBD patients?

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Background: Inflammatory bowel disease (IBD) patients are three times more prone to develop venous thromboembolism (VTE) compared to controls and reports on the influence of anti-TNF treatment (e.g. infliximab) have been controversial. Hypofibrinolysis is a possible mechanism for this extra-intestinal complication since increased Thrombin Activatable Fibrinolysis Inhibitor (TAFI) and Plasminogen Activator Inhibitor-1 (PAI-1) levels are associated with a risk for VTE.
Aim: To investigate the effect of infliximab induction therapy (week 0 vs. week 14) on TAFI and PAI-1 levels in IBD patients.
Methods: In a prospective study, 78 IBD patients (46 Crohn disease and 32 ulcerative colitis) and 60 healthy controls (HC) participated with informed consent. Trough Levels of Infliximab (TLI) were measured before therapy (w0) and after induction (w14). Levels of total PAI-1, active PAI-1, TAFI antigen (proenzyme) and activation peptide of TAFI (AP, marker for TAFI activation expressed as percentage relative to a normal plasmapool [%PP]) were determined by in-house developed ELISAs. Standard inflammation markers e.g. C-reactive protein, platelets and white blood cells were determined by routine analyses.
Results: Out of the 78 patients, nine were primary non-responders (did not show clinical benefit within 14 weeks) and 14 were secondary non-responders (had good initial response but lost response over time; median TLI of 0.3 μg/mL at w14 [IQR: 0.3–4.6]). These non-responders were excluded in the further analyses. The 55 responders had a median TLI of 4.3 μg/mL at w14 (IQR: 2.5–8.5).
At w0, responders had significantly higher total PAI-1 and active PAI-1 levels compared to HC (36 ng/mL vs. 28 ng/mL, $P = 0.01$ and 9.0 ng/mL vs. 5.6 ng/mL, $P = 0.02$ for total and active PAI-1, respectively). Neither TAFI antigen (16 μg/mL vs. 13 μg/mL) nor AP (117%PP vs. 108%PP) levels were elevated in responders compared to HC. At w0, the total and active PAI-1 levels were significantly higher in responders with a CRP level above 20 mg/L compared to responders with a CRP level below 5 mg/L (53 ng/mL vs. 33 ng/mL, $P < 0.01$ and 11 ng/mL vs. 6.7 ng/mL, $P = 0.03$ for total and active PAI-1, respectively).
During IFX treatment (w0 vs. w14) responders showed a significant decrease in CRP (8.0 mg/L vs. 1.4 mg/L, $P < 0.01$), WBC (7.3×10^9/L vs. 6.2×10^9/L, $P < 0.01$), platelets (336×10^9/L vs. 285×10^9/L, $P < 0.01$) and total PAI-1 levels (36 ng/mL vs. 31 ng/mL, $P < 0.01$). No difference was observed for TAFI, AP and active PAI-1 levels.
Conclusion: Before anti-TNF treatment, total and active PAI-1 levels are increased in IBD patients. Therapeutic levels of infliximab have an impact on total PAI-1 levels but not on TAFI, AP or active PAI-1 levels. The decrease of total PAI-1 after control of inflammation might be partially explained by the significant drop in platelets, containing a high concentration of PAI-1. In addition, none of the responders in this study was diagnosed with thrombosis.

PB 3.66-3

Pulmonary tuberculosis is associated with a systemic but not intrapulmonal procoagulant state

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Background: Tuberculosis (TB), caused by *Mycobacterium tuberculosis complex*, is a devastating infectious disease with a high mortality. Bangladesh is one of the most affected countries. Knowledge of the contribution of coagulation and fibrinolysis to the host response to TB is highly limited.
Aims: In this observational study performed in Chittagong, Bangladesh, we aimed to obtain insight into systemic and local (at the site of the infection) activation of coagulation during pulmonary TB.
Methods: Parameters of coagulation and fibrinolysis were measured in plasma of 64 patients with primary TB, 11 patients with recurrent TB and 37 healthy local controls. TB was proven by positive Ziehl-Neelsen staining of the sputum and confirmed by PCR. Additionally, nine patients underwent a bronchoscopy with a bilateral bronchoalveolar lavage (BAL) and measurements were performed in BAL fluid from TB-infected lung subsegments and contralateral control subsegments.
Results: Patients with pulmonary TB showed evidence for a systemic procoagulant state as reflected by enhanced coagulation (elevated plasma levels of thrombin-antithrombin complexes and D-dimer; prolonged PT and PTT; dysregulation of individual clotting factors) and impaired anticoagulation (reduced plasma levels of antithrombin and protein C). In addition, plasma tissue-type plasminogen activator, alpha-2-antiplasmin and von Willebrand factor levels were elevated, reflecting a fibrinolytic response and endothelial cell activation respectively. None of these alterations were detected in BAL fluid of TB-infected lung subsegments when compared to control subsegments.
Conclusion: Pulmonary tuberculosis is associated with a systemic but not intrapulmonal procoagulant state.

PB 3.66-4

Blood cell response to a fatty meal in healthy subjects at different degree of cardiovascular risk: effect of orange juice intake

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Background: A fatty meal has been proposed as a model of acute oxidative stress. Its effect on blood cells needs to be characterized and its possible modulation by polyphenols evaluated.
Methods: The acute effect of a standardized fatty meal on platelets and leukocytes and their interactions was evaluated on 61 apparently healthy subjects (48.6 ± 13.1 years old) at different degree of cardiovascular risk. Subsequently, 18 subjects were randomized, by a cross-over design, to drink during the meal, one liter of either blood or blond orange juice (OJ) (rich or poor, respectively, in anthocyanins), or water (control). Before and 2 h after fatty meal, blood cells were counted and markers of activation measured by flow-cytometry (intra-PMN myeloperoxidase-MPO; leukocyte Mac-1; platelet P-selectin; platelet-leukocyte conjugates).
Results: After the fatty meal, plasma triglycerides-TG (134.6 ± 68.8 vs. 181.5 ± 84.6 mg/dL, before vs after, $P < 0.0001$ by paired t; mean ± SD, $n = 61$) and both leukocyte and platelet counts significantly increased, more markedly in subjects with lower cardiovascular risk. Mac-1 expression too increased (32.2 ± 27.2% vs. 45.6 ± 29.0%, $P = 0.0016$), and MPO decreased (83.1 ± 16.3% vs. 64.5 ± 23.1%, $P < 0.0001$); platelet activation and interaction with leukocytes increase was not significant. Women were more susceptible to the fatty meal changes than men; age did not affect any cell response. BMI influenced platelet count increase, while waist-to-hip ratio affected PMN degranulation; the latter was smaller in subjects at higher risk (all $P < 0.01$). The meal-induced TG increase was reduced by blood OJ ($P < 0.05$); total cholesterol decreased after blood OJ ($P < 0.05$); glucose levels decreased after both OJ ($P < 0.01$). Leukocyte count increase and MPO decrease were significantly attenuated by both OJ. No OJ effect on platelet counts and activation.
Conclusions: Demographic variables and cardiovascular risk degree appear associated with cell response to fatty meal. OJ intake attenuated some of these responses, probably by anthocyanin-dependent and independent mechanisms.

PB 3.66-5

Higher thrombin generation during the luteal phase of a normal menstrual cycle does not depend on inflammatory activity

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Background/Aims: Higher thrombin generation (TG) during the luteal phase of a menstrual cycle cannot be explained by the effect of coagulation factors (submitted manuscript). In this report, we study inflammatory markers as a possible explanatory mechanism, and relate the results to fertility as expressed by the number of pregnancies and deliveries in a cohort of healthy women.
Methods: Our cohort consisted of 102 women not on hormonal medications. Blood samples were collected in the follicular (cycle day, cd 3–5) and the luteal (cd 22–25) phase. The inflammatory markers studied were PTX-3 (pentraxin-3), ICAM-1, VCAM-1, cathepsins L, B, S, hsCRP, e-selectin and p-selectin. Thrombin generation was assessed by the Calibrated Automated Thrombogram®. We used the paired *t*-test to study the differences in the values of the inflammatory markers during the two phases ($P < 0.05$), as well as multiple regression and correlation analysis (Pearson's) for the associations between markers.
Results: All inflammatory markers (except for CATHB and p-selectin) were higher during the follicular phase, however only PTX-3 and hs-CRP reached statistical significance ($P < 0.001$ respectively $P = 0.025$). There were no significant associations between TG and the inflammatory markers as well as the number of pregnancies and deliveries. PTX-3 during the follicular phase was associated with the number of reported pregnancies ($P = 0.014$).
Conclusion: Despite the close interactions between the inflammatory and haemostatic system, our results indicate that, during the menstrual cycle, higher inflammatory activity is associated with lower activation of the coagulation cascade, as expressed by TG. The rise in PTX-3 during the follicular phase was associated with the number of pregnancies for the women in our cohort, and thus indirectly linked with fertility. It is unclear whether our results can be explained by the action of a balancing mechanism that limits the activation of the coagulation cascade during the follicular phase, in order to protect from the higher thrombotic risk associated with inflammation.

PB 3.66-6

Coagulation profile in patients with H1N1 influenza A infection undergoing treatment for haematological malignancies

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Background: Immunocompromised patients, especially after allogeneic stem cell transplantation (HSCT) are prone to contract influenza A (H1N1) infection. This is a serious complication in patients with haematological malignancies most often resulting in low respiratory tract infection with progression to acute respiratory distress syndrome (ARDS) requiring mechanical ventilation and leading to increased mortality. Hemoptysis or low respiratory tract hemorrhage is frequently reported. Moreover, severe infection and inflammation are associated with activation of coagulation.
Aims: The aim of our study was to evaluate coagulation abnormalities in hemato-oncological patients with A (H1N1) influenza pneumonia by testing conventional coagulation parameters, as well as thromboelastometry and selected thrombophilia markers. The secondary objective was to identify coagulopathy abnormalities predicting a fatal influenza A (H1N1) infection outcome.
Methods: A total of 12 patients (median age 56, range 25–72) treated with conventional chemotherapy ($n = 10$) or allogeneic stem cell transplantation (two patients) were analysed. The underlying diseases included acute myeloid leukaemia ($n = 5$), acute lymphoblastic leukaemia ($n = 1$), myelodysplastic syndrome ($n = 1$), Hodgkin lymphoma ($n = 2$), non-Hodgkin lymphoma ($n = 2$) and chronic lymphocytic leukaemia ($n = 1$). None of the patients received pH1N1 vaccine. Influenza A (H1N1) was confirmed by real-time reverse-transcription polymerase chain reaction for pH1N1 of nasopharyngeal aspirates. All patients received antiviral therapy with oseltamivir in the dose 150 mg b.i.d. Thromboelastometry testing (ROTEM) was performed and selected coagulation parameters were measured at the time of diagnosis. The values of all the coagulation parameters were analyzed in two groups, the patients who survived and the ones who died in the course of the influenza.
Results: Hemoptysis was documented in 75% of the analyzed cases. ARDS developed in five patients. The course of the disease was complicated by Gram negative bloodstream infection in five patients and was fatal in four cases because of ARDS related to influenza. No difference in the mean value of prothrombin time, activated partial thromboplastin time, fibrinogen, fibrin degradation products, factor VIII activity, von Willebrand factor antigen (vWF) and in inhibitors of coagulation activities (free protein S, protein C, antithrombin) were observed between the analysed groups. A significant difference in the mean values of D-Dimer was observed between patients who survived and those who died (3258.1 ± 4187 vs. 7748 ± 7810 ng/mL; $P < 0.05$). Fatal cases had lower leukocyte counts compared with those who survived (0.50 ± 0.87 vs. 2.43 ± 1.51 G/L; $P = 0.0368$). The clot formation time (CFT) in INTEM, EXTEM, APTEM significantly differed between the groups and was longer in the patients who died. Similarly, C-reactive protein (CRP) was significantly higher in patients who developed ARDS and died (68.8 ± 85 vs. 179.12 ± 46.6 mg/L; $P = 0.0336$). In the cluster model, patients with lower leukocyte counts, lower protein C and free protein S activities with higher values of D-Dimer, CRP and vWF were assigned to the poorer outcome group.
Summary: In our cohort, patients with an increased risk of influenza complications and negative outcomes may be characterized by having higher D-Dimer, CRP and vWF and lower inhibitors of coagulation activities and lower leukocyte counts than the patients who survive.

PB3.67 – Inherited risk factors venous thrombosis: Clinical

PB 3.67-1

Molecular analysis of SERPINC1 abnormalities in 19 Japanese patients with hereditary antithrombin deficiency

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Background: Hereditary antithrombin (AT) deficiency is susceptible to thromboembolic diseases, particularly deep vein thrombosis of lower limb. To date, more than 200 different mutations responsible for AT deficiency have been identified. It is rare to find large deletions (>100-bp) of this gene (*SERPINC1*), very few cases have been analyzed in detail the deletion region. Traditionally, the direct sequencing analysis of *SERPINC1* is used for genetic survey of AT deficiency, but this method is not capable of detecting heterozygous large gene deletions because of the presence of a second normal allele. MLPA (Multiplex Ligation-dependent Probe Amplification) method came to be used for survey of candidate gene defects in recent years, large deletions came to be relatively easily detected.

Aims: To identify the causative gene abnormalities in patients with hereditary AT deficiency, we analyzed their *SERPINC1* genes.
Methods: The Ethics Committee of the Nagoya University School of Medicine approved this study. We analyzed all genomic sequences for exons and exon-intron junctions of *SERPINC1* gene by direct sequencing method. We also analyzed *SERPINC1* gene by MLPA method as well as real-time PCR to determine relative gene doses. We performed Long-PCR, Nested-PCR and mapping PCR in order to identify breakpoints of the deletion.
Results: We analyzed 19 unrelated Japanese cases with AT deficiency to detect *SERPINC1* abnormalities. We identified causative gene abnormalities in all cases; 8 distinct missense mutations in 11 cases, 1 nonsense mutation, 2 distinct small deletions in 3 cases, 1 small insertion, 1 splicing mutation, and 2 distinct large deletions, respectively. Both of the large deletions seemed to be unique gene abnormalities. One showed a 2094-bp deletion ranging from intron 4 to intron 5, and the other showed a deletion of approximately 130-kb losing the entire *SERPINC1* gene. Interestingly, the latter patient had an autoimmune symptom, and her deleted region of the genomic DNA contained *RC3H1* gene encoding the protein called 'roquin', which involved in repressing self-immune responses. It was reported that the mice with homozygous mutation in this gene developed antinuclear autoantibodies and had the typical features of systemic lupus erythematosus (SLE).
Summary/Conclusion: AT deficiency caused by heterozygous mutation of *SERPINC1* gene is susceptible to thromboembolic diseases, and most *SERPINC1* gene mutations such as the point mutations can be found by direct sequencing analysis. Some *SERPINC1* gene abnormality such as a heterozygous large deletion, however, cannot be detected by this method, because *SERPINC1* gene locates on chromosome 1 autosomally. By MLPA analysis, we identified 2 distinct heterozygous large deletions of *SERPINC1* gene, which showed normal patterns in PCR mediated direct sequencing analysis. MLPA method should be put into practice, when a heterozygous large gene deletion on autosomal chromosome is expected.

PB 3.67-2
Non-O blood group as a risk factor for cerebral venous thrombosis

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Background: The role of risk factors associated with the development of venous thromboses at unusual sites, including cerebral vein thrombosis (CVT), may be different compared to those of deep vein thrombosis of the lower limbs or pulmonary embolism, the most common manifestations of venous thromboembolism (VTE). In addition to pregnancy/puerperium, oral contraceptives, infection, hematological and autoimmune diseases, thrombophilic conditions are identified as predisposing conditions in patients with CVT. Increasing data recognize the ABO as a susceptibility locus for VTE, non-O blood group carriers sharing a two-fold increased VTE risk than O blood group carriers.
Aims: We addressed the role of ABO blood group as a risk factor of CVT. Methods. Blood groups from a retrospective-prospective cohort of 77 consecutive inpatients (21 men, 56 women, age at the event 39.97 ± 12.73 years, mean ± 1 SD) referred to our Centers from 1997 to 2012 because of a first episode of CVT were compared with data from a large population of asymptomatic Italian blood donors (n = 4272; 2277 males, 1995 females, mean age 34.7 + 10.08 years).
Results: Overall, the CVT episode was clearly associated with an established transient risk condition ('provoked' events) in more than half of patients (40/77, 51.9%). In particular, CVT occurred in 32/56 (57.1%) women while assuming oral contraceptives. Inherited thrombophilia, in the majority of cases due to the prothrombin G20210A polymorphism (n = 19), was detected in 23 subjects (29.9%), with higher rates in patients with apparently 'unprovoked' events (14/37, 37.8%, vs. 9/40, 22.5% in those with 'provoked' CVT). When ABO blood groups were considered, the observed distribution was significantly different from that in blood donors (P = 0.0003). The frequency of O blood group was significantly lower (24.7% vs. 44.5%), and conversely that of non-O blood groups was higher (75.3% vs. 55.5%), in patients with CVT than in blood donors. Therefore, having non-O blood group resulted in a 2.4-fold increase of risk of CVT (crude odds ratio, OR 2.44 95% CI, 1.42–4.26; P = 0.0008). Although the limitation of the sample size hampered reliable comparisons of subgroups, such a difference tended to be higher in CVT patients without thrombophilia (O vs. non-O blood groups: 12/54, 22.2% vs. 42/54, 77.8%; 7/23, 30.4% vs. 16/23, 69.6% in those carrying prothrombin G20210A or Factor V Leiden polymorphisms) and in patients with 'provoked' events (O vs. non-O blood groups: 8/40, 20% vs. 32/40, 80%; 11/37, 29.7% vs. 26/37, 70.3% in those with apparently unprovoked CVT). Indeed, the lowest prevalence of O blood group was seen in non-thrombophilic individuals with provoked CVT (4/26, 15%).
Conclusions: Our results show for the first time in the setting of venous thromboses at unusual sites an increase of risk related to non-O blood group. We were not able to perform a multivariate analysis and gain significant insight concerning the interaction of ABO blood group and other risk factors, including thrombophilia. However, our findings extend the interest in evaluating the ABO blood group in such rare and often challenging VTE through multicenter and prospective studies.

PB 3.67-3
Thrombophilic and systemic risk factors in patients with central retinal vein occlusion

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Introduction: Retinal vascular occlusions are common causes of visual loss. Few and contrasting data are available on the prevalence of risk factors in patients with central retinal vein occlusion (CRVO). Classic risk factors are commonly associated with CRVO, while unclear it is the role of the thrombophilic and coagulation disorders.
Aim: The aim of our study was to establish the prevalence of major and potential inherited and acquired thrombophilic risk factors in 120 patients (64 females, 56 males; age: 57.7 years; range 24–73) with CRVO. The control group consisted of 102 (55 females, 47 males; age: 56.9 years; range 19–72) healthy subjects, without any vascular, eye-related disease.
Methods: In all participants the prevalence of Leiden mutation (FV Leiden), protrombin variant (20210 G/A mutation), Leu 34 polymorphism of the factor (F) XIIIA-subunit, deficiency of protein C, S, antithrombin and antiphospholipid antibodies (aPL) were assessed. Also intima-media thickness (IMT) in carotid arteries were measured using ultrasonography.
Results: Elevated aPL antibody level (IgG>10 GPL; IgM>20MPL) were detected in 25 of 120 patients (20%) and in 8 of 102 controls (7.8%). The incidence of FV Leiden, deficiency of protein C were significantly more common in CRVO patients (P < 0.05) compared to controls. Patients with CRVO had greater IMT than healthy subjects (0.92 mm vs 0.75 mm; P < 0.05). There was no significant difference in the prevalence of deficiency of protein S, 20210 G/A mutation, FXIII Val34Leu polymorphism and antithrombin level between CRVO patients and controls. Among classic risk factors only hypertension, diabetes mellitus, hypercholesterolemia and cigarette smoking were significantly more frequent in CRVO patients than in control subjects.

Conclusions: These data show a potential role of some classic and thrombophilic risk factors in the pathophysiology of CRVO. Measurement of these parameters may be useful in preventing and treatment of such events.

PB 3.67-4

A case of protein S deficiency caused by compound heterozygous mutations in PROS1 gene

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Background: Protein S (PS) is a vitamin K-dependent plasma glycoprotein that plays an important role in the blood anticoagulant system. Inherited PS deficiency caused by *PROS1* mutations is associated with increased risk of venous thromboembolism. One of the most severe thrombotic manifestations (purpura fulminans) is related to the presence of homozygous or compound heterozygous genotypes. Up to date only a few cases with compound heterozygous PS deficiencies have been reported.

Aims: We present here a case of a 25-year-old male who developed massive deep vein thrombosis in the left leg, left and right iliac veins and vena cava inferior complicated by pulmonary embolism at the age of 16. Following the diagnosis of venous thromboembolism the patient was immediately put on indefinite anticoagulation treatment and no thrombo-embolic complications have been observed since.

Results: Laboratory results revealed markedly reduced free protein S antigen level – 15 IU/dL (ref.range 70–130 IU/dL); while other thrombophilic defects were excluded. Direct sequencing of *PROS1* gene indicated two already described causative heterozygous mutations; one in exon 2 (c.233C>T; p.Thr78Met) leading to lower expression of the affected protein and the other in exon 13 (c.1543C>T; p.Arg515Cys) responsible for impaired secretion of the mutant protein. Family studies showed reduced free protein S antigen level in four relatives (parents and two siblings). *PROS1* gene study identified heterozygous mutation p.Thr78Met in the mother (free PS antigen – 50 IU/dL) and sister (free PS antigen – 60 IU/dL), whereas p.Arg515Cys mutation was found in the father (free PS antigen – 19 IU/dL) and brother (free PS antigen – 28 IU/dL). Interestingly, in contrast to the propositus, none of his family members experienced thromboembolic events.

Conclusion: Our results may suggest that thrombosis risk in patient with inherited protein S deficiency is determined rather by the character of genetic defects than by protein S levels in plasma.

PB 3.67-5

Antithrombin-deficiency and pregnancy: report of four cases

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Background: Women with a history of thromboembolism or known thrombophilia have an increased risk for pregnancy-associated recurrent thromboembolism. The risk of fetal complications is also increased in thrombophilia. Antithrombin (AT) deficiency, among inherited thrombophilias, is considered to induce the highest risk of suffering a thromboembolic event during lifetime. The available data concerning relationship between AT deficiency and adverse pregnancy outcomes are conflicting.

Aim: To examine the optimal mode of antithrombotic management in pregnant women with established AT deficiency.

Methods and Results: Four pregnant women with AT deficiency were treated in our center in 2012. Two of them had homozygousBudapest3 mutation, in both cases the AT level was 17% (patients 1 and 2). In two other patients heterozygous mutations in the SERPNC1 gene were found (c.134A>T in patient 3 and a novel mutationdelc.1367–1368 GC, ins c.1367–1371 CTACA in patient 4). Their AT activity was 35–48%, respectively. Patient 1 had a history of recurrent venous thromboembolism and she also had a fetal loss despite heparin prophylaxis 5 years before. In her case the heterozygous form of FV Leiden mutation was also found. All four patients were put on therapeutic dose of enoxaparine upon recognition of pregnancy. The effectiveness of heparin treatment was evaluated by the measurement of anti – FXa activity. Patient 1, 2 and 3 required AT concentrate infusions in a dose of 1500–2000 IU, 2 or 3 times weekly to maintain therapeutic anti – FXa levels. In patients 1, 3 and 4 the pregnancy was uneventful and they had healthy babies born at terminuses. Patient 2 had a fetal loss on week 20.

Conclusion: There is evidence that heparin treatment is required for the prevention of maternal and fetal complications in AT deficient pregnant women. The effect of heparin should be monitored by the measurement of anti-FXa. If the effect is insufficient despite of the therapeutic dose of heparin, the application of AT-concentrate is required. Further data are needed to determine the anti-FXa level which is required to maintain an effective LMWH prophylaxis and to define the time when AT concentrate should be initiated. Furthermore, the dose and administration frequency of AT-concentrate is still an unresolved issue.

PB 3.67-6

Genetic background: analysis of protein C deficiency type I and type II in 26 Portuguese families

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Background: The inherited deficiency of Protein C (PC) is a well known risk factor for venous thrombosis (VT). Deficiency is classified as type I – both antigen and activity levels are reduced, or type II – antigen levels are normal and the activity is decreased. Plasma PC levels are reliable in moderate to severe deficiencies; however, in mild deficient individuals the levels may overlap with normal. Genetic studies of PROC could be helpful to identify the carriers, even though, the results are often not clear because the majority of the genetic variations are either rare mutations or common polymorphisms. Recently, GWAS shown that ≈50% of the PC deficiency phenotypic variation is caused by the additive effect of mutations in several other loci, namely in the PROCR gene.

Aim: To study the genotype/phenotype correlation of 58 individuals, from 26 unrelated families with a history of thrombosis, who had repeated low or borderline PC plasma levels. Fifty five had type I PC deficiency and three had type II. Clinical data: Among 26 probands, 22 had a personal history of thrombosis: VT (18), arterial thrombosis (2), stillbirths (1) and miscarriages (1) and four only had a family history of thrombosis. The mean age of probands at the time of first thromboembolic episode was 28.86 ± 12.50 years; 17F:9M; Nineteen probands had acquired risk factors – contraceptive drugs, varicose veins, trauma, pregnancy, dyslipidemia and immobilization. Three probands aged 24.33 ± 6.18 all with more than one thrombotic episode, had no identified risk factors and are considered as idiopathic DVT.

Methods: PC activity- amidolytic and coagulant assay; PC antigen/ELISA. Screening of mutations and promoter polymorphism in PROC, and polymorphism Ser219Gly in PROCR by direct sequencing; FVLeiden and FIIG20210A by multiplex ASPCR.

Results: Twelve different PROC mutations were identified: 9 missense: p.Cys105Arg (not previously described); p.Pro210Leu; p.Gly215Glu; p.Arg220Gln; p.Gly239Arg; p.Val339Met; p.Pro369Leu; P.Arg394Trp; p.Trp444Cys; 1 nonsense (p.Arg199X); 1 promoter

(-13A>G); and 1 small deletion (p.Leu212Hisfs*2). The last three mutations, the most frequent, were identified in 15 families, the ones with patients with the higher number of VT episodes. Only one patient was homozygous for a mutation (p.Arg220Gln), all the other were heterozygous. In one family with low PC levels, we could not find any mutation. FVLeiden was identified in six individuals; FII20210A in 3. One patient was double heterozygous for FII20210A and FVLeiden. A statistically significant difference in PC levels was observed between the control group and homozygous for haplotype CGT ($P = 0.0375$) or for polymorphism Ser219Gly ($P = 0.008$); 3/4 individuals with idiopathic DVT were homozygous for haplotype CGT; the presence/absence of Ser219Gly correlates with the different levels of PC in members of the same family.

Conclusion: The molecular studies of PROC and PROCR genes in 51 individuals with low or borderline PC plasma levels allowed the identification of the doubtful carriers. The type and location of the mutations correlates with the severity of the phenotype. Genetic family studies are important to evaluate the need for prophylactic measures, especially in the risk situations, and to offer the prenatal diagnosis in severe cases.

PB3.68 – Paediatric Thrombosis – III

PB 3.68-1

FondaKIDS II: long-term follow-up data of children receiving fondaparinux for treatment of venous thromboembolic events

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Background: The incidence of venous thrombotic events (VTE) in children has risen substantially over the past decade, resulting in increasing use of anticoagulation. Fondaparinux has several advantages over low molecular weight hpearins including a longer half-life allowing once daily dosing, no risk for heparin-induced thrombocytopenia and less effect on bone mineral metabolism. Recently, a PK, dose-finding, and safety study of fondaparinux in children was published, however it had only a short period of follow up.

Aims: The purpose of this study was to investigate the long-term safety, dosing, and efficacy of fondaparinux in children.

Methods: The following data were collected from all consecutive children (1–18 years) treated with fondaparinux in a single institution: demographics, location of initial VTE, fondaparinux dosing and fondparinux-based anti-Xa levels, bleeding events, other adverse events, status of VTE and VTE recurrence. Descriptive statistics are used to describe the patients and the outcomes.

Results: Thirty-five patients were included all of whom were included in the safety analysis while 34 were analyzed for dosing (one excluded due to only receiving two doses secondary to an allergic reaction) and 22 for efficacy (six excluded for resolution of DVT on a different anticoagulant, four because fondaparinux given prophylactically, two due to insufficient data, and one due to allergic reaction). There were 18 males (M) and 17 females (F) with a mean age of 9.11 years (median: 9 years; range: 1–17 years). The mean duration of treatment with fondaparinux was 371 days (d) (median: 152 days; range: 2–1566 days). The mean dose of fondaparinux was 0.1 mg/kg/dose (median: 0.1 mg/kg/dose; range: 0.04–0.15 mg/kg/dose). 14 of 22 evaluable patients (63.6%) had complete resolution of their thrombus while 6/22 (27.3%) had partial resolution, and 2/22 (9.1%) had no change. Thus, 20/22 (90.9%) patients had either a complete or partial response while none had progression. The mean time to best outcome from initiation of fondaparinux was 171.5 days (median: 92.5 days; range: 11–839 days). Eleven patients needed a total of 16 dose adjustments to achieve therapeutic levels. Two patients (9.1%) had a recurrent VTE: one was on fondaparinux at the time of recurrence and one was on warfarin. There were three major (intracranial hemorrhage- occurred prior to initiation of fondaparinux subsequently, pulmonary hemorrhage, and subretinal hemorrhage) and six minor (two with blood in stool, one with injection site, one CVC site, one tracheostomy bleed, one epistaxis) bleeding events. Importantly, only one of these events resulted in discontinuation of fondaparinux. One patient had an allergic reaction.

Conclusions: Given the advantages of fondaparinux over other low molecular weight heparins, this study suggests that fondaparinux could be considered a safe and effective alternative for the management of VTE in children.

PB 3.68-2

Safety and efficacy outcomes of home and hospital warfarin management within a paediatric anticoagulation clinic

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Background: Improved medical care and new surgical options for paediatric patients with chronic disease has led to an increase in the number of children requiring warfarin therapy. Time in Therapeutic range (TTR) refers to the amount of time a patient's International Normalised Ratio (INR) is within a designated target range. An increase in TTR is correlated with a decreased risk of bleeding or thrombosis. TTR is the most common method of determining the success of warfarin therapy.

Aims: This audit examined the TTR of warfarin patients managed by an anticoagulation clinic (AC) at the Royal Children's Hospital over 12 months. Aims included: determining TTR achievement and incidence of adverse events of all warfarin patients; and comparing TTR achievement between patients self-testing at home and those monitored at hospital.

Methods: INR results for children who currently have their warfarin therapy managed by the AC were analysed. Children were included in the study if warfarin therapy commenced before November 2012. INR results reported between January 1st 2012 and December 31st 2012 were collected. Due to the variation between the numbers of INRs each child required during the audit, cluster analysis of each child's INR results was used to calculate the mean percentage of INR's within TTR. Warfarin-related adverse events included major bleeding and/or thrombotic episodes that occurred during the audit period.

Results: 163 patients were included. 101 children were self-testing their INR and 62 tested their INR at a hospital or pathology service. The median age of patients was 10.6 years (range 0–19 years) and the mean number of days on warfarin for 2012 was 299 (range 80–363 days). The most common indication for warfarin was previous Fontan procedure ($n = 105$). Other indications included prosthetic heart valves ($n = 15$), pulmonary hypertension ($n = 9$), indwelling central venous line ($n = 5$) and deep venous thrombosis/stroke ($n = 8$).

Target range achievement for the cohort was 66.8% (95% CI 64 −69.6). 68.1% of INR tests conducted at home were within the TTR compared with 64.8% of INR tests conducted at a hospital or pathology service. The majority of patients ($n = 137$) achieved a TTR of >50%.

One major bleeding event occurred in a teenage girl with intra-abdominal bleeding and a sublingual haematoma following 3 days of vomiting. The patient's INR at the time of hospitalisation was >10. She required vitamin K, prothrombinex®, transfusion of red blood cells and fresh frozen plasma. There was one thrombotic episode; a non-occlusive thrombus in the Fontan circuit in a 6 year old female. The INR at the time was 1.8. Both patients monitored their INRs at hospital.

Summary: This audit demonstrates equivalence in safety and efficacy outcomes between home self-testing and hospital INR testing in children managed by an AC. Incidence of adverse events and TTR achievement is comparable to international paediatric centres. The size

of this cohort exceeds numbers previously reported when comparing self-testing and hospital monitoring. Routine outcome evaluation of paediatric anticoagulation management within single institutions is warranted due to the difficulties of multi-site studies assessing management strategies.

PB 3.68-3

Pediatric stroke: a single center experience

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Background: Pediatric stroke, defined as arterial ischemic stroke (AIS), cerebral sinovenous thrombosis (CSVT) and hemorrhagic stroke (HS) is a rare but serious event.

Aims: The aim of this study is to assess the clinical characteristics, risk factors, treatment and outcomes of pediatric stroke cases in a single center.

Methods: We reviewed the records of children who were diagnosed as pediatric stroke at our center between 2000 and 2011. Neonatal cases were excluded. The study was approved by medical ethics committee.

Results: We identified 67 patients with AIS (M/F: 33/34; median age: 2.9 years), 27 patients with CSVT (M/F: 20/7; median age: 8.8 years) and 24 patients with HS (M/F: 21/3), median age: 6.7 years). Main symptoms at presentation were hemiplegia (46%), convulsion (31%), headache (19%), vision defects and strabismus (13%), facial paralysis (11%), aphasia (10%), vomiting (7%), unconsciousness (4%). Hemiplegia was the most frequent symptom for arterial ischemic stroke. Most of the patients with hemorrhagic stroke had an underlying event or disease, 45% had late hemorrhagic disease of newborn (diagnosed >28 days after delivery) and 37% had a history of head trauma. An underlying acute or chronic illness and/or external trigger to thrombosis were present in 20 patients with CSVT and most frequent causes were acute otitis media-mastoiditis, acute leukemia and Behcet disease. Thirty five of 67 patients with AIS had an underlying disease and/or a trigger. The most frequent causes were congenital heart disease and trauma. Thrombophilia work-up was conducted in a group of patients with CSVT (*n*: 15/27), AIS (*n*: 41/67) and none of the patients with HS. At least one prothrombotic risk factor was positive in 78% of patients with CSVT and 37% patients with AIS. MTHFR A1298C heterozygote mutation was the most frequent finding in patients with CSVT. MTHFR C677T mutation was the most frequent finding in patients AIS. In total, 56 of the patients received antithrombotic treatment; no major complications were observed. In AIS, antithrombotic treatment was given to 34 of 67 children; the majority (*n*: 27) received LMWH and acetylsalicylic acid (*n*:20). In CSVT, antithrombotic was given to 22 of 27 children; LMWH treatment was used in 20 and warfarin was used in only 4. Two patients expired due to thrombosis; one of them had AIS and congenital heart disease and the other one had CSVT and macrocephaly telangiectasia syndrome. Neurological sequel was found in 28 of 67 patients (41%) with AIS, 5 of 27 patients (18%) with CSVT, 9 of 24 patients (37%) with HS.

Conclusion: We reviewed our experience of 11 years in diagnosis and treatment of pediatric stroke. Demographic findings in our study were consistent with the literature showing a high percentage of boys. Prothrombotic defects were more in common in children with CSVT compared to patients with AIS. We mainly use LMWH for anticoagulation and in our experience none of our patients had hemorrhage due to the treatment. Our results showed a low mortality rate mainly because only the survivors were referred to our center.

PB 3.68-4

Demonstration of construct validity of the KIDCLOT PAC QL©

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Background: Quality of life (QOL) as defined by the World Health Organization is 'an individual's perception of their position in life in the context of the culture and value systems in which they live, and in relation to their goals, expectations, standards and concerns'. QOL is an abstract entity which can be measured by a tool developed specific to the patient condition. Vitamin K antagonists (VKA) are known to affect quality of life and the only tool designed to measure its impact on QOL in children and families is the KIDCLOT PAC QL©. Reliability testing, face and content validity, and validation of the scoring system has been reported previously. In absence of a gold standard, further inventory validation can be achieved with construct validity. Construct validity in which the inventory actually measures the construct it is intended to measure can be supported with demonstration of discriminant and convergent validity. Convergent validity measures constructs that are theoretically related to each other to a pre-hypothesized extent. Discriminant validity measures constructs that are theoretically not related to each other. To establish construct validity one must establish both convergent and divergent validity.

Aim: To demonstrate construct validity of the KIDCLOT PAC QL©.

Methods: Convergent validity of the final version of the inventory was examined by measuring the association between the global scales of the KIDCLOT PAC QL ©and the PEDSQL © (an accepted, valid, and reliable inventory for pediatric QOL). The PEDSQL © was used with permission. Discriminant validity was examined by measuring the association between the KIDCLOT PAC QL © and knowledge retention scores (a test taken by parents and children to ascertain their knowledge of VKA) obtained by the same patients. The PEDSQL was selected to examine convergent validity as it is a well-accepted global measure of quality of life in children, has a child and parent-proxy version and takes <10 min to complete. As it is a QOL inventory with no questions specific to VKA the construct should be theoretically medium correlation. The knowledge retention test was selected as it is a test measuring knowledge only and therefore should be theoretically unrelated to the abstract concept of QOL with small correlation. Associations between scorings were tested with Spearman rank correlation because of the ordinal nature of the scales. Informed consent and ethics approval were obtained.

Results: Ninety parents and 42 children completed both the KIDCLOT PACQL and PEDSQL, 40 parents and 19 children completed the knowledge retention test. Convergent validity for the parent-proxy inventory $r = 0.528$ ($P < 0.001$) and for divergent validity $r = -0.213$ ($P = 0.186$). Convergent validity for the child/adolescent inventory convergent validity $r = 0.307$ ($P = 0.48$) and for divergent validity $r = -0.270$ ($P = 0.264$).

Summary: The KIDCLOT PAC QL © demonstrates predicted construct validity with medium correlation with a global QOL measure and small correlation with the knowledge retention test. The tool is now validated and ready for use.

PB 3.68-5

Decreased protein S activity in ulcerative colitis but not Crohn disease in a pediatric Cohort

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Background: Inflammatory bowel diseases (IBD), including ulcerative colitis (UC) and Crohn disease (CD) affect more than 1 million people in theUnited States. Growing evidence recognizes IBD as a chronic

inflammatory condition associated with a hypercoagulable/prothrombotic state, which represents one of the important extraintestinal manifestations, and can lead to increased mortality and morbidity in patients. Thromboembolism (TE) in patients with active IBD is well established with a 6.5% incidence of thrombosis in adults, a 3–4 fold higher risk than in the general population. TE can also occur in the pediatric IBD patients as well. The mechanisms for IBD associated thromboembolic disorders are extensively studied, but not clearly understood.

Aims: The aim of this work was to examine prothrombotic factors in hospitalized pediatric IBD patients.

Methods: Twenty five patients with the diagnosis of UC (n = 10) or CD (n = 15) were recruited between 2009 and 2012 during hospitalization for active disease. The patients were further subclassified based on treatment involving steroids and disease subtype (UC vs. CD). Peripheal blood was evaluated for hemostatic parameters including prothrombotic factors (PT/INR, PTT, fibrinogen, D-dimer, antithrombin, thrombin time, factor VIII, functional protein C, functional protein S, von Willebrand factor antigen and ristocetin co-factor, ADAMTS 13 activity, and lupus anticoagulant) using standard diagnostic procedures. The acquired data were analyzed and compared to an age and gender matched historical control group (n = 25).

Result: The assays for fibrinogen, D-dimer, thrombin time, factor VIII and protein S showed statistically significant ($P < 0.0001$) differences between IBD patients and the control group. Steroid therapy significantly increased fibrinogen ($P = 0.01$), and factor VIII activity ($P = 0.006$). Interestingly, protein S activity was significantly lower in the UC group ($P = 0.001$), while it was comparable to the control group in CD patients ($P = 0.14$).

Conclusions: IBD results in significant prothrombotic factor alterations that are likely a consequence of a complex systemic inflammatory process. Therapeutic interventions, such as steroids, can significantly affect these parameters. Interestingly, while protein C appears to be essentially unaffected in both UC and CD, protein S was significantly decreased only in UC patients within this cohort. Our findings may carry useful information for guiding possible prophylactic measures against thrombotic events in hospitalized pediatric IBD patients.

PB 3.68-6

Antithrombin concentrate in pediatric patients requiring heparin anticoagulation: a retrospective Cohort study

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Background: Heparins, unfractionated (UFH) and low molecular weight (LMWH), are used in children requiring anticoagulation. *In vivo*, low antithrombin (AT) levels often result in an inadequate anticoagulant effect with UFH. Effective anticoagulation is becoming increasingly important in the pediatric intensive care unit. Critically ill children require heparin anticoagulation for various indications including thrombosis, extracorporeal membrane oxygenation and ventricular assist devices. Developmental hemostasis, in combination with underlying risk factors in ill children, often result in physiologically decreased AT levels. Anticoagulation with UFH requires AT levels >~0.5 U/mL to achieve therapeutic heparin anticoagulation. Administration of antithrombin concentrate (ATC) increases AT levels and heparin effect. Dosing ATC per the manufacturer[1] (ATC dose = [desired level-baseline level] × patient weight in kg/2), results in wastage since ATC is supplied in 1000 Unit quantities.

Aims: The aim of this retrospective cohort study is to determine AT levels pre and post ATC administration, mean ATC dose/kg administered, bleeding, thrombotic or allergic events. During 2008–2011, ATC effect on heparin effect (anti-Xa and PTT) and the change in heparin dose was also collected.

Methods: From 2005 to 2011, the practice at Stollery Children's Hospital was to administer a vial of ATC in patients <20 kg when AT levels are <50% and therapeutic heparin effect is not achieved despite higher than age appropriate heparin doses. When ATC is administered, heparin dose is decreased by 50%, UFH doses, PTT, AT and anti-Xa levels were drawn 30 min post ATC infusion. Ethics approval was obtained.

Results: 121 patients received 246 doses of ATC. Median age (range) and weight (range) were 3.7 months (7 days–10.7 years) and 4.1 kg (2.1–20.0). Mean ATC dose was 222 IU/kg. Mean AT level increased from 0.39 (95% CI 0.37–0.42) to 1.20 U/mL (95% CI 1.10–1.30) post ATC administration. In the 2008–2011 subgroup, UFH doses to achieve a target anti-Xa level pre post ATC were 28 (95% CI 24–32) and 19 (95% CI 16–22) U/kg/h, respectively, for children ≤12 months and 25 (95% CI 19–30) and 19 (95% CI 11–19) U/kg/h, respectively, for children >12 months. The PTT was 110 (95% CI 90–131) and 136 (95% CI 116–156) seconds, pre/post ATC respectively, for children ≤12 months and 66 (95% CI 42–91) and 63 (95% CI 43–84) seconds, pre/post ATC respectively, for children >12 months. There were no hemorrhagic, thrombotic or allergic events.

Conclusions: This is the largest pediatric evaluation of ATC. Administration of high dose ATC appears safe in infants and children. Increasing AT concentration provides additional heparin binding sites as well as increased anti-Xa levels with lower age appropriate heparin doses. Further ATC was minimized due to a sustained effect resulting in more stable anticoagulation. Additional well designed studies are required to further evaluate safety and efficacy of ATC administration and to determine biochemical changes and dosing relationships between ATC and heparin. This data provides the basis for future investigations and the development of pediatric-specific, evidence-based guidelines for ATC use.

PB3.69 – Paediatric Thrombosis – IV

PB 3.69-1

Evaluation of a unique mHealth web-based VKA management system

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Mobile health (mHealth), supported by international health governing bodies, is a growing field that uses web based and mobile communication devices to improve health outcomes, healthcare services and health research, and may be used as a tool for reporting INR results. Patients prescribed long term vitamin K antagonist (VKA) therapy require close monitoring of their INRs. INR results are received by the patient directly from the laboratory or by self-testing using home INR meters and then are reported to the health provider for dose adjustment.

Aim: This study is to evaluate functionality and patient satisfaction with use of the INR reporting website www.kidclot.com.

Methods: The KIDCLOT© website was established as a tool for INR reporting using an SSL address. Two groups of patients prescribed VKA therapy used this website: (i) patients who self-managed (PSM) their VKA therapy and (ii) patients who self tested (PST) INRs with the KIDCLOT© health provider adjusting their VKA dose. Patients entered INR results into the website via computer or smartphone. Each was assigned a secure ID and password to log into the site. Usernames are only identifiable to the KIDCLOT© clinical team with no other patient identifiers stored in the database. Patient and administrative staff have different levels of access; therefore no patient has access to data outside of their own profile. This security is consistent with international health privacy policies.

All patients enter their INR result and VKA dose. PSM patients enter their INR, VKA dose adjustment, next INR testing date and comments. Whereas, PST patients enter their INR which triggers an email

to the KIDCLOT health provider to provide the VKA dose adjustment and next INR testing date. The KIDCLOT© health provider enters the dose plan which alerts the patient. All patients receive an INR test reminder alert the day the INR is due. When reported INRs are outside of the patient's therapeutic range an alert is sent to the KIDCLOT© health provider alerting the provider to review patient decision making, and allow immediate dosing support. Patient satisfaction surveys were completed by patients. Informed consent and ethics approval were obtained.

Results: 33 of 51 website users responded to the satisfaction survey. Results from the patient satisfaction survey found that 100% of respondents 'liked' the website format, found it easy to use, convenient, and preferred the website to any other method of INR reporting. 95% of patients reported that it assisted them to better manage their warfarin through INR alerts and ease of reporting. Patients reported the website to be a 'great improvement' 'the best thing ever' and 'outstanding' for INR reporting. Patients valued the ability to enter personal notes, and resource materials available on the website, and continued to feel supported by the KIDCLOT health care team. The KIDCLOT© health providers found the website to be an effective and efficient method for monitoring VKA therapy and for quality control for patients and health providers.

Conclusion: INR reporting using mHealth is a valued solution for patients and health providers.

PB 3.69-2

Anticoagulation of pulmonary hypertension in children

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Background: Pulmonary Artery Hypertension is characterised by increased pulmonary vascular resistance, right ventricular failure, low cardiac output and death. Without treatment, the long term prognosis of children with pulmonary arterial hypertension is poor, with an estimated median survival of <1 year, shorter than that which is seen in adults The reduced pulmonary blood flow leads to an increased risk of thrombosis. Anticoagulation is used in adults with pulmonary artery hypertension, and is recommended by the ACCP for use in children despite the lack of published primary evidence to support its use.

Aim: To determine the safety and efficacy of oral anticoagulant therapy (warfarin) for primary thromboprophylaxis in a cohort of children with Pulmonary Hypertension.

Methods: A retrospective clinical audit of the Royal Children's Hospital, Melbourne, pulmonary hypertension and warfarin INR databases was conducted with relevant outcomes including thrombosis and major haemorrhage, defined *a priori* according to internationally accepted definitions. We also *a priori* determined that a reduction in bone density would be classified as significant if a Z score of less than or equal to −1.0, was reported on a bone mineral density scan.

Results: 18 children (69.94 warfarin years) were examined with each child taking warfarin for an average 3.89 years (range: 0.5–7 years). The primary reasons for the discontinuation of warfarin therapy were death unrelated to warfarin thromboprophylaxis ($n = 4$), and transfer to adult care ($n = 5$), with eight patients remaining on continuous warfarin at the completion of the study. The median age of children starting warfarin was 7.63 years (Range: 0.670–16.4). The most common Target Therapeutic Range for warfarin therapy was 2.0–3.0. Of 1694 INR tests performed, TTR achievement was highly negatively skewed, and was achieved in a median of 48.75% of all tests. However, 89% of patients ($n = 16$) in this study achieved their TTR >30% of the time.

There were no deep vein clots in this cohort. Two patients experienced superficial vein thrombi (three thrombotic events); all at the site of line insertions. Therefore, despite the low TTR achievement numbers, treatment appeared effective. There were several warfarin related adverse events experienced during the study period; with two patients experiencing major bleeds. One was an episode of severe epistaxis, and the other a severe gastro intestinal bleed following a nasogastric tube resite. Of the 10 patients who had had bone mineral density scans, 8 (80%) had significant reductions in bone density in at least one site (hip or lumbar spine).

Summary/Conclusions: The high mortality rate of 22.5% ($n = 4$) in this cohort demonstrates the severity of this condition in children. However there were no clotting related deaths suggesting warfarin therapy can be effective, despite the low rate of TTR achievement in these children. The potential adverse events related to warfarin including bleeding and reduced bone density need to be considered.

PB 3.69-3

Clinical characteristics of pediatric and adolescent index cases with antithrombin deficiency: results of a cohort study

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Background: Venous thromboembolism [TE] is a multifactorial disease and antithrombin [AT] deficiency [AT] constitutes a major risk factor. In the present study the incidence of AT, the clinical presentation at TE onset and the recurrence rate in a cohort of pediatric index cases are reported.

Methods: In 205 unselected consecutively ascertained pediatric patients (neonate to < 18 years) with TE a comprehensive IT screening was performed along with recording of anamnestic data. Index patients were enrolled between January 1998 and December 2010 and were followed until December 2012 with the study endpoint symptomatic recurrence.

Results: Seventeen of 205 pediatric index patients [8.3%] were carriers of AT deficiency [heterozygous mutations, type 1: point mutation $n = 10$; deletion $n = 2$; stop $n = 2$; type 2: homozygous 'Budapest' $n = 2$; 'Charleville' $n = 1$]. Mean age at first TE onset was 14 years [range 0.1–17]. Thrombotic locations were neonatal renal vein thrombosis [$n = 1$]; cerebral venous thrombosis/stroke [$n = 6$], DVT of the leg [$n = 9$] and pulmonary embolism ($n = 1$]. AT deficiency was combined with FV G1691A mutation in three cases and FII G20210A variant in two. First TE onset was associated with underlying diseases in 70% of patients [use of OAC, smoking, immobilization, surgery]. Spontaneous TE occurred in a neonate with a heterozygous point mutation, a 9-year-old female with a stop mutation and a 14-year-old female homozygous for theBudapest variant. In five of 17 pediatric index patients [29.4%] recurrent TE occurred after withdrawal of oral anticoagulation [performed with LMWH and Warfarin according to CHEST guidelines] at a median follow-up time of 2.5 years [range: 2–8].

Conclusion: In an unselected cohort of pediatric patients with symptomatic TE, the prevalence of AT deficiency was 8.3%, followed by a high recurrence rate of 29.4%. Given its clinical implications thrombophilia testing should be performed in children with a first TE onset not to miss AT deficiency. Long time anticoagulation should be considered in pediatric AT carriers suffering from a first TE.

PB 3.69-4

Clinical experience with recombinant tissue plasminogen activator in the management of intracardiac and arterial thrombosis in children

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Background: Thrombotic events may complicate the clinical course of many neonatal and pediatric pathologic processes. Thrombolytic therapy is generally used for arterial thrombosis in selected patients. Drugs that have been used for therapeutic thrombolysis in children include streptokinase, urokinase and tissue plasminogen activator (t-PA). There are a number of practical advantages of recombinant t-PA (rt-PA) for non-invasive thrombolysis. However, experiences with rt-PA in pediatric age group are less than in adults. Many recommendations for children are based on extrapolation of adult data.
Aims: We aimed to present our experiences with rt-PA in children with intracardiac or peripheral arterial thrombus.
Methods: We conducted a retrospective review of 18 patients who received rt-PA because of thrombus.
Results: We evaluated 18 children (10 boy and 8 girl; age range from 1 day to 17 year) with intracardiac thrombus (n = 5), prosthetic heart valve thrombus (n = 2) and peripheral arterial occlusion (n = 11). Eight of the 18 patients had congenital heart disease and two had rheumatic heart disease. Three patients diagnosed with leukemia and five patients suffered from sepsis, prematurity or meconium aspiration syndrome. Seven of the 11 peripheral arterial thrombosis were observed following an intervention such as angiography. Three of the five intracardiac thrombi were detected in children with leukemia. All children initially received low molecular weight heparin (LMWH). Recombinant t-PA (alteplase) infusion (at a dose of 0.01–0.5 mg/kg/h) was administered in different time periods (3–66 h). Nose bleeding, melena and decreased serum fibrinogen concentration were observed in five patients during the rt-PA infusion. Ten of 11 patients with peripheral arterial occlusion and 3 of 5 patients with intracardiac thrombus showed full recovery. However, there was no response in two patients with intracardiac thrombus and in two children with heart valve thrombus. One patient with prosthetic mitral valve needed re-mitral valve replacement because of the thrombotic event. One newborn patient with aortic thrombus at the level of celiac artery died from sepsis without observing the fibrinolytic response.
Conclusion: Recombinant t-PA infusion seems effective and safe in treatment of peripheral arterial occlusion and intracardiac thrombus in children.

PB 3.69-5

Can we predict poor outcome in neonatal arterial ischemic stroke by testing for the presence of a prothrombotic risk factor (inherited or acquired)?

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Background: Childhood arterial ischemic stroke (AIS) is increasingly being recognized. A major proportion of childhood AIS occurs in newborns. The relevance of prothrombotic risk factors (thrombophilia) in neonatal AIS (NAIS) is unknown.
Aims: To describe the frequency of thrombophilia in NAIS and to evaluate the potential impact of its presence on recurrence risk and neurologic outcome.
Methods: A retrospective review of NAIS cases from July/1999-August/2009 at our institution was conducted. Thrombophilia testing included protein C (PC), protein S (PS), antithrombin, anticardiolipin antibodies (ACLA), lupus anticoagulant, lipoprotein-a, plasma homocystein, levels of FVIII, FIX, and FXI, and Factor V Leiden, prothrombin (PTG) and MTHFR mutations. Neurologic outcome was assessed by the validated Pediatric Stroke Outcome Measure (PSOM). Measures of association (Fisher's Exact) between thrombophilia and AIS recurrence and outcome were studied.
Results: Eighty-eight neonates with AIS were identified. Seventy-six had prothrombotic testing. Tests were abnormal in 9/76 (11.8%) [2 low PC, 1 elevated ACLA, 1 elevated ACLA plus a high FVIII level, 2 PTG, 1 elevated lipoprotein-a, 1 hyperhomocystinemia with MTHFR variant]. Recurrence was more frequent in neonates with at least one prothrombotic risk factor (OR 6.2 (95%: 1.18–32.56); P = 0.02) and the median time to recurrence was 13 days. Abnormal neurologic outcome (total PSOM $\geq$0.5) was also more frequent in children with than those without a prothrombotic abnormality (P = 0.04).
Conclusions: The presence of a prothrombotic risk factor is not necessarily common in NAIS. Nevertheless thrombophilia may be associated with unfavourable outcomes in neonates. A larger prospective study is needed to verify these findings.

PB 3.69-6

Anticoagulation therapy in pediatric Lemierre's syndrome

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Background: Lemierre's syndrome, most commonly caused by the anaerobic pathogen Fusobacterium necrophorum, is characterized by a recent history of oropharyngeal infection and ipsilateral internal jugular vein thrombosis. As Lemierre's syndrome progresses, complications arise in the form of septic thromboembolism and cerebral venous thrombosis. Although Lemierre's syndrome can affect all ages, demographically there is an incidence peak between the age of 10 and 35. The use of anticoagulation in patients with Lemierre's syndrome is controversial due to concerns regarding hemorrhagic complications.
Aim: To explore the efficacy and safety of therapeutic anticoagulation in pediatric patients diagnosed with Lemierre's syndrome.
Methods: A Pubmed search was performed, sourcing English publications since 2000 on diagnosed Lemierre's syndrome cases, aged 18 years or below. Two literature reviewers independently divided the cases presented in the publications into two groups: patients treated with anticoagulation therapy and patients treated without anticoagulants. For both groups, the development or progression of the jugular vein thrombosis into the cerebral venous sinus and/or development or progression of septic thromboembolism was determinded.
Results: Sixty-four relevant publications were identified, describing a total of 85 pediatric patients. No randomized controlled trials were found. The median age was 15 years (range 0–18) with a male preponderance of 55%. At diagnosis, 27/85 (32%) patients suffered from cerebral venous sinus thrombosis and 48/85 (57%) exhibited septic thromboembolism. Besides antibiotic therapy, 49/85 (58%) patients were treated with anticoagulation. Among anticoagulated patients, 1/49 (2%) demonstrated development or advancement of the thrombus into the cerebral venous sinus compared to 5/36 (14%) of patients not treated with anticoagulation. Development or progression of septic thromboembolism was observed in 10/49 (20%) cases in the group with anticoagulation vs. 12/36 (33%) in the group without anticoagulation. Unfractionated heparin, low molecular weight heparin as well as vitamin K antagonists were used as anticoagulants. The mean duration of anticoagulant therapy was 12 weeks (range 2–48). One patient in the anticoagulation group developed a major bleeding complication: A hemorrhagic pericardial effusion, which required pericardial drainage.
Conclusion: These limited data on anticoagulation in Lemierre's syndrome indicate that anticoagulation may decrease development and/or progression of thromboembolic events, with a low risk of hemorrhagic complications. These results should be confirmed in a prospective, randomized trial.

PB3.70 – Recurrent venous thrombosis – III

PB 3.70-1

The use of the REVERSE study clinical prediction rule for risk stratification after initial anticoagulation results in decreased recurrences in patients with idiopathic venous thromboembolism

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Background: The optimal duration of anticoagulation (AC) in patients with idiopathic venous thromboembolism (VTE) is uncertain but in general at least 6 months are needed. In 2008 our group published the findings of the REVERSE study (Rodger et al., CMAJ 2008), which showed that the group of patients with high risk of recurrence included all males and those females with 2 or more adverse risk factors (age ≥65, body mass index ≥30, VIDAS D-Dimer ≥250 mg/L, and signs of post-thrombotic syndrome). Since 2008 our center modified clinical practice based on such findings. Accordingly, patients in the high risk group have since remained on AC.

Aims: In this study we aimed to evaluate the impact of a practice change using the REVERSE clinical prediction rule on recurrence rates in patients with idiopathic VTE.

Methods: We conducted a single-center, retrospective cohort study of all patients with objectively confirmed VTE who were consecutively referred to our thrombosis clinic from January 1st, 1999 to December 31st 2011. Patients were excluded if the VTE was due to hospital stay, pregnancy, immobility, or surgery. The primary outcome was objectively documented VTE after the initial 6 months of AC. Patients were divided according to the year of diagnosis in two groups (before and after 2008). Groups were compared using χ^2 or Fisher's exact tests, as appropriate. Survival data was analyzed using the Kaplan-Meier method and Cox regression analysis adjusted for thrombophilia and use of AC.

Results: We included 1033 out of 1100 eligible patients. Mean age was 55.59 years (±17.96), the mean length of follow up was 55.5 months (range 0–229), and 48.11% were female. Overall, 569/1033 (55.1%) patients continued AC beyond the initial planned period. Patients continued AC based on REVERSE criteria in 47/371 (12.7%) and 146/198 (73.7%) of cases before and after 2008, respectively. VTE recurrences were observed in 168 (24.5%) patients before 2008 and in 9 (2.6%) after 2008 ($P < 0.001$). The proportion of VTE recurrences in patients continuing vs. stopping AC were 15.% vs. 35.6% ($P < 0.001$, Log-rank $P < 0.001$) before 2008 and 2.0% vs. 3.4% ($P = 0.506$, Log-rank $P = 0.214$) after 2008. Continuation of AC beyond the initial planned period resulted in 75% reduction in VTE recurrence risk (HR 0.25, 95% CI 0.18–0.35; $P < 0.001$) after adjusting for thrombophilia and year of diagnosis.

Summary/Conclusions: Although the present study has limitations mainly arising from its retrospective design, our findings support the use of the REVERSE clinical prediction rule to guide decisions on long term AC duration in patients with idiopathic VTE.

PB 3.70-2

Outcomes after vena cava filter (VCF) placement in patients with acute venous thromboembolism (VTE)

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Background: Evidence supporting use of VCFs to prevent death or recurrent VTE among patients with acute VTE is limited.

Aims: To determine the effect of VCF placement on the 30-day incidence of death and the incidence of recurrent pulmonary embolism (PE) and recurrent deep-vein thrombosis (DVT) among patients with acute-VTE who did not have cancer.

Methods: Using linked California Patient Discharge records, we performed a retrospective observational study that analyzed outcomes after VCF placement. All cases were hospitalized at one of the 400 non-federal public hospitals in California and had a principal diagnosis of acute VTE. After selecting the first acute-VTE hospitalization between 1/1/2005 and 12/31/2009 in each linked-patient record we excluded cases with, active cancer, prior VCF placement and all cases from hospitals that admitted fewer than 55 acute-VTE patients during the study period. Outcomes were death <30 days, recurrent PE, and recurrent DVT (within 180 days, and at median of 22 months). Three Methods: were used to analyze death <30 days, (i) standard risk-adjusted multivariable analysis, (ii) propensity score-matching (3:1) and (iii) instrumental variable (IV) analysis, using hospital as the instrument. Variables for adjustment included: age, race, sex, PE vs. DVT alone, prior VTE, acute bleeding, surgery, number of comorbidities, risk-of-mortality on admission and insurance status. Cox proportional hazard modeling was used to analyze the risk of post-hospital discharge recurrent VTE.

Results: Among 69,505 acute-VTE cases, the 30-day mortality was 4.3%; 5.4% had recurrent-PE and 6.2% had recurrent-DVT (median follow-up = 22 month). A VCF was placed in 7766 (11.2%) and 505 (6.5%) of these cases died <30 days; using risk-adjusted multivariable analysis of death <30 days, VCF use was associated with an 18% reduction in the odds of death (OR = 0.82, CI :0.74–0.92); but there was no significant difference using inverse-weighting propensity-score analysis (OR = 0.96, CI: 0.91–1.01, $P = 0.11$) or matched propensity-score analysis (OR = 0.91, CI: 0.79–1.05, $P = 0.18$). IV analysis showed no significant difference in the 30-day risk-adjusted mortality rate when the highest tertile VCF-utilizing hospitals (VCF use = 16.6%) and low VCF-utilizing hospitals (VCF use = 4.3%) were compared: Death = 4.3% vs. 4.1%, respectively; difference = 0.2%, $P = 0.31$). Cox modeling to predict recurrent VTE showed that VCF use was associated with a higher risk of recurrent-PE at 180-days (HR = 1.2, CI: 1.0–1.4, $P = 0.046$) but no effect after 22 months (HR = 0.91, CI: 0.81–1.02, $P = 0.09$); VCF use was associated with a 56% higher risk of recurrent DVT at both 180-days and 22 months (HR = 1.56, CI: 1.4–1.7).

Conclusions: Use of different methods: to analyze the effect of VCF use on death <30 days resulted in discrepant findings: simple multivariable regression showed a beneficial effect of VCF use, with an approximate 16–18% relative reduction in the 30-day risk of death (estimate = 0.6% absolute reduction), whereas propensity-score analysis and IV analysis showed no significant difference in the risk of death. For all methods, the effect of VCF use on the absolute risk of death was small, in part because the overall 30-day mortality rate was modest (4.0%). There was no significant effect of VCF use on the ~2-year incidence of post-discharge recurrent-PE, but the risk of post-discharge recurrent-DVT was increased by ~50%.

PB 3.70-3

External validation and updating of the Vienna Prediction Model for recurrent venous thromboembolism using a pooled individual patient data database

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Background: Guidelines recommend consideration of indefinite anticoagulation for patients with an unprovoked episode of venous thromboembolism (VTE) due to their overall high recurrence risk. At the individual level, many patients will never experience recurrence and will be exposed to unnecessary bleeding risk. The Vienna Prediction Model (VPM, Circulation 2010), based on Cox modelling, uses patient sex, VTE site and D-Dimer to stratify patients according to their recurrence risk.
Aims: To externally validate and possibly re-calibrate the VPM, using a large pooled database of individual patient data (IPD) from different prospective studies and validation techniques taking into account the time-to-event.
Methods: Validation cohort (Cohort V): IPD on 904 patients pooled from six prospective studies evaluating the effect of D-dimer on VTE recurrence risk. Both the most common but possibly misleading calibration comparing predictions to the observed raw proportions of events and the comparison of the cumulative recurrence rates predicted by the VPM to those observed in V (estimated by Nelson-Aalen method), at 12 and 60 months within risk strata (Harrell, 1996), were performed. By-study stratified Cox and Weibull regressions, including as covariate the Vienna's linear predictor $X\beta_{Vienna} = x_1\beta_1 + x_2\beta_2 + x_3\beta_3$ (where x_1-x_3 were sex, VTE site and D-Dimer), were also used to check the validity of the Vienna's overall hazard ratio and of the whole VPM (van Houwelingen, 2000). The updating of the VPM was achieved by adjusting the coefficient of each original predictor and including age as new predictor, through a stepwise forward procedure (Steyerberg, 2004). C-statistics, net reclassification improvement and discrimination slopes were used to explore model discrimination.
Results: In Cohort V, 123 (13.6%) patients had recurrent VTE (median follow-up 22 months, 3–71). According to the Cox and Weibull models, the calibrating coefficient for $X\beta_{Vienna}$ was always positive and not significantly different from 1, and the parameters describing the baseline recurrence risk function were similar to those found in the derivation cohort. Conversely, the analysis of goodness-of-fit of the non-recalibrated model showed that the performance of the whole VPM was less satisfying; indeed, the predicted and the observed cumulative rates for five risk strata were both increasing (at 12 months, predicted [95% CI]: 3.0 [1.3–3.7], 4.3 [3.7–4.9], 5.4 [4.9–6.0], 6.6 [6.0–7.5], 9.5 [7.5–18.5]; observed: 5.1 [2.7–9.9], 7.1 [4.0–12.5], 9.0 [5.4–14.9], 13.3 [8.8–20.3], 15.9 [10.9–23.4]), but with the predictions underestimating the observed recurrence rates. In updating the model, coefficients for VTE site and D-dimer, but not for sex, required significant adjustment. When added to this model, age was statistically significant. The c-statistics for the calibrating and the updated model were 0.626 and 0.674, respectively, with a significant higher ability of the updated model compared to the original VPM in reclassifying patients in agreement to their actual outcome.
Conclusions: Using a pooled IPD database as validation cohort we confirmed the validity of overall hazard ratio predicted by the VPM and improved its predictive performance by recalibrating the original coefficients and adding age, thus reinforcing its readiness for clinical application as a risk assessment tool.

PB 3.70-4
Risk factors of recurrent thromboembolism during pregnancy

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Background: Recurrent venous thromboembolism(VTE) is a frequent complication of an initial VTE. There are only few reports on recurrent VTE during pregnancy while on low-molecular-weight heparin (LMWH) prophylaxis.
Aims: To identify risk factors of recurrent antepartum VTE in women with LMWH prophylaxis due to at least one previous VTE episode.
Methods: This observational cohort study was undertaken at the Department of Obstetrics and Gynaecology, Helsinki University Central Hospital. The subgroup of 270 pregnant women with at least a single previous episode of VTE (369 pregnancies) was evaluated closely. All women delivered between 1994 and 2007, and received LMWH-prophylaxis due to prior VTE during pregnancy and postpartum. Antepartum VTE despite the LMWH prophylaxis in this study was defined as a VTE occurred at least 1 week after the initiation of the LMWH-prophylaxis. The risk factors between those women with LMWH-prophylaxis without recurrence were compared to those LMWH-users with recurrence.
Results: Twelve recurrencies (3.3%, $n = 10$ women) during early pregnancy before initiation of the LMWH-were excluded.
The incidence of recurrent VTE despite prophylaxis was 4.3% ($n = 16$ pregnancies in 15 women). Most of these were at high risk for VTE (thrombophilia or ≥2 previous VTE's). The mean gestational week (gw) at time of initiation of LMWH prophylaxis was 15 (range 4–39) in the group without recurrent VTE vs. 7(range 4–18) in the recurrent VTE group ($P < 0.001$). Duration of the medication was 24 (0–37) vs. 30 (range 16–36) gw ($P = 0.009$) respectively. The mean gw at diagnosis of recurrence was 22 (range 7–37) gw. Significant risk factors of recurrence of VTE were ≥2 previous VTE's (5.7% vs. 40.0%, $P < 0.001$) or thrombophilia (26.1% vs. 53.3%, $P = 0.034$). The strongest link existed in antiphospholipid antibodies carriers (2.6% vs. 20.0%, $P = 0.012$). Other risk factors for recurrence were history of VTE related to pregnancy and/or oral contraceptives with RR 8.5 (95% CI 1.1–62.5), but not age >35 years with RR 0.27 (95% CI 0.04–1.82) nor BMI >30 1.49 (95% CI 0.53–4.18).
Summary: Two main risk factors for recurrence of VTE during pregnancy seem to be thrombophilia and history of 2 or more VTE. In those women the LMWH prophylaxis should be initiated in the beginning of pregnancy with at least high prophylactic dose.

PB 3.70-5
Risk factors for recurrence after the first venous thromboembolic event in women

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Background: Despite the anticoagulant treatment substantial percentage (up to 25%) of patients with venous thromboembolic disease experience recurrence. The risk of DVT recurrence is particularly high in patients with unprovoked first thrombotic event. The assessment of the risk of recurrence is important for the decision of the optimal duration of treatment for each individual patient. Whether the risk of recurrence is related to a younger age of onset of VTE is unclear.
Aims: The aim of the study was to compare the recurrence rate of venous thromboembolic events (VTE) in women younger than 45 to women older than 45 years and to assess the predictive value of some laboratory (presence of thrombophilic abnormalities) and clinical thrombosis risk factors (pregnancy, surgery, oral contraceptive use, malignancy, obesity, infection).
Methods: Five hundred and eighty two women after a first objectively confirmed VTE were included in the study, 370 of them younger (mean age 30.2) and 212 older (mean age 58.3) than 45 years. All investigated women were outpatients at Anticoagulation Clinics at Clinical Center Novi Sad and Blood Transfusion Institute Belgrade. The signed informed consent was obtained from all investigated women and the study was approved by the local research ethics committee. All the data regarding the presence of risk factors and thrombophila (antithrombin, protein C, protein S, FV Leiden and FII G20210A mutation) were collected during their regular visits. Each recurrent event was confirmed by compression ultrasound examination, lung scintigraphy, computed tomography/angiography or magnetic resonance imaging.

Results: During a mean follow up of 9.6 years, 123 recurrent VTE events occurred in 100 patients (17.2%). The 1 year incidence of recurrent VTE was 4.3% in young women and 6.1% in older ($P = 0.33$), 5 year incidence of recurrence was 10.8% and 12.7% ($P = 0.51$), and 10 year incidence was 12.9% and 17.1% ($P = 1.0$). Thrombophilia was diagnosed in 163 young women, 56 of them had recurrent VTE, with no difference to the older group, with 10 recurrences out of 29 thrombophilic women ($P = 1.0$). The highest incidence (34 in 103 pts) of recurrences was observed in the group of women with unprovoked VTE as opposed to provoked VTE with 89 recurrences among 479 pts ($P = 0.002$) No significant associations between the recurrence of VTE and presence of risk factors such is pregnancy, surgery, hormone therapy, malignancy, obesity or infection was established in either group. We found no recurrences in the group of young women with oral contraceptive use related first VTE, due to the permanent discontinuation of their use.
Conclusion: The risk of VTE recurrence in women is highest after idiopathic first thrombotic event. According to our results the risk of recurrence is not age dependent. We have found neither laboratory nor clinical factors that could be useful in predicting the risk of VTE recurrence. Our findings could influence the decision on the duration of anticoagulant treatment of women with unprovoked VTE, which should be tailored individualy.

PB 3.70-6

Pulmonary embolism severity index accurately predicts long-term mortality rate in patients hospitalized for acute pulmonary embolism

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Background: The Pulmonary Embolism (PE) Severity Index (PESI) is a clinical prognostic rule that accurately classifies PE patients in five risk classes with increasing mortality. PESI score has been validated in studies with a relatively short-term follow-up and its accuracy in predicting long-term prognosis has never been established.
Aim of the study: To asses the accuracy of the original and simplified PESI to predict the 6-month and 1-year mortality rate in PE patients
Methods: Consecutive patients admitted to the tertiaryhospitalofVarese(Italy) with an objectively diagnosed PE between January 2005 and December 2009 were included. Information on clinical presentation, diagnostic work-up, risk factors, treatment, and mortality during a 1-year follow-up was collected.
Results: 538 patients were enrolled in this study. Mean age was 70.6 (±SD 15.2), 44.4% of patients were male, and 27.9% had known cancer. One-year follow up was available for 96.1% of patients. Overall mortality rate was 23.2% at 3 months, 30.2% at 6 months and 37.1% at 12 months. The discriminatory power of the PESI score to predict long-term mortality, expressed as the area under the ROC curve, was 0.77 (95% CI 0.72–0.81) at 3 months, 0.77 (95% CI 0.73–0.81) at 6 months and 0.79 (95% CI 0.75–0.82) at 12 months.
Anticoagulant treatment beyond 3 months did not influence mortality outcome at 6 or 12 months. Simplified PESI had a similar overall accuracy compared to the original PESI at 3 and 6 months, but this was significantly lower at 1 year.
Conclusions: The results of this study suggest that PESI score may also be an accurate tool to define the 6-month and 1-year mortality rates in PE patients.

PB3.71 – Regulation of gene expression in vascular cells

PB 3.71-1

Aspirin influences megakaryocytes gene expression leading to MRP4 up-regulation in human platelets

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Background: Drugs can trigger changes in the expression of mechanisms susceptible of favouring their elimination. Such mechanisms are related to transient induction of the corresponding gene transcriptional regulation. An important protein involved in drug resistances is Multidrug Resistance Proteins 4 (MRP4), an organic anion cellular detoxifier. We recently demonstrated that its up-regulation has a role for the reduced aspirin action in patients after by-pass surgery.
Aims : As it was demonstrated that aspirin enhances MRP4 mRNA levels in rat and this overexpression is PPARα dependent, the aim of our study is to verify whether aspirin induces MRP4 up-regulation in human platelets through a direct action on megakaryocytic gene expression and whether it is PPARα dependent.
Methodology: We evaluated the changes on MRP4 expression in: human megakacaryoblastic DAMI cells grown in the presence of aspirin or PPARα agonist, WY14643 (WY), in platelets derived from human megakaryocytic progenitor cell cultures and in platelets obtained from healthy volunteers, treated *in vivo* with aspirin for 15 days. MRP4 expression was analysed by Q-RT-PCR, Western blot and immunofluorescence.
Results : In DAMI cells, grown in the presence of aspirin or WY, we found a significant increase in MRP4 mRNA expression (1.5 fold increase for both aspirin and WY). We also revealed an increase in MRP4 proteins expression after treatment with aspirin and WY.
In human megakaryocytic progenitors, grown in the presence of aspirin or WY, MRP4 mRNA expression is higher than in mock culture (1.7 fold increase for aspirin and 2 fold increase for WY). Such treatment shows an MRP4 protein over-expression also in their derived platelets (1.6 fold increase and 1.4 fold increase respectively).
Aspirin induces MRP4 expression after *in vivo* administration, as MRP4 mRNA expression significantly increases in platelets obtained from healthy volunteers treated for 15 days with aspirin (300 mg/die) (approximately 30%, vs. platelets obtained after 1 day's treatment $P = 0.08$). Both Western Blot and immunofluorescence analysis confirm such enhancement.
Conclusion: The megakaryocytes have an adaptive response that leads to cell detoxification of aspirin; in fact, our results show, for the first time, that aspirin treatment induces genomic changes in megakaryocytes leading to MRP4 up-regulation and that PPARα is the nuclear receptor involved in this regulation.

PB 3.71-2

Deleterious effects of a mutant mitochondrial fission protein on calcium and energy homeostasis

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Background: Cardiovascular disease is the most common cause of morbidity and mortality worldwide, of which cardiomyopathies account for a proportion. One of the hallmarks of progressive heart disease is diminished energy metabolism associated with cardiac mitochondrial dysfunction. A recently identified mouse mutant in the *Dnm1 l* gene, Python, leads to the development of dilated cardiomyopathy. ATP levels were found to be reduced in the heart, but not in other tissues tested.

Aim: This project aimed at clarifying the role of mitochondrial dynamics in the development of heart failure in this model.
Methods: The effect of the mutation on protein function was assessed by measuring mRNA and protein levels, protein-protein interaction levels and direct measurement of mutant protein activity after purification from *E. coli*. The morphological effect of protein dysfunction was investigated by organelle imaging via immunocytochemical staining, while biochemical measurements included cytosolic and mitochondrial calcium levels and the efficiency of mitochondrial oxidative phosphorylation.
Results: Evidence was obtained of alteration in mitochondria, peroxisome and ER morphology in various cell types. There was a suggestion of altered physical interaction between the mitochondria and the ER. Increased cytosolic calcium levels after stimulated release from ER stores and reduced mitochondrial uptake of calcium were observed in Python fibroblasts. Mitochondrial membrane polarization was also altered. These changes were associated with reduced oxidative phosphorylation activity in the hearts of Python mice that increased in severity with age. Ultimately a decrease in cytosolic ATP levels occurred, which was insufficient for normal heart function. Dnm1 l expression was found to increase with age in hearts of wild type mice, but not other tissues. This may be suggestive of an increase in the importance of the Dnm1 l protein in the ageing heart.
Summary: A mutation in the mouse gene, *Dnm1 l*, leads to dilated cardiomyopathy. We hypothesize that the mutation impairs the ability of the mitochondrial fission protein to form stable higher order structures on the mitochondrial membrane, thereby impairing the functional ability of the protein. Mitochondrial fission and ER tethering are subsequently altered. The defect in mitochondrial-ER tethering leads to dysfunctional mitochondrial calcium uptake and altered mitochondrial membrane potential, leading to a progressive decline in oxidative phosphorylation activity and ATP production. The high-energy demanding cardiomyocytes are unable to cope with the decline in ATP levels resulting in cellular dysfunction and a dilative response.

PB 3.71-3

Diet modification in conjunction with regulatory immune response to a combination of ApoB and HSP60 peptides controls progression of atherosclerotic lesions in Apobtm2Sgy Ldlrtm1Her/J mice

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Background: The pathogenesis of atherosclerosis involves inflammation and immune reactions. Raised plasma cholesterol levels are required to accelerate atherogenesis in mice. Hypercholesterolemia results in accumulation of and oxidation of low density lipoproteins leading to activation of endothelial cells, macrophages and T cells. Several studies have demonstrated effective early reduction of atherosclerosis in hyperlipidemic mouse models by inducing immunological tolerance to modified lipids and peptides derived from apolipoprotein B (ApoB) 100, HSPs 60/65. However the effect of tolerance along with diet modification on plaque progression has not been studied in detail
Aim: The objective of the present study was to evaluate the effect of oral tolerance to a combination of ApoB and HSP60 peptides along with diet modification on atherosclerotic plaque progression in double-gene knockout ($Apob^{tm2Sgy}$ $Ldlr^{tm1Her}$/J) mouse model.
Methods: Groups of $Apob^{tm2Sgy}$ $Ldlr^{tm1Her}$/Jmice were fed a high-fat diet for 10 weeks, to establish atherosclerotic lesion. In the last 2 weeks, mice were orally dosed with combination of ApoB and HSp60 peptides or KLH followed by a shift to chow diet for the next 10 weeks. Plasma concentration of lipids were estimated using standard procedure. Quantification of atherosclerotic lesions was carried out in the aortic sinus sections stained with Elastic van Geison (EVG). Immunohistochemical analysis were carried out by indirect immunofluorescence and gene expression was quantified using real time polymerase chain reactions. Specific immune cells in the lymphoid organs were studied by flow cytometry
Results: Hypercholesterolemic mice at the end of 10 weeks of high fat diet feed had a plasma cholesterol level of 14.10 ± 0.52 mM which reduced to 10.79 ± 2.65 mM on shifting to chow diet. In spite of reduction in lipid levels, atherosclerotic lesion in the aortic sinus increased from $29.48 \pm 3.6\%$ to $42.93 \pm 2.9\%$ in control animals. Oral administration of ApoB and HSP60 peptides resulted in a 37.6% reduction in lesion progression compared to control in the aortic sinus ($42.93 \pm 2.9\%$ vs. $26.8 \pm 4.4\%$, $P = 0.01$) and descending aorta. Protection against plaque progression was associated with increase in $CD4^+$ $CD25^+$ T cells expressing Foxp3 ($P = 0.011$) and CTLA-4 ($P = 0.005$) cells in the lymphoid organs. Expression of regulatory cell markers;Foxp3 CTLA4 and TGF-β were significantly higher in aorta as shown by RT PCR analysis and the number of $CD4^+$ $Foxp3^+$ regulatory T cells was higher (2.53 ± 0.14 vs. 12.09 ± 0.43, $P < 0.001$) in the aortic sinus of the peptide-tolerized mice as observed by immunohistochemistry. Tolerance to peptides also resulted in 33.3% increase in collagen content in the lesion ($P = 0.008$), and reduction in macrophage (58.8%) and TNF-α (67.12%)in the plaque.
Conclusions: Our results suggest that diet modification in combination with oral tolerance can prevent progression of established lesion, mediated by regulatory T cells

PB 3.71-4

Aspirin inhibits the platelet-mediated expression of antithrombotic genes in monocytes

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Background: Activation of platelets *in vivo* leads to formation of monocyte platelet aggregates, which can be raised acutely in patients suffering a myocardial infarction (MI), and play a role in thrombus biology and atherogenesis. Genome wide expression profiling of monocytes stimulated *in vitro* by activated platelets found a number of antithrombotic genes are upregulated by the platelet stimulus, including tissue factor pathway inhibitor (*TFPI*) and protein C receptor (*PROCR*). GWE profiling also found that these two genes were expressed at lower levels in platelet-stimulated monocytes from MI patients compared to matched controls.
Aims: Since the MI patients were receiving aspirin the aim of this study was to determine the effect of aspirin on expression of these two genes.
Methods: Experiments were conducted in three stages using citrated blood from healthy non-medicated volunteers: (i) whole blood was incubated for 4 h with or without therapeutically relevant doses of aspirin (5.0×10^{-4} M and 8.3×10^{-5} M), and with cross-linked collagen-related peptide (CRP-XL; 0.5 μg/mL) to activate the platelets. Monocytes were then isolated using CD14 + ve magnetic Dynabeads®; (ii) washed platelets were treated for 15 min with aspirin before reconstituting with autologous monocytes (isolated using CD14 microbeads; Miltenyi), activating with CRP-XL and incubating for 6 h; (iii) washed platelets were activated with CRP-XL for 15 min and platelets and microparticles removed by serial centrifugation, leaving the platelet releasate. Releasate was separated into high and low molecular weight fractions using 10 kDa filters. The concentrated fraction was diluted back to the original volume. Arachidonic acid metabolites were isolated using a hexane based extraction solvent. The hexane was evaporated under nitrogen gas and the lipids re-suspended in 20 μL methanol before being made up to 1 mL in RPMI1640 + glutaMAX. All three fractions were added back to autologous monocytes that had been isolated using CD14 microbeads and incubated for 6 h. All incubations were carried out at 37 °C. Total RNA was extracted before reverse transcription and qRT-PCR was carried out on all samples.

Results: In whole blood (n = 7), *TFPI* and *PROCR*, were significantly decreased by 35–50% with both low and high therapeutic doses of aspirin (P = 0.0156 for all). Platelets that were treated with aspirin (n = 5) before reconstitution with autologous monocytes produced a similar decrease to that seen in whole blood, *TPFI* (34.5 ± 21.2%; P = 0.0386) and *PROCR* (46.3 ± 9.1%; P = 0.0127) with no further decrease in expression when monocytes were also treated with aspirin. The platelet-mediated increase in expression of these two genes was shown to be induced by the platelet releasate rather than by cell-cell contact. Using the fractionated platelet releasate, *TFPI* gene expression was significantly increased ($P \leq 0.0001$; n = 9) by the lipid soluble fraction whereas *PROCR* expression was significantly increased ($P \leq 0.0001$; n = 9) by the high MW protein fraction.
Conclusions: The increase in expression of *TFPI* and *PROCR* in monocytes induced by platelets is, in part, mediated through the effect of aspirin on platelets, which contrasts with aspirin's well-established cardio-protective properties. However, while regulation of TFPI appears to be via a reduction in COX-1 metabolites of arachidonic acid, regulation of PROCR may be through a different mechanism.

PB 3.71-5
Establishment of a Lentiviral vector encoding human HGF and the infection of human ADSCs

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Background: The delivery of adipose-derived stem cells (ADSCs) for promoting tissue repair has become a potential new therapy, while hepatocyte growth factor (HGF) is an important growth factor with angiogenic, anti-firotic, and anti-inflammatory benefis.
Aims: This paper was aimed to construct a recombinant lentiviral vector encoding human HGF and to examine hHGF expression in human ADSCs after infection.
Methods: The transfer of the target gene, hHGF, using the pcDNA3.0-hHGF plasmid was verified via endonuclease digestion and DNA sequence analysis. The HGF genomic fragment was isolated from the plasmid via polymerase chain reaction (PCR) and was subsequently cloned into multiple-cloning sites of the pGC-E1 vector to generate the recombinant lentiviral vector, pGC-E1-hHGF. The recombinant lentiviral vector was verified via PCR and DNA sequencing. The three lentiviral vectors, pGC-E1-hHGF, pHelper1.0, and pHelper2.0, were cotransfected into 293T cells using Lipofectamine 2000 to generate lentiviral particles. The viral titers were determined via a one-in-one whole dilution. hADSCs were separated, cultured, and identified based on the expression of cell surface antigens and multiple differentiation potential. The hADSCs were infected with the lentiviral vector, and the infection efficiency was assessed by flow cytometry. hHGF protein expression levels were detected by western blot analysis and the concentration of hHGF in cell culture medium was determined by enzyme-linked immunosorbent assay (ELISA).
Results: The hHGF lentiviral vector was successfully generated. The final viral titers were 1×10^8 TU/mL following viral concentration. The infection efficiency in hADSCs was 53 ± 15%. Western blot and ELISA results indicated that the hHGF protein levels were much higher in hADSCs infected with hHGF lentiviral vector than those of the cells infected with the empty lentiviral vector.
Conclusion: The hHGF lentiviral vector was successfully generated, and the lentiviral vector was able to safely infect hADSCs with high infection efficiency, thereby producing cells that overexpressed hHGF, which may provide a new strategy for the treatment of ischemic heart disease (IHD) and other ischemic diseases.

PB 3.71-6
Monocyte contribution to thrombus mass and stability through gene expression

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Background: Rupture of an atherosclerotic plaque and the consequent exposure of pro-thrombotic molecules to the flowing blood results in the formation of a platelet rich thrombus. Platelets become activated through G-protein coupled receptors and FcγR-linked tyrosine kinase receptors on their membrane. Their subsequent degranulation results in the translocation of P-selectin and other cell surface receptors to the membrane, which allows the recruitment of monocytes and other leukocytes into the growing thrombus. We have previously shown that the interaction of platelets with monocytes can induce gene expression, including some for proteins that regulate haemostasis and fibrinolysis. Whilst platelets are thought to be the main pro-thrombotic cell present within a thrombus, the contribution of monocytes to thrombus weight and stability through gene expression is yet to be determined.
Aims: We aimed to assess the role of monocytes in an *in vitro* model of thrombosis by depleting monocytes from whole blood before forming an arterial thrombus in a Chandler loop to assess the effect in weight and fibrinolysis, and to determine whether these effects are related to the expression of some key anti-thrombotic genes previously shown to be induced in monocytes by platelets; specifically tissue factor pathway inhibitor *(TFPI)*, endothelial protein C receptor *(PROCR)* and plasminogen activator inhibitor type 1 *(PAI-1)*.
Methods: Blood from healthy consented volunteers (n = 5) was collected into citrate anticoagulant and used to form thrombi in a Chandler loop by adding 1 mL recalcified blood to a 45 cm length of 3 mm diameter PVC tubing. Blood was used either untreated or after monocytes were depleted using CD14 Dynabeads®. The loops were incubated at 37 °C for 4 h, the resulting thrombi were removed from the tubes, blotted (to remove excess fluid) and weighed, and then either homogenised and used for RNA extraction, reverse transcription and qRT-PCR, or incubated for 24 h at 37 °C in Hanks buffered saline, after which the thrombi were weighed again to calculate weight loss and the supernatant analysed for D-dimer levels.
Results: Monocyte depletion was confirmed to be 95.5 ± 2.4% (P = 0.0032; n = 5) by flow cytometry using a CD14-FITC antibody (Serotec). Initial thrombus weight for the whole blood and monocyte depleted blood samples were similar (23.7 ± 4.6 mg and 21.4 ± 4.6 mg respectively), however, there was significantly more weight loss over a 24 h period in the monocyte depleted samples compared to controls (P = 0.0169; n = 4). Monocyte depleted thrombi also showed higher fibrinolysis, measured by D-dimer release, compared to the control samples (P = 0.0056; n = 4) suggesting monocytes stabilise thrombi. All four genes studied were expressed at significantly lower levels in the monocyte depleted thrombi compared to non-depleted thrombi (n = 5). For TFPI there was a decrease of 35.0 ± 23.0% (P = 0.0272), for PROCR the average decrease was 80.3 ± 11.4% ($P \leq 0.0001$) and for PAI-1 the average decrease was 78.0 ± 13.9% (P = 0.0002). Levels of CD14 mRNA were also decreased by an average of 61.6 ± 10.4% (P = 0.0002), confirming the thrombi were depleted of monocytes.
Conclusion: This study identifies monocytes as key players in thrombus stability that may be linked to the expression of anti-thrombotic genes through the interaction of monocytes with platelets within the thrombus.

PB3.72 – Thrombophilia – IV

PB 3.72-1

Peripheral arterial thrombosis and thrombophilia

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Background: Most peripheral arterial thromboses occur in association with atherosclerotic occlusive disease or cardiac dysrhythmia. In the absence of these co-morbid conditions, patients who develop arterial thromboembolism may undergo evaluation for an underlying thrombophilia.

Aims: The objective of this study was to determine the prevalence and spectrum of thrombophilia in patients who develop unexplained peripheral arterial thromboembolism.

Methods: After approval by the institutional review board, all patients at a tertiary referral center who underwent operative thromboembolectomy of upper extremity (UE), mesenteric and renal (MR), aorto-iliac (AI), and lower extremity (LE) arteries over a 7-year period were reviewed (January 2005-December 2012). Those with known peripheral atherosclerotic occlusive disease (PAOD) or arterial injury were excluded. Thrombus distribution, previous thrombotic events, cardiac evaluation, prevalence and type of thrombophilia, co-morbidities, and clinical outcomes (mortality and major amputation) were evaluated.

Results: 231 patients underwent upper or lower extremity, mesenteric, renal, or aorto-iliac thromboembolectomy during the study period, and PAOD was present in 119. In the remaining 112 patients without PAOD, 71 (63%) had a potential cardiac etiology (dysrhythmia, valvular heart disease, congenital heart defect, paradoxical embolism or aortic mass), 40 (35%) had an abnormal thrombophilia evaluation, and 14 (12.5%) had no detectable cardiac source or underlying thrombophilia. In the absence of a cardiogenic embolism, thrombophilia was present in 66% of patients, and 7 of 40 (17.5%) patients had multiple concurrent thrombophilias. The more commonly identified thrombophilias were antiphospholipid antibodies (APA) in 16, malignancy in 12, factor V Leiden in 4, protein S deficiency in 4, protein C deficiency in 3, factor VIII elevation in 3, heparin-induced thrombocytopenia in 2 and vasculitis in 2. Five additional patients presented either with pregnancy, prior splenectomy, nephrotic syndrome, hemolytic anemia, or lipoprotein (a) elevation. Thromboembolectomy was performed in 35 UE, 16 MR, 12 AI, and 69 LE arteries, and 14 had multiple sites of thromboembolism. Thrombus location was not significantly different when comparing patients with a cardiogenic vs. thrombophilic source. Patients with a cardiogenic etiology were significantly older than patients with thrombophilia (73.8 vs. 53.3 years, $P < .004$), and they had a significantly decreased survival ($P < .044$). Patients with thrombophilia had a significantly higher number of amputations or end-organ loss ($P < .023$) and cancer-associated thrombophilia was associated with a significantly decreased survival, when compared with non-cancer thrombophilia ($P < .046$). Twelve month survival was 73%, 84% and 58% in the cardiogenic, thrombophilia and malignancy groups respectively.

Conclusion: Most patients with non-cardiogenic peripheral arterial thrombosis have an underlying thrombophilia, with antiphospholipid antibodies and malignancy accounting for the majority. Better treatment strategies are needed to improve the high mortality associated with cardiogenic and cancer-associated arterial thrombosis.

PB 3.72-3

Thrombophilia screening as part of preventive medicine? Single center experience

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The need of screening healthy population for thrombophilia is debatable. Since 2005, a battery of screening assays has been offered as part of preventive health services at the Rambam Health Care Campus.

Thrombophilia screening includes protein C global assay, assessment of prothrombin G20210A mutation and homocysteine level, which amount to a third of the cost compared to complete thrombophilia evaluation.

Between 2005 and 2010, 15,668 subjects visited the Rambam preventive medicine center; only 910 (5.8%) of them decided to undergo thrombophilia screening.

The aim of this study was to characterize the group that chose to have screening tests and to evaluate, using a questionnaire, the effect of thrombophilia screen results on patients' health behavior. The questionnaires were distributed at least 1 year after receiving the test results.

The population that requested thrombophilia screening was older (52 ± 9 years) and included mostly males (76%) compared to the rest of the individuals attending the preventive medicine center (48 ± 11 years and 64% males, $P = 0.0001$). Out of the 910 subjects, 15.2% were found to have abnormal protein C global assay results, 6% had the prothrombin G20210A mutation and 18.5% had high homocysteine levels. Seventy percent of the subjects found positive for thrombophilia, referred to a coagulation or hematology specialist after receiving the test results. More than half of them (56%) reported receiving recommendations to engage in physical activity, 43% to reduce their body weight, 40% to start anticoagulant prophylaxis during hypercoagulable states, 15% to stop smoking and 12% to stop or avoid hormonal therapy. Among the persons found to have thrombophilia, 83% reported maintaining regular physical activity compared to 69% of those without thrombophilia ($P = 0.022$). Approximately 2/3 of the subjects who were recommended thromboprophylaxis reported following the instructions and receiving the therapy during hypercoagulable states.

None of the subjects with or without thrombophilia, who filled in the questionnaire, developed any thrombotic event after receiving the test results.

Interestingly, 47% of women with thrombophilic risk factors, experienced previous pregnancy complications compared to 9% in women without thrombophilic risk factors ($P = 0.005$). Most pregnancy complications were miscarriages (67%), premature birth (27%) and pre-eclampsia (6%).

Notably, 77% of the subjects found to have thrombophilia, would recommend others to undergo screening compared with 64% of the subjects in whom thrombophilia was not revealed ($P = 0.021$).

In conclusion, personal knowledge of thrombophilia test results raised patients' awareness and influenced their health behavior aiming to reduce the risk of thrombosis.

Thrombophilia screening might be an important part of preventive medicine; however further studies assessing this strategy are warranted.

PB 3.72-4

Aging induces a thrombophilic phenotype independently from acute medical conditions

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Aging is associated with an increasing prevalence of thrombotic complications, both in arterial (i.e. atherothrombosis, atrial fibrillation) and venous (i.e. venous thromboembolism) vascular bed. In fact, an activation of the coagulation cascade has been described in elderly people.

However, standard coagulation tests are not suitable to reveal a thrombotic tendency and markers of activation of coagulation have some relevant limitations. Instead, the measurement of an individual's capacity to generate thrombin captures the end result of the interaction between proteases and their inhibitors and therefore mirrors the thrombotic phenotype.

In this study we assayed plasma levels of PCR and markers of coagulation cascade activation as well as thrombin generation profile in a population of elderly patients admitted to the Emergency Department of our University Hospital. In order to take into account the possible effect of transient acute conditions, the results were compared with those obtained in healthy elderly subjects and in two younger populations, one obtained in the same setting and one composed of healthy adults.

The study included 45 subjects aged 70–106 years (mean 84.3 ± 12.6), 23 males and 22 females, consecutively admitted to the Emergency Department of Pisa University Hospital (group A). As a control group, we chose 35 subjects aged 18–69 years (mean 51.7 ± 15.9), 19 males and 16 females, also consecutively admitted to our Emergency Department (group B). Two further control groups consisted of 40 younger healthy subjects, 22 males and 18 females, aged <70 years (mean 41.0 ± 13.6) (group C) and 30 older healthy subjects, 16 males and 14 females, aged 70–95 years (mean 86.5 ± 11.8) (group D). None of the subjects participating to the study was being treated with antithrombotic or anticoagulant therapy at the time of enrollment.

Thrombin generation profile was assayed using a fluorimeter assisted by a dedicated software program (Technothrombin TGA, Technochlone, Wien).

High-sensitivity PCR (hsPCR) values in the four groups were 9.1 ± 1.5, 7.5 ± 1.5, 3.6 ± 1.1 and 8.2 ± 1.4 mg/L, respectively. The levels of fibrinogen ($P < 0.001$), D-dimer ($P < 0.01$), TAT complexes ($P < 0.05$), F1 + 2 fragment ($P < 0.02$) and tPA ($P < 0.05$) were significantly greater in the groups A and D in comparison with groups B and C, confirming the increase of coagulation activation with age; on the contrary, antithrombin III activity was lower in groups A ($P < 0.001$) and D ($P < 0.01$). Group D showed mean levels of fibrinogen ($P < 0.01$), D-dimer ($P < 0.05$), TAT complexes ($P < 0.05$) and F1 + 2 fragment ($P < 0.05$) significantly lower than those of group A.

The results of thrombin generation assay showed significant differences in lag time ($P < 0.001$), time to peak ($P < 0.01$), peak thrombin generation ($P < 0.05$), start tail ($P < 0.001$) and ETP ($P < 0.05$) in the four groups. In particular, there were highly significant differences between groups A or D and both group B and C, whereas the results of group A did not differ from those of group D and those of group B did not differ from those of group C.

These data suggest that aging induces a thrombophilic state independently from acute medical conditions.

PB 3.72-5

Congenital antithrombin deficiency-clinical phenotype in 40 patients

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Background: Inherited AT deficiency is regarded as the strongest genetic factor increasing the risk of venous thromboembolism (VTE) even 20–50 fold. Most patients have AT levels between 40% and 60%. Whether the risk for thrombosis correlates with the severity of AT decrease is still not clear.

Aim: To evaluate the clinical phenotype in patients with antithrombin deficiency registered at our centre with regard to the function levels of antithrombin and gender.

Methods: The diagnosis of antithrombin deficiency was based on repeated AT testing (chromogenic assay Anrithrombin Berichrom, Siemens) and exclusion of acquired causes of AT decrease. The prevalence, type and recurrence of thrombosis, age at first thrombosis, concomitant risk factors and indefinite anticoagulant therapy were evaluated.

Results: In the group of 40 patients with inherited AT deficiency (22F/18M) with a median age of 40 years (range 2 weeks–65 years) the AT activity was of 49% (18–65%). Familial thrombosis was present in 80% of patients, but only one and one patient had concomitant heterozygous FVLeiden and prothrombin mutation, respectively. Thrombosis developed 31(78%) patients with a first manifestation at median age of 25 years (2 weaks–52 years), 27/87% patients had deep vein thrombosis (DVT), and 2 and 4 developed sinus vein thrombosis and ischemic stroke, respectively. Out of 27 DVT patients 19/70% had proximal thrombosis; 13/48% developed PE and 13/48% suffered from recurrent thrombosis. Two patients died at age of 2 weeks and 13 years due to a massive VTE and recurrent PE, respectively.

Both, symptomatic females ($n = 16$) and males ($n = 15$) developed the first thrombosis at the same median age (25 years), with similar prevalence of PE (7/44% and 6/40%, respectively). However, recurrent DVT, idiopatic VTE and indefinite anticoagulation were more frequent in males than in females (60% v.s. 25%, $P = 0.053$; 67% v.s. 19%, $P < 0.05$; and 73% vs. 58%; $P < 0.05$, respectively). The first thrombotic event in 81% symptomatic females was triggered by concomitant risk factors with most frequent pregnancy (31%) and oral contraception (31%).

The proportion of symptomatic patients was similar in the patient′s group with AT ≤40% (11/13; 85%) and AT >40% (20/27; 74%). Comparison of symptomatic patients with the AT level ≤40% ($n = 11$) and >40% ($n = 20$) showed higher prevalence of PE (64% vs. 30%, RR:2.1), recurrent VTE (55% vs. 35%, RR 1.55), idiopatic VTE (64% vs. 30%, RR 2.1) and indefinite use of anticoagulants (64% and 50%, RR 1.27) in patients with AT levels ≤40%, however, the differences were not statistically significant.

Conclusion: Our results confirmed inherited AT deficiency to be a strong risk factor with an early manifestation of VTE. In women the use of contraceptives and pregnancy were the most frequent triggers of the first thrombosis. With regard to a high frequency of recurrent thrombotic events and a potential of early death we suggets that indefinite anticoagulant therapy is justified in AT deficient patients who developed idiopatic life threatening thrombosis in very young age.

PB 3.72-6

Multicenter evaluation of the INNOVANCE Free PS Ag assay

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Background: A particle-enhanced agglutination assay for the quantification of free protein S antigen in human plasma (INNOVANCE® Free PS Ag*) was developed by Siemens Healthcare Diagnostics Products GmbH (Marburg, Germany). Aim of this study was to investigate the new assay in different clinical settings.

Methods: The new assay was evaluated on BCS® XP, Sysmex® CA-7000, CA-1500 and CS-2100i* systems and compared with the STA® LIATEST® Free Protein S assay on the STA R EVOLUTION®

system (Diagnostica Stago, France) and the HemosIL™ Free Protein S assay on the ACL TOP® system (Instrumentation Laboratories, USA) by measuring >119 frozen samples. The following patient cohort was investigated: patients admitted for thrombophilia screening, patients with hereditary protein S deficiency, patients suspected for acquired protein S deficiency (e.g. Anti Vitamin K therapy, pregnancy, and severe liver disease), patients in the acute phase and patients with the FV Leiden mutation. Furthermore, diluted plasmas (<10% of all samples) were tested. Precision of the new assay was determined by testing two controls and three plasma pools covering the entire measuring range on 20 days in two runs with two single determinations ($20 \times 2 \times 2$ scheme).

Results: The overall correlation between the INNOVANCE Free PS Ag assay on the BCS XP system and comparative methods was high with a correlation coefficient of 0.966, slope of 0.95 and an intercept of 5.79% of norm for the comparison to the STA LIATEST Free Protein S assay and a correlation coefficient of 0.961, slope of 1.0 and an intercept of 6.44% of norm for the comparison to the HemosIL Free Protein S assay. Comparability of results obtained on the BCS XP system (Siemens) with results obtained on Sysmex CA-7000, CA-1500, and CS-2100i systems (all Sysmex Inc, Kobe, Japan) was excellent (correlation coefficients ≥0.994; slopes between 0.99 and 1.06; intercepts between −3.91% and −0.4% of norm).

Both, repeatability and within device CV were for all investigated controls and pools ≤2.5% on Sysmex CA-7000, CA-1500, and CS-2100i systems and ≤4.8% on the BCS XP system.

Conclusion: The new assay is well suited for the measurement of free protein S antigen. It demonstrated excellent precision, correlates well with other commercial available assays and showed an excellent comparability between different Siemens and Sysmex analyzers.

* Not available for sale in the U.S.

PB3.73 – Recurrent venous thrombosis – IV

PB 3.73-1

The risk of recurrent venous thromboembolism after a first surgery-related event a prospective cohort study

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Background: In contrast to patients with unprovoked venous thromboembolism (VTE), the risk of recurrence is low in patients in whom VTE occurred in association with a temporary risk factor/condition. Current guidelines recommend that these patients should receive secondary thromboprophylaxis for no longer than 3 months. The recurrence risk appears to be particularly low among patients with VTE related to surgery, but data are inconsistent and studies with a long follow-up are lacking.

Aim: To study the risk of recurrence in patients with a first VTE related to surgery.

Methods: The analysis was performed within the frame of the Austrian Study on Recurrent Venous Thromboembolism (AUREC), a prospective multi-center cohort study. Patients entered the study at the time of discontinuation of anticoagulant therapy. They were then seen at regular intervals at our study center and/or were contacted by the use of a questionnaire. Inclusion criteria were age >18 years, objectively verified symptomatic VTE, surgery within 3 months before VTE and anticoagulant treatment for at least 3 months. Exclusion criteria were previous VTE, VTE provoked by trauma or pregnancy, upper extremity DVT, cancer or requirement for extended antithrombotic treatment for other reasons. The study end point was symptomatic recurrent VTE objectively confirmed by imaging techniques.

Results: 157 patients with VTE after surgery were followed for a mean of 102 (+/−60) months. Patients were in average 49 (+/−14) years old and received anticoagulation for a mean of 8 (+/−9) months. Ninety patients (58%) were female. Ninety-four patients had DVT and 63 patients PE +/- DVT at presentation.

VTE recurred in 15 (10%) patients. Fourteen recurrences were unprovoked and one provoked by trauma (7 DVT, 8 PE +/− DVT). Nine were men, six were women. The mean age of patients with recurrence was 57 (+/−12) years as compared to 48 (+/−14) years among patients without recurrence ($P = 0.03$). The cumulative probability of recurrence was 2% (95% CI: 0–4) after 1 year, 3% (95% CI: 0–6) after 2 years, 6% (95% CI: 2–10) after 5 years and 12% (95% CI: 6–19) after 10 years.

Conclusion: The risk of recurrence among patients who had their first VTE event in association with surgery is low. Our findings support the concept of a limited duration of anticoagulation in this patient population.

PB 3.73-2

Prolonged clot lysis time as a potential risk factor for recurrent venous thrombosis; results from THE-VTE follow-up study

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Background: Hypofibrinolysis, as well as a high thrombin generating potential are associated with an increased risk of a first thrombotic event. However, whether they are also associated with the risk of a recurrent thrombotic event is unclear.

Aims : We studied the association of recurrent thrombosis with reduced fibrinolysis, as measured by a prolonged clot lysis time (CLT), and with high thrombin generation as measured by endogenous thrombin potential (ETP), and the combination of these two.

Methods: Between March 2004 and December 2008, consecutive patients with a first episode of deep venous thrombosis of the leg (DVT) or pulmonary embolism (PE) from the anticoagulant service at Addenbrooke's Hospital, Cambridge and the anticoagulation clinic in Leiden were invited to participate in THE-VTE case-control study ($N = 796$). Partners of patients were invited to participate as control subjects. The patients were subsequently followed-up for recurrent venous thrombosis. All recurrent events were objectively verified using hospital records. For the current analyses, we included patients who did not use oral anticoagulants at baseline and with a valid measurement for CLT and ETP ($N = 580$). To assess the risk of recurrent thrombosis, CLT and ETP were dichotomized at different cut-off points (80th, 90th, 95th percentiles as measured in the healthy controls. Hazard ratios (HR) for recurrent venous thrombosis were calculated using Cox regression analysis with 95% confidence intervals after adjustment for age, sex and study center. The study was approved by both the Medical Ethics Committee in the Leiden University Medical Center and Addenbrooke's Hospital. Informed consent was obtained by all participants.

Results: Mean duration of follow-up of 580 patients was 5.2 years. Eighty out of 580 patients were diagnosed with a recurrence. The overall incidence of recurrence was 27.0 per 1000 person years (95% CI: 21.8–32.2).

Hypofibrinolysis, i.e. CLT above the 90th percentile (>122 min), was associated with a 1.4-fold increased risk of recurrent thrombosis (HR = 1.4, 95% CI: 0.8–2.4). For high thrombin generation, i.e. ETP above the 90th percentile (>2025 nM/min), the risk was not affected (HR = 1.1, 95% CI: 0.6–2.0). Applying different cut-off points did not change the results.

Conclusion: In this study of 580 patients followed for recurrence over 5 years, we found no effect of a high thrombin potential on recurrence, while hypofibrinolysis yielded equivocal results.

PB 3.73-3

Risk factors for recurrent events in subjects with superficial vein thrombosis in the randomized clinical trial steflux (Superficial thromboembolism fluxum)

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Background/Aims: To evaluate risk factors for recurrent events in patients enrolled in the SteFlux (Superficial Thromboembolism Fluxum) clinical trial which compared different doses and duration of low molecular weight heparin (parnaparin) for superficial vein thrombosis (SVT).

Materials and Methods: Outpatients with acute SVT of at least 4 cm in length of the internal or external saphenous veins or their collaterals were randomized in a double blind fashion to receive either parnaparin 8500 UI aXa od for 10 days followed by placebo for 20 days or 8500 UI aXa od for 10 days followed by 6400 UI aXa od for 20 days or 4250 UI aXa od for 30 days. Outcomes were the composite of symptomatic and asymptomatic deep vein thrombosis, pulmonary embolism and SVT recurrence or extension in the first 30 days with a 60 day follow-up.

Results: Ninety-eight outcomes (14.7%) were recorded during 93 days among 664 patients (M/F: 246/418, mean age 65). After correction for treatment, outcomes during 93 days were associated with previous venous thromboembolism (VTE) and/or SVT and/or VTE family history or known thrombophilia (odds ratio-OR:1.2; 95% confidence interval – CI:1.1–2.0; $P = 0.011$), overweight)OR:1.9; 95% CI: 1.1–3.6; $P = 0.025$) and obesity (OR: 1.7; 95% CI: 0.9–3.4; $P = 0.09$). After stopping treatment, only absence of varicose veins (OR: 0.4; 95% CI 0.2–0.7; $P = 0.001$) and delay in diagnosis >6 days (OR: 2.2; 95% CI:1.2–4.2; $P = 0.001$) were associated with the risk for the developing an outcome.

Conclusions: Patients with these factors may deserve a higher intensity and/or longer treatment.

PB 3.73-4

Dyslipidemia and thrombotic complications in patients with venous thromboembolic disease (VTE)

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Background: Lipids and lipoproteins can affect hemostasis by modulating the expression and function of specific procoagulant, fibrinolytic and rheological factors, therefore they could contribute to venous thrombosis. The lipid-lowering therapy in some cases may prevent the complications of VTE, as recurrence and post-thrombotic syndrome.

Methods: We included 405 patients with VTE, mean age 55.1 ± 18.1 years. The patient group consisted of 53.6% women and 46.4% men. From each patient the following risk factors were collected: hypertension, diabetes, smoking, pregnancy, abortion, acute infection, AIDS, vascular disease, malignancy, COPD, trauma, thrombophilia, oral contraceptives, chemotherapy, antidepressants/antipsychotics, immobilization, surgery, family history, air travel. Cases of recurrence and post-thrombotic syndrome were recorded. A sample of serum lipid profile was collected from each patient for the study of: total cholesterol (CHT), triglycerides (TG), HDL cholesterol (HDL) and LDL cholesterol (LDL). Also we considered as dyslipidemic patients treated with lipid-lowering drugs at the time of thrombosis.

Results: The distribution of patients according to the type of thrombosis was: DVT-legs (45.9%), PE (23.4%), DVT + PE (7.9%), DVT-arms (2.1%), TV-superface(10.6%) and TV-unusual localization (8.1%). 30.4% ($n = 123$) of patients suffered recurrent thrombosis, while 20.4% ($n = 95$) developed a post-thrombotic syndrome. 47.4% ($n = 45$) of patients with recurrent thrombotic event was diagnosed with post-thrombotic syndrome, this represents 11.1% of the total group. 38.5% ($n = 156$) of patients included in the study had dyslipidemia, of which 34.1%% ($n = 53$) had a recurrent event and 29.5% ($n = 46$) was diagnosed with post-thrombotic syndrome. Of patients with dyslipidemia who had developed recurrent thrombosis, 35.8% ($n = 19$) were not being treated. While 26.1% ($n = 12$) of patients with post-thrombotic syndrome and dyslipidemia were no lipid-lowering therapy. Patients with alterations in levels of Cht had recurrent event more frequently than patients with normal Chtlevels, in patients who were receiving lipid-lowering ($P = 0.0001$) and in those who were not treated ($P = 0.03$).

Summary: A certain percentage of patients who develop post-thrombotic syndrome or a recurrent thrombotic event suffer dyslipidemia, some of them do not follow any specific treatment. According to these results, closer monitoring of lipid levels (Cht, TG, HDL, LDL), and adequate treatment of dyslipidemia could lead to a lower incidence of these complications in patients with VTE.

PB 3.73-5

Medical literature and vena cava filters: from weak to worse, with exceptions

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Background: Inferior vena cava (IVC) filters are designed to prevent the migration of venous clots toward the pulmonary circulation. Unlike anticoagulants, whose efficacy and safety have been evaluated extensively in prospective randomized controlled trials (RCT), a detailed literature analysis in 2001 found that only 1 (0.2%) of 568 articles regarding IVC filters reported a RCT (Girard et al., Chest 2002;122:963–7).

Aim: To investigate whether the literature about IVC filters has improved over the past 12 years.

Methods: An advanced search was performed in the PubMed® database (http://www.ncbi.nlm.nih.gov/pubmed, cava filter or interruption in title, items with abstract, publication date 2001–2012). All references were imported in an EndNote® library, and classified, one by one, according to predetermined criteria. These references (period B) were compared to those from the identical search and classification performed in 2001 (period A: 1975–2000).

Results: A total of 626 references for period B were analyzed, and compared to the 568 older references (period A). The mean annual number of articles increased from 21.8/year to 52.2/year ($P < 0.001$). The proportion of case reports remained stable (31.7% vs. 31.2%), whereas there was a significant increase in the proportions of retrospective series (from 33.3% to 38.5% [$P = 0.06$]) and reviews (from 6.7% to 15.5% [$P < 0.001$]). Significant decreases were observed in the proportions of animal/*in vitro* studies (from 12.9% to 7.3% [$P = 0.002$]) and prospective series or trials (from 7.4% to 4.2% [$P = 0.016$]). Articles mentioning temporary/retrievable filters increased from 20.2% to 59.1% ($P < 0.001$). Four RCTs were published during period B: one publication was an update of the PREPIC trial (the only RCT during period A), another RCT compared two techniques for filter insertion, and the remaining 2 RCTs were a feasibility study in 34 trauma patients (Rajasekhar et al., J Trauma 2011) and a RCT in 64 cancer patients that was stopped early due to poor accrual (Barginear et al., Support Care Cancer 2012). There were at least five population-based investigations (one during period A), including one study that linked IVC filter use and decreased case-fatality rate in patients with massive pulmonary embolism (PE) treated with thrombolytics (Stein et al., Am J Med 2012). In a similar search about heparin (heparin in title, items with abstracts, period 2001–2012), 247 of 3177 articles (7.7%) were

tagged as controlled trials in PubMed®. A search for trials of filters and venous thromboembolism at http://clinicaltrials.gov found 14 studies, of which four were RCTs, including three trials with published results (see above) and one completed trial whose results are awaited (the PREPIC2 trial).

Summary/Conclusion: Over the past 12 years (2001–2012), more articles about IVC filters have been published than in the preceding 25 years. This plethoric literature reflects the increasing experience with IVC (retrievable) filters, but did not report any new adequately powered RCT designed to assess filters efficacy. Only the awaited PREPIC2 trial results should provide fresh evidence regarding the efficacy of filters in selected patients with PE. In the foreseeable future, indications for IVC filter placement will remain mostly a matter of opinion.

PB 3.73-6

Vascular's involvements in Behçet's disease

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Introduction: Behcet's Disease is recognized as a thrombogenic disorder with venous tropism impairment. This vascular achievement justifies first and foremost the hospitalizations of these patients in internal medical and constitutes the first cause of morbi-mortality.

Objectives : To describe the topographies and the profile following up of this vascular Behçet's disease.

Patients and Methods: Retrospective study from 1996 to 2012, excluding the thrombotic vasculitides localized at the level of the small vessels (retina, cochleovestibular, digital).

Results: We have collected 397 patients, which sex ratio is 0.33 and average age is about 31.9 years We observed 97 vascular events (24.4%) characterized by 69 deep venous thrombosis 'DVT' events (17.4%), and 28 arterial events (7%). The average delay to diagnosis of the vascular manifestations from the date of the BD diagnosis is about 4.4 years (1–9), and 5 cases (7%) revealed the BD. The DVT are divides up essentially in lower limbs (25), on cerebral topography (4%), on abdominal area (2%), on superior vein cava (2%). We noted in the others cases some unusual localization of thrombosis (six cases of intra-cardiac, upper limbs and jugular veins…). The arterial infringements are located as followed: cerebral aneurism (05), pulmonary (10), abdominal (05), heart (4), inferior limb (3), and upper limb (1). The surgical treatment is required in 11 cases. The fatal course is deplored (10) linked in aneurysms of pulmonary arterial rupture and/or to the massive pulmonary embolisms (7), Budd-Chiari's syndrome (4) and to the intestinal ischemia (1)

Conclusion: As reported in the most studies BD affects the veins more than the arteries. The vascular involvement constitutes bad factor prognosis and the major cause of increased mortality. So it's must be prevented, detected and treated prematurely (embolization, correct anticoagulation, and reduction of thrombogenous's risks factors….)

PB 3.74-1

Overview of a global clinical trial programme with turoctocog alfa, a new recombinant factor VIII: the guardian™ programme

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Background: Congenital haemophilia A is a rare bleeding disorder that affects approximately 1 in every 5000 male births. In severe haemophilia bleeding can occur spontaneously or after minor trauma and serious complications result from bleeding into the joints, muscles, brain, or other internal organs. Novo Nordisk is developing turoctocog alfa, a new recombinant FVIII (rFVIII) for prevention and treatment of bleeding in haemophilia A. Extensive documentation of clinical safety and efficacy is a scientific and regulatory prerequisite due to the complicated structure and manufacture of this multi-domain protein. Because of the rarity of the condition, clinical trials investigating haemophilia therapeutic agents need to be multicentre/multinational, to enrol a sufficient number of patients, and to ensure that patients of different ethnicity are included.

Aims: To present an overview of a global clinical trial programme assessing the pharmacokinetics (PK), safety and efficacy of turoctocog alfa for the treatment and prevention of bleeding in relevant patient populations with severe congenital haemophilia A (baseline FVIII ≤1%).

Methods: The pre-registration clinical programme for turoctocog alfa involves seven clinical trials. The first human dose trial, performed during 2009, investigated turoctocog alfa PK and included a comparison with octocog (Advate®). The guardian[TRADEMARK]1 and guardian[TRADEMARK]3 trials were phase IIIa trials investigating safety and efficacy of turoctocog alfa in the prevention and treatment of bleeds in previously treated adult/adolescent (>12 years of age) and paediatric (<12 years of age) patients, respectively. Both trials included PK assessments, while guardian[TRADEMARK]1 also comprised a surgical subtrial. Guardian[TRADEMARK]1 and guardian[TRADEMARK]3 were both completed during 2011; participating patients were offered continued turoctocog alfa treatment in the guardian [TRADEMARK]2 extension trial, a long-term safety and efficacy trial which will continue until turoctocog alfa is commercially available in the respective countries. Two further PK trials investigated turoctocog alfa PK in Japanese subjects using different production lots. Finally, guardian[TRADEMARK]4, a phase IIIb safety and efficacy study in previously untreated patients (PUP), was recently initiated and is ongoing. Approximately 60 PUP will be enrolled and followed for ≥100 exposure days (initiated September 2012; estimated study completion date: September 2016).

Results: The PK properties of turoctocog alfa have been studied in >60 patients and shown to be comparable to those of commercially available rFVIII and plasma derived FVIII. To date, more than 210 patients have been dosed with turoctocog alfa in 18 countries worldwide (Brazil, Croatia, Germany, Israel, Italy, Japan, Lithuania, Macedonia, Malaysia, Poland, Russia, Serbia, Spain, Switzerland, Taiwan, Turkey, UK and US). The total turoctocog alfa clinical experience is in excess of 50,000 exposure days and 340 patient years. More than 110 patients have now been on preventive treatment with turoctocog alfa ≥18 months.

Summary/Conclusions: This comprehensive global clinical trials programme investigating the PK, safety and efficacy of turoctocog alfa will provide an extensive clinical database. On-going work includes defining some of the many possible further analyses of the available data. Future studies will add further important information to the

database on turoctocog alfa long-term safety and efficacy in specific patient populations.

PB 3.74-2

Coagulation Factor IX deficiency does not afford protection from pulmonary fibrosis in the experimental murine bleomycin model

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Background: Animal and human studies strongly suggest the importance of the coagulation cascade in acute and chronic lung injury. Indeed, beyond their role in hemostasis, coagulation factors can signal via their cellular receptors, the protease-activated receptors.
Aim: In this study, we hypothesized that the absence of coagulation Factor(F)IX, which is essential for the initiation, amplification and propagation phases of the coagulation cascade would reduce fibrosis development and progression.
Methods: We used the murine model of bleomycin-induced pulmonary fibrosis in wild-type (WT; $n = 14$) and FIX deficient mice ($n = 13$; a model for the severe bleeding disorder hemophilia B). After 14 days, we assessed histological markers of tissue fibrosis, inflammatory cell influx in the bronchoalveolar lavage fluid (BALF), and cytokines levels in the BALF, blood and lung homogenate of the animals.
Results: Mortality during the experiment was higher in the FIX deficient mice compared to wildtypes (23% vs. 7%). The remaining FIX deficient mice ($n = 10$) developed pulmonary fibrosis to a degree similar to WT ($n = 13$). There was no significant difference in the Ashcroft score between WT and FIX deficient mice (4.011 ± 0.4 vs. 4.2 ± 0.4), in the alpha-actin score (0.94 ± 0.09 vs. 0.70 ± 0.07) and in the inflammatory cell number. In contrast, we observed in the plasma of the FIX deficient mice significant elevations in levels of cytokines IL-12, TNFα, IFNγ, MCP-1 and IL-6.
Conclusion: Hemophilic mice with a congenital deficiency of FIX are not protected against bleomycin-induced pulmonary fibrosis. These data strongly argue against an important role of the blood coagulation cascade in the progression of pulmonary fibrosis, and raise important concerns about the use of anticoagulant therapy in patients.

PB 3.74-3

New anticoagulants and their effect on platetet function

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Background: For the last 70 years, vitamin K antagonists and heparins have been the treatment of choice for prophylactic and therapeutic anticoagulation. Over the past few years, several selectively acting anticoagulants such as the direct thrombin inhibitors argatroban (A) and dabigatran (D) and the factor Xa inhibitors rivaroxaban (R) and fondaparinux (F) have been developed. They are approved for various indications like heparin-induced thrombocytopenia type II (HIT), venous thromboembolism, stroke prevention in atrial fibrillation and acute coronary syndrome.
Aims: With their increasing clinical application, it is of interest to evaluate their interferences with the complex system of haemostasis and haemostaseological assays. Here we investigated the abovementioned anticoagulants concerning their influence on platelet function.
Methods: As platelet function assay light transmission aggregometry was used. Activation of platelets was induced by ADP, arachidonic acid, epinephrine, collagen, ristocetin, A23187, PAF C-16, PMA, TRAP, U46619 and thrombin. Further, the influence of the drugs on intracellular pathways was investigated by western blot analysis using immunostaining with VASP antibodies (M4, 5C6, 16C2).
Results: Our measurements by aggregometry revealed a distinct decrease of platelet aggregation after *in vitro* incubation with supratherapeutic concentrations of argatroban (1.0 mg/mL) compared to control. Western blot analysis showed that argatroban (c = 1.0 mg/mL) induces VASP-phosphorylation in platelets. Neither therapeutic nor supratherapeutic concentrations of dabigatran (20 µg/mL), rivaroxaban (20 µg/mL) and fondaparinux (50 µg/mL) revealed a comparable effect on platelet function.
Summary/Conclusion: Based on our investigations, under therapeutic conditions platelet aggregation assays, e.g. for the monitoring of ACS, seem to remain unaffected by the investigated drugs. Although argatroban inhibits the PKA-dependent pathway and induces VASP-phosphorylation, the reduced platelet aggregation was only observed using high concentrations, whereas therapeutic concentrations had no measurable effect.

PB 3.74-5

Significantly higher level of serum amyloid A among acute coronary syndrome (ACS) than stable angina pectoris (SAP) patients in indonesian population

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Background: The value of Serum Amyloid A (SAA) in predicting Coronary Heart Disease was still unclear in different population and ethnics, thus, we were triggered to investigate SAA level among two spectrum of Coronary Heart Diseases; the Acute Coronary Syndrome (ACS) and the Stable Angina Pectoris (SAP) in Indonesian population.
Aims: To know the difference of SAA level between ACS and SAP patients in Indonesia.
Methods: This cross sectional study was conducted in Sardjito General Hospital, Yogyakarta, Indonesia consisted of 43 participants in Acute Coronary Syndrome group and 30 participants in Stable Angina Pectoris. All patients provided witnessed informed consent, and the study was approved by the ethics committees. The Serum Amyloid A (SAA) was measured once by quantitative sandwich enzyme immunoassay and other laboratory results was obtained from medical record. Data were analyzed using Mann-Whitney test.
Results: We found that ACS group had higher median value of SAA than SAP and healthy subjects (8.05 mg/mL, 1.39 µg/mL, respectively, $P = 0.004$). Acute Coronary Syndrome group had higher leucocyte number (10.22×10^3 cell/mL vs. 7.05×10^3 cell/mL, $P = 0.001$), and creatinine level than SAP group (1.25 mg/dL, 0.95 mg/dL respectively, $P = 0.003$). There was no difference of age, lipid profile, blood glucose level and other hematology parameter among ACS compared to SAP groups.
Conclusion: Serum Amyloid A of ACS group is higher than SAP in Indonesian population.

PB 3.74-6

Elevated plasma microparticles in chronic obstructive pulmonary disease

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Background: Chronic obstructive pulmonary disease (COPD), which is characterized by poorly reversible airway obstruction, is now recognized to be a systemic disorder with extrapulmonary manifestations and significant systemic comorbidity. It is an independent risk factor for cardiovascular mortality and more than 50% patients die from cardiovascular causes, particularly from coronary artery disease. Although the association between COPD and atherothrombotic risk is established, the underlying mechanisms remain unexplored.
Aim: The present study aims to explore the possibility of platelet hyperactivity underlying the increased thrombotic events in COPD.
Methods: Levels of plasma microparticles and markers specific to activated platelets were assessed in whole blood of COPD patients and age-matched controls using flow cytometry. The study was approved by the Institutional Ethics Committee and due informed consent was obtained from participants in the study.
Results: 15 cases of stable COPD patients were selected for the study along with six age-matched controls. Current smokers were excluded from either cohort. No significant differences were noted in anthropometric features, routine hematological parameters and possible confounding variables between the two groups. The severity of COPD was staged by spirometry according to GOLD guidelines and most patients (10) were found to have moderate airway disease. Level of plasma microparticles were determined by flow cytometry and found to be significantly higher in COPD patients than normal healthy controls ($P = 0.03$). However, no significant difference was observed in markers of activated platelets including P-selectin exposure, PAC-1 binding and cytosolic reactive oxygen species between the two groups.
Conclusions: Elevated level of plasma microparticles in COPD is suggestive of prothrombotic state, and can possibly be employed as marker to assess thrombotic risk. Although enhanced level of circulating microparticles underscores possibility of platelet hyperactivity, the source of these microparticles needs to be ascertained.

PB4.21 – New antiplatelet agents – II

PB 4.21-1

Different effects of antiplatelet vs. anticoagulant agents in mouse bleeding models

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Background: Murine bleeding models are routinely used to investigate bleeding resulting from gene defects or administration of antithrombotic agents. Tail tip transections, which induce bleeding from venous, arterial, and microvascular sources, are most commonly used. However, alternative models, which assess bleeding in different vessels, vessel types and under different flow conditions are also used. It is not clear how such differences affect measurements of bleeding, or which methods are best suited for assessing disturbances in coagulation or platelet function.
Aims: We have used a saphenous vein bleeding model, a tail transaction model and developed a novel saphenous artery bleeding model in order to compare bleeding responses following haemostatic challenges to coagulation (unfractionated heparin [UFH]) or platelet function (intravenous infusion of an $\alpha_{IIb}\beta_3$ inhibitory antibody [Leo.H4]).
Methods: To induce bleeding from the saphenous vein, the vessel was transected, and a longitudinal incision was made along the medial surface as previously described (Buyue *et al*., (2008) *Blood* 112:3234). In the saphenous artery model, bleeding time is measured after transecting the vessel with a 27 gauge needle. Bleeding in the tail transection model was measured by blood loss over 30 min. Dose responses to intravenous infusion of UFH or Leo.H4 were derived and analyzed using GraphPad Prism assuming a sigmoidal dose response curve. Further, activated partial thromboplastin-initiated coagulation assays and impedance-based platelet aggregometry were used to characterize UFH and Leo.H4 doses, respectively.
Results: The tail, venous and arterial bleeding models were similarly sensitive in measuring bleeding after UFH infusion (EC50 = 0.07, 0.19 and 0.13 U/g, respectively). *In vitro*, an activated partial thromboplastin-initiated clotting assay showed that maximal inhibition of clotting could be achieved with doses of UFH above 0.01 U/g, which is below the EC50 values observed in all three bleeding models.
In contrast, Leo.H4 dose response curves showed that the saphenous vein bleeding model is less sensitive to $\alpha_{IIb}\beta_3$ inhibition (EC50 = 6.9 μg/mL) than either the tail transection or saphenous artery bleeding models (EC50 = 0.1 and 0.4 μg/mL respectively). A higher concentration of Leo.H4 (7.0 μg/mL) was required to inhibit ADP-induced platelet aggregation *in vitro* than to achieve EC50 in tail and arterial, but not venous bleeding models.
Summary: In conclusion, the findings suggest that coagulation is equally required for cessation of bleeding in isolated venous and arterial bleeding models, as well as in a tail tip transection model. In contrast, inhibition of platelet aggregation has a greater influence on limiting bleeding in arterial and tail bleeding models than in a venous model. In addition, *in vitro* coagulation assays are more sensitive to heparin inhibition than *in vivo* bleeding models, whereas arterial and tail bleeding models are more sensitive to $\alpha_{IIb}\beta_3$ inhibition than *in vitro* impedance-based platelet aggregometry.

PB 4.21-2

Relation between the mechanism of antiplatelet action of adenosine from *Solanum lycopersicum* and its derivate inosine

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Background: The inhibition of the platelet function has been used in an effort to primary and secondary prevention of cardiovascular diseases. One primary prevention strategy of this type of diseases is the fruit and vegetable consumption. In this sense, in *Solanum lycopersicum* (tomatoes), it has been observed antiplatelet and antioxidant effect, among other activities. In addition, our group has recently isolated and identified adenosine from *S. lycopersicum*. Adenosine showed a potent antiplatelet activity through the inhibition of platelet adhesion, aggregation and secretion. Inosine is a naturally occurring nucleoside degraded from adenosine and studies establish that the inhibitory effect of adenosine on platelet aggregation disappears after the addition of adenosine-deaminase.
Aims: The main aim of this work is to investigate antiplatelet action mechanisms of adenosine and inosine, and explain their differential biological effects using molecular modelling (docking). As well as investigating the protective mechanisms of aqueous fraction rich in adenosine from *S. lycopersicum* on platelet function.
Methods: For platelet activation, annexin V and P-selectin expression were measured by flow cytometry. Platelet aggregation and ATP secretion were monitored by lumi-aggregometer and luciferin/luciferase reagent. Platelet adhesion and aggregation under controlled flow were performed in a BioFlux 200 flow system. Platelet cAMP levels were measured using an immunoassay kit. The adenosine/inosine docking with respect to adenosine A2A receptor was determined by homology.
To study a possible application of an aqueous fraction in high concentration of adenosine, it was evaluated in a murine thrombosis model

and platelet aggregation in humans. The protocol was approved by the Institutional Review Board of Talca University in accordance with the Declaration of Helsinki. An informed consent was signed before blood was drawn.

Results: Adenosine and inosine concentration-dependently inhibited human platelet aggregation and ATP release stimulated by ADP and collagen. Only adenosine inhibited platelet activation by annexin V ($45 \pm 5\%$, $P < 0.05$) and P-selectin expression (80 ± 3, $P < 0.05$). Moreover, adenosine and inosine reduced collagen-induced platelet adhesion and aggregated formation under controlled flow, with IC_{50} values of 0.3 mM and 0.5 mM, respectively ($P < 0.05$). At the same concentrations that adenosine inhibited platelet aggregation, significantly increased the intraplatelet cAMP levels, but not inosine.

Based in docking experiments from adenosine receptor, the main difference is that adenosine, but not inosine, forms an additional hydrogen bond between the NH_2 of the adenine group and the residues Asn253 in H6 and Glu169 in EL2 of the A2A receptor.

Aqueous fraction inhibited thrombus formation in murine model ($75 \pm 8\%$, $P < 0.05$). Moreover, 4 h after treatment, we observed a significant inhibition of platelet aggregation induced by ADP for $19 \pm 7\%$ compared to basal ($P < 0.05$) in six healthy subjects who received the aqueous fraction.

Summary/Conclusion: It is known that adenosine presents antiplatelet activity. This study allows to conclude that deamination of this molecule to inosine does not involve a loss of the described activity, but a change in mechanism. Finally, we think that aqueous fraction from *S. lycopersicum* may be used as a functional ingredient that can be included in different food matrixes.

PB 4.21-3

Pharmacokinetics, pharmacodynamics, and tolerability of LC23-1306, a novel antiplatelet agent, in healthy subjects

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Background: LC23-1306, a reversible and active oral $P2Y_{12}$ receptor antagonist, is currently under development for the prevention of thrombotic events in patients with acute coronary diseases.

Aim: The aim of this study was to evaluate the pharmacokinetics, pharamacodynamics, and tolerability of LC23-1306 in healthy Korean subjects.

Methods: A dose-blocked, randomized, double-blind, and placebo-controlled, single ascending dose study was conducted in 60 healthy subjects, each 10 of whom received LC23-1306 at six dose levels ranging 10–600 mg or placebo in a ratio of 8:2. Blood samples were obtained for 48 h to assess the pharmacokinetics. Inhibition of platelet activity (IPA) was measured at 0 (pre-dose), 2, 8, and 24 h post-dose. The safety and tolerability were evaluated by adverse events, vital signs, ECG and clinical laboratory tests.

Results: LC23-1306 was rapidly absorbed with its median time to maximum concentration of 1.0–3.0 h. The mean terminal half-life was approximately 7.1–16.0 h, which showed an increasing tendency at higher doses. Maximum concentration and the area-under-the-concentration-time curve to the last measurable concentration increased as dose increased although it was less than dose proportional. IPA became greater as dose increased with platelet aggregation being completely inhibited at 2 h post-dose in the 100, 200, 400 and 600 mg cohorts. IPA >50% was sustained up to 24 h post-dose at doses of 200–600 mg. LC23-1306 was well-tolerated without serious adverse events. All adverse events resolved spontaneously without intervention.

Conclusions: LC23-1306 was well-tolerated after single oral administration at 10–600 mg in healthy Korean male subjects. The systemic exposure of LC23-1306 increased as dose increased. IPA was sustained effectively from 2 to 24 h in the 200–600 mg cohorts. LC23-1306 can be given once daily as an antiplatelet agent.

PB 4.21-4

Antiplatelet effect of black soybean mediated by adenosine-cAMP signaling pathway

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Background: Many clinical trials have demonstrated the beneficial effects of soybean (*Glycine max*) on general cardiovascular health. Among a variety of soybeans, black soybean is known to display diverse biological activities superior to yellow or green soybeans such as in antioxidant, anti-inflammatory and anticancer activities. However, few studies have been directed on the effect of black soybean on cardiovascular function.

Aims: In this study, we aimed to investigate the effect of black soybean extract (BB) on platelet activation, a key contributor to thrombotic diseases.

Methods and Results: In the freshly isolated human platelets, BB has shown potent inhibitory activity on collagen-induced platelet aggregation while yellow soybean extract had only marginal activity. BB also attenuated serotonin secretion and P-selectin expression, important factors for the platelet-tissue interaction along with thromboxane A_2 formation. These *in vitro* results were further confirmed in ex vivo platelet aggregation measurement and *in vivo* venous thrombosis model where oral administration of BB reduced collagen-induced platelet aggregation and $FeCl_3$-induced thrombus formation significantly. A potential active ingredient for anti-platelet effects of BB was isolated and identified to be adenosine through bioassay-directed fractionation and NMR and ESI-MS analysis. These results indicate that black soybean can be a novel dietary supplement for the prevention of cardiovascular risks and the improvement of blood circulation. We further elucidated the molecular mechanism of BB-mediated antiplatelet activity and demonstrated the significant role of adenosine in BB-induced health effects. Adenosine-cAMP signaling is found to be responsible for BB-mediated effects, resulting in inhibition of collagen-stimulated platelet activation such as intraplatelet calcium increase, adhesion receptor GP IIb/IIIa expression, fibrinogen binding and further platelet adhesion/aggregation.

Conclusion: Taken together, we provide a complete understanding for molecular mechanism of BB-induced anti-platelet activity and a new insight into the role of adenosine as a bioactive compound in soybeans.

PB 4.21-5

Anopheline anti-platelet protein from a malaria vector mosquito has anti-thrombotic effects in a pulmonary thromboembolism model without compromising hemostasis

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Background: Blood-feeding animals (e.g., mosquitoes, ticks, bats) inject saliva into hosts. The saliva has vasodilatory, anti-inflammatory and anti-thrombotic effects that facilitate smooth blood-sucking. Saliva components are considered to be attractive natural resources for drug development. We previously identified a unique anti-platelet protein, anopheline anti-platelet protein (AAPP), from the salivary gland

of female *Anopheles stephensi* (human malaria vector mosquito). AAPP specifically blocks platelet adhesion to collagen by binding directly to collagen and subsequently aggregating platelets.
Aims: To examine the potential of AAPP as a therapeutic agent, we investigated the *ex vivo* and *in vivo* anti-thrombotic effects using mice.
Methods: Effects of AAPP on whole-blood aggregation in mice were examined. AAPP was also challenged in an established model of pulmonary thromboembolism in mice. We simultaneously investigated the side-effects of the protein (prolongation of bleeding time and coagulation time). Aspirin was used as a positive control for comparison of anti-thrombotic effects.
Results: AAPP inhibits whole-blood aggregation induced by collagen at 10 mg/kg body weight. AAPP prevented pulmonary death at a lower dose (3 mg/kg) without prolongation of bleeding time compared with aspirin (100 mg/kg) that compromised hemostasis. AAPP and aspirin did not affect coagulation time. The pharmacodynamic study showed that the inhibitory effect of AAPP on whole blood aggregation decrease to 25% after 1 h from the administration.
Summary/Conclusions: These results indicate that AAPP has potential as a new anti-platelet agent with a better risk/benefit ratio than aspirin, which is the most widely used anti-platelet agent. Further study, optimization of its structure, is ongoing to improve pharmacokinetic profile.

PB 4.21-6
Interactions between vascular prostaglandins and antiplatelet agents result in profound inhibition of platelet function

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Background: It is believed that prostaglandins produced within the vasculature provide natural protection against platelet activation in man. These include the prostaglandins of the I series (especially PGI2) and of the E series (PGE1, PGE2 and PGE3). It is for this reason that high doses of aspirin, which prevent vascular prostaglandin synthesis, should be avoided.
Aims: Using antagonists selective for IP and EP receptors we have defined the receptors on platelets that mediate the effects of these four prostaglandins on platelet function. Also, we have explored the ways in which vascular prostaglandins interact with antiplatelet agents, specifically P2Y12 receptor antagonists and an EP3 antagonist, to provide enhanced inhibition of platelet function.
Methods: Platelet function was measured as platelet aggregation (platelet counting in PRP or whole blood) and P-selectin expression (flow cytometry). Intracellular VASP phosphorylation (bead assay in PRP or whole blood) was also measured. Platelet agonists were mainly U46619 and/or TRAP; receptor antagonists were CAY10441 (IP antagonist), DG-041 (EP3 antagonist) and ONO-AE3-208 (EP4 antagonist), ticagrelor, cangrelor and the active metabolite of prasugrel (P2Y12 antagonists).
Results: The overall effects of each of the prostaglandins on platelet function were inhibitory and this was accompanied by VASP phosphorylation. The inhibitory effect of PGI2 was reduced in the presence of CAY10441; that of PGE_1 was reduced in the presence of CAY10441 but not in the presence of ONO-AE3-208, demonstrating a role for IP but not EP4; in contrast the inhibitory effects of PGE_2 and PGE_3 were reduced in the presence of ONO-AE3-208 but unaffected by CAY10441, demonstrating a role for EP4 but not IP. The inhibitory effects of all prostaglandins of the E-series were enhanced in the presence of DG-041, indicating promotion of platelet function through EP3. All the P2Y12 antagonists combined with all vascular prostaglandins to provide enhanced inhibition of platelet function and in the case of the prostaglandins of the E-series there was further inhibition in the presence of the EP3 antagonist DG-041.
Conclusions: We have increased our understanding of the role of vascular prostaglandins in providing inhibition of platelet function and the ways in which they interact with existing (P2Y12 antagonists) and new (an EP3 antagonist) antiplatelet agents. The results demonstrate profound inhibition of platelet function by combinations of vascular prostaglandins and these pharmacological agents.

PB4.22 – Antiplatelet agents: miscellaneous

PB 4.22-3
A novel platelet aggregation inhibitor purified from Gloydius blomhoffii brevicaudus venom

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Background: Disintegrins are small, cysteine-rich proteins from various snake venoms with a high affinity to integrins which participate in platelet aggregation. Their inhibitory effect on platelet aggregation has been extensively studied and exploited in clinical interventions of many diseases including stroke and other cardiovascular disorders. *Gloydius* venom is a rich natural source of proteins possessing platelet aggregation inhibition activity.
Methods: This study presents production and purification process of a novel platelet aggregation inhibitor from *G. blomhoffii brevicaudus* snake venom and its biochemical and biological characterization. The platelet aggregation inhibitor was purified using three consecutive liquid chromatographic steps (*Blue sepharose, Q-sepharose* and *Superdex-200*). The purity and molecular weight were determined by SDS-PAGE, 2-D electrophoresis and MALDI-TOF mass spectrometry. The inhibitory activity on collagen- and ADP-induced platelet aggregation was examined in the platelet-rich rabbit plasma using the AP2110 'SOLAR' aggregometer. Proteolytic enzymatic and phospholipase activities were investigated by chromogenic (S-2251 and S-2238) and fluorogenic substrates (PED6), respectively.
Results: A single chain protein with molecular weight of 12 kDa and pI of 8.0 was purified with approximate purity of 99%. A dose-dependent activity curve analysis shows that the platelet aggregation inhibitory activity in the rabbit platelet-rich plasma was $ID_{50} = 3.26 \times 10^{-6}$ g or 542×10^{-9} M and no proteolytic activity detected.
Conclusion: In the present study, the purification process of the novel 12 kDa-protein with inhibitory activity on collagen- and ADP-induced platelet aggregation from the venom of the snake *G. blomhoffii brevicaudus* has been developed. The fact that no enzymatic activity found on chromogenic and fluorogenic substrates may indicate that our purified protein is potentially a new low molecular weight platelet aggregation inhibitor that inhibits a platelet aggregation receptor. Future work to investigate further structural and functional properties is required.

PB 4.22-4

Vipera lebetina (snake) venom components affecting hemostasis

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Background: *Vipera lebetina* venom is a complex mixture of biologically active components including coagulants and anticoagulants. Factor X and Factor V activating enzymes (procoagulants), lebetase (fibrinolytic and antiplatelet), L-amino acid oxidase, disintegrin viplebedin, phospholipase A_2 (antiplatelet), etc. have been isolated and characterized in our laboratory. Nucleases are ubiquitously represented in snake venoms but, especially 5'-nucleotidase, poorly specified.

Aims: The aim of this study is to extend the antiplatelet spectrum of *V. lebetina* venom via isolation and characterization of phosphodiesterase (PDE) and 5'-nucleotidase as potential antiplatelet agents, and cloning and sequencing corresponding cDNAs.

Methods: PDE and 5'-nucleotidase have been isolated by gel filtration (Sephadex G-100 sf) and ion exchange chromatography (CM52 cellulose and TSK-DEAE). The enzymatic activity of 5'-nucleotidase is determined with 5′-AMP as substrate. PDE is localised by hydrolysis of Na-bis(p-nitrophenyl)phosphate. Platelet aggregation is performed in PRP in a Chrono-Log aggregometer. Cloning and sequencing have been performed by standard procedures.

Results: *V. lebetina* venom PDE has been purified by gel filtration and CM52 cellulose chromatography, for purification of 5'-nucleotidase three-step procedure has been used including gel filtration, cation and anion exchange chromatography. PDE has molecular mass ca 120 kDa and 5′-nucleotidase ca 60 kDa (monomeric). Protein sequence translated from the PDE-encoding cDNA comprises 828 amino acids including eight potential glycosylation sites. The cDNA encoding 5'-nucleotidase is 5'-truncated and the translated protein sequence (406 amino acids) lacks about 160 amino acids from the N-terminus. Platelet aggregation studies with 5'-nucleotidase exhibited unfortunately no change in either ADP- or collagen-induced aggregation. Probably the enzyme preparation contains some unidentified impurities affecting platelets.

Conclusions: We have isolated and structurally characterized two potential antiplatelet components from *V. lebetina* venom. Their biological properties are under investigation.

PB 4.22-5

Sodium tungstate as a potential antiplatelet agent

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Background: Platelet inhibition is a key strategy for management of atherothrombotic complications. Cyclooxigenase-2 and the ADP receptors are currently the more widely used antiplatelet targets. However, the existence of large inter-individual response variability to both strategies, has led to the search for alternative inhibitors.

Aims: We have investigated the antiplatelet effect of the inorganic salt sodium tungstate, with potential effect on phosphotyrosine (PTP) 1B, with proven anti-obesity actions.

Methods: Wild-type and knockout mice for the phosphotyrosine phosphatase-1B (PTP1B), on a C57BL/6Jx129/svJ mixed genetic background, were untreated or treated for a week with sodium tungstate, at a concentration of 2 g/L in water intake, to study platelet function under flow with the PFA-100, acone plate analyzer (CPA), and a flat perfusion chamber using collagen coated surfaces. In addition, aliquots of human whole blood were incubated with sodium tungstate (from 100 to 400 μM, for 1 h at 37 °C) to evaluate its effect on platelet function using the platelet function analyzer (PFA-100) and the annular perfusion chamber. Aggregometry, with platelet rich plasma, and thromboelastometry, with whole blood, were also performed with the lowest more effective concentration.

Results: In studies in mice, treatment with sodium tungstate prolonged closure time in the PFA-100 using the Col/ADP cartridge (from 148 ± 17.4 s to 272.8 ± 12.4 s, $P < 0.01$), and decreased the surface covered by platelets (%SC) on a collagen surface exposed to flowing blood (45.5 ± 5.4% to 30.7 ± 1.3%). Results in the presence of sodium tungstate were similar to those obtained in KO mice in the absence of the salt. In studies with human blood, closure times in the PFA-100 were significantly prolonged in the Col/Epi cartridge (from 141 ± 6.6 s in controls to 193 ± 23.3 s; $P < 0.05$). In adhesion studies on denuded vascular segments, the%SC and the thrombus formation (%T) decreased in the presence of sodium tungstate (from 32.9 ± 1.4% in controls to 17.9 ± 3.2%, $P < 0.005$; and from 62.9 ± % in controls to 31.6 ± 5.0, $P < 0.002$, respectively). In contrast, neither the maximal aggregating response nor the thromboelastography properties of clots were affected by the presence of the tugstate salt.

Conclusion: Presence of sodium tungstate in blood decreases the hemostatic capacity of platelets by inhibiting their adhesive and cohesive properties under flow conditions in both a mice model and in human blood. Therefore, sodium tungstate exhibits a potential antiplatelet effect. Although it may be acting on platelet PTP-1B, the precise mechanisms through which sodium tungstate exerts its action should be further explored.

PB 4.22-6

Aspirin induces platelet apoptosis

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Background: Aspirin is widely used in the treatment of a number of clinical conditions, such as fever, pain, strokes, and heartattacks. Although aspirin is being thought of a relatively 'safety' medicine, it also has some side effects, particularly the risk of bleeding which may be severe and lead to death. The mechanisms, however, are not totally understood. The effectiveness of aspirin has been attributed to its ability to inhibit prostaglandin production by inhibiting the cyclooxygenase (COX) enzyme. Interestingly, it has been reported recently that aspirin induces apoptosis in many cell types. Platelet apoptosis induced by either physical or chemical compounds or platelet storage occurs widely. We have reported that the glycoprotein Ibα-von Willebrand factor interaction, hyperthermia, and calmodulin antagonists induce platelet apoptosis, suggesting that platelet apoptosis might play important roles in controlling the number and function of circulated platelet under physiological conditions, or in the development of platelet-related hemorrhagic diseases.

Aims: Thus, the aim of the current study is to explore whether aspirin induces platelet apoptosis.

Methods: Washed platelets (3×10^8 /mL) were incubated with various concentrations of aspirin (2.5 mM, 5 mM, 10 mM, 20 mM) at 37 °C for 2 h. The effect of aspirin on platelet mitochondrial membrane potential ($\Delta\Psi m$) depolarization and phosphatidylserine (PS) exposure were analyzed by cell-permeable lipophilic cationic dye tetramethylrhodamine ethylester (TMRE) and annexin V binding, respectively.

Results: The data show that $\Delta\Psi ms$ depolarization and PS exposures were dose-dependently induced by aspirin in platelets. In order to further confirm that aspirin incurs platelet apoptosis, caspase-3 activation was measured in platelets incubated with aspirin by detecting 32 kD pro-caspase-3 and 17 kD cleaved caspase-3 fragment, and the result shows that aspirin induced caspase-3 activation. Furthermore, platelet shrinkage appeared in platelets incubated with aspirin (20 mM). Caspase inhibitor z-VAD-fmk inhibited apoptotic platelet shrinkage, and

blocked ΔΨm depolarization, but had no effect on PS exposure, suggesting that caspase-3 acts upstream of ΔΨm depolarization, and plays a key role in aspirin-induced platelet apoptosis. Whereas, protein kinase C (PKC), protein kinase B (PKB), p38 mitogen-activated protein kinases (MAPK), protein 53 (p53), reactive oxygen species (ROS), and mitogen-activated protein kinase kinase (MAPKK) were not involved in aspirin-induced platelet apoptosis. In addition, platelets incubated with another COX inhibitor indomethacin did not incur ΔΨm depolarazation and PS exposure in platelets.
Conclusion: Taken together, the data indicate that aspirin induces platelet apoptosis via caspase-3 activation, and independent of the COX signaling.

PB4.23 – Platelet integrins – II

PB 4.23-1

The role of platelet actin polymerization and agonist-induced cortactin tyrosine phosphorylation in aIIbβ3 expression, activation, cytoskeleton association and fibrinogen binding

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Background: Inhibition of actin polymerization undermines stability of platelet aggregates and reduces cortactin tyrosine phosphorylation.
Aim: To investigate the role of actin polymerization and cortactin tyrosine phosphorylation in the regulation of αIIbβ3 cell surface expression and activation, actin cytoskeleton association and ligand (fibrinogen) binding in aggregating platelets.
Methods: Blood samples were obtained from adult healthy volunteers who signed an informed consent form (approved by the Sheba Medical Center ethics committee). Platelet-rich plasma (PRP) platelets were pretreated with 10 μM cytochalasin D (CytD) or DMSO vehicle (control) for 5 min and stimulated by 10 μM ADP or pervanadate (1 mM H_2O_2 + 1 mM sodium orthovanadate); aggregation was monitored at 37 °C under stirring. Affinity of αIIbβ3-fibrinogen interaction was evaluated by rate of platelet disaggregation following addition of 4.5 μM eptifibatide. To analyze cytoskeleton composition, PRP samples were lyzed with Triton X-100 lysis buffer, and the insoluble fraction was sedimented and subjected to SDS-PAGE followed by western blot analysis using mAbs against αIIb, pY^{466}-cortactin, cortactin and β-actin. Flow cytometry was performed on 50-fold diluted PRP samples using P2 and PAC-1 mAbs indicating the expression and the activation level of αIIbβ3, respectively.
Results: Stimulation of control platelets by ADP resulted in maximal platelet aggregation (77.6 ± 5.3%). CytD had no effect on the extent of platelet aggregation. Eptifibatide added 3 min after the addition of ADP did not change the course of platelet aggregation in control samples but caused disaggregation at a rate of 51.7 ± 10.2%/min in CytD-pretreated samples. Aggregation of control platelets was accompanied by actin polymerization, association of αIIb and cortactin with cytoskeleton and cortactin tyrosine phosphorylation. CytD-pretreated platelets demonstrated partial reduction (by 17.9%) in actin polymerization and absence of cortactin tyrosine phosphorylation while cytoskeleton association of αIIb and cortactin was not affected. In order to explore the role of cortactin tyrosine phosphorylation in αIIbβ3-fibrinogen affinity, CytD-pretreated platelets were stimulated by pervanadate, a phosphatase inhibitor. Pervanadate completely overcame the inhibitory effect of CytD on cortactin tyrosine phosphorylation and induced maximal platelet aggregation (83.4 ± 6.8%) but did not overcome the decrease in αIIbβ3-fibrinogen affinity. Pretreatment of resting platelets with CytD did not significantly change the basal levels of P2 and PAC-1 binding. However, ADP stimulation of both control and CytD-pretreated samples, increased the level of P2 binding by 30.5% and 34.2% ($P < 0.001$ for both), respectively, and increased the level of PAC-1 binding by 7.2-fold ($P < 0.001$) and 3.3-fold ($P < 0.05$), respectively.
Conclusions: Inhibition of actin polymerization by CytD in activated platelets results in: (i) cortactin-actin association without cortactin tyrosine phosphorylation; (ii) αIIbβ3-actin association and increased αIIbβ3 expression; and (iii) reduction of αIIbβ3 conformational activation concomitantly with reduction of αIIbβ3-fibrinogen affinity. Thus, platelet αIIbβ3 expression, activation and fibrinogen binding (at low affinity) are not dependent on actin polymerization or cortactin tyrosine phosphorylation; however, stable aggregate formation (high αIIbβ3-fibrinogen affinity) is highly dependent on actin polymerization.

PB 4.23-2

Fibronectin unfolding and assembly: the dual role of fibronectin in platelet adhesion and aggregation

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Background: Fibronectin (FN) has been shown to decrease platelet aggregation but enhance platelet adhesion, suggesting a dual role in hemostasis. To explore the suggested dual role of FN in further detail, we tested for differences of adherent platelet and suspended platelets with regard to their effect to unfold and assemble FN upon interaction.
Methods: Isolated human plasma FN was dually labeled with alexa fluor (AF) 488 and 546 for fluorescence resonance energy transfer (FRET) analysis. Ten μg/mL of FN mixture (unlabled FN: labeled FN, ratio 10:1) was incubated with platelets in suspension (10^6/mL) or platelets (10^8/mL) spead onto immobilized FN (50 μg/mL) in HEPES Tyrode buffer supplemented with 2 mM $CaCl_2$ and 40 nM PMA. FRET signals were recorded after 0–3 h of incubation to access the unfolding of FN on platelets from both preparations. For deoxycholate (DOC) solubility assay, platelets in suspension or FN-adherent platelets were incubated with 60 μg/mL AF488 conjugated FN (FN488) in the absence or presence of agonists (10 μM ADP or 40 nM PMA). Samples of both settings were washed with 2% DOC for 2 min. Microscopic analyses were performed before and after washing with 2% DOC.
Results: Labeled FN was exposed to increasing concentrations of GdnHCl (0–4 M) to evaluate the sensitivity of FRET indicative of the unfolding of FN. FRET signals of FN in its compact conformation (0 M GdnHCl) was set at 100% and decreased to 64%, as the FN molecule extended in 1 M GdnHCl solution. Further unfolding of FN at 2 M, 3 M, and 4 M concentrations of GdnHCl reduced FRET signals to 50%, 44%, and 40%, respectively. FRET signals decreased in a time-dependent manner by 4 ± 1.3% (mean ± SD) at 1 h, 5 ± 1.5% at 2 h, and 6 ± 1.1% at 3 h, when labeled FN was incubated with adherent platelets. In contrast, there was no significant decrease in FRET signals of labeled FN alone. The same observation was made in experiments with platelets in suspension. These data demonstrated that adherent platelets but not platelets in suspension were capable of unfolding FN during incubation. Fluorescence microscopic analyses revealed that after 3 h of incubation, bound FN488 formed a DOC-insoluble fibrillar matrix at the periphery of adherent platelets. The fibril formation of FN488 was enhanced on adherent platelets upon addition of ADP or PMA. In contrast, FN488 bound to activated platelets in suspension remained extractable by 2% DOC indicative of absent fibril formation.
Conclusions: Using fluorescence techniques and a DOC solubility assay, we demonstrated that adherent platelets can progressively unfold and assemble FN forming fibrils during incubation, whereas suspended platelets do not. This observation may help explain the dual role of FN in platelet adhesion and aggregation.

PB 4.23-3

FcgammaRII mediates the intrinsic platelet activation of disintegrin, probed by alphaIIbbeta3-specific monoclonal antibodies

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Thrombocytopenia, a common side effect of RGD-mimetic antiplatelet drugs, is thought to be caused by drug-dependent antibodies that recognize platelet conformation-altered alphaIIbbeta3. We investigated the possible mechanisms of intrinsic platelet activation of disintegrin in combination of alphaIIbbeta3-specific mAbs. Disintegrin (e.g. rhodostomin) and monoclonal antibody raised against integrin alphaIIbbeta3 (e.g. AP2 and 10E5) individually inhibited human platelet aggregation caused by collagen. However, when disintegrin (rhodostomin) and monoclonal antibody (AP2 or 10E5) were sequentially added into platelet suspension, platelet aggregation occurred, whereas other monoclonal antibodies (7E3 and AP5) did not induce platelet aggregation in the presence of disintegrin. 7E3 inhibited disintegrin/AP2 or 10E5-induced platelet aggregation, but AP5 inhibited only disintegrin/10E5 (not AP2)-induced platelet aggregation. In the flowcytometry assay, we also found that disintegrin enhanced AP2, AP5 and 10E5, but not 7E3, binding to platelets. FcgammaRII mAb attenuated disintegrin/AP2 or 10E5-induced platelet aggregation, and also inhibited the enhanced binding of AP2 to platelet caused by disintegrin. Immunoprecipitation of the lysates of rhodostomin/AP2-treated platelets using FcgammaRII Ab showed the complex formation of integrin beta3 and FcgammaRII. These results suggest that AP2 or 10E5 triggers platelet aggregation via binding to accessible FcgammaRII and the conformation-altered integrin alphaIIbbeta3 caused by disintegrin. FcgammaRII recuitment and the complex formation of integrin beta3/FcgammaRII mediate the intrinsic platelet activation of disintegrin.

PB 4.23-4

Platelet integrin aIIbβ3 (IIb-IIIa) and cytoskeleton modulate fibrin network formation and clot stiffness

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Background: The ability to clot *in vivo*, either inappropriately as in thrombosis, or insufficiently as in surgical bleeding, is fundamentally linked to the blood clot's mechanical stiffness, or lack there-of. While the active role of platelets in clot mechanics is well accepted, their role in organizing fibrin fibers and regulating their growth is less well understood. Platelets associate with fibrin through the platelet integrin $\alpha_{IIb}\beta_3$ (IIb-IIIa), initiating a pathway of $\alpha_{IIb}\beta_3$-associated tyrosine kinases that transmits signals to the cytoskeleton. After $\alpha_{IIb}\beta_3$ binds to fibrin, the integrin and ligand move selectively into integrin clusters, a process that could lead to changes in extracellular fibrin organization and likely requires modulation of cytoskeletal attachment of $\alpha_{IIb}\beta_3$.
Aims: Our work explores possible downstream effects of $\alpha_{IIb}\beta_3$ cytoskeletal signaling on fibrin fiber organization and clot stiffness. We hypothesize that changes in $\alpha_{IIb}\beta_3$ cytoskeletal signaling may organize extracellular fibrin network, which may affect overall clot stiffness.
Methods: Using a novel *in vitro* model of blood plasma clotting, fibrin-platelet interactions were visualized by DIC and fluorescence microscopy. Tracer particles were also added to measure stiffness by microrheological methods. The motion of the particles was tracked with a particle tracking program implemented in Image J.
Results: In this study, we observed a strong dependence of fibrin macro-fiber formation (300 nm and greater) on fibrin's active engagement with platelet $\alpha_{IIb}\beta_3$ integrin and on platelet actin cytoskeletal polymerization. Platelet concentration was strongly correlated with greater local fibrin macro-fiber formation, where a 2-fold greater local platelet concentration lead to 10× greater fibrin macro-fiber density. The correlation of platelet concentration with fibrin macro-fiber formation agreed with findings of a previous study by Collet et al 2002 and supports the hypothesis that the action of platelets influences the extracellular organization of the blood clot. Diffusivity of 300 nm particles within platelet rich plasma 45 min after clot initiation was significantly reduced in regions of high fibrin density (100 $\mu m^2/s$) relative to regions without visible fibrin (14,000 $\mu m^2/s$). Direct visualization by DIC indicated a striking correlation of clot stiffness and the appearance of fibrin macro-fibers. Abciximab (platelet integrin $\alpha_{IIb}\beta_3$ inhibitor) reduced fibrin macro-fibers in a dose-dependent manner. Similar reductions in fibrin macro-fiber formation were induced by treatment of platelets with cytochalasin D, an inhibitor of cortical actin polymerization.
Conclusions: The observations of the study support the hypothesis that fibrin macro-fiber formation correlates with clot stiffness and that the platelet integrin $\alpha_{IIb}\beta_3$ cytoskeletal signaling regulates fibrin network structure. A deeper understanding of the relationship between stiffness and platelet and fibrin interactions may lead to the evaluation of new anti-platelet drugs that might better navigate the balance between thrombosis and surgical bleeding.

PB 4.23-5

The potencies and mechanisms by which engineered nanoparticles induce platelet aggregation are dependent upon their precise physicochemistry

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Background: The emergence of nanotechnology has led to increased human exposure to engineered nanoscale structures (diameter ≤100 nm) through drug delivery systems, commercial products etc. Epidemiological and clinical work indicates that particulate matter present in air pollution is associated with increased incidence and risk of thrombotic events such as myocardial infarction and stroke. It is suggested that the nanoparticle fraction of this particulate matter may be responsible for some of the increased risk associated with ambient pollution. There is also evidence that nanoparticles can translocate the pulmonary epithelium and enter the systemic circulation. Platelet aggregation and activation underlies many thrombotic events, however there is limited information regarding the ability of engineered nanoparticles to directly influence platelet behaviour.
Aims: The primary aim was to investigate whether model engineered nanoparticles can interact with and functionally modulate platelets. The secondary aim was to assess whether this modulation is affected by particle size and surface chemistry by using 50 nm and 100 nm carboxyl, amine and unmodified model engineered polystyrene nanoparticles.
Methods: Optical aggregometry was used to assess function of platelets *in vitro*. An established murine model of platelet aggregation involving detection of radiolabelled platelets in the pulmonary circulation was employed to measure platelet function *in vivo*. Platelet activation was assessed by measuring P-selectin expression by flow cytometry.
Results: All nanoparticles tested induced concentration-dependent platelet aggregation, however carboxyl 50 nm and unmodified 100 nm particles were the most potent. All nanoparticle-induced aggregation, except for that induced by amine 50 nm particles, was inhibited by the nitric oxide donor sodium nitroprusside and the glycoprotein IIb/IIIa inhibitor eptifibatide. The ability of nanoparticles to induce concentration-dependent P-selectin surface expression reflected their ability to induce aggregation. Amine-modified 50 nm particles enhanced thrombin induced aggregation *in vitro* and this enhanced response was abolished by eptifibatide. Systemic infusion of a low concentration of amine 50 nm particles increased collagen- and thrombin-induced aggregation *in vivo*.

Summary/Conclusions: All nanoparticles investigated induced platelet aggregation with potencies and mechanisms that were dependent upon a distinct combination of size and surface chemistry. Anionic and uncharged particles induced GPIIb/IIIa-mediated platelet aggregation and signalling events similar to those occurring upon exposure to conventional platelet agonists. The sensitivity of nanoparticle-induced aggregation to the endogenous vascular regulator nitric oxide suggests a physiological protective mechanism mediated by the vascular endothelium that may reduce the cardiovascular impact of nanoparticle exposure in healthy individuals. In contrast, the ability of 50 nm cationic particles to enhance the effects of platelet agonists at low concentrations that more likely reflect potential nanoparticle exposure in humans suggest that these particles may present the largest risk to human health by either initiating or exacerbating thrombotic events. This work provides a potential mechanism by which nanoparticles with particular physicochemical properties could pose a thrombotic risk should they enter the blood either by translocating the lung or following direct introduction to the body during medical procedures.

PB 4.23-6

Role of heterotrimeric G proteins in regulation of aIIbβ3 fibrinogen affinity and aIIbβ3 cytoskeleton association

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Background: Upon platelet stimulation, integrin αIIbβ3 undergoes conformational change leading to fibrinogen binding on one end and to association with the cytoskeleton on the other end.
Aim: To investigate the role of heterotrimeric G proteins in regulation of αIIbβ3-fibrinogen interaction and αIIbβ3-cytoskeleton association in aggregating platelets.
Methods: Blood samples were obtained from adult healthy volunteers who signed an informed consent form (approved by the Sheba Medical Center ethics committee). Sole and combined activation of Gαq, Gαi and/or Gα12/13 proteins was achieved by using different combinations of the following platelet agonists and inhibitors: 10 μM ADP, 1.5 mM arachidonic acid (AA), 0.1 μM U46619 (stable thromboxane A_2 analogue), 10 μM AR-C66096 ($P2Y_{12}$ inhibitor), 10 μM MRS2500 ($P2Y_1$ inhibitor) and 0.5 mM acetylsalicylic acid (ASA; thromboxane A_2 synthesis inhibitor). Platelet aggregation was performed at 37 °C with stirring. Affinity of αIIbβ3-fibrinogen interaction was evaluated by rate of platelet disaggregation following addition of 4.5 μM eptifibatide 3 min after the agonist addition. Cytoskeleton composition (Triton X-100-insoluble fraction) was analyzed by western blot using anti-αIIb and anti-β-actin mAbs.
Results: (i) Combined stimulation of Gαq, Gαi and Gα12/13 (by ADP) leads to: maximal platelet aggregation (83.1%); maximal αIIbβ3-fibrinogen affinity – no platelet disaggregation upon eptifibatide addition; actin polymerization and maximal αIIb-cytoskeleton association. (ii) Combined stimulation of Gαq and Gα12/13 (by AA + ADP + AR-C66096) leads to: maximal platelet aggregation (87.2%); partial αIIbβ3-fibrinogen affinity (disaggregation rate, 13.2%/min); actin polymerization and marked αIIb cytoskeleton association (81.2%). (iii) Combined stimulation of Gαq and Gαi (by ADP + ASA) leads to: submaximal platelet aggregation (69.0%); decreased αIIbβ3-fibrinogen affinity (disaggregation rate, 21.6%/min); actin polymerization and mild αIIb cytoskeleton association (61.5%). (iv) Combined stimulation of Gαi and Gα12/13 (by ADP + U46619 + MRS2500 + ASA) leads to: decreased platelet aggregation (54.7%); much lower αIIbβ3-fibrinogen affinity (disaggregation rate, 62.0%/min); actin polymerization and decreased αIIb cytoskeleton association (45.3%). (v) Sole stimulation of Gαi (by ADP + MRS2500 + ASA) leads to: low-amplitude but sustained platelet aggregation (41.4%); low αIIbβ3-fibrinogen affinity (disaggregation rate, 118.5%/min); actin polymerization without αIIb cytoskeleton association. (vi) Sole stimulation of Gαq (by ADP + AR-C66096) leads to: low-amplitude and spontaneously reversible platelet aggregation; actin polymerization without αIIb-cytoskeleton association. (vii) Sole stimulation of Gα12/13 (by U46619 + ASA) leads to: no platelet aggregation but induced actin polymerization without αIIb-cytoskeleton association.
Summary: Affinity of αIIbβ3-fibrinogen binding increases in parallel to the aggregation amplitude; maximal affinity requires activation of all three G proteins. Interaction of αIIbβ3 with cytoskeleton requires activation of at least two of the G proteins and maximal affinity requires activation of all three G proteins. Sole activation of each of the G proteins is sufficient for actin polymerization. The order of contribution to platelet aggregation of combined and sole G protein activation is: Gαq/Gαi/Gα12/13 > Gαq/Gα12/13 > Gαq/Gαi > Gαi/Gα12/13 >G αi > Gαq > Gα12/13.

PB4.24 – New platelet agonists

PB 4.24-1

The endothelial cell surface enzyme, NPP-4 stimulates platelet aggregation via hydrolysis of diadenosine triphosphate to ADP and partially overcomes aspirin-mediated platelet inhibition

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Background: Platelet dense granules contain diadenosine triphosphate (Ap3A), which is released into the extracellular space during the secondary wave of platelet aggregation after platelet activation by ADP and other agonists. Ap3A has long been thought to stimulate platelet aggregation after release into the extracellular space by providing a prolonged source of ADP via hydrolysis by a slow extracellular enzyme present in human plasma. We have previously identified that enzyme as NPP-4, a member of the nucleotide pyrophosphatase/phosphodiesterase enzyme family and have shown that it is present on endothelial cell and monocyte surfaces. NPP-4 is a potent hydrolase of Ap3A capable of stimulating platelet aggregation and secretion at nanomolar concentrations via platelet ADP receptors. The aim of the current study was to determine whether NPP4, at physiologic concentrations, is capable of overcoming aspirin-mediated platelet inhibition.
Methods: We performed platelet aggregometry with citrated platelet-rich plasma (PRP) that was pre-incubated with 100 μM aspirin (ASA) or vehicle in the presence of increasing concentrations of NPP-4. Arachidonic acid (AA), ADP, Ap3A and U46619 were used as agonists to stimulate platelet aggregation. To determine whether certain NPP-4-mediated aggregation phenomena could be attributed to ADP-mediated pathways, experiments were conducted in the presence or absence of the ADP receptor antagonists MRS 2179 and MRS 2395 which block the P2Y1 and P2Y12 receptors, respectively, on platelet cell surfaces.
Results: As expected, 100 μM ASA completely inhibited platelet aggregation in response to AA and Ap3A, while it had no effect on aggregation with U46619 and resulted in attenuated aggregation with ADP compared to the ASA-free state. Addition of NPP-4 to ASA-treated PRP resulted in no improvement in either AA or U46619-stimulated aggregation, but led to an increase in aggregation amplitude in response to both ADP and Ap3A. These increases were prevented by pre-incubation of PRP with MRS 2179 and/or MRS 2395. These findings confirm an effect of ASA on ADP-mediated platelet activation pathways and suggest that NPP-4 may play a role in overcoming platelet inhibition by ASA via ADP-mediated pathways.
Conclusions: The results of these studies suggest that NPP-4 functions in hemostasis *in vivo* by augmenting platelet aggregation and release of granule contents at the site of vascular injury via ADP-mediated pathways. The ability of NPP-4 to partially overcome ASA-associated

platelet inhibition via ADP-mediated pathways suggests that NPP-4 may play a role in ASA resistance and be a target for development of novel antiplatelet therapies for use as adjuncts to currently available drugs that target the platelet directly.

PB 4.24-2

Histone deacetylase 6 (HDAC6)-mediated deacetylation of a-tubulin coordinates cytoskeletal and signaling events of platelet activation

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Background: The tubulin cytoskeleton plays a key role in maintaining the characteristic quiescent discoid shape of resting platelets. Upon activation, platelets undergo a dramatic change in shape to mediate thrombus formation; however, little is know how the microtubule system contributes to regulating platelet shape and function.
Aims: Here we investigate the role of the covalent modification of a-tubulin by acetylation in the regulation of platelet physiology in resting and activated conditions.
Results: Super resolution microscopy analysis of the platelet tubulin cytoskeleton shows the marginal band together with an interconnected web of finer tubulin structures that collapse upon platelet activation with the GPVI-agonist, collagen related peptide (CRP). Western blot analysis reveals that a-tubulin is acetylated in resting platelets and deacetylated following a time course of platelet activation. Tubacin, a specific inhibitor of the tubulin deacetylase HDAC6, prevents tubulin deacetylation upon platelet activation with CRP. Super resolution fluorescence microscopy shows that inhibition of HDAC6 upregulates tubulin acetylation and disrupts the organization of the platelet microtubule marginal band. HDAC6 inhibitors also inhibit platelet aggregation in response to CRP and block platelet signaling events upstream of platelet Rho GTPase activation.
Conclusions: Together these findings demonstrate that acetylation signaling controls the resting structure of the platelet tubulin marginal band and has roles in organizing signaling systems that drive platelet aggregation and function.

PB 4.24-3

Phosphorothioate oligodeoxynucleotides are potent platelet activators acting toll-like receptor 9 independently

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Background: The use of synthetic oligodeoxynucleotides (ODN) which function as immunotherapeutic agents has become common clinical practice in the treatment of some diseases. These ODNs contain unmethylated CpG motifs and function as toll-like receptor 9 (TLR9) agonists. There are three classes of ODNs (A-C) showing differences in structure and phenotype. Class C ODNs bear a phosphorothioate backbone and a CpG containing motif which is present in a manifold greater frequency in bacterial compared to mammalian DNA. The impact of ODNs on immune cells (e.g. lymphocytes) triggering innate and adaptive immunity is quite well investigated. However, little is known about the effect of ODNs on platelets.
Aims: Investigation of the impact of class C ODN 2395 which shares homologies with bacterial DNA on platelet activation.
Methods: Human platelets (washed platelets and PRP) were incubated with the class C ODN 2395 in different modifications and concentrations (0.5 μM/1.0 μM). Platelet activation was evaluated by flow cytometry (CD62P, PAC-1 binding) and light-transmittance aggregometry. The impact of TLR9 on activation was determined by ODN pre-incubation of wild-type (WT) and TLR9$^{-/-}$ mouse platelets. Moreover, C57BL6 mice were injected with class C ODN 2395 and then subjected to ferric chloride carotid artery injury.
Results: Platelets showed significantly increased activation following pre-incubation with class C ODN 2395 (MFI PAC-1 binding: unstimulated: 5.1 ± 0.9, 0.5 μM ODN: 238.4 ± 27.3, $P < 0.01$; MFI CD62P expression: unstimulated: 7.7 ± 0.9, 0.5 μM ODN: 429.9 ± 61.5, $P < 0.01$). However, platelet stimulation with the non-phosphorothioate modified ODN 2395 did not result in increased platelet activation. TLR9 independency was observed as there was no significant difference in the extent of platelet activation between WT and TLR9$^{-/-}$ mouse platelets after stimulation with ODN 2395. Furthermore, C57BL6 mice showed a significantly shortened occlusion time after ODN injection compared to placebo treated mice following ferric chloride carotid artery injury (ODN: 267.8 ± 40 s, placebo: 606.5 ± 45 s, $P < 0.01$).
Conclusion: Phosphorothioate ODNs are potent platelet activators. As some pathogenic bacteria share homologies with ODNs which have a phosphorothioate backbone, this could constitute an additional mechanism for platelet activation in bacteremia.

PB 4.24-4

High resolution structure determination and small molecule inhibitor identification of NPP4, a procoagulant enzyme localized to brain vascular endothelium

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Background: We recently identified the enzyme NPP4, an uncharacterized member of the nucleotide pyrophosphatase/phosphodiesterase family, as present at high concentrations on the vascular surface of the brain, and that NPP4 promotes platelet aggregation in a dose dependent manner at physiologic concentrations of AP3A. AP3A is a small molecule stored at high concentrations in platelet dense core granules and is released upon platelet activation at high concentrations in the vicinity of the growing thrombus. Ap3A has a significantly longer lifespan in whole blood than ADP and has long been thought to promote stable thrombus formation by serving as a 'chemically masked' source of ADP. NPP4 functions upstream of the endovascular enzymes CD39 and CD73 in the metabolism adenine nucleotide phosphates, and collectively the three enzymes sequentially metabolize AP3A into ADP, Adenosine, and inorganic Phosphate. The goal of the present study was threefold: 1. To examine other sites of hemostasis for the presence of NPP4, 2. To determine the high-resolution structure of NPP4, and 3. To identify small molecule inhibitors of NPP4 useful for the *in vitro* and *in vivo* validation of the physiologic role of the enzyme.
Methods: We used flow cytometry to examine circulating hematopoietic cells for the presence of NPP4, X-ray crystallography to determine the high-resolution structure of the enzyme, and high-throughput small molecule screening to identify lead small molecules as lead inhibitors.
Results: Approximately two-thirds of human monocytes present in the bone marrow express NPP4 on their membrane surfaces. We determined the 1.5 Å molecular structure of NPP4 by X-ray crystallography in the presence and absence of its enzymatic product (AMP). Finally, we identified a high-affinity (nM) small molecule inhibitor of NPP4 that is an FDA approved drug for another indication.
Conclusions: Monocytes have a defined role in hemostasis, and the presence of NPP4 on monocyte surfaces further supports a physiologic role of NPP4 in platelet aggregation. The high-resolution structure of NPP4 allows us to understand the molecular determinants of catalysis and substrate specificity, and provides a reagent for the discovery and optimization of small molecule inhibitors. The identification of a high-affinity NPP4 small molecule which is an FDA approved drug identifies a critical reagent for establishing the physiologic role of NPP4.

Our combined studies lay the foundation for the validation of the biological role of NPP4 in hemostasis and establishes the necessary tools and reagents for the translational application of our findings into the clinic.

PB 4.24-5

Functional implications of histone deacetylases in platelet activation: a role for alpha-tubulin acetylation?

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Background: Acetylation of proteins with an emergent role in the regulation of cellular metabolism is regulated by acetylase(HAT)/deacetylase (HDAC) activities, although the role of these enzymes in platelet function remains unidentified. In other cells, several deacetylases have been identified and classified into four families, including zinc-dependent deacetylases (I, II and IV) and NAD^+-dependent (family III or sirtuins). α-Tubulin is one of the proteins first recognized to be regulated by acetylation/deacetylation processes through the action of the deacetylases HDAC6 and sirtuin2. The presence of acetylated α-tubulin has been described in platelets, although its role in platelet function is not well understood. It is known that in platelets α-tubulin participates in the processes of cytoskeleton reorganization, granule release and cytosolic calcium increases (1), but it is unknown if the α-tubulin acetylation/deacetylation cycle participates in these processes.
Aim: Evaluate if deacetylases have a regulatory role on platelet function and α-tubulin acetylation.
Methods: For detection of acetylated α-tubulin, washed human platelets lysates were immunodetected with a specific antibody against the acetylated form of α-tubulin. The role of deacetylases in α-tubulin acetylation was assayed by incubating platelets (1 h, 37 °C) with specific inhibitors: trichostatin A (TSA) (inhibitor of all HDAC except sirtuins) and cambinol (sirtuin inhibitor). Functional studies of washed human platelets were performed according to previously published methodologies (2). To study dense granule release, platelets were previously loaded with ^{14}C-5HT. For the determination of calcium concentration in the cytoplasm, platelets were loaded with FURA 2AM. Platelet function was assayed with a panel of physiological agonits (collagen 0.5 μg/mL, thrombin 0.1 U/mL, U46619 0.5 μM, ADP 10 μM).
Results: In resting human platelets, several proteins were acetylated, including α-tubulin. To identify the deacetylase(s) responsible of α-tubulin acetylation/deacetylation, resting platelets were incubated with TSA or cambinol; only incubation with TSA strongly increased α-tubulin acetylation while cambinol was without effect. Furthermore, inmunoblotting experiments demonstrated the presence of HDAC6 in platelet lysates. In addition, when platelets were aggregated with collagen or U46619, HDAC6 was time-dependently incorporated to the reorganized cytoskeleton. When platelets were aggregated, α-tubulin acetylation was strongly decreased with all the agonists used. In contrast, blocking platelet aggregation with RGDS abolished the process of α-tubulin deacetylation, demonstrating the participation of platelet aggregation in this process. When we explored the functional implication of HDAC inhibition on platelets reactivity, we found that TSA treatment partially reduced platelet aggregation, dense granule release, calcium movement and cytoskeletal reorganization concomitantly with blockade of the agonist-induced α-tubulin deacetylation. Importantly, when the concentration of agonist employed overcomes the action of TSA on platelet function, α-tubulin was deacetylated, suggesting a connection between the level of α-tubulin acetylation and the platelet function.
Conclusion: We describe here for the first time that HDAC6 participates in the process of platelet activation; this effect could be mediated by the acetylation status of α-tubulin. Grants: PI07/0463;Retics06/0026.

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PB 4.24-6

The role of peroxisome proliferator-activated receptor (PPAR)-r? ligands in platelet activation

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Background: The mechanism of platelet production and activation is not fully understood. Recently, the ligand-activated transcription factor peroxisome proliferator-activated receptor gamma (PPARγ) has been reported to promote the formation of platelets from megakaryocytes and accelerate platelet recovery after radiation-induced bone marrow injuries.
Aims: In the present study, we analyzed whether and how PPARγ ligand and its antagonist affect platelet function *in vitro*.
Methods: Platelets were obtained from plateletpheresis products by centrifugation. Western blotting for PPARγ was performed, and bands were visualized by chemiluminescence. The effects of the potent PPARγ antagonist GW9662 and the anti-oxidant brown algae-based polyphenol complex Seanol were also evaluated. Platelet flow cytometry and aggregation tests were performed after each treatment in order to analyze the effects of the treatment on platelets.
Results: Treatment with 10 μM PPARγ ligand (15-deoxy-$D^{12,14}$ prostaglandin J_2, 15d-PGJ_2) did not significantly affect platelet expression of CD61, CD41, CD42b, CD62p, CD63, or PAC1 as measured by flow cytometry. However, the PPARγ antagonist GW9662 at a concentration of 20 μM decreased CD42b and CD62p expression, while Seanol at 25 μg/mL decreased CD63 and PAC1 expression. Studies of the effects of these treatments on other platelet function parameters including platelet aggregation, Multiplate and PFA-100 tests, are ongoing.
Conclusions: Understanding the mechanisms that underlie megakaryocyte maturation and platelet production will provide insight into new ways to enhance the production of platelets from precursor cells. Furthermore, rapid platelet recovery after stem cell transplantation can reduce both the cost of supportive therapy and the risk for bleeding. Our findings support the hypothesis that PPARγ plays a role in platelet activation, and we are now planning to investigate its involvement in areas including thrombopoiesis from cord blood and diseases such as myelodysplastic syndromes.

PB4.25 – Thrombus formation – II

PB 4.25-1

Convection of platelets to a growing thrombus is governed by vessel size, shear rate, and platelet size and stiffness

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Rapid formation of arterial thrombi requires a high platelet concentration at the walls. Despite the low concentration of platelets in blood, margination of platelets towards the walls may increase the near-wall platelet concentration several fold. Vasoconstriction in response to arterial injury and stenotic atherosclerotic plaques lead to a reduction of the lumen with a subsequent increase in the local shear rate. This study explores the relative role of vessel size, shear rate, and platelet size and stiffness on margination.
We have performed large-scale computational simulations of particulate whole blood flow. Our model includes flow of many soft red blood

cells (RBC) and platelet mechanics and particle-particle and particle-fluid interactions in blood flow. In addition to stiff platelets, we use soft platelets and large, stiff platelets, as 'model' particles to examine the physics of margination. The distance for platelet margination and platelet concentration near the wall are used as the quantitative endpoints. We found that large stiff particles marginate to the wall slowly. Next, small soft particles marginated faster, and small stiff particles marginated fastest. Thus, the smaller size of platelets is the dominant parameter driving platelet margination within the range studied. Our results further show that a high near-wall concentration (70% of the platelets in the control volume are within 6 μm from the walls) of stiff small platelets develops after relatively short lengths of 12 mm for a 40 μm channel. For comparison, 50% of soft platelet-sized particles and 30% of stiff large particles marginate to the near-wall region after 12 mm of length. The high platelet concentration in the plasma skimming layer forms much more rapidly as the lumen size is decreased. For a larger 80 μm channel, only 30% of platelets marginate to the near-wall region within 12 mm. Platelet margination rate also increases linearly with shear rate over a range of 1000–20,000 1/s; however, the development length of platelet concentration profile remains about constant as the travel distances increase with high shear. As platelet concentration near the wall may be the rate-limiting step in occlusive thrombus formation, identification of hemodynamic parameters governing platelet margination may guide us in identifying risk factors associated with atherothrombosis and suggest new ways to prevent arterial thrombosis.

PB 4.25-2

A novel approach to reduce variability in platelet flow chamber experiments by utilizing an internal control

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Background: The use of microfluidic devices is an established tool in studying shear-dependent platelet adhesion and aggregation. However the use of these devices and their construction are poorly standardized. Most systems utilize single-use components and thus enable only one blood sample per run and system, and the inherent variability in overall adhesion/aggregation between different runs is usually high. This complicates any attempts to compare different samples. This variability may stem from small changes in flow chamber geometries, especially since it is not uncommon with 'homemade' chamber solutions, image capture in different regions of the chamber, and the possible effect of time between runs since each sample has to be run individually.

Aims: To develop a method using an internal control for increasing experimental control and decreasing variability in platelet adhesion/aggregation assays in microfluidic devices.

Methods: Heparinized whole blood was drawn from healthy volunteers. Platelet-rich plasma (PRP) was prepared by centrifugation at 140 ***g*** for 12 min. PRP was obtained from the supernatant and RBC-rich plasma from the bottom. PRP was divided and platelets were either labeled with $DiOC_6$ or rhodamine 6G, respectively. These two markers have different excitation and emission spectra and can therefore be well separated during fluorescence microscopy. One of the portions is untreated and used as the internal control while the other can be treated with inhibitors/agonists of choice. Both the labeled platelet populations are added to RBC-rich plasma immediately before the start of the experiment.

The method was evaluated with adhesion assays performed with fibrinogen or von Willebrand factor (vWf) coated surfaces in commercial flow chambers (Ibidi), where the adhesion receptors $\alpha_{IIb}\beta$ and GPIb were blocked with abciximab and Mouse Anti-Human MoAb (Clone HIP1), respectively.

Further evaluation was performed by aggregation experiments under elevated shear stress in an in-house constructed PDMS channel with a geometry resembling a stenosed vessel. Also in this experiment the adhesion receptors $\alpha_{IIb}\beta$ and GPIb were inhibited to evaluate their participation in high-shear aggregation.

Results: This procedure eliminated the variability between flow chamber runs and therefore decreased the overall variability when comparing two different samples. The method also provided a direct and visual indication of the results. The standard deviation was lowered in comparison to the use of an external control, in the case of the vWF surface the SD was in average 155% higher without internal control for the first 3 min of measurement. The SD was calculated based on six runs, where the ratio of the control and actual sample were compared. Blocking $\alpha_{IIb}\beta$ and GPIb gave a distinct reduction in platelet adhesion, also GPIb inhibition alone, but not $\alpha_{IIb}\beta$ alone, prevented shear induced aggregation in the PDMS channel.

Summary/Conclusion: We present a method for internal control during flow experiments. This procedure will give a more reliable and precise result as well as save both time and material. As the control is performed in the same environment as the treated sample itself non-relevant variables are excluded. The method is therefore well suited for screening assays.

PB 4.25-3

Anfibatide, a novel GPIb complex antagonist inhibits platelet adhesion and thrombus formation *in vitro* and *in vivo* murine models of thrombosis

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Background: Platelet adhesion and subsequent aggregation at sites of vascular injury are required to maintain normal hemostasis; however, these processes also contribute to pathologic occlusive thrombosis. Interaction of platelet surface GPIb complex with VWF is key for initiation of platelet adhesion, particularly at high shear stress. This interaction is also essential for platelet aggregation at sites of vascular stenosis (e.g. shear rate >10,000 s^{-1}), leading to vessel occlusion. Interaction of platelet surface integrin aIIbβ3 with its ligands (fibrinogen or other ligands) mediates platelet aggregation and contributes to platelet adhesion at lower shear stress. Thus, both GPIb complex and integrin aIIbb3 are important targets for anti-thrombotic therapy. Interestingly, although several inhibitors of aIIbβ3 have been developed for antithrombotic therapies with some limitations due to bleeding complications, anti-GPIb therapy has not been developed.

Aims: To evaluate the effect of a novel GPIb complex antagonist, snake venom anfibatide, on platelet function *in vitro* and anti-thrombotic activity *in vivo*.

Methods: The effect of anfibatide on platelet function was assessed in platelet-rich plasma (PRP) and gel-filtered platelets using a platelet aggregometer. Platelet surface P-selectin expression and integrin aIIbb3 activation was assessed on anfibatide-treated platelets *in vitro* by flow cytometry. Platelet adhesion, aggregation, and thrombus formation on a collagen-coated surface were studied by perfusing anfibatide-treated or control whole blood through a perfusion chamber system at shear rates of 100–5000 s^{-1}. *In vivo* thrombus growth was monitored in real-time under intravital microscopy in both $FeCl_3$-injured mesenteric arterioles and laser-injured cremaster muscle arterioles.

Results: Anfibatide specifically inhibited ristocetin-induced human platelet aggregation. Interestingly, anfibatide did not inhibit botrocetin-induced murine platelet aggregation in PRP, suggesting its unique binding site on GPIbα. It had no significant effect on ADP- or collagen-induced aggregation in PRP, nor on thrombin-induced gel-filtered platelet aggregation. There were no detectible differences in platelet

surface P-selectin expression or β3 integrin activation when platelets were incubated with anfibatide in static condition. In *ex vivo* perfusion, anfibatide strongly inhibited murine platelet adhesion, aggregation, and thrombus formation at high shear rate (600 s^{-1} or above). It also inhibited thrombus growth at lower shear rate (300 s^{-1} or less) although its effect was less potent. In the mesenteric arteriole thrombosis model, anfibatide markedly inhibited platelet adhesion, aggregation and thrombus formation, and prevented vessel occlusion in response to $FeCl_3$ injury ($P < 0.05$). Consistent with these results, anfibatide also dramatically inhibited platelet accumulation and thrombus growth at sites of laser-injured cremaster arterioles. Importantly, we did not observe prolonged tail bleeding time or increased bleeding diathesis during surgeries in anfibatide-treated mice, which is consistent with our observation in an ongoing Phase I clinical trial in human volunteers.

Conclusions: Anfibatide inhibits ristocetin-induced human platelet aggregation but not botrocetin-induced murine platelet aggregation, suggesting its binding site may be different from other snake venoms. Anfibatide effectively inhibits platelet adhesion, aggregation, and thrombus formation and prevents vessel occlusion without significantly impairing hemostasis, indicating that anfibatide may have great potential as a new anti-thrombotic agent.

PB 4.25-4

Kinetics of thrombus formation and further characterization of the bleeding phenotype in PT-VWD hTgG233V mouse model

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Background: The interaction between the platelet GPIb*a* and Von Willebrand factor is critical to platelet adherence to the subendothelial vasculature and thrombus formation at sites of injury. Stabilization of thrombus is supported by signaling events that are not completely defined. Platelet-type von Willebrand disease (PT-VWD) is a rare bleeding disorder caused by gain-of-function mutations in the platelet *GP1BA gene* resulting in a GPIb*a* protein that is a hyper-responsive to VWF. Mice expressing the human GPIb*a* mutation G233V showed impaired thrombus formation and bleeding phenotype similar to human PT-VWD.

Aim: To study the kinetics of thrombus formation in hTg^{G233V} mice for better assessment of the bleeding phenotype in PT-VWD and to investigate the effect of inhibition of the hyper-responsive platelet GPIb*a* on the phenotype.

Methods: *In vivo* thrombus formation was visualized using intravital microscopy following laser injury to the cremaster artery. Platelets were labelled *in vivo* by injecting Rhodamine 6G. Images were taken over 20 min to visualize platelet deposition on the arteriolar wall and occlusive thrombus formation. To assess the initial adherence of the platelets, attachment was defined as continuous interaction (for 5 s) with the endothelium by a platelet after laser-induced injury. Mice tail bleeding times were determined and measurements were terminated at 10 min. The effect of GPIba inhibition was evaluated using 6B4; a monoclonal antibody that blocks the GPIba-binding site for VWF. The inhibitor was injected through jugular vein in intravital studies and tail vein in tail bleeding time experiments. Global haemostasis was assessed by thrombelastography using citrated whole blood obtained via cardiac puncture from hTg^{WT} and hTg^{G233V} animals. In addition, blood was incubated with the inhibitor *in vitro* for 5 min prior to TEG.

Results: Intravital microscopy revealed severe impairment of occlusive thrombus in any of the hTg^{G233V} mice, whereas stable clots were formed and vessels were occluded in hTg^{WT} mice ($n = 4$). Kinetics of clot formation was determined through accumulated fluorescence intensity as a surrogate for platelet accumulation. All time points were examined and hTg^{G233V} mice showed significantly less platelet accumulation than hTg^{WT} mice $P < 0.001$ ($n = 4$). In hTg^{WT}, GPIba inhibitor had no significant effect on the time to form initial clots but significantly reduced clots' stability ($P < 0.02$). In hTg^{G233V}, there was a significant increase in the number of formed clots but these clots were not stable. Considerable individual variation in TEG parameters was shown and GPIb*a* inhibitor shortened the R and K times, increased alpha angle and MA in each of the hTg^{WT} hTg^{G233V}. All hTg^{G233V} ($n = 5$) mice had a bleeding time 10 min. On the other hand, hTg^{WT} mice ($n = 7$) had widely variable bleeding times ~3–10 min. GPIb*a* inhibitor had no effect on bleeding time in hTg^{G233V} and in hTg^{WT}.

Conclusions: This study provides further insights into the bleeding phenotype in PT-VWD mouse model and new understanding of the molecular behaviour of the hyper-responsive GPIb*a* associated in this bleeding disorder.

PB 4.25-5

The Rho GTPase effector PAK regulates thrombin- and collagen-stimulated platelet aggregation, lamellipodia formation and aggregate stability under shear

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Rho GTPase proteins regulate the dynamics of the platelet actin cytoskeleton. However, little is known regarding how Rho GTPases coordinate platelet activation and function. In this study, we characterize the role of the Rho GTPase effector p21 activated kinase (PAK) in platelet activation, lamellipodia formation and aggregate formation under shear. Stimulation of platelets with the GPVI agonist, collagen-related peptide (CRP), rapidly activated PAK in a time course preceding phosphorylation of PAK substrates LIMK1 and MEK and the subsequent activation of MAPKs and Akt. Pharmacological inhibitors of PAK blocked signaling events downstream of PAK and prevented platelet secretion as well as platelet aggregation in response to CRP. PAK inhibition also prevented PAK activation and platelet spreading on collagen surfaces. Platelet responses to thrombin, including the activation of a set of Rac1-associated PAK effectors were also abrogated by PAK inhibition. PAK was also required for platelet aggregate formation to maintain aggregate stability under physiological shear flow conditions. These results suggest that PAK serves an orchestrator of platelet functional responses following activation downstream of GPVI and PAR engagement.

PB 4.25-6

Light-controlled coagulation – aptamer-templated synthesis facilitating masking and photochemical liberation of thrombin function

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The serine protease thrombin is one of the central proteins in the blood coagulation cascade. Its enzymatic activity regulates various mechanisms connected to hemostasis and blood clot formation. The direction of coagulation processes to blood vessel lesions and prevention of thrombus by noninvasive systems is of great interest in hematology. Furthermore, spatiotemporal regulation of thrombin activity is not solely important in preventing thrombosis but could be a powerful tool to restrict tumor blood supply or stop uncontrolled bleeding in emergency surgeries.

Here, we present an aptamer-templated synthesis approach of a light-inducible thrombin release complex using the advantages of the existing thrombin inhibitor aptamer HD1 to form an ON switch for coagulation in human blood plasma. HD1 is functionalized with a photocleavable extension and a cross-linking moiety. The recognition of thrombin exosite I, responsible for the procoagulant functions of thrombin such as conversion of fibrinogen to fibrin and platelet aggregation, enables the aptamer to covalently cross-link to nucleophilic amino acid residues of the protein. Thereby, fibrinogen binding to exosite I is inhibited and fibrin formation, essential for blood clot formation, is prohibited. UV-irradiation of the aptamer-protein complex causes photocleavage of the linker, which results in HD1 dissociation from exosite I. Consequently the blood clotting activity of thrombin is restored. Photo-cleavage is visualized by western blot analysis and light-activated coagulation is measured in human plasma.
In summary, we designed an aptamer-based light-inducible ON-switch for thrombin function, demonstrating the great potential of aptamer-mediated, spatiotemporal control of coagulation.

PB4.26 – Platelet activation mechanisms

PB 4.26-1

A critical role of thrombin/PAR-1 in ADP-induced platelet secretion and the second wave of aggregation

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Background: The stable or second wave of platelet aggregation often observed in ADP-stimulated platelet-rich plasma (PRP) with artificially lowered extracellular calcium level have been attributed to enhanced thromboxane A_2 (TXA_2) generation and inhibition of ecto-nucleotidases activity. However, the role of thrombin in ADP-induced platelet secretion and second wave aggregation are unknown.
Aims: To investigate the role of thrombin in ADP-induced platelet activation.
Methods: We employed aggregometry, flow cytometry, immunoblotting and ELISA to determine whether and how thrombin participates in ADP-induced platelet secretion and second wave aggregation.
Results: ADP induces a phosphoinositide 3-kinase (PI3K) pathway-dependent thrombin generation, presumably resulted from the cleavage of $\alpha_{IIb}\beta_3$-associated prothrombin. Generated thrombin subsequently activates protease-activated receptor-1 (PAR-1) and mediates dense granule secretion and second wave of platelet aggregation in ADP-stimulated citrated PRP. Thus, ADP-induced dense granule secretion and second wave of platelet aggregation in PRP were similarly and non-additively blocked by thrombin inhibitor hirudin, PAR-1 antagonist SCH-79797, or PI3K inhibitor wortmannin. Moreover, ADP-stimulation caused the dissociation of prothrombin from $\alpha_{IIb}\beta_3$ and increased plasma thrombin level, both were prevented by wortmannin. Furthermore, wortmannin-inhibited second wave of platelet aggregation by ADP was restored by a subaggregation concentration of PAR-1 activating peptide SFLLRN. Blocking TXA_2 production with indomethacin or restoring extracellular calcium to physiological concentration did not influence this thrombin/PAR-1-dependence.
Conclusions: A PI3K-dependent thrombin generation and the resultant PAR-1 activation serve as an indispensable mechanism to relay the platelet activation process induced by ADP.

PB 4.26-2

Oxidised LDL activates blood platelets through NADPH oxidase-dependent modulation of the cGMP/Protein kinase G signalling cascade.

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Background: Blood platelets are critical for haemostasis, but require regulation to prevent vascular occlusion and thrombosis. The cyclic guanosine-5'-monophosphate (cGMP) signalling pathway is a potent endogenous regulator of platelet function. Oxidised low density lipoproteins (oxLDL) may contribute to unregulated platelet activation in atherothrombosis by unknown mechanisms. We hypothesised that oxLDL increases platelet activation through modulating platelet sensitivity to cGMP signalling.
Aims: We investigated whether oxLDL activated platelets by reducing platelet sensitivity to cGMP-mediated inhibition.
Methods and Results: Platelet aggregation and accrual under physiological conditions of flow were inhibited by 8-pCPT-cGMP, a direct activator of protein kinase G (PKG). OxLDL, but not native LDL, reduced the inhibition of platelet aggregation and accrual by 8-pCPT-cGMP, without itself causing significant platelet activation. OxLDL prevented cGMP-stimulated phosphorylation of the PKG substrate, VASP (serine239), through a mechanism requiring the scavenger receptor CD36 and the generation of reactive oxygen species (ROS). To explore the role of these ROS in platelet function we used a multi-pronged approach. Firstly we found that the ability of oxLDL to modulate PKG signalling and stimulate platelet activation was prevented by cell-permeable superoxide scavengers, MnTMPyP and TEMPOL, and the NADPH oxidase (NOX) inhibitor gp91ds-tat, suggesting a role for gp91phox/NOX2. Secondly, we found that oxLDL generated ROS through ligation of CD36. Thirdly, oxLDL stimulated gp91phox/NOX2-mediated ROS generation through sequential activation of Src kinases, Syk, PLCγ2 and PKC. Fourthly, PKC-dependent phosphorylation of p47phox resulted in its translocation to the membrane compartment where it associates with p22 phox to form an active NADPH oxidase holoenzyme.
Conclusion: We show for the first time that oxLDL stimulates the generation of intracellular ROS through the activation of gp91phox/NOX2 in platelets, downstream of a CD36/tyrosine kinase signalling pathway. These data reveal a new role for oxLDL in promoting platelet aggregation through modulation of the PKG signaling pathway.

PB 4.26-3

P2X1-mediated p38 signaling enhances U46619-induced platelet secretion and aggregation

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Background: Platelet ATP receptor $P2X_1$ is an ion channel that evokes a rapid calcium influx and sequential signal events, such as activation of calmodulin, ERK2, and myosin light chain kinase. Through autocrine and/or paracrine mechanisms, ATP induces $P2X_1$ activation, and can subsequently amplify platelet reactions initiated by other agonists, including Thromboxane A2 (TXA_2). It is, however, poorly understood how $P2X_1$ activities influence TXA_2-stimulated platelet acativation
Aims: To elaborate the molecular mechanisms of $P2X_1$ engagements in U46619-induced platelet secretion and aggregation.
Methods: Platelet aggregation was monitored by aggregometry. Platelet secretion was measured as P-selectin expression using flow cytometry. Immunoblotting was employed to monitored platelet proteins phosphorylation.
Results: The $P2X_1$ inhibitor NF449 (1 μM) inhibited platelet P-selectin expression induced by a low concentration of U46619 (0.3 μM) (32.0 ± 2.0% vs. 43.4 ± 3.0%, approximately 26% relative inhibi-

tion; $n = 5$; $P < 0.05$). Similar to NF449, the p38 inhibitor SB203580 (10 μM), but not the ERK inhibitor U0126 (10 μM), inhibited U46619-induced platelet P-selectin expression (16.2 ± 2.8% vs. 27.5 ± 4.7% of vehicle; $n = 4$; $P < 0.05$). No additional inhibition was observed when NF449 and SB203580 were combined (16.1 ± 2.2%; $n = 4$). Adding exogenous α,β-Me-ATP (0.5 μM) at 1 min after U46619 stimulus increased platelet P-selectin expression by 26% ($n = 6$; $P < 0.05$). The enhancement was abolished in the presence of SB203580. In consistence with above results, 0.3 μM U46619-induced platelet aggregation was similarly decreased by NF449 (1 μM), SB203580 (10 μM), or $P2X_1$ predesensitization with α,β-Me-ATP (0.5 μM). Immunoblot analysis showed that α,β-Me-ATP (2 μM) caused rapid and reversible p38 phosphorylation. U46619-induced platelet p38 phosphorylation was decreased by $P2X_1$ predesensitization with α,β-Me-ATP. Intriguingly, $P2X_1$-p38 pathway seems to be effective to only platelet activation induced by low concentrations of U46619 but not high concentrations. The latter was evidenced by the results showing that α,β-Me-ATP supplementation or p38 blockade had no effect on platelet activation induced by higher concentration of U46619, such as 3 μM.

Conclusions: Activation of $P2X_1$ ion channel potentiates platelet secretion and aggregation initiated by low doses of U46619 and via p38 signaling pathway. Hence, the $P2X_1$ action may be an important mechanism for more robust platelet responses when only mild stimuli are present.

PB 4.26-4

Characterization of losartan effects on platelets

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Background: Losartan, a selective, competitive inhibitor of the angiotensin II type I receptor, is routinely used to treat hypertension. It has been reported to have antithrombotic effects in animal models dependent on NO/prostacyclin and direct antiplatelet effects were described *in vitro*: competitive inhibition of U46619 binding to the thromboxane A2 receptor (TPR) *(Guerra-Cuesta JI et al. J Hypertens 1999)* and interaction with GPVI *(Grothusen C et al. Arterioscler Thromb Vasc Biol, 2007*; *Ono K et al. J Med Chem 2010*).

Aims: This study was aimed to better characterize *in vitro* the effect of losartan on platelets and to elucidate its mechanism of action.

Methods: Experiments were performed on PRP and washed platelets from healthy donors who had not taken medication for at least for 10 days. Samples were preincubated with increasing concentrations of Losartan (0–50 μg/mL) before measuring platelet aggregation and P-selectin exposure. Platelet activation was triggered by arachidonic acid (AA, 1.5 mM), APD (10 μM), thrombin-receptor activating peptide (TRAP 20 μM), the thromboxane A2 analogue U46619 (1 μM), type I collagen (Horm, 1 μg/mL), convulxin (Cvx, 0.5 nM), or the anti-FcgRIIA antibody IV.3 cross-linked by anti-mouse IgG Fab'2. Platelet adhesion to immobilized collagen was analysed using whole anticoagulated blood in flow conditions (1500 s^{-1}). GPVI binding to collagen was measured using recombinant soluble dimeric GPVI (GPVI-Fc). GPVI dimerisation/clustering was analysed by flow cytometry using the specific antibody 9E18 (*Loyau S et al. Arterioscler Thromb Vasc Biol 2012*).

Results: Losartan up to 50 μg/mL had no effect on AA, TRAP or Cvx-induced platelet aggregation and P-selectin exposure. Losartan (50 μg/mL) inhibited the second wave of aggregation in response to ADP and dose-dependently inhibited U69919- and collagen-induced platelet aggregation. However, the losartan IC50 was six times higher for U46619 than for collagen-induced platelet aggregation (18 vs. 3 μg/mL) suggesting that the inhibition of collagen-induced platelet activation was partly independent of the effect of losartan on TPR. This was confirmed by the observation that losartan still inhibited the collagen-induced residual platelet aggregation of indomethacin-treated platelets. When blood preincubated with losartan (10 μg/mL) was perfused over collagen, less platelet aggregates were formed as compared to control conditions. Losartan up to 100 μg/mL slightly reduced the binding of the anti-GPVI monoclonal antibody 9O12 to GPVI-Fc and had no effect on the high affinity binding of GPVI-Fc to collagen suggesting that losartan does not occupy the collagen-binding site on GPVI. However, interestingly, losartan (10 μg/mL) inhibited the binding of the 9E18 antibody to platelets incubated with collagen suggesting that losartan blocks collagen-induced GPVI clustering.

Conclusion: Losartan inhibits collagen-induced platelet adhesion, activation and aggregation. This effect is, at least partly, independent of TPR inhibition, and does not rely on the inhibition of the protein tyrosine kinase pathway since it does not impact FcgRIIA activation. Our data suggest that the inhibition, by losartan, of collagen-induced GPVI clustering could reduce platelet reactivity and contribute to its antithrombotic effect. The relevance of this effect in losartan-treated patients is under investigation.

PB 4.26-5

Sex-specific differences in platelet activity

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Background: Women and men have important differences to the pharmacodynamic response and clinical outcomes following the use of antiplatelet therapy. A sex-specific difference in baseline platelet activity is one potential mechanism.

Aim: We sought to examine platelet activity when stratified by sex.

Methods: A total of 542 healthy volunteers (349 women and 232 men) underwent light transmission aggregometry in response to epinephrine, ADP and collagen. Platelet aggregation >60% was considered to be hyperreactive. Principal Components Analysis (PCA) was used to infer principal combinations of the three platelet activity measures.

Results: Women were older (29 ± 9.1 vs. 26 ± 7.0, $P < 0.001$) and less frequently white (60% vs. 73%, $P = 0.002$) compared with men. Smoking and body mass index was similar between groups. In the overall population, stimulation with submaximal epinephrine (2 μM), ADP (1 μM) and collagen (2 μM) concentrations elicited a distinct, bimodal pattern of platelet aggregation in this population. Maximum aggregation was greater in women than men for epinephrine (43.7% ± 33.8 vs. 38.6% ± 31.6, $P = 0.07$), ADP (26.7% ± 25.8 vs. 19% ± 19.5, $P < 0.01$), and collagen (72% ± 25.7% vs. 69.6 ± 23.3, $P = 0.25$). Compared with men, women were significantly more likely to be hyperreactive to epinephrine and ADP. After adjustment for age and race, sex was independently associated with platelet hyperreactivity to epinephrine and ADP. Platelet aggregation in response to epinephrine, ADP and collagen using PCA is significantly associated with sex ($P = 0.01$).

Conclusion: Women have significantly increased baseline platelet activity compared with men. Studies are needed to determine whether a differential sex-specific response to antiplatelet therapy can be attributed to this underlying sex-specific difference in baseline platelet activity.

PB 4.26-6

The cellular prion protein PrPC regulates platelet signaling and activation

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Background: Prion diseases manifest through the conversion of the cellular prion protein, PrPC, to its pathogenic scrapie form, PrPSc, to result in protein aggregation and disease. In healthy cells, PrPC serves as a signaling scaffold at the cell surface to regulate signaling processes

in an undetermined manner. Interestingly, platelets express abundant PrPC which has hypothesized roles in platelet physiology.
Aim: Our study was designed to characterize the role of PrPC in platelet function.
Results: Upon stimulation of platelets in solution with agonists including collagen and thrombin, PrPC is externalized to the platelet surface. PrPC also localizes to the cell surface as platelets encounter immobilized surfaces of fibrinogen or collagen. Immunofluorescence microscopy supports a model of PrPC externalization in which PrPC traffics in granules first to platelet filopodia and ultimately to the platelet surface, suggesting that PrPC has roles in signaling complex formation at sites of filopodial anchoring and actin organization. PrPC upregulation at the platelet surface was abolished by tyrosine kinase inhibition but not Rho GTPase inhibitors, suggesting that PrPC mobilization is an early event in platelet activation.
Conclusions: These signaling studies, together with studies of platelets from PrPC mouse models suggest that PrPC may regulate signals downstream of platelet agonist and substrate receptor binding to early phases of platelet activation.

PB4.27 – Platelet activation: miscellaneous – II

PB 4.27-1

Non-uniform distribution of coagulation factors on the membrane of activated platelets

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Introduction: All major reactions of blood coagulation proceed on the negatively charged phospholipid membranes. The interaction proteins of with phospholipid membranes leads to a dramatic acceleration of the reaction rate. A possible mechanism for this acceleration of the reaction rate is increase of the local concentrations of the factors. To investigate further this phenomenon, we examined binding of fluorescentlylabeled coagulation factors with the phosphatidylserine-exposing platelets using confocal microscopy.
Aims: To Investigate distribution of coagulation factors on the membrane of activated platelets.
Materials and Methods: FX was covalently labeled with fluorescein. Platelets were activated at 2×10^{7}/mL with thrombin (100 nM), thrombin(100 nM) + CRP (10 μg/mL) or ionophore(10 μM) in the presence of 2.5 mM $CaCl^{2+}$ for 10 min. They were incubated with FITC-labeled 2% (v/v) annexin V, 500 nM fX, fIXa or prothrombin for 5 min, and were imaged with microscope Zeiss Axio Z1.
Results: Annexin V, which was the specific marker for phosphatidylserine, was non-uniformly distributed on the membrane of activated platelets. There was a small convex area region with high concentrations of annexin V. That region was observed for different types of activation (ionophore, thrombin, thrombin + CRP). fX, fIXa and prothrombin were distributed simularly. The area region with high concentration of factor usually had surfase 10–20% of the platelets membrane. Mathematical modeling showed that this distribution of the factors entering into tenase accelerates the reaction at lease by an order of magnitude.
Conclusions: The coagulation factors are distributed non-uniformly on the membrane of activated platelets. The location of clotting factors in a small area region of the membrane of activated platelets can lead to a significant acceleration of coagulation reactions.

PB 4.27-2

Assessment of platelet inhibition and its stability during aspirin and clopidogrel treatment

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Background: Assessment of platelet inhibition and its stability is crucial for patients with coronary syndromes and those undergoing percutaneous coronary intervention (PCI).
Aims: To assess platelet inhibition and its stability using different platelet function assays and to seek correlation and agreement between these tests.
Methods: Samples from 58 patients who underwent PCI and were on both aspirin and clopidogrel were collected at hospital discharge following PCI (visit-1) and at 30–90 days (visit-2). Platelet function was measured using light transmission aggregometry with arachidonic acid (LTA-AA) and ADP (LTA-ADP), VerifyNow (Aspirin; ARU and P2Y12; PRU), *ex vivo* TxB_2, urinary 11dhTxB_2, and VASP (PRI) assays. Patients were categorized as poor responder (PR) to aspirin based on ARU ≥550, LTA-AA ≥20%, TxB_2 ≥1 ng/mL or 11dh TxB_2 ≥1500 pg/mg of creatinine and PR to clopidogrel with PRU ≥240, PRU ≥208, LTA-ADP ≥40%, PRI ≥50%, or PRI ≥66%.
Results: Depending on platelet function assay, 3–33% of patients in visit-1 and 5–28% in visit-2 were aspirin PR while persistent aspirin PR (PR in both visits) were 2–9%. Similarly, 10–35% of patients in visit-1 and 14–45% in visit-2 were clopidogrel PR and 5–28% were persistent clopidogrel PR. There was a significant difference between visit-1 and visit-2 measures of PRU, LTA-ADP, and PRI which were higher in the second visit (P-value <0.05). Analysis of data quartiles between visits revealed moderate but the highest agreement for PRU (κ = 0.46, 95% CI = 0.29, 0.63, P-value <0.001) and PRI (κ = 0.4, 95% CI = 0.23, 0.57, P-value <0.001). Based on binary data, agreement between two visits was: high for PRU (cut-off 240; κ = 0.7, 95% CI = 0.47, 0.93, P-value <0.001) and PRI (cut-off 66%; κ = 0.69, 95% CI = 0.42, 0.95, P-value <0.001), moderate for LTA-ADP (κ = 0.57, 95% CI = 0.22, 0.93, P-value <0.001), poor for 11dhTxB_2 (κ = 0.31, 95% CI = −0.1, 0.72, P-value = 0.019), and absent for ARU, LTA-AA, and TxB_2. Comparison of platelet function assays in a visit indicated a poor agreement between PRU and LTA-ADP (κ = 0.26, 95% CI = 0.08, 0.44, P-value <0.001), and PRU and PRI data quartiles (κ = 0.36, 95% CI = 0.19, 0.54, P-value <0.001). A poor to moderate agreement was also present between PRU (cut-off 240) and LTA-ADP (κ = 0.33, 95% CI = −0.07, 0.73, P-value <0.001), and PRU (cut-off 208) and PRI (cut-off 50%) (κ = 0.42, 95% CI = 0.16, 0.68, P-value <0.001) binary data.
Conclusion: There is overall stability in platelet inhibition. However, its assessment is influenced by the platelet function assay utilized. Clopidogrel-related assays display a higher agreement in the assessment of platelet function compared to aspirin-related assays. Compliance and effective dose of the regimens should be strictly followed.

PB 4.27-3

Platelet aggregation behavior in the perioperative period of vascular surgery

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Background: Cardiovascular complications are common after vascular surgeries and atherothrombosis is a determinant factor. The perioperative maintenance of aspirin is recommended for risk control.

Aims: Our purpose is to evaluate the behavior of platelet aggregation (PA) and its relation with cardiovascular events (CVE) in the perioperative period of vascular surgical patients under chronic aspirin therapy.
Methods: The protocol was approved by the local ethics committee and we obtained written informed consent from the patients. We analyzed PA before surgery for 191 patients and in 163 of them PA was reassessed after surgery. Maximum extent aggregation was evaluated on an impedance aggregometer (Chrono-log/USA) after blood exposure to different agonists: collagen 1 μg/mL; collagen 2 μg/mL; collagen 5 μg/mL and arachidonic acid (AA) 0.5 mM. We defined the following CVE, during hospitalization: acute coronary syndromes, isolated troponin elevation, ischemic cerebrovascular event, cardiovascular death and vascular reoperation. The independent pre-operative predictors of CVE were identified in a logistic regression model and the paired T-test was used for PA behavior evaluation. The mean of the individual differences between post and pre-operative PA tests (delta PA) were compared between patients with and without CVE.
Results: The incidence of CVE was 22% and its predictors were: dyslipidemia (OR 3.90; IC95% 1.32–1.51; $P = 0.014$), anemia (OR 2.64; IC95% 1.19–5.85; $P = 0.017$), trans operative hemodynamic instability (OR 4.12; IC95% 1.87–9.06; $P < 0.001$) and PA with AA >11 $^{\Omega}$ (OR 2.48; IC95% 1.07–5.76; $P = 0.034$). There was a significant decrease in PA after exposure to collagen 2 μL/mL ($10.43^{\Omega \pm 5.14\ X\ 8.14\ \Omega} \pm 4.45$; $P < 0.001$), collagen 5 μL/mL ($16.75^{\Omega \pm 5.79\ X\ 14.68\ \Omega} \pm 5.51$; $P < 0.001$) and AA ($5.80^{\Omega \pm 5.74\ X\ 3.83\ \Omega} \pm 5.30$; $P < 0.001$). Delta PA was higher among patients with CVE, when compared to patients without CVE: $-4.58^{\Omega \pm 7.33\ X\ -1.35\ \Omega \pm} 5.89$; $P = 0.007$(response to collagen 5 μL/mL) and $-3.76^{\Omega \pm 6.18\ X\ -1.44\ \Omega} \pm 6.12$; $P = 0.045$ (response to AA).
Conclusions: Highest PA in response to AA is a predictor of CVE, indicating that a pro-thrombotic status is a determinant factor toward the occurrence of perioperative complications. The decrease in PA after surgery is possibly related to perioperative consumption that is greater in patients with thrombotic complications.

PB 4.27-4

Binding of factor X to the activated platelet membrane demonstrates a multistep dissociation process that allows hysteresis effects

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Introduction: Activation of blood coagulation factor X (fX) by fIXa occurs at physiologically significant rates only in the presence of Ca^{2+}, fVIIIa and negatively charged phospholipid membrane. The activated platelets are physiological source of negatively charged phospholipid membranes. Binding to platelets is the first step in the activation of factor X and its understanding is a prerequisite for the analysis of their mechanisms.
Predominant binding of factor X (fX), a major coagulation zymogen, to the phosphatidylserine(PS)-positive subpopulation of platelets was shown earlier, but was poorly characterized. In additionwe recently discovered a new PS-positive subpopulation of platelets (Topalov et al. Arterioscler Thromb Vasc Biol 2012, 32:2475–2483). Here we studied interaction of factor X with all three subpopulations of activated platelets.
Methods: FX was covalently labeled with fluorescein. Platelets were activated at 2×10^8/mL with 100 nM thrombin in the presence of 2.5 mM $CaCl_2$ for 10 min. They were incubated with FITC-labeled fX at for different time periods, diluted (if required), and immediately analyzed with an Accuri C6 cytometer. Fluorescence intensity was converted to a mean number of molecules per platelet using a calibration curve prepared with Green Flow Cytometry Intensity Calibration Kits.
Results: In this work was shown, factor X binds with the two subpopulations of PS-positive platelets predominantly (50,000–60,000 molecules fX per platelet, $K_d = 1340 \pm 290$ nM, $n = 3$). Binding, was calcium -depended, reversible andspecific as determined in the experiments in the presence of excess unlabeled factor. Prothrombin competed with fX for binding sites on the membrane. Unexpectedly, kinetics of dilution-induced fX dissociation from the PS-positive platelets revealed a multistep process, with a rapid (1–2 min) step followed by a plato. As a result, the overall binding and dissociation process was a hysteresis-like process possing a kind of 'memory'.
Conclusions: Binding of fX to both PS-positive subpopulations is calcium-depended, reversible, specific, but with a possibility of competition with prothrombin. Most importantly, this is a hysteresis-like process possessing a 'memory' that can be important for reactions in flowing blood.

PB 4.27-5

Mean platelet volume interactions with glycoprotein IIb-IIIa and Ib content and platelet aggregation in acute coronary syndrome patients and healthy volunteers

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Increased mean platelet volume (MPV) is an independent risk factor of cardiovascular thrombotic events. In this study we investigated interactions of MPV with glycoprotein (GP) IIb-IIIa and GP Ib content and platelet aggregation in patients with acute coronary syndrome (ACS, $n = 116$) and healthy volunteers ($n = 31$). Patients and healthy volunteers gave written informed consent for participation in the study. In ACS patients blood was collected at days 1, 3–5 and 8–12 after disease onset. MPV was measured in haematological analyzer. GP IIb-IIIa and GP Ib numbers on platelet surface were measured using ^{125}I-labeled monoclonal antibodies. Platelet aggregation was induced by 5 and 20 μM ADP in ACS patients and by 1.25, 2.5, 5 and 20 μM ADP in healthy volunteers and was evaluated by turbidimetric method. GP IIb-IIIa and GP Ib genetic polymorphisms were identified using RT-PCR or PCR-RFLP. Plasma thrombopoietin (TPO) was measured by ELISA. Strong direct interactions were detected between MPV and GP IIb-IIIa and GP Ib content in both ACS patients (at all time points after disease onset) and healthy volunteers – correlation coefficients (r) from 0.439 to 0.645, $P < 0.005$ (for all correlations). Direct correlations were also revealed between the numbers of both glycoproteins – r from 0.504 to 0.566, $P < 0.002$ (for all correlations). GP IIb-IIIa and GP Ib genetic polymorphisms (GP IIIa Leu33Pro, GP Ibα Thr145Met and GPIbα (-5)T/C [Kozak]) were identified in ACS patients and appeared to have no significant impact on GP IIb-IIIa and GP Ib expression. Modest direct correlations between MPV and platelet aggregation (maximal level) were registered at day 1 of ACS in patients whose blood was collected after aspirin but before clopidogrel uptake – $r = 0.445$, $P = 0.003$ ($n = 43$) and $r = 0.318$, $P = 0.036$ ($n = 44$) for 5 and 20 μM ADP respectively. No correlations between these parameters were observed when patients received clopidogrel in addition to aspirin (patients who had already received clopidogrel at day 1 and all patients at later time points). No effects of GP IIIa Leu33Pro polymorphism on platelet aggregation were detected at all time points. In healthy volunteers significant correlation was revealed between MPV and platelet aggregation only for 2.5 μM ADP ($r = 0.404$, $P = 0.022$) and for other ADP doses it did not reach significant level ($r = 0.320$, $P = 0.074$, $r = 0.328$, $P = 0.067$ and $r = 0.245$, $P = 0.183$ for 1.25, 5 and 20 μM ADP respectively). Weak but significant direct correlation was revealed between MPV and plasma TPO which was determined in patients at day 1 of ACS ($r = 0.286$,

$P = 0.005$). The data obtained indicated that increased MPV is associated with increased amount of GP IIb-IIIa and GP Ib and increased platelet aggregation activity. At least in some ACS patients production of large hyper-reactive platelets, containing high amounts of GP IIb-IIIa and GP Ib, might be stimulated by increased TPO.

PB 4.27-6

Elevated prevalence and stability over time of high on-treatment platelet reactivity in patients with ischemic cerebrovascular disease

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Background: Ischemic stroke and transient ischemic attack (TIA) are a main cause of morbidity and mortality worldwide, and up to 25% of them are recurrent. Aspirin is the most used drug for secondary prevention of acute and long-term treatment of cerebrovascular disease (CVD). However, evidence that many patients have recurrent vascular events while on aspirin, led to alternative therapies such as clopidogrel or, more controversial, aspirin plus clopidogrel combination therapy. High on-treatment platelet reactivity (HTPR), referred to as limited inhibition of platelet function achieved by an antiplatelet therapy, has recently emerged as a potential risk factor for recurrent vascular events, and whether patients with HTPR phenotype may benefit from intensified antiplatelet treatment is under debate. In the setting of CVD there is scarce information on the prevalence HTPR and on the stability over time of on-treatment platelet reactivity phenotype (HTPR or No-HTPR).

Objective: To investigate, by means of different platelet function tests, the frequency and stability of a HTPR phenotype in patients with ischemic stroke/TIA, under treatment with antiplatelet therapy.

Patients and Methods: Platelet reactivity was assessed on 18 patients (50%M, 72.4 ± 9.9 years) with ischemic stroke/TIA 1 week (D7) after prescription of clopidogrel (75 mg/day), in monotherapy (39%) or associated to aspirin (61%), and again at day 90th (D90), by means of four methods Light transmission aggregometry (LTA), VASP, Verify Now® $P2Y_{12}$, and INNOVANCE® PFA P2Y. HTPR was defined as: LTA-ADP 5 μM >46%, LTA-ADP 10 μM >70%; PFA-100-P2Y closure time <106s; VerifyNow $P2Y_{12}$, PRU >235, VASP, PRI >50%.

Results: All tests detected a marked inhibition of platelet reactivity towards ADP in these CVD patients, as a result of clopidogrel therapy, that was similar at D7 and D90. Correlations between results of platelet reactivity with either test at D7 and D90, and between measures on either day with the different assays were low-to-moderate. The prevalence of HTPR in these CVD patients was highly assay dependent: with 5 mM LTA-ADP, 50% both on D7 and D90; 10 mM LTA-ADP, 5.5% at D7 and 16.6% at D90; PFA-100-P2Y, 38.9% at D7 and 55.5% at D90; Verify Now $P2Y_{12}$ 55.5% at D7 and 61.1% at D90; VASP, 44.4% at D7 and 27.7% at D90. There was, at best, only moderate agreement (k statistic <0.5) between these tests in categorizing patients with or without HTPR both at D7 and D90. Interestingly, our study also showed that 55–90%, depending on the platelet method/criteria used, maintained their platelet reactivity phenotype, HTPR or no-HTPR, over time.

Conclusions: A high prevalence of HTPR is observed in CVD patients, although the value is highly influenced by the method used to assess platelet function and the HTPR definition. Different methods correlate and agree poor-to-moderately between each other. The new PFA-100 P2Y point-of-care system is equivalent to other assays in evaluating HTPR on CVD patients. In most CVD patients the platelet reactivity phenotype perceived soon after prescription of clopidogrel seems to remain stable over time. As the clinical meaning of HTPR is uncertain, our data do not apply to individualizing therapy in CVD.

PB4.28 – Platelet function in health and disease

PB 4.28-1

The platelet delta granule storage pool increases in pregnancy

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Background: Pregnancy induces numerous systemic physiological changes including hematologic adaptations for the labor and delivery process. Postpartum hemorrhage (PPH) is a major cause of maternal morbidity and mortality and most often related to atony. Other risk factors have been established for the etiology of PPH, but bleeding diatheses, while recognized, appear to be rare. Few studies of PPH or other bleeding complications related to parturition and platelet dysfunction have been published, most of which have been case reports. We have become aware that patients diagnosed with delta granule storage pool deficiency (δ-SPD) have not experienced bleeding complications related to parturition at our hospital. Platelet δ-SPD is a bleeding diathesis related to a decreased number of platelet dense granules presenting with common symptoms of platelet dysfunction including epistaxis, easy bruising, mucosal bleeding and heavy menstrual bleeding. To our knowledge, a prospective study of the platelet dense granule storage pool during the state of pregnancy and parturition bleeding complications has not been evaluated.

Aims: The purpose of our study was intended to evaluate the platelet dense granule storage pool as a potential risk factor for PPH and anesthesia related hemorrhagic complications.

Methods: Pregnant women being seen in a tertiary hospital obstetrics clinic for routine prenatal check-up were approached for participation in our Institutional Review Board approved study. Participants included by informed consent were asked to provide a blood sample and to complete a pictorial menses score-sheet and a bleeding checklist (based upon the Vincenza bleeding questionnaire). To date 157 women have been enrolled. Platelet dense granules were enumerated using established electron microscopy (EM) protocols. CBCs collected for routine prenatal workup as well as immediately post partum will be assessed including estimated blood loss at parturition. Blood obtained from non-pregnant age matched women ($n = 40$) served as controls.

Results: The mean number of dense granules/platelet (DG/PL) has been found to be significantly increased (5.12 ± 0.14) in pregnant women when compared to non-pregnant controls (4.21 ± 0.09 DG/PLT). The average age of participants is 24.2 ± 4. Completed bleeding scores average 0.96 ± 0.10 (normal <5) and pictorial menorrhagia scores average 197.9 ± 15.14 (normal <185). Of the women with a score >186, all had a normal bleeding score but 22.6% (31/137) had menses pictorial scores consistent with a clinical diagnosis of heavy menstrual bleeding (ranging from 198 to 660). Blood for EM was diluted in ACD venipuncture tubes and thus unsuitable for CBC determination. Not all participants have delivered to date and data is currently being collected from patient medical records to assess hemoglobin and hematocrit values obtained during prenatal care and post partum and will be presented.

Summary/Conclusions: These data suggest that the platelet δ-storage pool is altered in pregnancy and may be responsible for the maternal thrombotic response needed during labor and delivery to control blood loss. Our preliminary results were unexpected and have stimulated future plans for which we will obtain multiple blood samples during and after pregnancy in a more focused and controlled study.

PB 4.28-2

Essential thrombocythemia and polycythemia vera thrombotic or bleeding diathesis? flow study results

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Background: Thrombocythemia (ET) and polycythemia vera (PCV) exhibit increased platelet counts that are thought to explain their major complications, i.e. thrombotic events and embolism, and ensuing morbidity and mortality. Use of anti-aggregatory drugs is recommended to prevent these thromboembolic complications. However at platelet counts exceeding 1 mio/μL the thrombotic diathesis reverses into a spontaneous bleeding tendency due to an acquired von Willebrand factor deficiency, especially of the large VWF multimers. The von Willebrand factor usually mediates the interaction between platelets and vascular injury site, especially in areas of high haemodynamic shear stress.

Aim: We performed an *in vitro* flow study with ET or PCV blood to assess the overall effect of VWF large multimer deficiency and simultaneous acetylsalicylic acid treatment on platelet aggregation. To determine the role of the single contributing factors we crosswise exchanged plasma and platelets of patients and healthy donors.

Methods: Our study involved 12 patients with essential thrombocythemia or polycythemia vera with platelet counts over 1 mio/μL on low dose acetylsalicylic acid treatment. None of them had any prior thrombotic or bleeding event. As control group we analyzed blood from 11 healthy volunteers. Platelet aggregation on fibrillar collagen type I was recorded with videomicroscopy in a wall parallel flow chamber with subsequent off-line image analysis. Platelet receptor expression was analyzed with flow cytometry.

Results: Patient platelets tested in patient plasma demonstrated reduced aggregate forming capacity on collagen under high shear stress in contrast to the healthy control. Replacing patient platelets with platelets of healthy volunteers (at normal platelet counts) and keeping the patient plasma increased aggregate formation under moderate shear rates. Flow experiments using healthy subject plasma and platelets, but adjusting platelet counts to over 1 mio/μL, demonstrated maximal aggregation in less than half the time of normal conditions. In addition, prior acetylsalicylic acid intake in healthy controls led to a 50% aggregate reduction in spite of high platelet counts. Flow cytometry of ET and PCV platelets demonstrated reduced expression of the platelet receptor integrin alpha-2bbeta3. Analysis of plasma VWF confirmed lack of large multimers.

Conclusion: We hypothesize that in ET and PCV patients two mechanisms are operational that balance the underlying thromboembolic diathesis: deficiency of the large VWF multimers and reduced expression of integrin alpha-2bbeta3 receptors. Our results demonstrate that these compensatory mechanisms are effective at high shear rates but not sufficient at moderate shear rates to counter the prothrombotic tendency. Therefore we consider the use of antiaggregatory drugs an important therapeutic option for the prevention of thrombosis and embolism in such patients.

PB 4.28-3

Risk-stratification of essential thrombocythemia patients for arterial, venous thromboses and for microcirculatory disturbances

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Background: Advanced age (over 60 years) and previous thromboses are well established risk factors for thrombosis in essential thrombocythemia (ET) patients. Recently, the impact of novel risk factors, such as JAK2 V617F; MPL W515L/K mutational status or leukocytosis has been investigated.

Aims: To evaluate the cardiovascular major risk factors (hypertension, cigarette smoking, diabetes mellitus, and hyperlipidemia) JAK2 V617F, MPL W515L mutations impact on the thrombotic events of ET patients.

Methods: *Study participants:* One hundred and one patients (29 males; 72 females) with median age: 61 years [range: 14–95 years] diagnosed with essential thrombocythemia between 1999 and 2011 at our Department were enrolled to the study.

Laboratory methods DNA was isolated from EDTA-stabilized peripheral blood samples, and screened for the JAK2 V617F, MPL W515L mutations with an allele-specific PCR method.

Statistical analysis: Patients were stratified into subgroups according the presence or absence of thrombotic events which were compared by a series of variables such as age, presence of JAK2 V617F and MPL W515L mutations, measured platelet and leukocyte counts at diagnosis, cardiovascular risk factors, and thrombotic events before and after diagnosis. Mann-Whitney tests were performed to explore overall effects of these variables. Multivariate binary logistic regression was also run to estimate the probability of thrombotic events.

Results: JAK2 V617F-positivity was proven in 61 patients. MPL W515L mutations could be detected in 16 patients. Compared to the data published so far, the incidence of thrombosis was higher in our study group. Fifty-one thrombotic events were recorded in the prior history of ET patients, (before the clinical diagnosis of ET): myocardial infarction events in 16 cases (15.8%), ischemic stroke or transient ischemic attack in six cases (5.9%), venous thrombotic events in 11 cases (10.9%), and microcirculatory disturbances in 26 cases (25.7%). During the follow up period, 23 new thrombotic events were recorded: myocardial infarction in three cases (3%), ischemic stroke or transient ischemic attack in 10 cases (9.9%), venous thrombosis in three cases (3%) and microcirculatory disturbances in 13 patients (12.9%).

The univariate analysis of the individual cardiovascular risk factors, revealed that the presence of diabetes mellitus ($P = 0.581$), high blood preassure ($P = 0.119$), and cigarette smoking ($P = 0.293$) were not associated with an increased risk of thrombosis. However, hyperlipidaemia ($P = 0.032$) was associated with a significantly increased risk of thrombotic events. Multivariate binary logistic regression analysis confirmed that the probability of recurrent thrombotic events is significantly higher in patients who had prior history of thrombosis ($P = 0.071$), especially in the view of prior myocardial infarction events ($P = 0.030$). Nevertheless, the expected prognostic value of JAK2 V617F, MPL W515L mutations for thrombosis could not be detected in our patients.

Summary/Conclusions: Despite the low number of patients, it might be seen that further risk factors also play a role in the development of thrombotic events. Therefore, we purpose that identifying and eliminating modifiable risk factors such as cardiovascular risk factors might be greatly beneficial in the complex management of ET patients.

PB 4.28-4

Omega-3 Alpha linolenic acid interferes with platelet rolling and adhesion to von Willebrand factor

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Background: The plant-derived omega-3 fatty acid (FA) alpha-linolenic acid (ALA) may constitute an attractive cardioprotective alternative to fish-derived n-3 FA. We have previously shown that ALA reduces experimental atherosclerosis and platelet-dependent thrombosis in mice.

Aim: To further analyse the inhibitory effect of ALA on platelet function, specifically platelet rolling and adhesion to vWF and collagen under flow.

Methods: For platelets adhesion to collagen, citrate-anticoagulated blood from healthy donors was incubated with vehicle (ethanol) or ALA (30 μM) for 1 h at room temperature. Platelets were labelled in parallel with the fluorescent dye calcein. 48-wells plates (Bioflux) were coated with equine collagen (100 μg/mL) for 1 h, followed by blocking with 0.5% BSA in PBS for 10 min. Blood was added to the well of the plate and a flow shear of 10 dyne/cm^2 was applied for 10 min. At the end of the experiment 6–8 pictures were taken along the whole channel in order to visualize the thrombi. The total thrombus area was calculated with the Bioflux software. For platelet adhesion to vWF, citrate- and EDTA-anticoagulated blood were used (the latter to analyse exclusively GPIb-mediated adhesion). Aliquots from each sample were incubated with vehicle (ethanol) or ALA at 7.5/15/30 μM final concentrations for 1 h at room temperature. Platelets were stained with calcein in parallel with the vehicle/ALA treatment. Plates were coated with human vWF at 100 μg/mL for 1 h at room temperature, then blocked with 0.5% BSA in PBS. At the end of the treatment, blood was added to the plate and a shear flow of 100 dyne/cm^2 (corresponding to a shear force of 2500 s^{-1}) was applied for 10 min. Pictures were taken every 5 s. The platelet-covered area and the platelet rolling velocity were calculated with the Bioflux software.

Results: After incubation with ALA, thrombus formation on collagen (measured as thrombus-covered area) was reduced (162,193 μm^2 vehicle vs. 87,883 μm^2 ALA, $n = 5$). Similarly, platelet adhesion to vWF and platelet aggregation were dose-dependently reduced by ALA, the difference becoming statistically significant at the higher ALA concentration (platelet-positive area: 64,112 μm^2 vehicle vs. 21,102 μm^2 ALA, $n = 6$, $P = 0.018$). A reduction in GPIb-mediated platelet adhesion was also observed with EDTA-anticoagulated blood (platelet-positive area: 99,781 μm^2 vehicle vs. 70,405 μm^2 ALA, $n = 6$, $P = 0.016$). Analysis of single platelets showed an increased rolling speed in platelets from the ALA-treated samples compared to vehicle (velocity: 0.75 μm/s vehicle vs. 1.56 μm/s ALA, $n = 10$, $P = 0.023$). Incubation with ALA did not change GPIb surface expression, as shown by flow cytometry (MFI: 3217 vehicle vs. 3477 ALA, $P = 0.3$) and glycocalicin in plasma as measured by ELISA (2.29 μg/mL vehicle vs. 2.37 μg/mL ALA, $P = 0.35$).

Conclusions: ALA inhibits platelet adhesion and aggregation to collagen and vWF, and it reduces GPIb-vWF binding without affecting the receptor expression. Possible mechanisms include alteration of the membrane microdomains (lipid-rafts) and of GPIb clustering-signalling from/to these domains.

PB 4.28-5

Platelet aggregations in thrombus and thrombopoiesis in bone marrows visualized by *in vivo* molecular imaging and contribution of inflammatory cytokines

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Objective: The mechanism by which thrombotic vessel occlusion occurs independently of plaque development or endothelial cell (EC) disruption remains unclear, largely because of an inability to visualize thrombus formation, especially at the single-platelet level in real time.

Methods: Therefore, we developed *in vivo* imaging technique based on single- and multi-photon microscopy, and we assessed dynamic cellular interplay in thrombosis models. We visualized that rapidly developing thrombi composed of discoid platelets without EC disruption was triggered by ROS photochemically induced by moderate power laser irradiation.

Results: Using this technique, we elucidated that Lnk (adapter protein) regulates integrin signaling leading to stabilization of developing thrombus *in vivo*. We established the efficient culture system of human iPS-derived platelets, and we confirmed the functional role *in vivo*. These artificial platelets can circulate, and contribute to their thrombus formation, indicating the clinical usefulness considering the cell therapy for future.

In addition, we elucidated the contribution of inflammatory cytokines, ROS, and integrin signaling to our thrombosis models. The inflammatory cytokines TNF-alpha and IL-1 could be key components of the EC response, acting through regulation of von Willebrand factor mobilization to the cell surface. Thrombus formation was then initiated by the binding of platelet GPIb-alpha to endothelial von Willebrand Factor in our model, and this effect was inhibited by the ROS scavenger N-acetylcysteine. Actin linker talin-dependent activation of alphaIIb-beta3 integrin in platelets was required for late phase thrombus stability.

We also visualized the megakaryocyte dynamics during thombopoiesis in living bone marrows, and both proplatelet productions and fragmentation were revealed. We identified novel humoral factors which programmed these steps, which synergistically work with thrombopoietin for efficient platelet production *in vivo*.

Summary: In sum, using our imaging system can be a powerful tool to analyze thrombus formation. We clarified the mechanism of discoid platelet aggregations on undisputed endothelium. The initial platelet aggregation subsequently leads to irreversible integrin- and actin-dependent thrombus development. Inflammatory cytokine signaling in ECs also played pivotal role.

PB 4.28-6

Plasma levels of soluble receptor protein-tyrosine kinases Mer and Tyro3 are increased in acute coronary syndrome patients

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Backgroud: Growth arrest-specific protein (Gas6) and its receptors Axl, Mer, and Tyro3 have numerous roles in inflammatory diseases, cancer, and thrombosis. These receptors could be cleaved by metalloproteinases to form soluble receptors. Recent reports suggested they might have important roles in the progress of Acute Coronary Syndrome (ACS).

Aims: To investigate the roles of Gas6 and its receptors in the progress of acute coronary syndrome and their correlation with thrombin generation and atherosclerotic plaque stability.

Methods: Sixty-six ACS patients and 42 healthy controls were recruited in this study. Plasma levels of Gas6, sAxl, sMer, and sTyro3 in ACS patients were examined by enzyme-linked immunosorbent assay (ELISA) and compared to those in the gender and age compared control group. The correlation of plasma levels of those proteins with clinical indices was further examined.

Results: Plasma levels of sMer, sTyro and sAxl were significantly increased in ACS patients compared to those in age and gender compared controls ($P < 0.01$, $P = 0.0006$ and $P = 0.0387$, respectively). However, there was no significant difference in plasma Gas6 level between ACS group and control group ($P = 0.6568$). After percutaneous coronary intervention (PCI), there was no significant difference in plasma levels of Gas6, sMer, sTyro3 and sAXL between ACS group and control group ($P > 0.5$). Plasma levels of sMer and sTyro3 were significantly correlated with thrombin-antithrombin complex (TAT) formation ($P = 0.0138$ and 0.0255, respectively).

Conclusions: Compared to control group, plasma levels of sMer and sTyro3 were significantly increased in ACS patients. In ACS, atherosclerotic plaques may not be the source of sMer and sTyro 3. However, plasma levels of sMer and sTyro3 were correlated with thrombin-antithrombin complex generation. They may come from thrombin stimulated inflammatory cells in circulation.

PB4.29 – Acquired and immune thrombocytopenia

PB 4.29-1

The immature platelet fraction is susceptible to the platelet size and useful for screening of macrothrombocytopenia from immune thrombocytopenia

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Background: The immature platelet fraction (IPF) is a useful parameter indicating thrombopoietic activity to differentiate the causes of thrombocytopenia. We previously reported that the percentage of IPF (%IPF) is negatively correlated to the platelet count among ITP patients, and not among myelodysplastic syndrome (MDS) patients. We also noticed that some MDS patients exhibited extremely high% IPF values, which were dissociated from the percentages of reticulated platelets (%RP) measured by flow cytometry. Such discrepancies were also observed in hereditary macrothrombocytopenias, which are sometimes difficult to be distinguished from ITP, because ITP also exhibits increased number of reticulated platelets in a slightly larger size. Once misdiagnosed, a hereditary macrothrombocytopenia patient might be subjected to an invasive treatment such as splenectomy. In order to avoid such mistreatments, a clear marker to differentiate macrothrombocytopenia is desperately needed.
Aims: To elucidate the mechanisms of the aberrant increase of IPF among macrothrombocytpenic patients, and search for a useful parameter to distinguish macrothrombocytopenia from ITP
Methods: The IPF values and other platelet indices of various hereditary macrothrombocytopenia were determined using Sysmex XE-2100 automatic hematology analyser in the blood samples. Platelet count and other parameters of platelet, such as MPV, PDW and P-LCR were measured simultaneously. Phosphorylation of myosin light chain was also examined to estimate activation status. Sixteen individuals from 12 families of hereditary macrothrombocytopenia were enroled in this study. We also monitored the IPF during EDTA-induced aggregation, platelet agglutination by macroglobulinemia and cold-storage. The morphological changes of platelets were also examined on the blood film.
Results: The IPF values were about five times higher in MYH9 disorders (%IPF 48.0 ± 1.8) and about 1.5 times higher in other macrothrombocytopenias (%IPF 17.0 ± 2.2) than immune thrombocytopenic patients with similar platelet counts (%IPF 9.3 ± 0.4). These results suggested that the platelet size affect the IPF value. However it still remains the possibility that some factors other than the size might make an influence on the IPF. No one knows whether large platelets are functionally identical to normal platelets except for the size. In order to exclude the possibility, we next examined the changes of IPF values during EDTA aggregation, agglutination by macroglobulinemia and cold-storage. The IPF was significantly increased under these conditions in a time dependent manner along with forming platelet clumps. Durnig cold-storage, each platelet increased in size with fewer granules probably due to degranulation, and a couple of platelets stuck to each other to form a few tiny clumps. The IPF was strongly influenced by a few tiny platelet aggregates rather than other platelet indices, such as mean platelet volume (MPV), platelet-large cell ratio (P-LCR) and platelet distribution width (PDW).
Conclusion: IPF is susceptible to the platelet size, and could be a useful parameter for screening of macrothrombocytopenia from ITP.

PB 4.29-2

Abnormal lipid rafts related signaling in T lymphocytes in immune thrombocytopenia patients

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Backgrounds: The lipid rafts are dynamically variable cell membrane microdomains containing special lipid. The receptor-mediated reaction between the proteins promotes the aggregation of the lipid rafts and the signal transduction. Any lymphocytes state changes may result in the accumulation of lipids in the cell membrane lipid rafts, causing a greater area of lipid rafts formation. Therefore, abnormal changes in the size and structure of the lipid rafts will affect their stability, and may lead to abnormal signal transduction and pathological states. Immune thrombocytopenia (ITP), as an autoimmune disease, has confirmed cellular immune abnormalities associated with T lymphocytes. So far, no study on the relationship of lipid rafts and ITP was reported.
Aims: To further clarify the important role of lipid rafts in immune signaling processes, and to provide new clues to new treatments for ITP.
Methods: The $CD4^+$ and $CD8^+$ T lymphocytes were isolated by flow cytometry from the ITP patients and the healthy controls, and cells were stained with fluorescence-labeled cholera toxin subunit B(CTB), which binds the raft-associated glycosphingolipid GM1. GM1 levels on the lipid rafts of the $CD4^+$ and $CD8^+$ T lymphocytes were measured by confocal microscope. To verify whether the T-cell activation may cause the aggregation of lipid rafts, peripheral blood T lymphocytes were stimulated by anti-CD3/CD28 antibodies after 48 h culture. To determine whether MβCD inhibits TCR-mediated lipid raft clustering, anti-CD3/CD28 antibodies-activated $CD4^+$ and $CD8^+$ T lymphocytes were treated with MβCD. The T cell proliferation and the secretion of the IFN-γ in the culture supernatant were also measured.
Results: Twenty-five patients and 20 healthy controls were enrolled in this research. Compared to the healthy controls, in ITP patients, GM1 levels on the lipid rafts of the $CD4^+$ and $CD8^+$ T lymphocytes were both significantly elevated, indicating that T lympocytes in ITP patients are automatically and strongly activated(CD4: $P = 0.014$; CD8: $P = 0.045$). When activated by anti-CD3/CD28 antibodies for 48 h culture, GM1 levels on T lymphocytes of healthy controls also increased when compared with unactivated normal T lymphocytes (CD4: $P = 0.553$;CD8: $P = 0.313$), but not as significantly as the activated T lymphocytes of ITP patients(CD4: $P = 0.005$;CD8: $P = 0.003$). Meanwhile, it demonstrated that the anti-CD3/CD28 antibodies strongly enhanced lipid raft clustering on T lymphocytes in ITP patients when compared with the unactivated patients group(CD4: $P = 0.047$;CD8: $P = 0.007$), suggesting that the threshold of the T cells activation from patients with ITP decreased. There were no significant differences on the GM1 levels between the unactivated ITP patients and the healthy controls(CD4: $P = 0.064$;CD8: $P = 0.150$), which might indictate that after 48-h 'rest' the T cell lipid raft aggregation were reversed. As we expected, MβCD could blockade the T lymphocytes activation, both in the lipid raft aggregation and the T cell proliferation. No significant differences were found in the same patients who were previously untreated and then got remission(CD4: $P = 0.140$;CD8: $P = 0.100$), indicating the lipid raft aggregation might be not related with the disease progression.
Conclusion: In ITP patients, the T lymphocytes abnormal lipid raft aggregation is proved to be associated with the pathological T cell activation. And the depletion of the lipid raft can reverse the over-reacted T lymphocytes in ITP patients.

PB 4.29-3

Alloimmune thrombocytopenia masking a bernard soulier syndrome in a congenital nephrotic syndrome of the Finnish type (nphs1)

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Background: A male child developed a nephrotic syndrome due to glomerular sclerosis soon after birth. He carries double homozygote mutations on the chromosome 19 in the major podocyte slit diaphragm protein, the nephrin, and was diagnosed NPHS1. A high level of consanguinity was evidenced in his family and his parents are first cousins. Few days after birth, the platelets count was 9 G/L. Hb level was 182 g/L. No infection, disseminated intravascular coagulation, haemangioma, splenomegaly were evidenced. In the bone marrow, megakaryocytes were normally present and with a normal morphology. A thrombocytopenia has never been described in NPHS1 patients; In addition one of his cousins, carrying the same nephrin mutations, did not exhibit thrombocytopenia indicating no relation between NPHS1 and the observed thrombocytopenia.

Aims: To investigate the etiology of this thrombocytopenia.

Results: Platelet immunology investigation detected alloantibodies against HPA5b antigen in the mother. Maternofoetal alloimmunization was straightforward with genotyping in the two parents and the propositus.

Platelet transfusions (apheresis platelet concentrates) were rapidly initiated twice a week, in parallel with treatment for NPHS1. The child was daily supplemented with albumin, thyroxin, antithrombin and IV immunoglobulins, received angiotensin converting enzyme inhibitor and non steroidal anti inflammatory drug. A nephrectomy is already plan and he will be placed on the waiting list for renal transplantation.

Intriguingly 2 months after birth, severe thrombocytopenia was still present and managed with platelet transfusions every 6 days. This prompted us to pursue platelet exploration. Study of platelet morphology occasionally revealed giant platelets. Parents' platelet counts and mean platelet volume were normal. Flow cytometry revealed a moderate decrease in GPIb levels in both parents (MFI mother and father vs. range of control values: 16.8 and 17.9 vs. [21.2–39.9] AU) suggesting that parents can carry GPIb/IX heterozygous mutation. By immunofluorescence we confirmed the absence of GPIb expression in the propositus's megakaryocytes whereas GPIIbIIIa was normally expressed. At the age of 4 months, the propositus was free of platelet transfusions for 3 weeks with a platelets count between 30 and 50 G/L. This allows us to confirm a total absence of GpIb expression at the platelets' surface but a normal expression of GPII/IIIa. GPIb/IX genes (Chr 22 and 3) are under sequencing.

Conclusion: We described a case carrying an association of hereditary diseases in a context of high consanguinity level. Immunofluorescence on megakaryocytes helped us to detect GPIb/IX deficiency in this complex situation involving platelet polytransfusion and immunization.

PB 4.29-4

Type I interferon dampen platelet production and function

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Background: We have previously shown that infection of human $CD34^+$ cells with Junin virus (arenavirus causative of Argentine hemorrhagic fever) or Poly(I:C), a synthetic double-stranded RNA that mimics viral replication, results in an impaired platelet production with no quantitative changes in megakaryocyte generation that was associated with the production of type I interferon (IFN-I). These findings identified IFN-I as a new regulator that selectively affects the last steps of megakaryocyte lifespan, and suggested a potential mechanism for thrombocytopenia in Argentine hemorrhagic fever and other diseases associated with increased type I IFN levels.

Aim: To further analyze the megakaryocyte/platelet function abnormalities mediated by IFN-I and explore whether the levels of IFN-I produced during a viral infection are associated with quanti/qualitative alterations of thrombopoiesis.

Methods: *In-vitro:* Cord blood human $CD34^+$ cells were stimulated with thrombopoietin (50 ng/mL) and treated with different concentrations of Poly(I:C), strong inducer of IFN-I, or with human recombinant IFN-beta at day 7 of culture. After 14 days, CD61 expression and fibrinogen binding in platelets were evaluated by FACS. *In-vivo*: 8–10 week old C57BL/6 female mice were daily intravenously inoculated with Poly(I:C) (100 μg/mouse) and blood samples were taken at 24, 48 and 72 h. Platelet count was determined with a veterinary cell counter. P-selectin expression and fibrinogen binding in peripheral platelets were evaluated by FACS. Plasmatic IFN-beta levels were determined by ELISA. Results represent the mean ± SEM of 5–6 experiments.

Results: While the number of CD61 copies on the membrane of *in-vitro*-generated platelets after Poly(I:C) or IFN-I treatment was similar to control samples, fibrinogen binding of thrombin-stimulated platelets was significantly inhibited (85% and 69% of maximal inhibition respectively, IC_{50}: 6.5 mg/mL and 7.6 U/mL for Poly(I:C) and IFN-I respectively). When mice were inoculated with Poly(I:C), a reduction of 28, 54 and 67% in the platelet count was observed at 24, 48 and 72 h respectively. The mean platelet volume was 8 and 13% higher than control values at 48 and 72 h respectively ($P < 0.01$), indicating a slight but significant bone marrow response to the decreased platelet count. While thrombin-induced fibrinogen binding and P-selectin externalization of platelets from mice that were treated for 24 or 48 h with Poly(I:C) were not different from control animals, both responses were significantly reduced after 72 h (complete turnover of the platelet population) (51 and 33% lower than the control value respectively, $P < 0.05$) suggesting an inhibitory effect at the bone marrow level rather than an effect on peripheral platelets. The IFN-beta levels in plasma from treated animals were significantly higher than those found in controls (89 ± 19, 101 ± 26, 126 ± 21 pg/mL at 24, 48 and 72 h respectively, vs. 41 ± 9).

Conclusions: Our data suggest that treatment of mice with Poly(I:C) inhibits platelet generation and function associated with an increase in the levels of plasmatic IFN-I. These findings were also supported by the *in-vitro* data in human cells and may explain the defects observed in platelet production/function as a consequence of viral infections.

PB 4.29-5

Lack of association between NR3C1 polymorphism and glucocorticoid resistance in Chinese patients with immune thrombocytopenia

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Background: Immune thrombocytopenia (ITP) is an autoimmune disorder involving autoantibody- and cell-mediated increased platelet destruction and/or decreased platelet production. First-line therapy of ITP comprises applying glucocorticoid and/or intravenous immunoglobulin. But resistance to glucocorticoids (GCs) is an intractable problem in therapy for ITP. Because GCs exert their effects through glucocorticoid receptor (GR), being a GR gene, NR3C1 is thought to connect with individual differences in glucocorticoid responsiveness during GCs treatments.

Aims : The purpose of this study was to analyze the correlations between three novel SNPs of NR3C1 and GCs responsiveness in Chinese patients (Han ethnic) with ITP.
Methods: We analyzed the frequency of three novel single nucleotide polymorphisms (SNPs) of NR3C1 in ITP patients and evaluated the role of these genetic variants in GCs therapy. Four hundred and seventy-three patients with ITP and 160 healthy controls were recruited. Patients were allocated into GCs-responsive (n = 358) and -non-responsive group (n = 115). All subjects of the three groups were genotyped by PCR-RFLP (restriction fragment length polymorphism) method for the *Bcl*I, N363S and ER22/23EK polymorphisms. Assess the statistical differences of genotypes between ITP and controls, and those between GCs- responsive and non-responsive group.
Results: In healthy controls, *Bcl*I-GG/GC/CC occurred with 0.581/0.35/0.069 frequency. In ITP patients, *Bcl*I-GG/GC/CC was found with 0.617/0.353/0.03 frequency. There was no statistically differences between ITP and controls (P = 0.070). In GCs-responsive and -non-responsive group, *Bcl*I-GG, GC, CC occurred with frequencies of 0.628/0.352/0.02 and 0.583/0.357/0.061 respectively. No correlations in the variants of *Bcl*I was found between the GCs-responsive and -non-responsive group (P = 0.086). Neither N363S nor ER22/23EK polymorphism was observed in all 636 participants.
Conclusion: The *Bcl*I polymorphism is not related to response of glucocorticoids in patients with ITP. Furthermore, we did not observe N363S and ER22/23EK polymorphism in Chinese Han population.

PB 4.29-6

Liver X receptor (LXR) activation influences murine platelet count only under normolipidemic conditions

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Background: We recently showed that hypercholesterolemia induces altered platelet characteristics as well as hyperreactivity in mice and humans[1,2]. Activation of the cellular cholesterol sensors liver X receptor (LXR) β, which is an oxysterol-activated nuclear receptor that tightly regulates intra- and extracellular cholesterol homeostasis, also modifies platelet responsiveness[3]. Therefore, LXRβ might be a potential target for the treatment of atherothrombosis.
Aim: The current study aims to elucidate the influence of hyperlipidemia on LXR-induced changes of platelet characteristics.
Methods: Experiments were performed with apolipoprotein E knockout (apoE KO) mice, a commonly used hyperlipidemic mouse model, or their wildtype (WT) littermates. ApoE deficiency in mice results in extremely elevated plasma VLDL and LDL cholesterol levels on regular chow diet (5.7% (w/w) fat and no added cholesterol). To study the effect of the synthetic LXR agonist T0901317 on platelets during normalization of plasma cholesterol levels, apoE KO mice were fed a Western-type diet (WTD; 15% (w/w) fat, 0.25% cholesterol) for 3 weeks and were subsequently transplanted with WT bone marrow (BM) to restore plasma apoE levels and normalize plasma lipid levels. Then the mice were fed T0907317 (10 mg/kg/day) mixed with regular chow diet for 6 weeks.
Results: The elevated plasma total cholesterol (TC) levels of ApoE KO mice on chow (546 ± 58 mg/dL vs. 61 ± 6 mg/dL in WT mice, $P < 0.001$) or WTD (764 ± 118 mg/dL, $P < 0.001$) were associated with significantly lower platelet counts (73% and 84% of control, respectively; $P < 0.001$), suggesting that hyperlipidemia affects platelet turnover. Normalization of plasma TC levels by transplantation of WT BM into apoE KO mice (from 984 ± 90 mg/dL to 81 ± 6 mg/dL; $P < 0.001$) resulted in a slightly increased platelet count (865 ± 83 × 10^9/L) compared to apoE KO BM-transplanted mice (TC = 436 ± 45 mg/dL, platelet count = 692 ± 106 × 10^9/L, ns). LXR activation by T0901317 raised plasma TC levels in both WT BM- and apoE KO BM-transplanted mice (182 ± 11 mg/dL and 859 ± 78 mg/dL, respectively). However, T0901317 significantly raised platelet count in WT BM-transplanted apoE KO mice (1325 ± 107 × 10^9/L vs. 865 ± 83 × 10^9/L, $P < 0.01$) but left the platelet count in apoE KO BM-transplanted mice unchanged (664 ± 74 × 10^9/L vs. 692 ± 107 × 10^9/L).
Summary/Conclusions: Activation of LXR by T0901317 leads to increased platelet counts, but only under normolipidemic conditions. This effect of hyperlipidemia on LXR function should be taken into account when evaluating LXR as potential target for prevention of atherothrombosis.
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PB4.30 – Microparticles and disease – III

PB 4.30-1

Thrombin generation caused by microparticles in patients with atherosclerosis of the vessels of the lower extremities

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Background: Atherosclerotic diseases are accompanied by activation of blood cells and endothelium that can lead to microparticles (MP) release. MP expresses tissue factor (TF) and procoagulant phospholipids on their surface that can enhance prothrombogenic potential of blood plasma.
Aim: To estimate MP-associated thrombin generation in the group of patients with atherosclerosis of the vessels of the lower extremities
Methods: This research included 29 patients (10 females and 19 males) at the age from 44 to 77 with atherosclerosis of the vessels of the lower extremities. Reference blood samples were obtained from 54 healthy controls. Thrombin generation was measured in platelet-free plasma with Calibrated Automated Thrombinogram Assay method (CAT). Following reagents (Trombinoscope BV, The Netherlands) were used: «MP-reagent», containing negatively charged phospholipids (4 μM), «PRP-reagent», containing rTF (1 pM), and «FluCa"�kit». CTI (40 mg/mL) was added to inhibit contact activation. Following parameters were derived: endogenous thrombin potential (ETP, nM-min), peak thrombin activity (Peak, nM), and rate of thrombin generation (R, nM/min). Data were described by non-parametric methods using the median (Me), 50% confidence interval (CI) and Mann-Whitney U test (Statistica 6.0). $P < 0.05$ was considered sighnificant.
Results: The role of procoagulant phospholipids of MP in thrombin generation was studied with «PRP-reagent» but there were no reliable differences between patients and controls when it was used. Significant differences was found when was used «MP-Reagent» with CTI. This reagent makes it possible to estimate thrombin generation which depends on TF. There was increase ETP, Peak and R in the patients group compared with controls (ETP: Me-807.50, CI: 712.50–1282.00 vs. Me-566.50, CI: 462.25–708.00, $P < 0.01$; Peak: Me-92.09, CI: 60.04–129.62 vs. Me: 46.25, CI:32.91–67.21, $P < 0.001$; R: Me-20.98, CI: 6.76–33.18 vs. Me-7.34, CI: 5.17–12.58, $P < 0.01$).
Conclusion: MP enhances thrombin generation and increase prothrombogenic potential of patients with atherosclerosis of the vessels of the lower extremities due to TF expressed on their surfaces.

PB 4.30-2

Circulating microparticle number and function vary with age: a study of 120 healthy blood donors

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Background and Aims: Information on circulating Micrparticles(MP) in normal subjects is fragmented and incomplete. The aim was to conduct a thorough evaluation of MP subsets and pro-coagulant function in healthy individuals and to determine how general clinical and biochemical demographics influence MP levels and/or function.

Methods: Citrated blood sample were collected from 120 healthy volunteer blood donors after receiving informed consent. Platelet free plasma was prepared using a standardized published protocol. MP subsets were enumerated by Flow cytometry(BDFACS Canto) after staining with specific antibodies for platelets(CD41), endothelial cells (CD105), tissue factor(CD142), white blood cells(CD45), monocytes (CD14) and red cells(CD235a). Counting beads using an ISTH protocol was employed. Coagulant function was assessed by the Xact assay on a BCS coagulation analyser and by thrombin generation using a Calibrated Automated Thrombogram(CAT) on a thrombinoscope. An ELISA was undertaken for Annexin V expression. Hormonal and lipid analysis – FSH, LH, testosterone, oestradiol, progesterone as well as HDL, LDL, cholesterol and HDL/LDL ratios were measured by standard biochemistry techniques. A total of 108 samples were finally analysed according to age, gender, body mass index (BMI), full blood counts, smoking status, hormones and lipid profile. For analysis, the cohort was divided into three age groups (G): ≤29 years(G1, $n = 31$), 30–59 years(G2, $n = 60$) and ≥60 years(G3, $n = 17$). STATISTICA software was used for all analyses.

Results: A total of 108 subjects were finally analysed which included 60 males and 48 females. Twenty were smokers. The mean age was 40 years (range 16–73). The mean BMI was 26.5(range 17.5–46.9). All full blood count parameters were within normal range. Platelet MP (median 38.8/μL) were highest at ages ≤29 and ≥60 and significantly lower($P = 0.00013$) in G2(30–59 years). A similar ''U'' shaped curve was noted for red cell, tissue factor and Annexin V expressing MP, while the opposite trend was noted for endothelial MP. In the functional analysis, CAT results were similar to that of platelet MP, with ETP, time to peak, and lag time showing a significant reduction in the middle aged G2 group but elevated in G1 and G3. There were no significant differences in the Xact test or the ELISA for phospholipid content between the three groups. There were no gender differences in levels of circulating MP except for monocyte microparticles that were significantly ($P = 0.0014$) higher in males. Multivariate modelling did not show any specific variable, including BMI, full blood counts, lipid or gender specific hormonal parameters, predictive of microparticle number. Interestingly, smokers showed a reduction in red cell, TF and Annexin V expressing MP.

Conclusions: Considerable variability with age was observed in MP counts and thrombin generation, but not in phospholipid dependent pro-coagulant properties. The most significant finding was that of lower MP levels in those between ages 30–59 years compared to those either younger or older than this group. This is an important finding, which has bearing on interpretation of levels of circulating MP in pathological states. The physiological explanation for this finding is not yet apparent. The differences noted in smokers need more extensive evaluation.

PB 4.30-3

Microparticles in aneurismal subarachnoid hemorrhage: role in acute and delayed cerebral ischemia

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Background: Microthrombosis has been demonstrated in early and delayed cerebral ischemia after aneurismal subarachnoid hemorrhage (aSAH). Markers of coagulation activation as microparticles (MPs) are an established risk factor for thrombosis. We tested the hypothesis that levels of microparticles might correlate with (i) aSAH severity (ii) early cerebral ischemia (ECI) and (iii) delayed cerebral ischemia (DCI).

Methods: Consecutive aSAH patients (age 18–80) admitted to our department between November 2011 and September 2012 were included in the study. Total Mps (AnnexinV+), platelet Mps (PLT-MPs, AnnexinV+/CD41+), tissue factor MPs (TF-MPs, AnnexinV+/CD142+), and endothelial MPs (E-MPs, AnnexinV+/CD144+) were measured by flow cytometry in venous blood samples, obtained at early (<3 day) and delayed phase (7–10 days). Multi-parameter MRI was performed to evaluate ischemic damage and vasospasm at the same timepoints. Levels of MPs were evaluated comparing patients according to (i) SAH severity (World Federation of Neurological Surgeons score) (ii) ECI severity and (iii) occurrence of delayed complications (vasospasm and DCI).

Results: Twenty-five patients (age 57 ± 12 years) were included in the analysis. Overall increased levels of MPs were observed at early phase after aSAH when compared to controls (334, IQR 80–775 vs. 64 IQR 40–147, $P = 0.0024$). In the early phase no significant differences in MPs level were observed in patients with different aSAH severity (MPs tot 436, IQR 76–918 vs. 313 and IQR 83–638 respectively, $P = 0.56$). Interestingly patients with most severe ECI (MPs tot 95, IQR 47–178 and 69, IQR 28–95 vs. 37 IQR 34–39, Kruskal-Wallis test $P = 0.19$) and DCI (MPs tot 131, IQR 74–281 and 53 IQR 35–361 vs. 46 IQR 19–117, Kruskal-Wallis test $P = 0.29$) showed the highest values of MPs, but this trend did not reach statistical significance, probably because of the the still small sample size small number of patients included in the study.

Conclusions: Microparticles and microthrombosis may increase the severity of early and delayed ischemic damage after aneurismal SAH. A larger number of patients are required to confirm this preliminary observation.

PB 4.30-4

Platelet microparticles: biomarkers of arterial thrombus formation?

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Background: Most platelet microparticles (PMP) in fresh plasma of healthy humans do not bind annexin V or lactadherin, thus questioning the role of PMP as a procoagulant risk factor. Because the release of PMP requires not only platelet activation (Blood 2009; 113: 1112–1121) but also the binding of fibrinogen to glycoprotein IIb (J Biol Chem 1993; 268: 14586–9), we hypothesize that PMP may reflect arterial thrombus formation/degradation.

Aim: To investigate the role of fibrinogen binding on the release and composition of PMP after *in vitro* thrombus formation

Methods: Citrate-anticoagulated blood was collected from healthy subjects ($n = 4$–6). Platelet rich plasma was prepared, and incubated

for 5 min at 37 °C under stirring conditions in a multiplate aggregometer. After 5 min, ADP (10 mM) was added, and after 30 min the incubation was stopped, supernatant was collected and analyzed by flow cytometry.

Results: In fresh plasma 13,980 ± 8419 CD61+ PMP ($n = 6$) were detectable, of which only 2% stained for lactadherin and 5.6% for P-selectin (CD62p). Upon addition of ADP, platelet aggregation occurred immediately, and the number of CD61+ PMP increased to 21,624 ± 5310. The newly formed PMP all expose P-selectin, do not bind lactadherin, do not support coagulation (fibrin generation test), inflammation (whole blood test), or fibrinolysis (plasmin formation). In the presence of abciximab (reopro), the formation of novel PMP is entirely abolished. Thus, the release of PMP exposing P-selectin requires not only platelet activation but also fibrinogen binding *in vitro*.

Conclusions: Our results confirm earlier studies that both platelet activation and fibrinogen binding are both essential for the release of PMP. Because the concentration of PMP exposing P-selectin is associated with aging, peripheral arterial disease and myocardial infarction (Clin Chem 2006; 52: 657–64), we hypothesize that PMP exposing P-selectin may by a marker of arterial thrombus formation/degradation rather than being a risk factor for thrombosis.

PB 4.30-5

Elevated circulating platelet microparticles determine thrombus occlusion in acute coronary syndrome

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Background: Early steps of coronary artery thrombosis involve platelet activation. Activated platelet generates microparticles which convey prothrombotic activity. Platelet microparticles (PMP) may provide additional thrombus burden after coronary plaque rupture, thus leads to aggravated coronary thrombus occlusion in culprit vessel in patients with acute coronary syndrome.

Aims: To investigate whether elevated circulating PMP associated with level of coronary thrombus occlucion in acute coronary syndrome.

Methods: Consecutive patients admitted to Intensive Coronary Care Unit with acute coronary syndrome were enrolled. Circulating PMP were isolated from platelet-poor plasma (PPP) of citrated-venous blood which was drawn within 24 h of admission. CD42b-PE was labelled into PPP and PMP was detected with flow cytometry. Platelet counts, mean platelet volume (MPV) and platelet distribution width (PDW) were measured. Acute coronary syndromes were classified as ST-elevation myocardial infarction (STEMI), non-STEMI and unstable angina, respectively reflect coronary thrombus occlusion levels. Major adverse cardiac events were also documented.

Results: Forty-one patients were analysed. STEMI, indicating coronary total thrombus occlusion and distal myocardial infarction, had significantly highest circulating PMP (6329.74/μL; $P < 0.05$). Non-STEMI, the less severe coronary thrombus, had lower circulating PMP (3990.18/μL). Whereas unstable angina, the mildest event, had lowest circulating PMP (2051.62/μL). Circulating PMP did not significantly correlate with platelet count ($r = 0.220$), MPV ($r = -0.192$) or PDW ($r = -0.071$). Major adverse cardiac events during hospitalisation were not affected by circulating PMP (3852.99 vs. 4268.70/μL; $P = 0.81$).

Conclusion: Elevated circulating PMP determines aggravated thrombus occlusion following acute coronary syndrome. However, it does not associate with major adverse cardiac events.

PB 4.30-6

Increased procoagulant microparticle levels in women with recurrent pregnancy loss

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Background: Pregnancy loss is the most common complication of pregnancy affecting up to 15% of reproducing couples and recurs in 2% to 3% of them. Despite a wide range of investigations, no apparent cause can be found in large number of cases. A defective maternal hemostatic response leading to hypoxia secondary to thrombosis of the uteroplacental vasculature has been hypothesized to subsequently lead to fetal loss. This may include impairment of trophoblast invasion, villitis, and placental microthrombi. Hereditary thrombophilia and antiphospholipid antibodies have been extensively described as risk factors for pregnancy loss. More recently, a new maker has emerged: the cell-derived circulating procoagulant microparticles, which have been reported to have a major role in many thrombosis complicated diseases.

Aim: To analyze the prevalence and significance of circulating procoagulant microparticles (MP) in women suffering from pregnancy loss (PL), both recurrent and unexplained fetal loss and characterize their cellular origin.

Methods: 115 women (60 with early losses, 24 with late and 31 with both early and late PL) with no other presumptive etiological causes (anatomic, genetic or hormonal) were analyzed in the present study for the common thrombophilia markers (i.e. lupus anticoagulant, anti-cardiolipin antibodies, anti-b2GP1 antibodies, annexinV antibodies, protein C, protein S, antithrombin III, factor V Leiden mutation and MTHFR C677T) and for MPs of different cellular types i.e. total procoagulant annexin V MPs, procoagulant platelet(CD41a), endothelial(CD146, CD62e), leukocyte(CD45), erythrocyte(CD235a) MPs as well as tissue factor (TF) expressing MPs. The results were compared with 20 non pregnant healthy women. Methodology for analysis of MPs has been standardized on BD FACS Aria flow cytometer by participating in the 'Vascular Biology SSC workshop: standardization of FCM-based PMP enumeration'.

Results: Thirty seven women were found positive for any of the thrombophilia markers analyzed. Nine were positive for lupus anticoagulant, five for anti-cardiolipin antibodies, three for anti-b2GPI antibodies, 10 for annexin V antibodies and in all, 20 patients had one or more acquired thrombophilia markers. Among the hereditary thrombophilia, seven had reduced protein C levels, 10 had reduced protein S, five had reduced antithrombin III, three were factor V leiden heterozygotes and three were homozygous for MTHFR. No significant differences were seen in the MP profile of women with or without any thrombophilia marker. Total annexin MPs, total TF expressing MPs and endothelial MPs, both constitutive and activated, were found significantly increased($P < 0.05$) in women with PL of all categories (early, late & early and late) when compared to the control group. Differences in platelet and leukocyte MP levels were not found to be significant.

Conclusion: Our findings suggest that MPs are associated with the pathogenesis of PL and may contribute in uteroplacental thrombosis. In addition, the presence of elevated endothelial and TF expressing MPs at a distance (3–24 months) from the adverse pregnancy event suggests a continued chronic endothelial damage or activation which might be one of the causes of recurrent pregnancy loss. Although none of the patients in this study had any thrombotic episode, it is possible that this might become apparent in pregnancy.

PB4.31 – Atherosclerosis: risk factors

PB 4.31-1

Can we identify patients at risk of recurrent coronary artery disease?

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Background: Premature coronary artery disease (CAD) occurs at an earlier age in South Asians compared with other ethnic groups. Infection and inflammation show a positive association with the disease. In our previous studies we have shown that pathogen burden specifically Cytomegalovirus (CMV) infection in combination with inflammatory markers show significant association with CAD and recurrent CAD in Indians (Heart. 2012;98(13):982–987). Further global proteome analysis may give more biomarkers for better risk assessment for these events.
Aim: This study was aimed at identifying new biomarkers by performing comparative global proteome analysis for improvement of risk assessment of recurrent CAD.
Methods: 240 age and gender matched subjects (60 healthy controls, 120 CAD affected and 60 recurrent CAD) were selected for a case control study from ongoing Indian Atherosclerosis Research Study (IARS). The information regarding inclusion, exclusion criteria and process of sample collection, and data storage have been published (Indian Heart J, 2010;6:286–95). All the blood samples were collected after overnight fasting of 12–14 h and stored at −80 °C. The sero positivity for CMV was established using neutralizing antibody titer values for each sample. Global proteomic analysis was performed using Surface Enhanced Laser Desorption/Ionization CM10 arrays on serum samples of these subjects. Support Vector Machine method for risk stratification of CAD affected and Tagident software for protein identification. STRING database was used to build the network topology followed by analysis using Cytoscape v.2.8.3 software.
Results: We have identified 39 significantly differentially expressed protein peak (m/z) patterns of which 19 peaks were detected for CAD affected and 20 peaks in recurrent CAD subjects. We used the 20 significant peaks from affected vs. recurrent disease subjects for risk assessment and found that two peaks corresponding to beta-defensin and histatin-3 were significantly associated with recurrent CAD. The odds ratio for beta-defensin was 7.49 (95% CI 2.57–21.9, *P*-value 0.0001) and for histatin-3 it was 1.4 (95% CI 1.02–2.13, *P*-value 0.042) in recurrent CAD subjects. Furthermore, when these markers were combined with conventional risk factors the odds ratio improved to 8.97 (95% CI 2.87–28.04, *P*-value 0.0001). All the 39 proteins were used in network construction, the comparison of the two networks (CAD affected vs. controls and CAD affected vs. recurrent), suggests that the proteins like serpin peptidase inhibitor A1 (), compliment C3 (C3), compliment C5AR1, were significant in both the networks. Proteins like amyloid beta A4 (APP), Pro-opiomelanocortin (POMC) and vasoactive intestinal peptide (VIP peptides) were significant in CAD affected vs. controls but not in those with recurrent disease. New proteins representing infection pathway beta-defensin, histatin-3 and others such as vascular endothelial growth factor A (VEGF-A), growth related alpha protein (GROA), natriuretic peptide B (BNP), and cardiac phospholamban (PPLA) were identified only in the recurrent subjects.
Conclusions: These findings suggest that beta-defensin and histatin-3 may add value to risk assessment for recurrent CAD and further validation in larger cohort is needed. Our data also suggests that infection and immune system proteins may play a major role in recurrent disease.

PB 4.31-2

Risk factors associated with myocardial infarction in venous thromboembolism patients

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Background: Some of the newer oral anticoagulants have been associated with a heightened risk of myocardial ischemic (MI) events. Data is currently lacking in evaluating risk factors associated with MI in venous thromboembolism (VTE) patients, a population which is commonly treated with anticoagulants.
Aims: The purpose of this study was to identify risk factors associated with MI in VTE patients.
Methods: Health insurance claims between 01/2004 and 09/2008 from the Ingenix IMPACT database were analyzed. Subjects aged ≥18 years with ≥1 year of continuous insurance coverage before the index VTE were identified as of the date of their first VTE diagnosis (index deep vein thrombosis, pulmonary embolism, or both). Patients' histories were analyzed up to the earliest date between health plan disenrollment and lack of follow-up data. The risk of MI for VTE patients with 1, 2, and ≥3 major risk factors (age, gender, hypertension, diabetes, hyperlipidemia, cardiovascular diseases, family history of cardiovascular diseases, chronic kidney disease, obesity, and smoking) as identified by published guidelines was reported. Multivariate Cox proportional hazards models were conducted to identify the most predictive risk factors associated with MI, adjusting for region, insurance, years (2005–2008), baseline medications and healthcare costs.
Results: Of 177,885 VTE patients identified, 4412 (2.5%) developed an MI during a mean follow-up period of 1.3 years. The most frequent major risk factors for MI at baseline were hypertension (46.4%), cardiovascular disease (40.1%), and hyperlipidemia (39.2%). The proportions of patients with 0, 1, 2, or ≥3 major risk factors were 28.9%, 19.1%, 16.2%, and 35.8%. Previous MI, age (≥65 years), coronary artery disease, and hypertension were the risk factors most predictive of MI, with adjusted hazard ratios (HRs; 95% CI) of 5.47 (5.01–5.97), 1.78 (1.66–1.91), 1.60 (1.48–1.74), and 1.42 (1.31–1.55), respectively. Adjusted HRs (95% CI) for VTE patients with 1, 2, ≥3 major risk factors relative to no major risk factor were 2.34 (1.94–2.81), 3.21 (2.67–3.85), and 6.93 (5.85–8.22), respectively.
Conclusions: Traditional major cardiovascular risk factors are very prevalent and predicative of MI in VTE patients. Having multiple major risk factors significantly increases the probability of developing MI events in VTE patients.

PB 4.31-3

Further evidence in support of the association between atherosclerosis and venous thrombosis

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Background: Ten years ago a strong association between subclinical atherosclerosis and venous thrombosis was found (Prandoni et al., N Engl J Med 2003;348:1435–41). In that study patients with primary pulmonary embolism (PE) and those with previous venous thromboembolism (VTE) were excluded.
Aims: In a new unselected cohort of patients with VTE older than 50 years, including patients with primary PE and patients with previous VTE, we assessed the prevalence of symptomatic and that of subclinical atherosclerosis. In addition, we compared the prevalence of atherosclerotic disease between patients with unprovoked VTE and patients with VTE triggered by acquired risk factors.

Methods: Patients meeting the eligibility criteria were interviewed for history of previous episodes of symptomatic atherosclerosis (including coronary heart disease, transitory or well defined ischemic stroke and arteriopathy of the lower limbs). If the medical history was negative, the presence of vessel wall plaques was investigated through the bilateral ultrasound assessment of carotid arteries with the adoption of standard methods and criteria. After adjusting for age, risk factors for atherosclerosis, thrombophilia and previous VTE, we calculated the odds ratio (OR) and its 95% CI for (either symptomatic or subclinical) atherosclerosis in patients with unprovoked VTE vs. those with a secondary event. The study was approved by the local ethics committee and for all patients written informed consent has been obtained.
Results: Out of 202 consecutive VTE patients meeting the eligibility criteria, 119 (58.9%) referred with an unprovoked VTE, and 83 (41.1%) with a secondary VTE. The mean age (±standard deviation) of study patients was 73.8 ± 10.6 years, 90 (45.1%) were males, 54 (26.7%) presented with primary PE and 40 (19.8%) with previous VTE. No appreciable differences were seen between the two patients' groups in terms of baseline features. A history of symptomatic atherosclerosis was recorded in 42 (35.3%) patients with unprovoked VTE and in 26 (31.3%) with a secondary event. Of the remaining 134 patients, the presence of at least one carotid plaque was recorded in 42 of the 77 (54.5%) patients with unprovoked VTE and in 22 of the 57 (38.5%) with secondary thrombosis. The adjusted OR for either symptomatic or subclinical atherosclerosis in patients with unprovoked as compared to those with secondary VTE was 1.8 (95% CI 1.2–4.1).
Conclusions: The study results, obtained in a broad number of unselected patients over 50, expand those of our previous publication and confirm that an important association does indeed exist between atherosclerosis and unprovoked VTE.

PB 4.31-4

The relationship of FII, FVII, FXIII and fibrinogen levels with conventional risk factors in patients with and without coronary artery disease

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Background: A number of coagulation factors are required for the cascade of biochemical reactions leading to fibrin formation. The aim of the study was to investigate the relationship between haemostatic factors II(FII), VII(FVII), XIII(FXIII), fibrinogen and conventional coronary artery disease (CAD) risk factors in order to enhance understanding of phenotypic variation in haemostatic factor levels in patients and clinical controls.
Materials and Methods: The study was performed according to Helsinki Declaration and was approved by the *Magdalena* Special Hospital for Cardiovascular Surgery and Cardiology Ethic Committee. Association analysis was carried out in 242 subjects with ³ 50% stenosis in at least one of the major coronary vessels (CAD+) and 138 subjects with angiographically excluded disease (CAD-). Data regarding body mass index (BMI), presence of arterial hypertension (AH), diabetes, smoking status or history of myocardial infarction (MI) was obtained on admission. Blood collection and processing were performed according to standardized procedures. Established risk factor ratio (ERF) was calculated by dividing total cholesterol by HLD-cholesterol. Fibrinogen was determined by Clauss method; FII and FVII by single step clotting assays and FXIII by chromogenic assay on a BCS analyzer (Siemens Diagnostics, Marburg, Germany). Bivariate correlations between variables were analyzed with Pearson's correlation coefficient, and trendlines were calculated with linear regression analysis. Significance was taken as $P < 0.05$.
Results: Median of the FII, FVII, FXIII and fibrinogen levels was not significantly different between the CAD- and CAD+ patients (108.5 vs.111.0; 120.6 vs.117.1, 93.2 vs.90.9, 2.5 vs.2.6, respectively) ($P > 0.05$). Females have higher values in compare to males (FII:113.6 vs. 105.9, $P = 0.005$; FVII:131.5 vs. 117.9, $P = 0.006$, FXIII:100.6 vs. 92.0, $P = 0.001$, FIB:3.18 vs. 2.8, $P = 0.006$). Fibrinogen correlated with age (R:0.124, $P = 0.05$), FXIII(R:0.285, $P = 0.01$) and FII (R:0.162, $P = 0.01$). Furthermore, FII correlate with BMI (R:0.117, $P = 0.05$), ERF(R:0.174, $P = 0.01$) and FVII (R:0.741, $P = 0.01$) whereas FVII values correlated with ERF(R:0.118, $P = 0.05$) and smoking($R = -0.105$, $P = 0.01$). In clinical controls FII, FXIII, FVII activities correlated with AH (R:0.192; 0.263, 0.296 at $P < 0.05$, respectively). Obtained fibrinogen values were higher in females than in males(3.1 vs 2.4, $P = 0.03$). Fibrinogen correlated with FXIII (R:0.405; $P < 0.01$), but in males also with age(R:0.234) and smoking (R:0.243). In CAD patients FII correlated with age(R:0.130, $P < 0.05$), AH(R:0.195, $P = 0.01$), ERF(R:0.205, $P = 0.01$), FVII (R:0.795, $P = 0.01$) and fibrinogen(R:0.154, $P < 0.05$). Females had a higher FII levels(115.7 vs. 107.8, $P = 0.07$) that correlated with BMI (R:0.279, $P < 0.05$), and higher FXIII in compare to males (99.9 vs. 88.6, $P = 0.02$). FVII was higher in smokers(121.3 vs. 112.3, $P = 0.030$). Fibrinogen correlated with FXIII (0.195, $P = 0.01$). Overall, multiple regression analysis revealed age, sex, AH, EFR and fibrinogen to be independent predictors of FII clotting activity, predicting ~11% of the variability; AH, ERF and fibrinogen to be independent predictors of FVII clotting activity, predicting ~4% of the variability; age, sex, AH and fibrinogen to be independent predictors of FXIII activity, predicting ~11% of the variability; smoking, sex and age to be independent predictors, that explain ~15% of the fibrinogen variability.
Conclusion: The obtained data suggest that different combination of conventional risk factors may have different effects on coagulation factor activities in different patient's subgroups. However, more extensive study has to be performed in order to determine a combination of relevant factors contributing to the difference in clotting factor activities.

PB 4.31-5

Subclinical atherosclerosis in patients infected with Human Immunodeficiency Virus. Early predictors

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Background: Patients infected with Human Immunodeficiency Virus (HIV+) have a greater burden of clinical and sub-clinical atherosclerotic disease (SA) compared to the general population. The main causes of SA in HIV+ are multiple pro-atherogenic metabolic abnormalities related to Antiretroviral Therapy, early immune senescence (consequence of chronic immune activation), and an individual's genetic background. Subsequently, early biomarkers might help to identify HIV+ subjects who are at an elevated risk of SA and guide the development of therapeutic interventions.
Aims: The purpose of this study was to identify early predictors of SA in HIV+, specifically endothelial and genetic markers.
Methods: We measured plasma von Willebrand Factor (vWF) (LIATEST-Stago), the −2518 A/G polymorphism in the monocyte chemoattractant protein (MCP-1) gene (PCR/RFLP) and blood lipids. SA was considered to be present when carotid intimate media thickness (IMT) was >1.0 mm at any site. IMT was measured by echo-doppler in 70 HIV+. Patients were divided according to the absence (SA-, $n = 40$) or presence (SA+, $n = 30$) of SA. We also assessed traditional coronary risk factors, HIV disease characteristics and treatment. The study was approved by the ethics committee and subjects gave informed consent.
Results: The groups were comparable in sex, waist circumference, duration of HIV infection, viral load, smoking habits, alcohol use, illegal drug use, co-infection by hepatitis virus C and the use of protease

inhibitors ($P > 0.05$). There were no significant differences in the −2518 A/G MCP-1 genotypic ($P = 0.915$) distributions between the groups.

Significant differences ($P < 0.05$) were noted between SA- and SA+ patients, specifically, age (37 vs. 47 years old), co-infection with hepatitis virus B (15 vs. 40%), current CD4+ T cell count (398 vs. 498 cells/μL), LDL cholesterol (85 vs. 109 mg/dL) and triglycerides (105 vs. 159 mg/dL). The nadir of CD4+ (207 vs. 54 cells/μL), months of exposure to treatment (56 vs. 72), total cholesterol (163 vs. 179 mg/dL) and vWF (111 vs. 140%) were different between SA- and SA+ patients, although these differences did not reach statistical significance. We included those variables in a logistic regression model. Previously, we categorized the variables according to the median values obtained or the international criteria as follows: nadir of CD4+ (50 cells/μL), current CD4+ T cell count (350 cells/μL), triglycerides (150 mg/dL), age (37 years old) and vWF (110%).

Predictive factors for the development of SA included an age of more than 37 years (OR = 16.69; 95% CI: 2.67–104.36, $P = 0.003$), vWF of more than 110% (OR = 13.05; 95% CI: 2.36–72.12, $P = 0.003$), CD4+ cells of more than 350 cells/μL (OR = 27.75; 95% CI: 3.87–198.80, $P = 0.001$), and triglycerides levels of more than 150 mg/dL (OR = 3.94; 95% CI: 1.02–15.26, $P = 0.047$).

Conclusions: In our HIV+, age and the level of triglycerides were risk factors for the development of SA, as has been previously reported. High current CD4+ levels as a consequence of immune status improvement could be a key factor in the progression of atherosclerosis via a synergistic effect with inflammation. The level of vWF has the promise to be an attractive study variable that could be used to differentiate between SA risk groups.

PB 4.31-6

Comprehensive analysis of global gene expression and microRNA profile in coronary artery disease

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Background: Studying the global gene expression and microRNA (miRNA) profile can provide a better understanding on the genetic basis of Coronary Artery Disease (CAD)

Aims: The aim of the present study was to perform global transcriptome profiling to identify novel genes/pathways for CAD, supported by miRNA profiling to understand epigenetic regulation of candidate gene expression.

Methodology: Blood whole transcriptome profiling was undertaken in 13 CAD patients and 11 asymptomatic controls while miNA profile was analyzed in 14 cases and 10 controls, respectively, using the Agilent microarray platform. The study participants were selected from the ongoing Indian Atherosclerosis Research Study (IARS). Information on both gene expression and miRNA was available for 10 cases and 8 controls. Validation of microarray findings was done in 100 cases and 100 matched controls for 10 putative candidate genes showing high differential expression. Gene expression and miRNA data were analyzed with 'R' program and Gene Spring software respectively.

Results: 343 genes were significantly up regulated and 151 genes were down regulated in cases relative to the controls (Fold change >2, $P < 0.05$). Gene assigned based on functional category primarily belonged to inflammation and immune response pathways, cell signaling, regulation and proliferation, coagulation, lipid and insulin metabolism, matrix degeneration and early growth response. Down-regulated genes included those associated with anti proliferation, protein and calcium signaling and other basic cellular processes.

Analysis of candidate gene expression showed *CXCL1, EGR3, IL8, PTGS2* and *CD69* to be up regulated and *IFNG* and *FASLG* to be down regulated among cases ($P \leq 0.05$), the trend being comparable to the microarray findings. *EGR3* and *IL8* genes in particular showed 9-fold and 8-fold differential expression. Three miRNAs, miR-144*, miR-96 and miR-1260, were significantly up regulated while miR-185* was found to be down regulated in the cases as compared to the controls. Of note, the expression levels of *ADAMTS18* and *KCTD16*, which are predicted gene targets of miR-96, were lower in CAD patients in the microarray dataset.

Summary/Conclusion: Combining global gene expression and miRNA data can improve our understanding on the underlying molecular pathogenesis of atherosclerosis.

PB4.32 – Atherosclerosis: miscellaneous – II

PB 4.32-1

Association of obesity markers and atherothrombotic biomarkers in coronary artery disease

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Background: Metabolic disturbances mediated by obesity and associated biomarkers can increase the risk of Coronary Artery Disease (CAD). The Adipose tissue releases a number of important hormones such as Adiponectin and Leptin, pro-inflammatory biomarkers that interact with other blood-borne atherothrombotic biomarkers and thus regulate the progression of heart disease.

Aims: We investigated the association between obesity markers and key circulating biomarkers in a family-based cohort of angiographically confirmed CAD patients and unaffected family members participating in the Indian Atherosclerosis Research Study (IARS)*.

Methods: Obesity markers depicting central obesity (body mass index-BMI) and visceral obesity (waist circumference -WC, Hip circumference – HC and waist-hip-ratio – WHR) were measured in 287 CAD patients and 477 unaffected family members. A wide panel of plasma/serum biomarkers were measured by standard methods and included the following – Lipids (total cholesterol-TC, triglycerides, High density lipoprotein cholesterol-HDL-c, Low density lipoprotein-cholesterol-LDL-c, Apolipoprotein-Apo- A1 and -B100, Lipoprotein (a)-Lp(a)), Coagulation factors (Factor VII.c, Fibrinogen), Inflammatory markers (IL6, hsCRP), Adipokines (Adiponectin, Leptin), Adhesion molecules (P-selectin, sICAM), Oxidative stress markers (OxLDL, Myeloperoxidase-MPO), antibody titers against common pathogens (cytomegalovirus, C pneumonia, H pylori and HSV), heat shock proteins (HSP-60, -65, -70) and other novel biomarkers (Cystatin C, secretory phospholipase A2-sPLA2, Neopterin). Multivariate analysis and correlations were performed to test the association of the biomarker panel across the obesity marker quartiles using SPSS version 17.0 software. Age and gender were considered as covariates.

Results: Mean levels of BMI, WC and WHR were higher in CAD affected than unaffected subjects, but statistically significant difference was seen only for WHR ($P = 0.002$). There was stronger correlation of BMI with WC ($r = 0.73$, $P < 0.0001$) than with WHR ($P = 0.20$). The best linear association was seen of BMI and WC with Adiponectin, Leptin and PAI-1 followed by hsCRP, FVII.c, Fibrinogen and lipids, particularly triglycerides and HDL-c ($P < 0.001$). In addition, WC showed significant association with ApoA1, ApoB100 ($P = 0.0001$), sICAM and p-selectin ($P <\leq 0.04$), Ox-LDL ($P < 0.0001$) and sPLA2 ($P < 0.0001$). This data was supported by strong correlation of WC and BMI with Leptin (0.56), Adiponectin (−0.28), PAI-1(0.32), sPLA2 (0.22), hsCRP (0.17) and Fibrinogen (0.22). Further analysis is in progress.

Summary/Conclusion: Waist circumference appears to be an excellent representative marker of visceral adiposity and shows strong association with a number of established and new atherothrombotic biomarkers in Asian Indians. Significance of these biomarkers in predicting new/recurrent coronary events will also be discussed.

*The rationale and design of the IARS has been published in Indian Heart journal 2010, 62:286–95.

PB 4.32-2

Free leptin, carotid plaque phenotype and relevance to related symptomatology: insights from the OPAL-Lille carotid endarterectomy study

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Background: Free leptin, an adipose tissue-derived cytokine, exerts important cardiovascular effects, but its role on human atherosclerotic carotid plaque phenotype and related symptomatology is unknown.
Aims : This relationship was assessed in 146 consecutive patients (71 asymptomatic and 75 symptomatic) scheduled for carotid endarterectomy.
Methods and Results: Free leptin was assessed in serum (9.7(4.6–19.7) ng/mL, range 0.47–107) and carotid plaques (0.075(0.040–0.163) ng/mg protein) (median [percentiles 25–75%]). Serum and plaque free leptin concentrations were positively correlated to each other ($P < 0.0001$, $r = 0.726$). Serum free leptin concentrations were independently and negatively associated with plaque-related symptomatology (ß = −0.066, 95% CI: −0.124; −0.007). Regarding plaque phenotype, histological examination revealed plaque Vascular Smooth Muscle Cell (VSMC) content and VSMC to macrophage area ratio to be significantly higher in asymptomatic patients compared to symptomatic ones (39.3(35.9–55.0) vs. 20.4(17.7–26.3), $P = 0.0003$, 6.8(2.6–31.4) vs. 1.4(0.6–2.0), $P = 0.014$, respectively). When serum or plaque leptin concentrations were above median value, plaque VSMC to macrophage area ratio and VSMC content or proliferation index were significantly higher in carotid plaque compared to concentrations below median value ($P < 0.019$). Interestingly, via ERK, Smad-2 and Akt activation, at 20 ng/mL, leptin induced a migratory signal and higher collagen synthesis in human VSMC, followed by a VSMC proliferative response at the concentration of 75 ng/mL.
Conclusions: We reported serum free leptin to be a marker of carotid plaque-related symptomatology. Free leptin could modulate plaque phenotype via direct effects on human VSMC.

PB 4.32-3

Nicotinic acid/laropiprant: negative effects on vascular function

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Background: Lipoprotein (a) is an atherothrombotic marker, damaging vessels indirectly by its capability to alter fibrinolysis, and directly, by its similarity with an LDL protein. Nowadays, the only pharmacological approach able to modify Lp(a) levels is niacin at high doses.
Aims: To assess the behaviour of lipid profile, haemoreological profile and endothelial function in secondary prevention patients treated with niacin/laropiprant.
Methods: 45 consecutive patients, all presenting Lp(a) >300 mg/dL, with previous adverse cardiovascular event (acute coronary syndrome, stroke, peripheral artery disease, retinal occlusion, sudden hypoacousia) and multiple comorbidities, were treated daily with 2000 mg of nicotinic acid/40 mg laropiprant and optimal statin therapy, in addition to cardiovascular medications according to current guidelines, for 12 months. Cholesterol, triglyceride, HDL-c, LDL-c, Lp(a), high-sensitive-PCR levels and haemoreological profile were analysed by fasting venous sampling. Endothelial function was assessed by peripheral arterial tonometry (PAT).
Results: After 12-month treatment the levels of triglycerides (118.71 ± 84.58 mg/dL vs. 88.86 ± 61.14 mg/dL), LDL-c (119.20 ± 35.81 mg/dL vs. 96.41 ± 36.98 mg/dL) and Lp(a) (1146.19 ± 451.54 mg/L vs. 776.79 ± 326.26 mg/L) were significantly reduced ($P = 0.002$, $P = 0.002$, $P < 0.0001$), instead HDL-c levels (52.89 ± 18.04 mg/dL vs. 60.14 ± 17.46 mg/dL) increased ($P = 0.029$). No differences in hsPCR levels were found. Unexpectedly, regarding blood viscosity, red cell deformability (0.3351 ± 0.3422 vs. 0.3128 ± 0.0392) and aggregation (63.80 ± 8.90% vs. 60.78 ± 5.70%) were impaired ($P = 0.023$ and $P = 0.077$, respectively) after 12 months. Accordingly, PAT values were lower after treatment (2.12 ± 0.77 vs. 1.77 ± 0.38, $P = 0.044$).
Conclusion: Niacin is significantly able to improve lipid profile, particularly by reduction of Lp(a). Despite these behavioural effects, nicotinic acid/laropiprant seems to negatively affect blood viscosity and endothelial function, suggesting a possible explanation of the negative results of HPS2-THRIVE study.

PB 4.32-4

Oxidized LDL: a link between atherosclerosis and Alzheimer's disease?

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Background: Shared risk factors for both atherosclerosis and Alzheimer's disease (AD) have raised interest in understanding vascular mechanisms probably involved in neurodegeneration. Atherosclerosis of intracranial arteries has been described as a risk factor for dementia which points to the role of vascular changes in AD development. A vascular and/or inflammatory biomarker present in both atherosclerotic diseases and AD, could not only unveil pathological pathways but also help in Alzheimer's diagnosis. Oxidized LDL (oxLDL) is a known mediator of vascular injury and atherosclerosis as it stimulates endothelial cells to produce chemoattractants that recruit monocytes into the vessel wall in addition to inducing smooth muscle cells proliferation.
Aims: After we observed in our studies that AD patients presented increased circulating products of lipid-peroxidation and a higher frequency of the epsilon4 APOE gene allele (strongly associated with both atherosclerotic cardiovascular disease and AD), we aimed to investigate the lipid profile of those patients and whether their oxLDL circulating levels were also increased. Our ultimate goal was to observe if oxLDL could represent an abnormal parameter in AD, linking its pathological pathways with atherosclerosis.
Methods: Through *Enzyme-linked Immunosorbent Assay* (ELISA), we measured the oxLDL levels in the serum of 40 elderly individuals with no cognitive impairment (controls) and 40 AD patients, selected in the Academic Hospital at the Federal University of Minas Gerais, Brazil, after informed consent by themselves or relatives. We also evaluated the participants' lipid profile through biochemical analysis. The study was approved by the ethics committee of the respective university.
Results: Surprisingly, the oxLDL levels for controls (126.32 ± 122.30 mg/dL) were higher than those for AD patients (71.05 ± 100.50 mg/dL), $P = 0.016$.
The levels of LDL, HDL and total cholesterol were similar for both groups. VLDL levels in the control group (22.10 ± 15.35 mg/dL) were higher than in the AD group (13.91 ± 7.13 mg/dL), $P < 0.0001$, and triglyceride levels were also higher for the controls (110.50 ± 76.80 mg/dL) compared to AD patients (69.57 ± 35.70 mg/dL), $P < 0.0001$.
Although higher for the control group, the VLDL and triglyceride levels were still under their clinical cut-offs.
Studies by other researchers have associated statin use with decreased oxLDL levels, but in our study statin use was more prevalent in the control group, which presented higher levels of oxLDL compared to AD patients. This unexpected lower oxLDL levels could be a conse-

quence of a more controlled diet for the AD patients; thus, it would be interesting to investigate the oxLDL levels of individuals with mild cognitive impairment who progressed to AD after follow up. It would also be beneficial to investigate if microglial cells of AD patients express more scavenger receptors that bind to oxLDL than the ones of healthy elderly controls.

Conclusion: The oxLDL serum levels were not increased in Alzheimer's disease patients compared to controls; therefore, we were unable to describe a link between atherosclerosis and this disease based on this parameter.

PB 4.32-5

Plasma salusin-beta levels in health and atherosclerotic cardio- and cerebro-vascular diseases

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Background: Salusin-a and salusin-b are related vasoactive peptides biosynthesized from the same precursor, prosalusin. Salusin-a has been demonstrated to exert anti-atherogenic effects. While Salusin-b has the ability to temporally induce potent hypotension, this peptide also accelerates the development of atherosclerosis by enhancing macrophage foam cell formation and vascular smooth muscle cell proliferation. Salusin-b is highly expressed in human coronary atherosclerotic lesions. Despite the potent hemodynamic and pro-atherosclerotic activities of salusin-b, its presence in human circulation remains largely undetermined, because of its unique physicochemical properties.

Aims: We recently established a sandwich ELISA for salusin-b, overcoming technical difficulties by including a low dose of NP-40 in the assay system. We measured salusin-b in healthy human plasma and examined whether there were physiological variations using the sandwich ELISA. Furthermore, we investigated plasma salusin-b levels in patients with coronary artery disease (CAD) or cerebrovascular disease (CVD).

Methods: After obtaining informed consent, venous blood samples were collected from 106 healthy volunteers and 80 patients with CAD or CVD. The plasma was then pretreated and salusin-b levels were measured using the sandwich ELISA.

Results: Plasma salusin-b levels were 4.1 $\pm$ 0.9 nM in healthy volunteers, and did not differ significantly between males (n = 64) and females (n = 42). Age was not associated with the levels. Plasma salusin-b levels were lower in the morning than in the daytime. Both patients with CAD and CVD had significantly higher levels of plasma salusin-b compared with healthy volunteers (5.4 $\pm$ 1.3 and 5.3 $\pm$ 1.4 nM, both $P < 0.0001$).

Summary/Conclusion: These results indicate that plasma levels of salusin-b exhibit a circadian rhythm with levels lower in the morning. In addition, salusin-b is significantly higher in patients with atherosclerotic CAD or CVD. We provide insights into the potential use of salusin-b as a reliable biomarker and therapeutic target for atherosclerotic cardio- and cerebrovascular diseases.

PB 4.32-6

Urinary Biomarker discovery for coronary artery disease risk prediction using global proteomic analysis

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Background: Diagnosis of premature coronary artery disease (CAD) is a major concern and several studies have focused on using the urinary proteome approach due to non-invasive biofluid collection, low complexity and stability of proteins. These studies are more important in Indian population where premature CAD is increasing at alarming rate.

Aim: In this study we have analyzed global proteomic profile analysis of urinary proteome of Asian Indians to identify biomarker profile for premature CAD risk prediction.

Methods: 407 age and gender matched subjects were selected for a case control study from ongoing Indian Atherosclerosis Research Study (IARS) (Indian Heart J, 2010;6:286–95) which was designed according to the guidelines of World Medical Association Declaration of Helsinki and Indian Council of Medical Research and approved by Institutional Ethics Committee. Patients living in India for at least two generations were enrolled after signed informed consent was obtained (male $\leq$60 years and female $\leq$63 years at onset of disease, diagnosed as CAD without any major diseases like cancer or liver failure) All the urine samples were collected after overnight fasting of 12–14 h and stored at -80 °C. Global proteomic analysis of urine samples were performed using Surface Enhanced Laser Desorption/Ionization-Time of Flight (SELDI-TOF) on three different arrays cationic CM10 (204 CAD affected and 203 unaffected), anionic Q10 (80 CAD affected and 79 unaffected) and metal binding IMAC30 (196 CAD affected and 195 unaffected) chips. For risk prediction model development, we used four different statistical methods namely Support Vector Machine (SVM), Multilayer perception Artificial Neural Network (ANN), Discrimination Analysis (DA) and Logistic Regression (LR).

Results: 118 significantly differently expressed protein profiles were identified from SELDI-TOF analysis in all the arrays. The CM10 array gave 55 peaks, 29 peaks in Q10 and 34 in IMAC array chips. Four different statistical analyses (SVM, ANN, DA and LR) were performed for developing risk assessment algorithm for each array type. In each of the statistical methods half of the samples were used for validation in a blind test. In all the array types, SVM based algorithm gave the significant risk assessment. In CM10 array the SVM based risk stratification had sensitivity of 91.22%, specificity of 67.13%, AUC of 0.961, Q10 array sensitivity of 82.93%, specificity of 79.22%, AUC of 0.921 and IMAC: sensitivity of 78.76%, specificity of 80.30%, AUC of 0.878. Furthermore, we identified that five protein peaks corresponding to myotropin, prepronociceptin, StAR-related lipid transfer protein 5, D-amino acid oxidase activator, and integral membrane protein 2C as significant proteins in the above SVM based model for risk assessment.

Conclusions: Our data suggests that urinary proteome profile can be used to identify new biomarkers which may be related to premature CAD in Asian Indians. Further studies of integrating the genomic data (gene expression and single nucleotide polymorphism) using bioinformatics tools are underway for developing systems biology based risk assessment.

PB4.33 – Fibrinolytic system: Basic – IV

PB 4.33-1

Homocysteine plasma levels influence fibrinolytic capacity but not *in vitro* cleavage of fibrin by exogenous plasmin in patients with pulmonary embolism

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Background: Hyperhomocysteinemia (HHcy) affects haemostasis and shifts its balance in favour of thrombosis. *In vitro* and *in vivo* studies suggested that HHcy may impair fibrinolysis either by influencing the plasma levels of fibrinolytic factors or by altering the fibrinogen structure.

Aims: We investigated the influence of mild HHcy levels on plasma fibrinolytic potential by using Clot Lysis Time (CLT) and fibrin sus-

ceptibility to plasmin-induced lysis in 94 patients with previous pulmonary embolism (PE) and no pulmonary hypertension.
Methods: CLT was measured as lysis time of tissue factor (TF)-induced clots exposed to exogenous tissue Plasminogen Activator (t-PA). The rate of '*in vitro*' plasmin-mediated cleavage of fibrin β-chain was assessed over a 6-h period on fibrin clots, which were obtained by exposition to thrombin of purified fibrinogen. Homocysteine plasma levels were measured by Abbott Imx immunoassay and we considered as altered values the plasma levels above 15 μM according to the literature. Fibrinolytic components were also measured by ELISA or functional tests.
Results: In 68 patients homocysteine levels were below 15 μM (NHcy) and in 26 they were above (HHhcy). Significant differences were observed between the two groups regarding plasma fibrinolytic potential ($P = 0.016$), TAFIact ($P = 0.02$), t-PA(0.008) and PLG (0.037), but not for the other fibrinolytic components and for the rate of plasmin-mediated cleavage of fibrin β-chain. The HHcy-patients had a three-fold higher risk to have an impaired fibrinolysis. Instead, a multivariate logistic regression analysis adjusted for significances of univariate showed that HHcy(OR:5.2 95% CI: 1.7–15.9; $P = 0.003$) and BMI (OR:1.1 95% CI: 1.0–1.3; $P = 0.01$) resulted indipendently associated with impaired fibrinolytic activity.
Conclusions: HHcy affects TAFI-mediated hypofibrinolysis but not fibrin(ogen) structure or function as documented by fibrin degradation analysis.

PB 4.33-2
Real time imaging of plasminogen binding to platelet-rich micro-thrombus and its effective lysis by tPA infusion *in vivo*

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Background: The detail how thrombolysis takes place after microthrombus is formed at vascular injury site.
Aim: To elucidate the mechanism of thrombolysis in the vasculature, we analyzed the process of plasminogen-binding to laser-induced microthrombus and tPA-induced its lysis in mice using intra-vital fluorescence microscopy.
Method: Mesenteric vein of green fluorescent protein expressing transgenic mouse (GFP-mouse) was irradiated by laser-beam through objective lens of fluorescence confocal microscopy and platelet-rich micro-thrombus was formed. The accumulations of GFP expressing platelets as well as Alexafluor 568 labeled plasminogen (Glu- or mini-plg) on injured vessel wall were monitored by an increase in the corresponding fluorescent intensity with time course, and their distributions were analyzed. The process of tPA-induced effective clot lysis was also analyzed.
Results: (i) Glu-plg accumulated in a time-dependent manner to the center of micro-thrombus, where phosphatidylserine (PS) was exposed on platelets surface and fibrin was formed. (ii) The bindings of Glu-plg in the presence of EACA (approximately 20 mM) or after treatment by carboxy-peptidase (approximately 10 U/mL), and that of mini-plg were significantly less than that of Glu-plg alone. (iii) Though the micro-thrombus was resistant to clot lysis for 3 h, the effective clot lysis was initiated by intra-venous tPA infusion.
Summary: Glu-plg appeared to accumulate in the center of micro-thrombus in a time-dependent manner by binding to either activated platelets' surface or formed fibirn in a lysine binding site dependent mechanism. Such accumulation of Glu-plg, however, was not sufficient and the subsequent tPA infusion was required for the effective clot lysis.

PB 4.33-3
Kinetic and thrombolytic properties of streptokinase-polyethylene glycol conjugates

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Background: Streptokinase (SK), a widely used thrombolytic drug, is an effective plasminogen activator. Some limitations to SK therapy have been imposed by its significant side effects on plasma proteins, rapid clearance from the circulation, antigenicity and instability of plasmin (Pm)*SK complex to proteolysis. SK therapy is accompanied by bleeding as a result of the exhaustion of plasma fibrinogen.
Aims: To improve of SK properties by its modification with nonantigenic polyethylene glycol (PEG).
Methods: PEG of 2 and 4 kDa (PEG2 and PEG4) were activated with 1,1'-carbonyldiimidazole and imidazole carbamate derivatives of the polymers were dialyzed. Purified SK was modified with activated PEG in the ratio 1:500 (M:M) during 0.5, 2, 3 and 4 h. Stability of SK и SK-PEG conjugates in human plasma was traced by fall of their fibrinolytic activity using Christensen method. Kinetics of plasmin (Pm)*SK и Pm*SK-PEG stoichiometric complexes formation was measured by rise in the hydrolysis rate of Pm substrate. Kinetic parameters (k_{pg} and K_{Pg}) of Glu-Pg activation by Pm*SK и Pm*SK-PEG complexes were determined using conjugated method in the presence of Pm substrate. Thrombolytic activity of SK and SK-PEG conjugates was measured by lysis kinetics of human plasma clot immersed in plasma using cathetometer, fibrinogen level in plasma was measured using developed by us method.
Results: Optimal durations for SK conjugation with activated PEG2 and PEG4 were found to be 1 and 2 h owing to the loss of SK fibrinolytic activity did not exceed 15–25% at modification degree of its lysine ε-amino groups 49–52% in 1 h and 52–53% in 2 h with PEG2 and PEG4. These modification degrees of SK had no effect on the formation rate and amidase activity of stoichometric complexes of Pm*SK-PEG2 and Pm*SK-PEG4 but increased in stability of SK-PEG conjugates in human plasma *in vitro*: the $t_{1/2}$ of fibrinolytic activities decline were 60 and 110 min for two SK-PEG2, 90 and 120 min for two SK-PEG4 derivatives, 50 min for unmodified SK. As compared with Pm*SK, both k_{pg} and K_{Pg} values of Glu-Pg activation by Pm*SK-PEG2 and Pm*SK-PEG4 complexes with two modification degrees of SK decreased and the catalytic efficiency of Pg activation (k_{pg}/K_{Pg}) by these complexes was some lower or higher than that by Pm*SK. The SK-PEG2 and SK-PEG4 derivatives retained *in vitro* 88–85% and 68–62.5% of SK thrombolytic activity, respectively. The SK-PEG conjugates caused the lesser depletion of plasma fibrinogen than equal dose of unmodified SK: $t_{1/2}$ of plasma fibrinogen level fall was 26 min for SK, 36 and 38 min for SK-PEG2, 90 and 120 min for SK-PEG4 derivatives.
Conclusions: Conjugation of SK by PEG extends its half-life in plasma and protects Pm*SK-PEG complexes from proteolysis. Obtained SK-PEG2 и SK-PEG4 derivatives retained great bulk of SK thrombolytic activity and caused a lesser side effects on plasma proteins *in vitro* than equal dose of unmodified SK. Moreover, according to literature data derivatization of SK by PEG substantially reduced the SK antigenicity.

PB 4.33-4
Insights from a mathematical model of fibrinolysis

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Background: Mathematical models can be powerful tools when laboratory experimentation is impossible or inconvenient (due to technologi-

cal limitations, cost, time constraints, etc.) We have developed a 3-dimensional multiscale mathematical model that accurately predicts many observed characteristics of both single fiber and clot lysis. Fibrinolytic therapy for stroke patients often involves administration of tissue-type plasminogen activator (tPA), however, bleeding complications can arise post-treatment. More recently, local administration of plasmin via catheter has been suggested as a possible treatment strategy.

Aims: The aim of this research is to develop mathematical models of fibrinolysis that can be used to test potential fibrinolytic therapies. Specifically, we aim to accomplish the following: identify a safer, more effective tPA-variant that could be bioengineered; test the effectiveness of both tPA-variants and plasmin as thrombolytic agents under various conditions (e.g., in different clot structures, or in the presence of fibrinolytic inhibitors).

Methods: We use a 3-dimensional multiscale mathematical model to investigate fibrinolysis of pre-formed clots, initiated by various types of molecules. The microscale model contains detailed biochemistry and represents a single fiber cross section. Data from the microscale model are used in a macroscale model of a 3-dimensional fibrin clot. To test different treatment strategies, we include different molecules in the macroscale model to initiate lysis. Patterns and rates of lysis are recorded. Promising results from the theoretical experiments can be tested in a laboratory.

Results: We find that a tPA-variant with a larger-than-physiological dissociation constant results in faster lysis rates. Interestingly, there is an optimal K_d value, as lysis rates begin to decrease if the dissociation constant is too large. Lysis rates are often larger when degradation does not proceed as a front moving across the clot, but rather occurs in many places throughout the clot simultaneously. We also find that activated thrombin activatable fibrinolysis inhibitor (TAFIa) has only a small effect on tPA-mediated lysis in the absence of α_2-antiplasmin (α_2-AP), but can almost completely stop lysis in the presence of α_2-AP.

Summary/Conclusions: We use a mathematical model of fibrinolysis to assess potential fibrinolytic treatments. Model results suggest that a tPA-variant that binds less strongly to fibrin will elicit faster lysis. The presence of inhibitors can greatly affect the progression of lysis, and should be considered when assessing thrombolytic therapies.

PB 4.33-5

The impact of bradykinin-potentiating peptide on fibrinolytic activity of blood plasma on the model of diabetes mellitus 1 type

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Background: The development of diabetes mellitus type 1 (DM), accompanied by hyperglycemia and glucosuria, leads to negative changes in the system of hemostasis – increase of coagulation potential and depression of fibrinolytic activity of blood. Therefore, the search for new approaches to prevention and treatment of complications in this pathology is very timely. Bradykinin-potentiating peptides (BPP) by preventing the splitting of bradikinina and inhibition of the angiotensin-converting enzyme able to influence positively on the hemostasis system and endothelial function, which is violated in diabetes.

Research objective: To evaluate the impact of a single intravenous injection bradykinin-potentiating peptide 9a (BPP9a) on indicators fibrinolysis and blood sugar level of rats on the model of diabetes mellitus type 1 (DM).

Materials and Methods: The experience was carried out on non-linear white male rats weighing 200–350 g. DM caused by the single-injection to v. jugularis solution alloxan in a dose of 37.5 mg/kg. The analyzed peptide (Serva) provided an experimental animal with DM single intravenous dose of 5*10–4 mg/kg. The control rats with diabetes similarly provided equal amount of saline solution. Blood for examination was taken: before the introduction of alloxan and on the background of the development Board: after 1 h, one and 2 weeks after the introduction of the peptide. In the blood plasma was determined: the of euglobulin clot lysis (ECL), the total fibrinolytic activity (TFA), plasmin activity (PA) and plasminogen activator activity (tPA). Quantitative determination of glucose in the blood was carried out with the help of biochemical analyzer One Touch Horizon (USA).

Results: About 1 h after injection BPP9a in the experimental group significantly ($P < 0.05$) reduced the level of glucose in the blood compared with the value in these rats prior to its adoption. The normal level of glucose (4.7 mM) in the experimental group was observed in about 1 week in comparison with the control (of 8.0 mM), and then the difference between the groups is leveled. In rats of experimental group after 1 h after the introduction of the peptide ECL unreliably lengthens in comparison with the control, while in the blood plasma, a decrease of TFA, PA-and tPA. One week ECL in both groups did not differ, in the test there was an increase in TFA in six times, PA 14 times and tPA in 3.5 times compared with the control. Two weeks after the introduction of peptide ECL in experimental animals correspond to the normal values, and values TFA, PA, tPA were three times higher than the control group.

Conclusion: The data obtained suggest that a single intravenous injection of the BPP9a in the dose of 5*10–4 mg/kg to rats with alloxan, got diabetes caused a decrease of the level of glucose in the blood within 1 h after injection, which has been stable for 1 week, and also increase of enzymatic fibrinolytic activity within 2 weeks of the experiment.

PB 4.33-6

Diagnosis performance of thromboelastography to assess tissue plasminogen activator induced fibrinolysis

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Background: Fibrinolytic activation is a component of acute coagulopathy of trauma and the extent of activation correlates with disease injury score and clinical outcome [1]. It is not possible to measure the extent of fibrinolytic activation with standard emergency laboratory tests, but thromboelastography is able to detect fibrinolytic activation but this has not been fully validated.

Aims: To investigate the diagnostic performance of thromboelastography (TEGÒ Haemonetics Corp., MA, US) to detect tissue plasminogen activator-induced fibrinolysis in an *in vitro* study.

Methods: Blood was drawn from 10 healthy volunteers using flawless venepuncture into 0.105 M sodium citrate and divided into aliquots. Fibrinolytic activation was generated by adding tissue plasminogen activator (tPA), at four different concentrations: 0; 0.2; 1 (values similar to the concentrations seen in plasma drawn from bleeding trauma patients) and 4 nM.

TEG assays were performed in triplicate on each whole blood sample: KaolinTEG, RapidTEG (tissue factor is added to kaolin-activated TEG to activate both intrinsic and extrinsic coagulation pathways) and functional fibrinogenTEG. Recorded parameters were Reaction time (*R*), time to clot formation (K), Maximal Amplitude (MA), percentage lysis LY30, LY60 and functional fibrinogen level (FLEV).

To assess if the delay between sampling and performing TEG test is relevant, tests were performed immediately after spiking tPA, 15 and 30 min later. Platelet poor plasma was produced and frozen immediately. Plasmin anti-plasmin complex (PAP) were measured by ELISA and fibrinogen levels by Clauss assay.

Results: Adding 1 and 4 nM tPA increased tPA antigen concentration and PAP, whereas 0.2 nM tPA had no effect. Clauss fibrinogen levels decreased from 2.72 g/L to 2.47 g/L with 4 nM tPA.

In KaolinTEG, only 4 nM tPA increased R and decreased MA. K was not modified whatever tPA concentrations. Hyperfibrinolysis was detected only with 4 nM tPA: LY30 and LY60 were higher than 7.5%

(respectively 48 ± 14 and 74 ± 8%). 1 nM tPA significantly increase lysis but in a non relevant way (<2% and <6% respectively for LY30 and LY60). With RapidTEG, all values were in normal range, except LY30 and LY60, which were increased with 4 nM tPA. 4 nM tPA induced a decrease in FLEV, from 3.37 to 2.46 g/L as well as in Clauss fibrinogen concentration from 2.72 to 2.47 g/L. Increasing delay between sampling and performing tests increased PAP levels, percentage lysis and decreased fibrinogen concentration.

Summary/Conclusions: TEG tests did not detect moderate fibrinolytic activation. Clot lysis parameters are the most sensible to detect fibrinolysis. RapidTEG detected fibrinolytic activation more rapidly than kaolinTEG. Hyperfibrinolysis induced fibrinogen decrease. Delay between sampling and performing tests modified tests results.

Raza I. J Thromb Haemost. 2012.

PB4.34 – Fibrinolytic system: Basic – V

PB 4.34-1

Assessing the extent of fibrinogenolysis by t-PA seen in bleeding trauma patients and what is the extent of its inhibition by tranexamic acid using an *in vitro* model

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Background: Systemic fibrinolysis contributes to the acute coagulopathy of trauma (ATC), and is known to correlate with the extent of injury and clinical outcome [1]. ATC is also characterized by a decrease in fibrinogen concentration [2], although its cause has not been adequately studied.

Aims: To investigate the effects of a tissue-plasminogen activator (t-PA) on fibrinogen levels in an *in vitro* model and the effects of tranexamic acid (TXA) on this model.

Methods: Ethical committee approval was obtained to draw blood from healthy volunteers and collected into 0.105 M sodium citrate. Fourteen different concentrations of tPA between 0.05 and 60 nM were added to aliquots of whole blood. Thromboelastography (TEGÒ Haemonetics Corp., MA, US) was performed, assessment included measuring functional fibrinogen level (FLEV). At the same time a sample was drawn off to prepare platelet poor plasma (PPP), which was frozen immediately to later perform a Clauss fibrinogen assay.

The effects of TXA were assessed in this model on four different tPA concentrations: 0; 1 nM (close to the concentrations observed in severe trauma patients), 4 nM and 20 nM (close to the concentrations used for thrombolysis), using 4 TXA concentrations: 0; 0.003 mg/mL; 0.03 mg/mL and 0.3 mg/mL (equivalent to the TXA concentration of the CRASH2 trial [3]). Kaolin TEG was performed on each of the 16 samples. Recorded parameters were Reaction time (*R*), Maximal Amplitude (MA), clot strength (G), percentage lysis LY30 and Maximum rate of Lysis (MRL). Fibrinolysis was assessed on PPP by plasmin anti-plasmin complex (PAP) measurement and fibrinogen concentration was measured with the Clauss method.

Results: tPA induced fibrinogenolysis, with a concentration-dependant decrease in fibrinogen level, as measured by FLEV (from 3.8 ± 0.6 to 1.3 ± 0.2 g/L and for Clauss assay from 2.9 ± 0.5 to 0.9 ± 0.1 g/L at 60 nM tPA). FLEV and Clauss fibrinogen were well correlated ($r^2 = 0.734$). tPA induced fibrinolysis as it increased PAP, LY30 and MRL, but decreased MA and G. It had no effect on R.

TXA alone had no effect on TEG parameters, FLEV, Clauss fibrinogen concentration or PAP levels. The lowest TXA concentration partly corrected MRL whereas the others normalized MRL and LY30. TXA corrected MA and G and partly protected fibrinogen against fibrinogenolysis in a concentration-dependant manner. TXA had no effect on PAP.

Summary/Conclusions: tPA induced fibrinolysis and fibrinogenolysis in a concentration dependant manner. TXA did not affect plasmin activation but reduced fibrinogenolysis, consistent with its known action as a competitive inhibitor of plasminogen. These results suggest that TXA should be given in ATC to prevent fibrinogenolysis and thus potentially may reduce the need for fibrinogen supplementation.

1. Raza I. J Thromb Haemost. 2012.
2. Curry NS. Blood Rev. 2012.
3. CRASH-2 trial collaborators. Lancet. 2010

PB 4.34-3

Increased fibrinolysis on blood outgrowth endothelial cells (BOEC) from chronic thromboembolic pulmonary hypertension (CTEPH) patients

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Pulmonary hypertension (PH) is a rare but disabling disorder estimated to affect 30–50 individuals per million. PH is characterised by increased pulmonary vascular resistance, causing an increase in pulmonary artery pressure leading to right ventricular heart failure, progressive disability and death. Chronic thromboembolic pulmonary hypertension (CTEPH), a distinct category of PH, is a condition in which the initiating event is either overt or occult pulmonary embolism. Why some patients develop CTEPH after PE is not clear. It is believed that the persistence of thrombus is a prerequisite for the development of PH following PE. Failure to lyse thrombi is thought to create altered blood flow triggering abnormal vascular remodelling. We hypothesised that defective fibrinolysis on the surface of the pulmonary endothelium causes progression from PE to CTEPH.

The study of a patient's endothelium is complicated by the difficulty in accessing tissue for analysis. We used a recently developed technique to study the endothelial cells in patients with CTEPH in comparison to healthy controls. We isolated and cultured blood outgrowth endothelial cells (BOEC) from the mononuclear fraction of peripheral blood. Discrete colonies of BOEC appeared between days 7–21 in culture. Cells were then passaged to obtain confluent BOEC monolayers with characteristic endothelial cobblestone morphology. Endothelial identity was confirmed with a range of endothelial markers including VWF and VE-Cadherin. 17 patients (M = 7, F = 10) and 10 (M = 8, F = 2) controls were recruited. BOECs were isolated from 10 patients and six controls. All experiments were carried out between passages 4–7 in culture.

Thrombin generation and fibrinolysis was investigated on BOEC from patients or controls. Clot formation was triggered by exogenous tissue factor (TF) (5 pM) or by upregulating endogenous TF expression in BOEC with tumour necrosis factor (TNF-α) treatment (1.0 nM for 5 h). Clot lysis was promoted by addition of exogenous tPA (150 ng/mL). Lysis time was calculated as described (Lisman et al Gastroenterology 2001;121:131–139). Thrombin generation was measured using a modified calibrated automated thrombogram (CAT) assay as described (Hemker et al Curr Opin Haematol 2004;11:170–175).

Following addition of TF, mean lysis time on unstimulated CTEPH BOECs was shorter than that of control BOECs (minutes +/-SEM, 53.68+/−2.15 and 62.69+/−2.79 respectively; $P = 0.04$). Mean lysis time on TNF-α-stimulated CTEPH BOECS (34.42 min) was also significantly shorter than control BOECs (minutes +/-SEM, 35.86+/−3.11 and 52.22+/−4.67 respectively; $P = 0.016$). No difference was seen between patient and controls in the peak thrombin generated

after addition of TF (Control 248.74 nM thrombin, CTEPH 218.25 nM thrombin) or after TNF-α stimulation (Control 80.03 nM thrombin, CTEPH 79.46 nM thrombin). Thus the difference in lysis times is not attributable to differences in thrombin generation.
In conclusion we successfully isolated BOEC from patients with CTEPH and healthy controls in order to evaluate thrombin generation and fibrinolysis. We observed no difference in thrombin generation but surprisingly we observed a shorter clot lysis time, i.e. faster fibrinolysis on CTEPH patient BOECs compared to controls. Further investigation at the molecular level is ongoing to establish the mechanism for this increase fibrinolysis and its relationship to the development of CTEPH.

PB 4.34-4

Spatial clot lysis rate is regulated by clot growth rate

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Background: The clot growth is a spatially heterogeneous process, and when it occurs in the patients with stroke, deep vein thrombosis or thromboembolism under fibrinolytic therapy, it is followed by rapid clot lysis due to artificial overactivation of lytic system. Clot growth activator is located on the vessel wall, while fibrinolytic activator is distributed all over the blood volume, so the patterns of clot lysis, as well as its interactions with blood coagulation system, are not explored yet.
Aims: In order to investigate the spatial aspects of clot lysis and its interaction with clot growth, we developed an in silico model of fibrin clot formation and dissolution, which was validated using *in vitro* experiments.
Methods: Mathematical model consisted of 35 PDEs simulated spatial clot growth and lysis in a 1D area 3 mm long. Clot growth was initiated by TF located at $x = 0$ coordinate, and tissue plasminogen activator (TPA) was distributed evenly in the whole area of simulation. Our model included the whole coagulation cascade, save the FXII contact pathway, leading to thrombin generation, which activated fibrinogen, as well as the fibrinolytic system equations describing TPA, 2 forms of plasminogen (lys- and glu- forms), plasmin, their complexes with fibrin, PAI, antiplasmin. The model described coagulation and fibrinolysis factors diffusion and reaction fluxes. Equation system was solved numerically with the COMSOL 4.3a software package using the finite element method. The relative tolerance was 0.1%; the element size was 0.01
Results: Clot lysis rate was not sensitive to any alterations of fibrinolytic system parameters, like changes in plasmin affinity with fibrin, rate of fibrin degradation, rate of plasmin activation or inhibition(<3% change for all cases). In contrast, increase of clot growth rate by supplementing the system with phospholipids or factor XIa caused increase of the clot lysis rate ($r = 0.997$). Also, we predicted in our in silico simulations that high level of TPA will cause the decrease of clot lysis rate and increase the lysis lagtime due to depletion of plasminogen before the clot is formed. These results were confirmed in our *in vitro* model.
Conclusions: We assume that spatial lysis of fibrin clot cannot be absconded from the clot formation: the latter not only forms the media where the first can propagate, but it also defines how fast lysis will occur. All that makes one suggest that spatial clot lysis can work like a time delayed clot termination, and the activation signal cannot facilitate its efficacy.

PB 4.34-5

Hemostatic alterations induced by Micrurus tener tener venom in C57BL/6 mice

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Background: *Micrurus* genus is mainly known for the neurotoxic effect produced in the victims. However, it have been described experimentally cardiotoxicity, myotoxicity, edematogenic effect and hemorrhagic activity in mouse skin. Recently, we described the hemostatic activity *in vitro* induced by *Micrurus* venoms (*M. tener tener*, *M. fulvius fulvius* and *M. isozonus*).
Aim: Due to the poor knowledge of the hemostatic effects induced *in vivo* by these venoms, we evaluated the alterations in this system by *M. tener tener* in C57BL/6 mouse.
Methods: Lethality of crude venom was determined by intraperitoneal (IP) injections into mice and expressed as LD_{50}, which was calculated according to the method of Spearman-Karber (1978). Male mice were injected with a sub-lethal dose in 200 μL of sterile saline solution and control mice were injected with 200 μL of sterile saline solution. Blood samples, about 1 mL per mouse, were collected by cardiac puncture under anaesthesia in CO_2 chamber. Hematologic (hemoglobin, hematocrit, leukocyte count, differential leukocyte count and platelets count) and hemostatic parameters (fibrinogen and endogenous fibrinolytic activity) were measured at 72 h after venom injection.
Results: LD_{50} of *M. tener tener* crude venom was 0.99 mg/kg (19.8 μg/mice). After IP injection of 8 μg/animal crude venom, the mice presented hemorrhage in the abdominal cavity. In comparison with control mice, the treatment with crude venom induced a decrease in hemoglobin concentration (11.9 ± 2.0 vs. 14.1 ± 2.2 g/dL; $P < 0.05$) and hematocrit ($32 \pm 17\%$ vs. $38 \pm 2\%$), but there was not an important change in the other hematological parameters. The venom also decreased fibrinogen values (211 ± 71 vs. 277 ± 38 mg/dL; $P < 0.05$). Additionally was observed an increase in the endogenous fibrinolytic activity (26.00 ± 37.40 vs. 158.71 ± 44.41 mm^2; $P < 0.05$).
Conclusions: Our results indicate that *M. tener tener* venom has toxins that are able to alter the vascular integrity and can affect directly the hemostatic system inducing an exacerbated fibrinolytic process that may be associated with the hemorrhagic manifestation in mice. These findings are related with fibrino(geno)lytic activity described previously for this venom. This is the first report *in vivo* of the effects induced by *M. tener tener* on the hemostatic system, particularly on the fibrinolytic system.

PB 4.34-6

The Factor VII activating protease (FSAP) regulates fibrin clot structure and fibrinolysis by direct interaction with fibrinogen

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Background: Factor VII activating protease (FSAP) is a plasma protease that can activate FVII and plasminogen activators as well as inhibit tissue factor pathway inhibitor (TFPI), thereby accelerating coagulation and fibrinolysis. Cleavage of the fibrinogen Aa and Bb chain by FSAP has also been reported, but its impact is not clear.
Aims: Systematic investigation of FSAP's influence on clot structure and fibrinolysis.
Methods: Interaction of FSAP with plasmin(ogen) and fibrin(ogen) was measured by ELISA, and processing of fibrinogen by FSAP was

analyzed by SDS-PAGE. Plasmin activity was measured chromogenically. Clot formation and fibrinolysis of FSAP-treated purified fibrinogen or plasma was studied by turbidity as prothrombin time and as clot lysis time, respectively, or by thrombelastometry. For fibrinolysis studies, fibrinogen was pre-incubated with FSAP after which thrombin/tPA and $CaCl_2$ were added to initiate clotting and fibrinolysis. The clot structure formed by FSAP-treated or control fibrinogen in the presence of fluorescence-labeled fibrinogen was visualized by laser scanning confocal microscopy (LSM).

Results: Binding of FSAP to plasminogen was seen without preference for any particular domains within plasminogen. FSAP did not activate plasminogen, nor did FSAP significantly increase plasminogen activation in the presence of pro-uPA or pro-tPA. Interaction of FSAP with fibrin and fibrinogen could be observed, but was decreased in the presence of plasminogen. Vice versa, FSAP decreased binding of plasminogen to fibrinogen and fibrin. Incubation of fibrinogen with FSAP led to the disappearance of the Aα- and the Bβ-chains with apparently unchanged γ-chain. Upon pre-treatment with FSAP, purified fibrinogen and diluted plasma, as well as plasma depleted of uPA or tPA, showed strongly reduced clot lysis time values. A reduced clot firmness and shorter lysis time of FSAP-pretreated plasma could be seen by ROTEM analysis. In confocal microscopy FSAP-treated fibrinogen showed a different fibrin clot structure with thinner fibers compared to untreated fibrinogen. This change in fibrin structure was not seen with aprotinin-treated FSAP.

Summary/Conclusion: FSAP binds to plasmin(ogen), but plasminogen activation is not affected. Despite this, a clear acceleration of fibrinolysis of purified fibrinogen or plasma can be observed in the presence of FSAP. This effect seemed to be independent of FSAP's pro-uPA and pro-tPA-activating properties, but is more likely a direct influence of FSAP on fibrinogen. Specific binding of FSAP to fibrin(ogen) was also observed. In the presence of FSAP purified fibrinogen formed clots with thinner and denser fibrin fibers than control clots. Presumably the cleavage of the Aα chain by FSAP causes an impaired lateral aggregation of fibrin fibers, thus preventing the formation of thicker fibrin fibers, and may account for an accelerated clot lysis time. In conclusion, FSAP's influence on clot structure is a new parameter that deserves attention when evaluating the contribution of FSAP to thrombosis and haemostasis.

PB4.35 – Haemophilia A: Basic – IV

PB 4.35-1

Analysis of neutralizing and non-neutralizing anti-canine FVIII antibodies in hemophilia A dogs treated with FVIII gene therapy

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Background: Intravenous infusions of canine factor VIII (cFVIII), and various gene delivery strategies in hemophilia A dogs have generated anti-FVIII antibodies.

Aim: To evaluate the anti-FVIII immune response in three hemophilia A dogs following cFVIII gene therapy.

Methods: Prior to entering this study, each dog had received a single infusion of canine cryoprecipitate. To prevent an immune response to cFVIII, dog #2 received six weekly infusions of cyclophosphamide beginning the day before gene delivery and dog #3 was pre-treated with eight infusions (10 U/kg) of recombinant BDD cFVIII. The gene delivery involved autologous blood outgrowth endothelial cells (BOECs) that were genetically modified to express BDD-cFVIII. Cells were implanted in a fibrin-based scaffold in the omentum. cFVIII activity and antigen, anti-cFVIII IgG2, and inhibitory FVIII antibodies were measured for at least 1 year after cFVIII gene delivery.

Results: No functional cFVIII was detected in any of the gene therapy treated dogs and in dogs #1 and #2 this was attributed to the appearance of a transient, low level (<4.5 BU) inhibitor. However, no inhibitors were ever detected in dog #3. In dogs #1 and #3, cFVIII antigen levels peaked at 47% and 20% respectively and although levels slowly declined, they persisted for a year post-gene delivery. IgG2 anti-cFVIII antibodies were not initially detected in dogs #1 and #2 however, levels rapidly increased to >100,000 ng/mL with the appearance of inhibitors. Flow cytometry of BOECs exposed to thrombin indicated that this component of the fibrin matrix cleaves PAR-1 leading to increased expression of IL-6 and MCP-1, both of which contribute to the immune response in the treated dogs. With a view to induce immunological tolerance to cFVIII, dog #3 received eight intravenous infusions of recombinant BDD cFVIII prior to receiving gene delivery. Although no inhibitors were detected, low levels (83 ng/mL) of anti-FVIII IgG2 appeared after the first infusion and rose to a maximum level of 546 ng/mL by the 7th cFVIII infusion. Levels increased to >1000 ng/mL after gene delivery but dropped to <1.000 ng/mL within 7 days and only rose above 1000 ng/mL when the dog received subsequent infusions of cFVIII. After each of the last three infusions, FVIII:Ag did not return to baseline levels and surprisingly, FVIII:Ag levels peaked to 300% normal levels within 30 min of infusing cFVIII. We used a canine FVIII peptide library to identify antibody binding sites. Nine binding sites were identified, six of which were mapped to non-functional sites in the A2, A3 and C1 domains and 3 to canine residues 32–56, 313–347 and 2312–2331 which respectively encompass a FXa inactivation cleavage site, the APC binding/cleavage site and the phospholipid binding site.

Conclusion: Thrombin used to produce the fibrin-based scaffold contributes to the development of low-level inhibitors while pre-infusions of cFVIII appear to modulate this response. IgG2 antibodies bind to the FXa inactivation and APC binding sites to prolong circulation of cFVIII antigen but also bind to the phospholipid binding site, rendering the FVIII inactive.

PB 4.35-2

Structural and functional characterization of clinical phase 1 and phase 2/3 material of BAX 855, a PEGylated recombinant FVIII

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Background: Baxter and Nektar have developed a longer acting recombinant FVIII (BAX 855), which is manufactured by stably coupling PEG using Nektar technology to Baxter's full-length rFVIII bulk drug substance from its protein-free rFVIII manufacturing process.

Aims: To corroborate the controlled production process of BAX 855, batches from clinical Phase 1 and Phase 2/3 production were characterized with respect to their structural and functional properties.

Methods: The primary structure of BAX 855 was investigated using a tryptic peptide mapping approach. The composition of the N-linked oligosaccharides were determined by normal phase chromatography with fluorescence detection of released and labeled N-glycans. PEGylation site distribution and detailed analysis of the consistency of PEGylation was investigated by activating BAX 855 with thrombin. The resulting PEGylated and non-PEGylated fragments were separated using RP-HPLC and the bound PEG was measured for each thrombin fragment. PEGylation site distribution was confirmed by two-dimensional electrophoresis (2-D DIGE). Dynamic light scattering and Fourier-transformed infrared spectroscopy (FTIR) were used to monitor the consistency of the 3-dimensional structure of BAX 855. Assays to functionally characterize BAX 855 included FIXa cofactor activity, time course of thrombin-mediated activation and inactiva-

tion, thrombin generation capacity and susceptibility to activated protein C. Finally, the binding affinity and capacity of BAX 855 to VWF and the clearance receptor LRP were determined.
Results: Tryptic peptide mapping of BAX 855 resulted in a sequence coverage of 94% with high consistency between batches. Composition of the N-linked oligosaccharides showed a similar pattern for BAX 855 and unmodified rFVIII. Importantly, BAX 855 batches from clinical Phase 1 and Phase 2/3 production showed comparable distribution and extent of PEGylation. Two-dimensional electrophoresis confirmed high similarity of the spot and band patterns of clinical Phase 1 and 2/3 batches, indicative of high consistency of the manufacturing process. The mean hydrodynamic diameter of BAX 855 was between 35 and 38 nm as measured by dynamic light scattering; several BAX 855 batches showed almost overlapping FTIR absorbance spectra. Functional characterization of BAX 855 revealed characteristics similar to those of unmodified rFVIII in all assays used. Binding affinity and capacity to VWF was similar between BAX 855 and rFVIII. Interaction with the clearance receptor LRP was altered by PEGylation of rFVIII, shown by a decreased binding capacity.
Summary/Conclusions: BAX 855, a PEGylated rFVIII development product, can be manufactured reproducibly without changes to the protein structure and retaining its functional activities.

PB 4.35-3

Global haemostatic assays in monitoring the effect of bypassing agent therapy in haemophilia A patients with inhibitors- an in-vivo prospective crossover study

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Background: The optimal dosing of bypassing agent (BPA) therapy in haemophilia patients with inhibitors is challenging due to a lack of standardized method for monitoring its haemostatic response. Several small case-reports have demonstrated the potential of global haemostatic assays, thromboelastography (TEG) and thrombin generation assay (TGA), in monitoring the effect of BPA therapy but prospective clinical study is lacking. Moreover, the adjuvant therapy of tranexamic acid may improve haemostatic outcomes of BPA treatment but has been little studied due to safety concerns.
Aims: We aimed to study the ability of TEG and TGA in monitoring the effect of the BPA, activated prothrombin complex concentrates (aPCC) and recombinant FVIIa (rFVIIa), given with and without tranexamic acid (TXA) in haemophilia A with inhibitors. In addition, the individual response to each treatment arm was evaluated.
Methods: We conducted a prospective clinical crossover study including haemophilia A patients with high titer inhibiors ($n = 6$), age between 22 and 6 that had signed informed consent. The study was approved by the Regional Committee for Medical and Health Research Ethics. On the first study day the participants received aPCC (75 IU/kg intravenously) while on the second study day aPCC was given concomitantly with TXA (20 mg/kg orally). Blood sampling was performed at baseline, 15, 30, 60, 120, 180 and 240 min post-treatment on both days for TEG, TGA analysis and DIC monitoring. After a washout period of 14 days the subjects were crossed over to rFVIIa (90 μg/kg intravenously) without and with TXA. Blood sampling and experimental set up was repeated as for the aPCC study arm. Time response effect was evaluated using TEG parameters clotting time (CT/second), maximum clot firmness (MCF/mm^{-1}), maximum-velocity (maxV/mm/second) and time to maxV (ttMaxV/second). Similarly, TGA parameters such as lagtime (LT/minute) endogenous thrombin potential (ETP/nM), time to peak (ttpeak/minute) and peak thrombin (nM) were analysed.
Results: TEG: Mean CT and ttMaxV declined rapidly at 15 min timepoint after infusion of BPA, thereafter it increased almost linearly whereas MaxV raised and reach its maximum at this timepoint. MCF and AUC increased following the treatment as well but less than with other parameters. The most significant difference in MCF and AUC were achieved in blood containing tPa. Here, MCF and AUC increased by threefold in the presence of TXA compared to without.
TGA: The LT and ttpeak was decreased after the infusion of BPA whereas the ETP and peak thrombin increased rapidly and reached its maximum at 15 min timepoint. There was no difference in TGA result following the treatment with or without TXA. The individual haemostatic response was observed for study participants.
Conclusions: The study demonstrated that TEG and TGA are valuable methods in assessing the haemostatic effect of BPA therapy, providing an opportunity to individualized the treatment. Some parameters seemed to be more robust than other in monitoring the effect such as ETP, peak thrombin in both PRP and PPP in TGA and CT and MCF/AUC in TEG.

PB 4.35-4

Improved strategy for rapid genetic analysis of hemophilia A

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Background: Hemophilia A is a bleeding disorder caused by deficiency or impaired function of factor VIII (FVIII). FVIII is encoded by a large and complex gene (F8) comprised of 26 exons spanning 186 kb, located at the most distal region of the long arm of the X chromosome (Xq28). About 50% of severe hemophilia cases are caused by intron 22 inversions in the F8 gene. The remaining 50% include mainly missense mutations spread over the gene, with about 1500 unique mutations causing hemophilia A reported up to date. In view of this mutation heterogeneity, examination of potential hemophilia A carriers for definite determination of their carrier status and further prenatal diagnosis offered to the carriers are commonly based on linkage analysis using short tandem repeat (STR) markers. Accurate genetic analysis of hemophilia A using STR markers should be based on at least 2 markers: one intragenic and one extragenic marker, or two extragenic markers located at the 3' and 5' ends of the F8 gene. The aim of this approach is to exclude recombination between the marker used for analysis and the hemophilia-causing mutation.
Aims: 1. To improve the sensitivity of Inverse-shifting PCR (IS-PCR) assay for analysis of intron 22 inversions in the FVIII gene.
2. To identify additional extragenic STR markers proximal to the 5' region of the FVIII gene, which is very close to the chromosome X telomere, since to date, the majority of extragenic STR markers useful for analysis of hemophilia A are located at the 3' region of the F8 gene.
Methods: Genomic DNA samples were prepared for IS-PCR as described elsewhere (Fujita et al., J. Thromb. Haemost. 2012, 10:2099–2107). PCR fragments specific for the normal, inversion type I and inversion type II patterns were amplified separately, using two sets of primers for each ('nested' PCR system).
Potential new STR markers were identified by searching the telomeric region of the X chromosome for dinucleotide repeats (UCSC website, http://genome.ucsc.edu/). The polymorphic nature of the potentially polymorphic regions was either confirmed or excluded by fluorescent PCR analysis of 20 chromosomes.
Results: 1. Using the 'nested' PCR system, we significantly improved the sensitivity of IS-PCR assay for detection/exclusion of intron 22 inversions in the FVIII gene.
2. Using the above approach, we identified five new STR markers at the distance of 100–1 Mb from the 5' end of the F8 gene, in addition to only 2 STR markers previously identified in this region.

Summary: We suggest an improved strategy for rapid genetic analysis of hemophilia A, based on a 'nested' PCR system for detection/exclusion of intron 22 inversions, followed by fluorescent PCR analysis of 14 highly polymorphic STR markers (2 intragenic, 7 and 5 at the 5' and 3' ends of the F8 gene, respectively). During the period of 2009–2012, this approach enabled us to perform accurate genetic diagnosis of hemophilia A, complying with the requirement of at least 2 polymorphic markers, in 239 potential carriers and 36 male fetuses of established carriers.

PB 4.35-5

The inhibition mechanism of factor ? Trp1707Ser mutation associated inhibitors

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Background: Inhibitor formation and development following replacement therapy has become a major complication in the treatment of hemophilia A patients. F8 gene mutation has been elucidated as a key factor corresponded to the inhibitor formation. The inhibition mechanism of various gene mutation types may also differ as the different specificities of formed inhibitors.
Aims: Here we investigated the inhibitor mechanism of FVIII Trp1707Ser missense mutation associated inhibitors.
Methods: Plasma contained inhibitors were interacted with different fragments of FVIII (Heavy chain, light chain, A1, A2, A3, C1, C2), and corrected test was used to measure the remaining FVIII:C(%)by adding pooled normal plasma. Western blot was applied to further confirm the binding reactions which employed fragments as antigens and biotin-labeled purified inhibitors as antibodies. After completely interacting recombinant FVIII with inhibitors, the complex was added to microparticles coated with vWF or phospholipids. Then Anti-FVIII A1 domain antibodies were used to detect the FVIII binded to vWF or phospholipids. The FIXa and FVIIIa complex formed by interacting FIX with different concentrations of FVIIIa and inhibitor complexes was used to activate FX, and the apparent dissociation constant (Kd) for FVIIIa binding was calculated by the kinetic results.
Results: Corrected method detected that the remaining FVIII:C were increased when inhibitors interacted with heavy chain, light chain, A2 and C2 domain. No significant differences were seen in the A1, A3 and C1 domain reactions. Western blot further confirmed the previous results as bands were seen when heavy chain, light chain, A2 and C2 were used as antigens. No significant binding was detected when FVIII and inhibitor complex was added compared with only FVIII. Kd for FVIIIa binding showed that the binding capacity of FVIII and inhibitor complex were significantly lower than only FVIII.
Conclusion: The binding sites of the inhibitors of FVIIITrp1707Ser mutation were in the A2 domain of heavy chain and C2 domain of light chain. Inhibitors binding to A2 domain may affect FVIII to activate FIX as a cofactor, and inhibitors binding to C2 domain may block the interations of fVIII with phospholipids and vWF.

PB 4.35-6

Copy number variation (CNV) within the F8 gene causing haemophilia A; gene dosage determination of gross gene deletions/duplications by MLPA

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Background: Haemophilia A (HA) is caused by genetic lesions in the factor 8 (*F8*) gene. The most common mutations causing severe haemophilia A are inversion events involving either *F8* intron 22 or intron 1 repeats. Typically other causative mutations are detected by re-sequencing of the entire *F8* coding regions. Gross gene deletions or duplications in *F8* are rare molecular events that render direct sequencing methods uninformative and complicate molecular characterisation and diagnosis of carrier status. Multiplex Ligation-Dependant Probe Amplification (MLPA) allows the determination of gene dosage by simultaneously screening for the loss or duplication of intragenic target sequences using gene specific probes. Comparison of patient data with normal controls allows for the determination of copy number variation (CNV) within patients and potential carriers.
Aims: To identify copy number variation (gene dosage) in patients with gross deletions and suspected duplications within the *F8* gene, and determine carrier status in female relatives.
Method: The SALSA MLPA P178-B1 *F8* probe mix (MRC Holland) was used to determine gene dosage in a group of HA patients whose *F8* gene failed to amplify or where direct sequencing was uninformative. The probe mix contains 36 probes spanning all 26 *F8* exons, DNA specific and sex specific controls, and nine extragenic controls. Gene dosage of each exon was determined using Coffalyser software (MRC Holland) and the U.K. National Genetics Reference Laboratory (NGRL, Manchester) MLPA macro spreadsheet. Copy number variation was determined by comparing patient and carrier data with a series of sex matched normal controls. Peak height ratios (Coffalyser) and dosage quotients (DQ's; NGRL) were subject to statistical analysis within each software package. Coffalyser predicted the homozygous loss (HO Loss) or gene amplification in male patients and the loss of heterozygosity (LOH) in female carriers. The NGRL spreadsheet calculated the probability of deviating from the 'normal' dosage hypothesis.
Results: MLPA analysis allowed confirmation of gross exonic deletions in four families affected by severe HA. Deletions of exons 1–14, deletion of exon 15, and deletion of exon 26 in two unrelated cases were confirmed. MLPA analysis of female relatives of the affected patients was performed and data analysed using both Coffalyser and NGRL macros. Identical predictions of carrier status and normal gene dosage were obtained using both macros. A family with mild HA was also investigated as two affected males appeared to be heterozygous for a known HA point mutation within exon 14 (p.Arg717Trp). On analysis, both patients exhibited duplication of exon 14 and exon 18. The well established mild phenotype in this family is in accordance with the point mutation. This suggests that the duplicated regions are not pathogenic and do not disrupt *F8* mRNA synthesis.
Conclusion: MLPA is a simple and useful technique for identifying gene deletions and duplications, and provides a straightforward method for carrier determination for X-linked disorders. Variations in gene copy number are not always necessarily pathogenic as described here for a family with mild HA and a gene duplication event.

PB4.36 – Haemophilia A: Clinical – XIV

PB 4.36-1

Correlation between phenotype and genotype in a large unselected cohort of 621 PUPS (previously untreated patients) with severe haemophilia A

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Background: Phenotypic variability is well recognized in patients with severe hemophilia A (SHA). A few studies mainly in adults treated lifelong on demand suggest that bleeding phenotype correlates with factor VIII gene (F8) mutation; patients with non-null mutations (e.g. mis-

sense) appear to have a milder phenotype than those with null mutations (e.g. inversions). Nowadays, the widespread use of prophylaxis started at very young ages has changed the natural history of SHA. Even patients not started on prophylaxis but treated on-demand may be treated with different on-demand regimens thus affecting their disease phenotype. In this light, the period from birth to start of prophylaxis may represent the most suitable period to define the intrinsic bleeding phenotype of a patient with SHA.

Aims: Our aim was to analyze the very early phenotypic expression of SHA in 621 consecutively enrolled, well characterized previously untreated patients (PUPs) and to correlate this with patients' (F8) mutation type.

Patients and Methods: Patients were born between 1/01/2000 and 31/12/2009 and were enrolled, with informed consent, into the Rodin/Pednet Hemophilia Registries. Detailed information was collected on bleeds and treatment for the first 75 exposure days or until inhibitor development. F8 mutation type was known for 531 patients; 402 (76%) had null mutations and 129 (24%) had non-null mutations. We considered as markers of bleeding phenotype: ages (i) at diagnosis (only for patients with a negative family history of hemophilia); (ii) at first bleed; (iii) at first joint bleed; (iv) at second joint bleed and (v) at start of prophylaxis. Additionally the time elapsed between first and second joint bleed was determined. All outcomes were censored for start of prophylaxis and inhibitor development.

Results: Median age (in months) for all patients (including those with unknown mutations) at diagnosis was 8.8; at first bleed 9.6; at first joint bleed 14.8; at second joint bleed 20.2; and at start of prophylaxis 16.7. Median time elapsed between the first and second joint bleeds was 2.3 months. Compared to children with non-null mutations children with F8 null mutations were diagnosed at a median of 1.8 months earlier (8.3 vs. 10.1; $P = 0.04$), experienced a first bleed at a median of 1.2 months earlier (9.7 vs. 10.9; $P = 0.009$), and experienced a first joint bleed at a median of 2.3 months earlier (13.8 vs. 16.1; $P = 0.05$). No statistically significant differences were observed between mutation groups in age at second joint bleed, time elapsed between the first and the second joint bleeds and in age at start of prophylaxis.

Summary/Conclusions: PUPS with F8 null mutations show a slightly more severe phenotype (vs. PUPS with non-null mutations) as evidenced by earlier ages at diagnosis, at first bleed and at first joint bleed. However, these differences were modest and as such not likely to impact on decisions regarding when and how to start prophylaxis. We speculate that these relatively modest differences in disease phenotype at very young ages might, become larger as the children age. Further follow-up is needed to confirm this hypothesis.

PB 4.36-2

Evaluation of unfavourable cardiovascular risk factors and metabolic syndrome in young haemophilics

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Background: Increased risk of unfavourable cardiovascular risk factors has been defined in the aging population of haemophilia however they were less investigated in young haemophilics.

Aim: The purpose of this study is to assess obesity, hypertension, metabolic variables, insulin resistance and metabolic syndrome in young people with haemophilia.

Methods: From February 2010 to November 2010, 48 haemophilia A and B patients (87% severe factor deficiency) and 35 age and sex matched healthy controls <40 years old were included. Blood pressure, anthropometric measurements, body mass index, waist circumference, blood pressure, fasting glucose and insulin levels, serum lipids and diet history were evaluated. Insulin resistance was estimated by HOMA-IR (Homeostasis model assessment of insulin resistance) formula. According to the criteria of International Diabetes Federation, metabolic syndrome was defined. The study was approved by the ethics committe.

Results: The mean age of the haemophilics was 21 years (range 6–40 years). Most of them were on prophylaxis (66% of haemophilia A and 50% of haemophlia B), others were on demand therapy. Artropathy was present in 62% of haemophlia A and 67% of haemophilia B patients and mainly in patients over 18 years old. Only few of these were getting physiotherapy (5% of haemophilia A and none of the haemophilia B patients). Forty-six percent of the haemophilics over 18 years old were obese, however none of the patients younger than 18 years old had obesity. Obesity was more prevalent in haemophilics with arthropathy ($P = 0.017$). When metabolic syndrome was assessed in different age groups, none of the patients <10 years old had metabolic syndrome, 7% of patients between 10 and 18 years old and 25% of haemophilics between 18 and 40 years old had metabolic syndrome. Fifty percent of the haemophilics between 18 and 40 years old were prehypertensive or hypertensive. Fasting blood glucose levels of haemophiliacs were higher ($P = 0.02$) compared to controls. When haemophilics and controls over 10 years old were compared for metabolic syndrome, no significant statistical difference was found (19.5% and 10% respectively, $P = 0.34$).

Conclusion: Overall life expectancy and quality of life among people with haemophilia have increased in recent years, primarily because of the advances in factor replacement therapy. However older patients who had been treated with on demand therapy have still a variety of orthopedic problems. Our study showed that obesity, hypertension and metabolic syndrome are frequent problems especially in haemophilics with arthropathy. Early prevention and management of overweight, obesity, and their sequelae must be addressed in clinical practice in order to maximize the overall health of the haemophilia population. Our sudy showed that prehypertension and hypertension are common in young haemophilics. Blood pressure measurements should be a part of standard care in haemophilia patients early in their life.

PB 4.36-3

Development of Factor VIII inhibitors and its association with HLA Class I and II alleles in Pakistani hemophilia A patients

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Background: Severe form of hemophilia A is a major cause of life threatening hemorrhages. Factor VIII inhibitors are developed in approximately 15–25% of hemophiliacs who receive the Intravenous Factor VIII products. These neutralizing antibodies inhibit the substituted F VIII. A lot of research is under going globally to determine the environmental and genetic predictors of F VIII neutralizing antibodies.

Objective: To investigate the association between HLA class I and II alleles and the development of Factor VIII inhibitors in Pakistani Hemophilia A patients.

Material and Method: A case control study was carried out in the department of thrombosis and hemostasis, National Institute of Blood Disease and Bone Marrow Transplantation after approval of Institutional review board. Patients of Hemophilia A were divided into two categories one that had developed inhibitors and other without inhibitors. Healthy donors of bone marrow transplantation were included as control group. Genomic DNA of all participants was extracted from peripheral blood using Qiagen column based technique. Identification of HLA A, B and DRB1 alleles was amplified by Polymerase chain reaction sequence specific primers (PCR-SSP) of low resolution kit

(Olerup SSP HLA A-B-DR SSP, Stockholm, Sweden). Simple descriptive and chi square test were applied for statistical analysis of data using SPSS version 17.0.

Result: Total 125 participants were studied. Of 75 hemophilia A patients with mean age 11.5 ± 8.25 were categorized into two groups, one who had developed inhibitors (n = 25) exhibiting from >3.0 to 192.0 Bethesda units and other who had no inhibitors (n = 50). The frequency of HLA A3, A32, B40, DRB1*11 alleles was high in patients with no inhibitors as compared to control group and patients with inhibitors ($P < 0.01$). On the other hand only HLA B8 allele showed an increased association with patients with inhibitors as compared to patients without inhibitors and control group ($P < 0.01$).

Conclusion: HLA Class I and II alleles can be used as a predictor of development or non-development of inhibitors in patients with severe hemophilia A in Pakistan. A prospective study with larger sample size employing high resolution HLA typing will help in substantiating the results.

PB 4.36-4

Laboratory control of replacement therapy in severe hemophilia A patients

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Background: Some hemophilia A patients exhibit different bleeding phenotypes despite the similar base factor VIII concentration (FVIII:C) and need a different strategy of the factor replacement therapy.

Aims: To estimate the utility of different hemostasis assays for laboratory control of replacement therapy in severe hemophilia A patients under prophylactic and on-demand regimes as well as during surgery.

Methods: 17 severe hemophilia A patients (FVIII:C < 1%) were included in the study; three of them were examined before the surgery. Frequency of bleedings was 0–6 per month. Activated partial thromboplastin time (aPTT), FVIII:C, thrombin generation test (TGT), thromboelastography (TEG) and Thrombodynamics were performed. Thrombodynamics is a new assay based on a spatial fibrin clot growth registration; the stationary clot growth rate (Vst) was registered. Test of pharmacokinetics included 4–5 days washing period and blood sampling before and 0.5, 1, 3, 6, 24, 48 h after 50 IU/kg of FVIII infusion.

Results: Seven of nine patients on the prophylaxis therapy had 0–2 bleeding case per month, two patients had more than three bleedings per month. Thrombodynamics in 1 h after FVIII infusion demonstrated Vst ≥22 μm/min in all patients with low bleeding frequency and Vst <22 μm/min in patients with high bleeding frequency. $T_{1/2}$ for FVIII:C was more than 18 h in 6 of 7 patients with low bleeding frequency, $T_{1/2}$ <18 h in others. Endogenous thrombin potential (ETP) in TGT in 24 h after infusion was more than 800 nM*min in 6 of 7 patients with low bleeding frequency and ETP <800 nM*min in others. No clinical correlation with aPTT or TEG was observed for any time points.

In 5 patients with the therapy on-demand $T_{1/2}$ was in agreement with bleeding tendencies, while Vst and ETP showed this agreement only in three of them.

Three patients underwent a major orthopedic surgery. The surgical blood loss was 1.4, 2.1 and 0.85 L for patients #1, 2 and 3 respectively; bleeding period was prolonged in patient #2 to two days in comparison with 1 day for patients #1 and 3. Vst values correlated with surgical blood loss: Vst was 21.5 μm/min for patient #2 and 30.0, 24.0 μm/min for patients #1 and 3 respectively. $T_{1/2}$ and ETP did not show any agreement with the clinical observations ($T_{1/2}$ was 10.2, 10.4 and 12.0 h, ETP was 740, 847 and 784 nM*min for patients #1, 2 and 3 respectively).

Conclusions: Thrombodynamics, TGT and pharmacokinetics can be used for laboratory control of replacement therapy in severe hemophilia A patients.

PB 4.36-5

ORTHem 15–25: French retrospective national survey of different treatment regimens in patients with severe (factor VIII or IX = 2%) haemophilia A and B between 15 and 25 years old

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Background: Prophylaxis has been widely used in young children to preserve joint status. Arguments to further maintain prophylaxis in adulthood are scarce.

Aim: To assess the treatment modalities and their determinants in persons with severe (FVIII/IX ≤2%) haemophilia A (HA) or B (HB), without inhibitor at inclusion, aged 15–25 years.

Methods: A national retrospective multicenter survey was offered to all French HTCs. Patients with haemophilia diary at least partially completed and receiving over the past 12 months and up to 3 years, one of the three following regimens could be included: Long-Term Prophylaxis (LTP), On-Demand (OD) treatment or 'Other' regimen. Distribution of patients was analyzed regarding age class ([15–18],] 18–21], >21), type (A or B) and severity (<1%, [1–2%]) of haemophilia, type of mutation (null, non null, inversions), absence or presence of family history of haemophilia or viral infection, history of intracranial haemorrhages (ICH), history of inhibitors, age at first treatment, first haemarthrosis and first LTP.

Results: 212 patients were analyzed, 169 (79.7%) in LTP, 40 (18.9%) in OD and 1.4% in 'other' regimen. The analysis focused only on LTP and OD groups. The median age was 18.9 years in the LTP vs. 22.0 years in the OD group ($P < 0.001$). LTP was correlated with younger age ($P < 0.003$), HA ($P < 0.007$) and FVIII/IX <1% ($P < 0.002$). Patients with null mutations tend to use more often LTP ($P = 0.058$). No correlation with the intron 22 inversion ($P = 0.386$), family history of haemophilia ($P = 0.081$), IHC ($P = 0.581$) or inhibitors ($P = 0.121$) could be found.

The younger age class (15–18 years) started the first period of prophylaxis earlier ($P < 0.001$) compared to the two others. LTP patients were younger at first treatment, first haemarthrosis and first prophylactic treatment: 1.45 vs. 2.44 ($P = 0.002$), 3.25 vs. 4.88 ($P = 0.002$), 7.15 vs. 11.15 ($P = 0.001$) (years), respectively. The average duration of LTP was 11.0 ± 4.0 years and that of OD regimen was 11.3 ± 7.9 years. The main reasons for initiation of LTP were appearance of haemarthrosis (50.9%), a target joint (42.0%) or a high number of bleeding (38.1%); reasons for OD regimen were a mild bleeding phenotype (47.5%), constraint related to prophylaxis (40.0%) or absence of arthropathy (35.9%). The median dose in LTP was 34 IU/kg/injection (IQ 30–40) with a median frequency of three times a week. The median number (0.00) of days of hospitalization per year for bleeding events was similar for the two regimens ($P = 0.067$). The total number of bleeding episodes recorded for a period of 12 months was smaller in the LTP group ($P = 0.014$), but the number of target joints and haemarthrosis were similar (LTP/OD: 5 vs. 4 and 1 vs. 1, respectively).

Conclusions: In this French survey, almost 80% of 212 patients with severe haemophilia (FVIII/IX ≤2%) aged 15–25 years received LTP

for an average of 11 years. LTP was more frequent in younger age class, severe and HA types. Overvalue of the proportion of patients receiving LTP cannot be excluded due to the inclusion criteria.

PB 4.36-6

Implementing pharmacokinetic (PK)-guided dosing in hemophilia; a discrete choice experiment (DCE) in an international cohort of hemophilia professionals ('OPTI-CLOT' studies)

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Background: Hemophilia is a rare X-linked bleeding disorder, caused by a deficiency of clotting factor VIII (hemophilia A) or IX (hemophilia B). The cost of treatment of hemophilia weights heavily on national health care budgets and is estimated at €130 million annually in the Netherlands, mainly clotting factor concentrates. Treatment includes prophylactic intravenous administration of the deficient clotting factor several times a week to prevent bleedings. These infusions are roughly aimed at raising clotting factor plasma levels above the threshold of 1% to prevent joint damage and subsequent long term disability. Pharmacokinetic (PK)-guided therapy in hemophilia using a population-based model generated by Bayesian analysis is available. This makes individualized dosing regimens based on PK-guided dosing possible. Earlier studies have reported a 30% reduction of clotting factor consumption without an increase in bleeding when prophylaxis is dosed according to a PK-profile. Despite guidelines supporting PK-guided dosing, it has still not been implemented as standard of care for a number of hypothetical reasons.
Aims: Quantification of barriers and facilitators of individualized PK-guided dosing of clotting factor as part of standard hemophilia care in hemophilia professionals. Currently, this study is also being performed in patients and parents in a multicenter setting in theNetherlands.
Methods: An international DCE survey was conducted during the World Federation of Hemophilia Conference inParis, 2012 among 91 hemophilia professionals from 28 countries with varying prophylactic treatment strategies. A panel latent class model was used to quantify the trade-offs between five barriers and facilitators for PK guided dosing: number of blood samples necessary to construct PK-profile; frequency of prophylactic infusions after PK-profiling; frequency of repeated PK-profiling; risk of bleeding; and cost for society.
Results: All barriers and facilitators of PK-guided dosing proved to be important for hemophilia professionals' preferences, except for the number of blood samples necessary to construct PK-profile. Reduction of risk of bleeding was the most important facilitator to implement PK-guided dosing; the most important barrier was a high frequency of prophylactic infusions as a consequence of PK-profiling. In general, hemophilia professionals displayed a positive attitude towards PK-guided dosing, although preference heterogeneity was substantial. Hemophilia professionals required a cost saving for society of 34.2% (CI: 21.7% to 57.3%) and 5.1% (CI: −2.7% to 12.9%) to compensate for daily prophylactic infusions ór infusions every other day, instead of twice a week. Hemophilia professionals from countries without a prophylactic dosing strategy had a lower probability to accept daily PK-guided dosing than hemophilia professionals from countries with a prophylactic dosing strategy.
Conclusions: Hemophilia professionals show an overall favourable attitude towards PK-guided dosing, especially if the risk of bleeding or cost for society is reduced, and if a frequency of daily PK-guided dosing is avoided. Hemophilia professionals from countries without a prophylactic dosing strategy had a lower probability to accept daily PK-guided dosing.

PB4.37 – Haemophilia A: Clinical – XV

PB 4.37-1

Different neutralizing effects of factor VIII concentrates associated with von willebrand factor and the inhibitor epitopes

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Background: Factor (F)VIII inhibitors, whose epitopes are localized to the A2 and C2 domain(s), develop as alloantibodies in patients with hemophilia A. The neutralization therapy by FVIII replacement is utilized as hemostatic treatment for hemophilia A patients with inhibitors classed as low responder, while the bypassing agents as recombinant (r)FVIIa and activated prothrombin complex concentrates (APCC) are used for those classed as high responder, but the effects may depend on various properties of these inhibitors. Some group reported the anti-C2 type 2 inhibitors associated with an antibody-dependent reduced rate of FVIIIa release from von willebrand factor (VWF) (Blood 1999). We have recently reported that the bypassing agent rFVIIa could activate FVIII directly even in the presence of anti-FVIII inhibitors and the inactivation was moderated by anti-C2 type 1 antibodies (Thromb Haemost. 2011), and another bypassing agent APCC could also activate FVIII in the presence of anti-FVIII inhibitors similarly to rFVIIa (ASH abstract #1178, 2011).
Methods: In this study, to investigate the effects associated with the properties of the inhibitors in the neutralization therapy, the coagulation function in timed-reaction of mixtures with FVIII (1 IU/mL) and anti-FVIII alloantibodies with distinct epitopes (2.5 BU/mL) were evaluated by global coagulation assays as thrombin generation test and clot waveform analysis.
Results: Thrombin generation assay revealed that the decrease rates of peak thrombin in anti-C2 type 1 inhibitors were ~3-fold or 10–20-fold greater than those in anti-A2 type 1 or anti-C2 type 2, respectively. Compared to FVIII alone, the mixture of FVIII/VWF with anti-C2 type 1 or anti-A2 type 1 mildly or little reduced the decrease rates by ~10% or <5%, respectively, whilst that with anti-C2 type 2 enhanced by ~2-fold, reflecting the property of anti-FVIII antibodies on the FVIII-VWF association. Anti-FVIII monoclonal antibodies showed similar patterns of reaction with anti-FVIII polyclonal. Clot waveform analysis also showed that coagulation parameters in 2-h reaction of FVIII with anti-A2 type 1 or with anti-C2 type 2 were ~3- or ~6-fold greater compared to anti-C2 type 1, respectively.
Conclusion: We demonstrated that the neutralizing effects of FVIII in the presence of anti-FVIII inhibitors differed with epitope of inhibitors. It should be important to determine the properties of inhibitors for prediction of the effects on FVIII-neutralization therapy and choice of the treatment using FVIII concentrates or FVIII/VWF complex concentrates, and the results could have significant therapeutic implications.

PB 4.37-2

Pharmacokinetics, efficacy, and safety of BAY 81-8973, a full-length plasma-protein-free recombinant factor VIII product: results from the LEOPOLD trial

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Background: BAY 81-8973 is a full-length recombinant factor VIII (rFVIII) product manufactured without use of animal- or human-derived proteins in the fermentation and purification steps.

Aims: To demonstrate the pharmacokinetic (PK) noninferiority of BAY 81-8973 vs. sucrose-formulated rFVIII (rFVIII-FS) and to assess the efficacy and safety of BAY 81-8973 for treatment of bleeds and prophylaxis in hemophilia A

Methods: This multinational, randomized, open-label study is part of the Long-Term Efficacy Open-Label Program in Severe Hemophilia A Disease (LEOPOLD; ClinicalTrials.gov identifier: NCT01029340). Males aged 12–65 years with severe hemophilia A were eligible if they had ≥150 exposure days to any FVIII product and no history of FVIII inhibitors. For the phase 1 PK study, patients received a single 50-IU/kg dose of BAY 81-8973 and rFVIII-FS, separated by ≥3-day washout. Blood samples for PK testing were collected periodically up to 48 h postdose. PK parameters analyzed included maximum concentration (C_{max}), C_{max} divided by dose per body weight ($C_{max\ norm}$), area under the curve (AUC), half-life ($t_{½}$), volume of distribution at steady state (V_{SS}), mean residence time (MRT_{IV}), and clearance (CL). For the phase 2/3 efficacy and safety study, patients received 20–50 IU/kg BAY 81-8973 2–3 times/week for 12 months. BAY 81-8973 potency was based on chromogenic substrate assay per European Pharmacopoeia (CS/EP) and chromogenic substrate assay/label adjusted to mimic one-stage assay potency (CS/ADJ; dose was ~18% higher because of potency differences between the two assays). Patients crossed over from one potency group to the other after 6 months. Repeat BAY 81-8973 PK analysis was conducted at 6 or 12 months. The primary efficacy endpoint was the annualized number of total bleeds in each 6-month potency period. Patients also received BAY 81-8973 during major surgeries. All patients provided written informed consent; the protocol was approved by each site's independent ethics committee/institutional review board.

Results: Valid PK measurements were available for 26 patients (mean age, 30.6 years). The 90% CIs for the AUC and C_{max} ratios of BAY 81-8973 to rFVIII-FS were above the lower limit of the prespecified interval of 0.80–1.25 demonstrating noninferiority. Analysis of variance using 95% CIs showed that AUC, $t_{½}$, and MRT_{IV} were significantly higher and CL was lower after administration of BAY 81-8973 vs. rFVIII-FS ($P < 0.02$). No significant differences between treatments were seen for $C_{max\ norm}$ or V_{SS}. No relevant changes were seen in BAY 81-8973 PK after 6 or 12 months of treatment. Sixty-two patients (mean age, 31.5 years) received BAY 81-8973 prophylaxis for 12 months. Median annualized number of total bleeding episodes was 1.0 in the combined CS/EP and CS/ADJ groups (mean, 3.8; range, 0–26.1), without clinically relevant differences between potency periods. BAY 81-8973 maintained hemostasis in seven patients undergoing eight major surgeries. Incidence of treatment-related adverse events was low in all study phases (≤7%); no patient developed inhibitors.

Conclusions: In previously treated patients with severe hemophilia A, BAY 81-8973 PK were noninferior to those of rFVIII-FS. BAY 81-8973 was efficacious in preventing and treating bleeding episodes, with few treatment-related adverse events.

PB 4.37-3

Treatment of bleeding episodes in subjects with haemophilia A with long-lasting recombinant Factor VIII Fc fusion protein (rFVIIIFc) in the Phase 3 A-LONG study

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Background: A long-lasting monomeric recombinant factor VIII Fc fusion protein (rFVIIIFc) for the treatment of haemophilia A has been developed using Fc technology to extend half-life over currently available rFVIII products. rFVIIIFc is comprised of a rFVIII molecule genetically linked to the Fc domain of human immunoglobulin G_1 (IgG) with no intervening sequence. Fc fusion technology utilises the endogenous IgG cycling pathway to prolong the half-life of therapeutic proteins. A 1.5-fold increase in half-life was demonstrated in the recently completed pivotal phase 3 study (A-LONG), which evaluated the safety, efficacy, and pharmacokinetics (PK) of rFVIIIFc when used for prophylaxis, to treat acute bleeds, and in the surgical setting, in previously treated subjects with severe haemophilia A.

Aims: To evaluate the treatment of bleeding episodes with rFVIIIFc in the pivotal A-LONG study.

Methods: Eligible male subjects ≥12 years old, with severe haemophilia A (<1 IU/dL [1%] endogenous FVIII), a history of ≥150 documented prior exposure days (ED) to FVIII, and no history of FVIII inhibitor, received either individualised prophylaxis (25–65 IU/kg every 3–5 days; Arm 1), weekly prophylaxis (65 IU/kg dose; Arm 2), or episodic (20–50 IU/kg; Arm 3) treatment. The number of bleeding episodes, number of injections, and median dose required to resolve bleeding episodes were evaluated. Efficacy assessments by subject and investigators were recorded.

Results: Overall, 165 subjects from 60 centres were enrolled (Arm 1, $n = 118$; Arm 2, $n = 24$; Arm 3, $n = 23$) and 92.7% of subjects completed the study. In total, 757 bleeding episodes (Arm 1 = 209; Arm 2 = 92; Arm 3 = 456) were treated in 106 of 163 subjects. To achieve resolution of a bleed, 87.3% of bleeds required a single injection of rFVIIIFc, and 97.8% required ≤2 injections. By study arm, 85.6%, 80.4% and 89.5% of bleeding episodes in Arms 1, 2 and 3, respectively, were resolved with 1 injection of rFVIIIFc. The median dose per injection required for resolution of bleeding was 27.4 IU/kg, and the median total dose required was 28.2 IU/kg. The median number of injections required for resolution of a bleeding episode was consistently 1.0 across treatment arms, regardless of type or location of bleed, or time of administration of treatment. Overall, subject assessment of response to treatment with rFVIIIFc was excellent or good for 78.8%, 64.8% and 79.7% of injections in Arms 1, 2, and 3, respectively. The investigators' global assessment of subject response to their assigned rFVIIIFc regimen was rated as excellent or effective for 99.4%, 100% and 98.1% of responses in Arms 1, 2 and 3, respectively.

Summary/Conclusions: Long-lasting rFVIIIFc provided effective control of bleeding episodes in the pivotal A-LONG study. The majority of bleeding episodes required only one injection. Subject response to rFVIIIFc regimen (rated by investigators) and response to bleeding events rated by subjects, were excellent or good/effective. rFVIIIFc offers the potential of a reduced injection frequency for treatment of bleeds in patients with severe haemophilia A.

PB 4.37-4

Does prophylactic factor replacement prevent asymptomatic microscopic haematuria in adult people with severe haemophilia?

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Background: Asymptomatic microscopic haematuria has been reported to occur in up to 20% of people with moderate/severe haemophilia before the introduction of coagulation factor replacement therapy. In the era of modern haemophilia treatment, however, the significance and frequency of haematuria is unknown.

Aim: The aim of the present study was to establish the frequency of asymptomatic microscopic haematuria among adult people with severe haemophilia and to determine the impact of prophylactic factor replacement on the occurrence of haematuria.

Methods: Fifty-five adult people with severe haemophilia A and B have been evaluated on 69 outpatient admissions for microscopic haematuria by urine examination. Microscopic haematuria was defined as the presence of ≥3 red blood cells in the urine sediment prepared via standard methods. Patients with urinary symptoms were excluded. None of the patients were positive for inhibitors. Factor levels and the treatment modality (on demand vs. prophylactic) of the patients, as well as the time (in days) from the last factor infusion were recorded.

Results and Conclusion. : In 9 of the 69 admissions patients were found to have asymptomatic haematuria (13%). No significant difference in the frequency of haematuria was observed between the patients with on demand treatment and those on prophylaxis ($P = 0.268$). These findings suggest that about 1/10 of people with severe haemophilia present with microscopic haematuria without any urinary symptoms. Prophylactic factor replacement does not necessarily prevent haematuria. This should be considered in the management of bleeding in people with severe haemophilia. The absence of haematuria should always be confirmed prior prescribing antifibrinolytic agents for the treatment or prevention of bleeding episodes.

PB 4.37-5

Ageing, haemophilia A and associated bleeding disorders – whose problem is it?

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Background: Due to the success of treatment and care within haemophilia and associated bleeding disorders, the UK have a growing population who are now enjoying living longer and entering a unique period of history in relation to their own health and care which needs to be explored and understood.

Aims: A pilot study set out to investigate patients, carers and health professional's attitudes towards ageing, health and social care needs with a view to developing a consistent and high quality health and social care framework for haemophilia and associated bleeding disorders in the UK.

The impact these attitudes have upon self-management and ageing was investigated with a view to informing discussion around the development of associated guidelines relating to caring for a senior population within their own homes, clinics, hospital, nursing and retirements homes, therefore covering the full spectrum of bio psychosocial care.

Methods: The phenomenological approach has been used to examine experiences in ageing and bleeding disorders. Sixty patients (age range 40+), seven carers and six health professionals participated in focus group discussions on haemophilia, ageing and current available health and care support services and preferences relating to desired services for the future.

Results: The results indicate uncertainties around solutions to increasing difficulty with venous access as a result of lack of self-efficacy and memory problems. Concerns over access to haemophilia centres as a consequence of increasing disability and poor mobility were stressed. A reluctance to accept dependency on others and attitudes related to poor knowledge base within the general nursing community/nursing/care homes were associated with fear and scepticism of quality of service and treatment delivered for current and future co-morbid conditions.

A lack of skilled general community nurses who can give administer IV treatments has been echoed throughout the meetings. Preferences for further development into E Health and multi-disciplinary meetings to ensure effective communication with other health teams were expressed.

Conclusions: Guidelines should provide standards for both health and social care; they should also be developed to promote the wellbeing of all those aging and living with haemophilia for which these services are destined. Upgrade existing services which should include an increase of community haemophilia nurses and better communication with GP'S. The development of screening and preventative services within haemophilia centres, training manuals should be disseminated as a guide to competent authorities, accredited bodies and care agencies operating in this area.

PB 4.37-6

Impact of children with inhibitors on caregiver burden

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Background: Haemophilia is a bleeding disorder that can be classified as mild, moderate or severe depending on plasma level of clotting factors. Inhibitors are alloantibodies that can develop after exposure to replacement therapy with coagulation factor VIII or IX products and are presently considered the most serious complication in the treatment of haemophilia. Since treatment of haemophilia is typically provided at home by parents and relatives (i.e. caregivers), caregiver burden may be impacted by inhibitor complications.

Aims: To compare the overall burden of caregivers of children with and without inhibitors.

Methods: A questionnaire with six domains (emotional stress, financial, sacrifice, medical management, child's pain, and transportation) and burden visual analogue scales (B-VAS) was developed based upon the peer-reviewed literature and previous survey findings. Questions for each domain were answered using a scale of 1–5 where '1'represented 'never burdened' and '5' represented 'nearly always' burdened. B-VAS utilized a scale from 0 to 10 with 0 indicating 'no burden' and 10 indicating 'worst burden'. High burden was defined as those with a total burden score greater than the observed mean score of 80. Logistic regression was used to adjust for confounders including age, gender, income, education, marital status, disease severity, haemophilia type, inhibitor status, bleeding episodes, time taken off work, treatment type, time since diagnosis and distance from haemophilia treatment center. IRB approval was obtained.

Results: 310 caregivers completed the survey; 30 reported taking care of a child with an inhibitor. Most respondents ($N = 274$, 88%) were mothers between 18 and 54 years. The average age of the child for whom care was provided was 10.04 (SD 4.57). Mean total burden scores for all respondents were 80.79 (SD 20.14). Unadjusted results showed that compared to non-inhibitor patients, caregivers of children with inhibitors had statistically significant higher total burden scores (96.17 vs. 78.65, $P < 0.0001$), B-VAS scores (5.57 vs. 3.44, $P < 0.0001$) and all six burden domains: Child's pain (3.90 vs. 3.48, $P < 0.0096$);

emotional stress (3.15 vs. 2.73, $P < 0.0005$); financial burden (3.01 vs. 2.46, $P < 0017$); transportation (3.22 vs. 2.42, $P < 0.0001$); sacrifice (2.98 vs. 2.13, $P < 0.0001$); medical management (2.38 vs. 2.00, $P < 0.0009$). After controlling for confounders, having a household income >$100,000 USD ($P < 0.05$) was associated with less burden whereas taking time off work in the previous month ($P < 0.003$) and caring for a child with inhibitors ($P < 0.001$) were associated with greater burden. Adjusted odds ratios indicated that caregivers of children with inhibitors were 5.37 (95% CI 1.98–14.58) times more likely to have high burden compared to caregivers of children without inhibitors.

Conclusions: Caregivers of children affected by inhibitors to FVIII/FIX replacement therapy were significantly more burdened than caregivers of children without inhibitors. The burden of caregivers should be considered when assessing the psychosocial aspects of managing patients with inhibitors.

PB4.38 – Haemophilia A: Clinical – XVI

PB 4.38-1

Immunological detection of factor VIII antibodies in congenital and acquired haemophilia A

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Background: The Nijmegen Bethesda assay is the gold standard for detection of Factor VIII (FVIII) antibodies, albeit with significant inter-laboratory variability. However, it may miss low titre antibodies or those without direct inhibitory capacity. This may not have immediate clinical repercussions but the true rate of anti-FVIII immunological activity in clinical studies may be underestimated. This could compromise the significance of surrogate markers of inhibitor risk. A FVIII ELISA kit (Hologic, previously GTI Diagnostics) has previously been described. This detects both inhibitory and non-inhibitory FVIII antibodies. This assay does not form part of standard laboratory practice in the United Kingdom. We present a large cohort describing the application of this FVIII ELISA in routine laboratory practice.

Aims: To compare anti-FVIII antibody detection by the Bethesda assay and FVIII ELISA in congenital and acquired haemophilia A in two large haemophilia treatment centres.

Methods: All routine inhibitor samples from patients with congenital and acquired haemophilia A were tested in parallel using the Bethesda assay and FVIII ELISA. These were subdivided based on diagnostic information provided at the time of venepuncture. Samples were defined as being positive if the Bethesda assay was ≥0.7 BU/mL and/or if the ELISA optical density (OD) was greater than that of the kit control.

Results: Four hundred and ninety seven samples (240 patients) were tested in parallel by Bethesda and ELISA: 140 patients with severe haemophilia A (291 samples); 86 patients with non-severe haemophilia A (129 samples); 14 patients with acquired haemophilia A (77 samples). Sixty three samples were positive by the Bethesda assay, median 1.2 BU/mL (0.7–978) and 75 positive by ELISA, median OD 0.978 (0.204–3.363). There were 40 discrepant samples: 14 Bethesda positive/ELISA negative; 26 Bethesda negative/ELISA positive. Comparing the assays for all samples the ELISA demonstrated a sensitivity of 77.8%, specificity 94.0%, positive predictive value (PPV) 65.3% and negative predictive value (NPV) of 96.7%. In the sub-group analysis of patients with acquired haemophilia A the ELISA demonstrated a sensitivity of 100%, specificity 82.9%, PPV 36.8% and NPV 100%, with 12 samples found to be positive by ELISA, but negative by the Bethesda assay.

Conclusions: Detection of FVIII antibodies by ELISA offers a simple method of batch testing, complementing the Bethesda assay. This test does not require modification for residual FVIII levels. Immunological detection of anti-FVIII antibodies may have as yet unforeseen predictive applications, e.g. risk of relapse in acquired haemophilia A. The reasons for ELISA and Bethesda assay discrepancy and resulting clinical significance needs to be defined through prospective collaborative studies.

PB 4.38-2

Long-term anti-FVIII antibody response in Bethesda-negative haemophilia A patients on continuous replacement therapy

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Background: Patients with haemophilia A may develop non-neutralizing anti-FVIII antibodies (NNA) that escape detection by the Bethesda assay, but are detected using immune-based assays. Recently, we and others found NNAs to be directed, not only towards non-functional parts of the protein, but towards all regions of the FVIII protein. We also showed in a cohort of brother pairs with haemophilia A, a heterogeneous antibody response towards different FVIII products. However, the clinical relevance and the natural history of NNA remain unclear.

Aims and Method: We have followed a cohort of unrelated subjects with haemophilia A, on continuous replacement therapy, for 4 years with the goal of exploring the long-term development of NNA using an ELISA assay.

Results: Ten of 78 subjects (12.8%) exhibited an immune response that was transient and heterogeneous, and none of the subjects developed an inhibitor towards FVIII during the study period. In order to investigate the potential clinical relevance of NNA, the result of the ELISA assay was examined in relation to clinical variables. No associations between a positive ELISA assay and age, *F8* mutation or PAC implantation was found. We found a trend towards an increased NNA risk in HCV positive subjects. Interestingly, patients with NNA had significantly fewer bleeding episodes ($P = 0.048$) compared with NNA-negative subjects.

Conclusion: The results indicate that the immune response towards FVIII within an individual may vary over time, and that anti-FVIII antibodies may potentially exert not only detrimental effect on FVIII protein, but hypothetically protective or potentiating effects as well. However, the clinical impact of NNA still remains unclear and requires further investigation.

The study was approved by the regional research ethics committee for southern Sweden.

PB 4.38-3

Carrier and prenatal diagnosis in sporadic haemophilia A and B families in China

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Background: About 1/3 of haemophilia families were sporadic families. Carrier and prenatal diagnosis in these families is necessary and it is important to determine the origins of the mutations.

Aims: In this report, we made genetic diagnosis of 40 sporadic HA families and three sporadic HB families.

Methods: LD-PCR and PCR were adopted for the screening of the INV22 and INV1 respectively. The F8/F9 coding and boundary sequences were analyzed by sequencing. Seven STR sites related to F8/F9 gene were combined together to apply to the linkage analysis

respectively. ABI PRISM SNaPshot ddNTP Primer Extension kit was used to analyze the chimerism. We assessed the sensitivity of the primer extension PCR method for detecting the mosaicisms by a mutant/wild DNA mix experiment.

Results: Tests showed that these 40 HA families were caused by INV22 (13/40), small insertions(6/40), small deletions(5/40), missense mutations(10/40) and nonsense mutations(6/40). The three sporadic HB families were all caused by missense mutations. From the linkage analysis, we confirmed that the origin of the mutations. For INV22 positive families, eight were from the maternal grandfathers (MGF) with no INV22 mutations and the others from the carrier maternal grandmothers (MGM). For the other INV22 negative families, 13 index's mothers were normal according to the DNA sequencing, the other 14 were carriers, in which six of the mutations were from MGM and the others from the MGF. The origin of the mutations were form the maternal grandfathers in all of the HB families. Mosaicism was identified in two families with the mutation of c.G96085T, c.T32874A in HA families and c.A32772T in HB familiy respectively. For the HA families, somatic mosaicism was identified in both of the grandmother's blood and oral cells with the percentage of 10.62% and 11.01% mutant (c.G96085T) respectively. 33% mutant (c.T32874A) was detected in the index's mother's blood cells while the formal DNA sequencing demonstrated the normal results. In HB family, the maternal grandfather's oral mucosa cells possessed wild-type as well as mutant F9 genes and the percentage of the mutation was 14.4%. We estimated the limit of the mosaicism detection as 2% mutant allele.

Conclusions: Based on the present study, we can reasonably suggest that intron 22 and 1 inversions screening combined with the linkage analysis using seven STR sites are available for carrier detection and prenatal diagnosis in Chinese HA families and the direct sequencing of F9 gene combined with the linkage analysis are available for diagnosis in Chinese HB families. The extention primer PCR method is a good approach to detect the mosaicism and can give information which may prove valuable in determining the origin of new haemophilia mutations, and in counselling relatives as well.

PB 4.38-4

Quality of hemophiliacs treatment in India – a survey

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Background: India with a population of 1.2 billion would have approximately 75,000 hemophiliacs. The problems with management of hemophilia in developing countries are poor awareness, inadequate diagnostic facilities and scarce factor concentrates for therapy.

Aims: This study describes the epidemiology and health services offered to hemophiliacs in India.

Methods: Survey was done through organizing hemophilia awareness camps with workshops in 12 cities of North India in last 2 years by expert hemophilia team. A detailed survey form was filled to know the quality of care in hemophiliacs.

Results: The survey included 403 patients with median age of 13 years (range 1.5–65 years) (73.7% were <20 years of age). Hemophilia A and B constituted 90.3% and 9.7% with severe, moderate and mild disease in 70.5%, 22.3% and 7.2% respectively. Half of patients had family history of hemophilia. Median age of clinical presentation was 1 year (range: birth to 20 year). At the time of first diagnosis of hemophilia, 67%, 29.5% and 3.5% patients had spontaneous, traumatic and procedural bleeding respectively. Majority of patients (90.4%) were being treated on demand, 39/403 (9.6%) were not even exposed to factor therapy yet (receiving alternative treatment). At the time of diagnosis 22.3% patients received factor replacement therapy, 61% received alternate form of treatment in the form of blood transfusion, fresh frozen plasma or cryoprecipitate and 16.7% received minimal or no treatment. The most common manifestation seen was target joint in 247/403 (61.3%) of patients. Single target joint was involved in 215/403 (53.3%) patients with knee joint in 43.7%, elbow 7.4%, hip 2%, shoulder 1.2% and ankle in 0.7%. Two and three target joints were present in 28/403 (6.9%) and 4/403 (1%) respectively. Clinical (Child instrument) and Orthopedic joint scores were pathetic in these patients. Permanent disability was present in 155/403 (38.5%), of these 15.1% were using crutches. Inhibitor levels were not checked in majority (>95%) of patients. Most of patients were using ice packs (63%) and tranexemic acid (55%) for their bleeding episodes, crutches (15.1%) for disability, 27.8% different NSAIDS, 8.2% codeine, 20% paracetamol, 1% homeopathic and 1% antispasmodic as pain killers. Virological studies for HIV, hepatitis B and C were performed in 10.6%, 11.4% and 10.2% of patients respectively. Two patients were positive for HIV, Hep B and HCV each. Not more than 10 patients were covered with insurance or reimbursement policies. Only few states governmental agencies are providing factor replacement free of cost. Major reasons for not using regular factor replacement were economical, followed by illiteracy. Secondary prophylaxis was opted by 6.7% of patients and none opted for primary prophylaxis.

Conclusion: Although this survey does not cover the whole country uniformly, it gives a snap view of health care services offered to 403 patients with hemophilia in India. Access to factor replacement is limited by economical constraints. More emphasis should be put on comprehensive services and prophylactic factor replacement to improve quality of life in developing countries.

PB 4.38-5

The impact of hemarthropathy on the QoL of Korean patients with severe hemophilia A: The critical level of hemarthropathy for the QoL

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Background: It is acknowledged that as the degree of hemarthropathy approaches a certain critical level, the disabled physical state may markedly affect the QoL of hemophiliacs. However, the critical level of hemarthropathy may be different in different countries because the QoL could be influenced not only by the physical state but also by socio-cultural environments across countries.

Aims: The impact of hemarthropathy on the QoL in Korean hemophilia A patients were investigated to find out the critical level of hemarthropathy in these patients.

Methods: This study was conducted in 27 severe hemophilia A patients over 17 years old who were registered at a single hemophilia treatment center. Depending on observed Pettersson scores of these patients at the start of study, they were divided into three groups, P (Pettersson score) £10, P 11~19 and P³20 groups. The QoL of each patient, assessed by the SF 36 (Korean version), was compared between the groups. In addition, a correlation analysis was conducted between Pettersson scores and scales of the SF36 to assess the impact of hemarthropathy on the QoL.

Results: In the present study, Pettersson scores were significantly correlated with physical health scales (PF, BP, GH) and physical component summary (PCS). None of the scores of the SF36 scales were different between the P£10 and P11~19 groups. In contrast, the scores of PF and MH scales were significantly different between the P11~19 and P³20 groups. When changes in the scores of each scale in the SF36 were observed according to changes in Pettersson scores, the average P score of 13.0 ± 2.7 was thought to be the critical level of hemarthropathy because above that level, hemarthropathy and physical and mental health of the patients rapidly deteriorated.

Conclusions: The progression of hemarthropathy to the critical level should be delayed as long as possible to prevent or to delay a rapid deterioration of the QoL of Korean patients with hemophilia.

PB 4.38-6

Social determinants of quality of life in persons with hemophilia

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Background: The availability of safe and effective factor replacement therapies, in persons with hemophilia (PWH), has in some countries answered the basic need for treatment of these patients. In countries and regions where factor replacement and other treatment facilities are not enough available, Psychosocial support may help PWH, to cope with their disease and related problems. Meanwhile, most of the social determinants evaluations in PWH have been a component of quality of life (QoL) questionnaire – based studies.

Aims: This study is designed to evaluate the QoL in adult PWH, by focusing on social determinants of QoL and their relationship with health related dimensions, in Tabriz hemophilia treatment center (HTC) of Iran.

Material and Methods: The survey instrument was a self-report 36 items questionnaire, 'A36 Hemofilia – QoL', which is a disease specific questionnaire for the assessment of the health-related QoL in adults living with hemophilia. A total of 100 Hemophilia A and B, mild to severe, over 17 years old participated in this study within 1 year.

Results: A36 Hemofilia-QoL Total score (TO) was 71.88(±26.89 SD), which was in the range of moderate to good. Dimensions covered by this survey were classified as good to moderate for Physical Health 16.37 (51.15%), Treatment Satisfaction 5.39 (67.37%), Treatment Difficulties (60.62%). Other dimensions were classified as moderate to poor for Daily Activities 7.20 (45%), Joint Damage (5.52%), Pain 3.50 (43.75%), Emotional Functioning 9.26(46.3%), Mental Health 5.8 (48.41%), Relationships and Social Activities 9.58 (47.9%). Patients who treat in our HTC, had better QoL score ($P = 0.000$), and education has a significant impact on the social aspects of QoL ($P = 0.18$). The QoL was very poor in urban area in contrast to patients who lived in the city (54.45 vs. 74.21, respectively). Single patients have a better QoL than married patients (76.56 vs. 68.50, respectively).

Conclusion: Our results showed that PWH who live in urban areas have very poor QoL and more disease complications, because of less replacement therapies and lack of awareness of their diseases. Serious designed works strongly recommend for this group of PWH.

PB4.39 – Haemophilia A: Clinical – XVII

PB 4.39-1

Peak FVIII levels and time spent in hemostatically effective FVIII range post-infusion correlates with improved efficacy for prophylaxis in hemophilia A: a closer look at the other end of the curve

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Background and Aims: We previously demonstrated that increasing time with a FVIII concentration below 1 IU dL(−1) is associated with increased total hemorrhages and hemarthroses in severe hemophilia A patients treated with regular prophylaxis regimens (Collins PW, et al. J Thromb Haemost 2009; 7: 413–20). Targeting trough levels at ≥1% above baseline using PK-guided dosing at 72 h intervals has been demonstrated to be an effective treatment strategy (Valentino LA, et al. J Thromb Haemost 2012; 10: 359–367). In this study, subjects on PK-guided dosing experienced a median annual bleed rate (ABR) of 2.0 (range 0–17.1) representing a 96% reduction in ABR from on-demand therapy. The individual FVIII half-lives (median: 11.7 h; range: 7.3–30.7; IQR: 10.1–13.6; 5–95% percentiles: 7.7–21.4), and therefore, the FVIII dose/infusion (median 41.3 (IU/kg), range 18.9–84.9) varied widely in the study cohort. This allowed examination of the role of treatment- and patient-related variables other than FVIII trough in achieving low ABRs in patients using individualized regimens.

Methods: Data from subjects prescribed PK-guided dosing given every third day ($n = 34$) were examined. Average Cmax was estimated using the individual IVR values for each subject and their average dose per prophylactic infusion. AUC/week and time spent above 5, 10, 20, 30, or 40% FVIII levels (i.e, within hemostatically effective, non-hemophilic range) in each subject were extrapolated using parameters from individual PK profiles and actual infusion records. Negative binomial multivariate regression model was used for analysis with age and BMI as covariates.

Results: The estimate for Cmax ranged from 24.3 to 167.5% (median 70.9%). A significant relationship between lower Cmax and increased risk for bleeding was seen (All bleeding $P = 0.0004$; joint bleeding $P = 0.0013$). Similarly, time spent above 30% and 40% FVIII levels showed a significant relationship with lower ABR (all bleeds, $P = 0.0034$ and $P = 0.0012$, respectively). Similar significant relationships were found in all AUC/week variables tested (e.g., above 5%, 10%, and 20%); both AUC and time spent variables are strongly correlated with average Cmax. A substantial reduction of ABR during prophylaxis as compared with on-demand therapy was seen in each subject however many of those with higher ABR on prophylaxis appeared to have had more bleeding episodes during the preceding on-demand period and had a lower% ABR reduction on prophylaxis. Otherwise no other parameters examined (e.g., age, BMI, number target joints, compliance) appeared different amongst these subjects.

Summary and Conclusion: These results demonstrate a relationship between higher Cmax values and/or time spent within 'hemostatically effective' FVIII range and better prophylactic efficacy in subjects on PK-guided dosing given every third day. While targeting FVIII trough at >1% above baseline is generally effective, this strategy *alone* may not be suitable for all patients, especially those with high ABRs on on-demand therapy, and with longer FVIII half-lives, who require lower FVIII doses to maintain the specified trough levels. These findings may have significant implications for the development of optimal individualized PK-guided prophylactic dosing regimens with unmodified FVIII concentrates as well as with the modified longer-acting FVIII molecules currently in development.

PB 4.39-2

Self-efficacy in the Dutch pediatric population; Reliability and validity of the Hemophilia Self-Efficacy Scale (HSES)

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Background: In all patients with chronic diseases, self management capacities of the patient and caretakers with regard to the disease are of importance. Especially in hemophilia patients on prophylaxis, adequate judgement and motivation considering adherence to prescribed

therapy, and behaviour are important skills; summarized by the term self-efficacy. Previous studies have demonstrated that a well developed capacity towards self-efficacy is associated with greater adherence and less clinical manifestations of disease. Therefore, it is important to quantify a patients' self-efficacy and to formulate interventions to modify self-efficacy skills.

Aims: To assess the reliability and validity of a novel scale measuring disease-specific perceptions of self-efficacy in patients with hemophilia on prophylactic clotting factor replacement therapy.

Patients and Methods: Children from three Dutch Hemophilia Treatment Centers with severe and moderate hemophilia on prophylactic treatment were included. Exclusion criteria were: prophylactic treatment <1 year, presence of inhibitors and language difficulties. Parents and children aged 10–18 years filled out the study questionnaire. Written informed consent and ethical approval was obtained.

The Hemophilia Self-Efficacy Scale (HSES) consists of 12 items relating to participants' perceptions of their ability to function on a day-to-day basis and to manage symptoms (e.g. bleedings episodes) and therapeutic interventions in hemophilia. This instrument is based on the validated Sickle Cell Self-Efficacy Scale with the concept of the ability towards 'self-care'. This incorporates an individuals' perceptions of both disease symptoms and the patients' abilities to cope with or reduce these symptoms.

Response choice ranged from 'not at all sure' to 'very sure' on a 5-point Likert scale. Higher scores reflect greater self-efficacy, the total score range is 12–60. Psychometric properties (construct validity including Cronbach's alpha, discriminative validity, test-retest reliability) were empirically assessed. Convergent validity was tested with previously validated generic measure of related construct (General Self-Efficacy Scale (GSES). Predictive validity was assessed by computing correlations between HSES total score and reported health-related quality of life in the previous month (Haemo-QoL).

Results: A total of 53 parents and 28 adolescents filled out the questionnaire (response rate 79%). Median age of the children was 11.4 years (IQR 8.1–13.9). Test-retest reliability was measured in a subgroup ($n = 55$). Mean of total score was 55.5 (SD 4.7, range 38.0–60.0). Construct validity: mean Cronbach's alpha was good for the total score (0.8). The scale had no floor effects but did have a ceiling effect of 21%. Test-retest Pearson correlation was positively significant (0.8; $P < 0.001$). Correlation between disease specific self-efficacy and GSES was statistically significant (Pearson correlation 0.5, $P < 0.001$). The reported Haemo-QoL correlated significantly to HSES scores (Pearson correlation −0.33, $P = 0.02$), indicating that higher self-efficacy was associated with higher quality of life.

Conclusions: HSES is a reliable and valid tool to asses patients' self-efficacy in patients with severe or moderate hemophilia and prophylactic treatment. In our study, high self-efficacy correlates with higher quality of life reported by HaemoQol, underlining the importance of evaluation of capacity towards self-efficacy.

PB 4.39-3

Recombinant activated factor VII use for surgical/invasive procedures in congenital haemophilia with inhibitors and acquired hemophilia: analysis from 10-year Japanese post-marketing surveillance

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Background: Patients with congenital haemophilia with inhibitors (CHwI) and acquired haemophilia (AH) are at risk of bleeding complications during surgical/invasive procedures, since replacement therapy for the missing coagulation factor is ineffective. Therefore, management of bleeding requires the use of bypassing agents, such as recombinant activated FVII (rFVIIa, NovoSeven®). To investigate the safety and efficacy of rFVIIa in the treatment of bleeding during surgical/invasive procedures in Japanese patients, data from Japanese post-marketing surveillance was analysed.

Methods: A multi-centre, observational study was conducted from May 2000 to March 2010. The dosing regimen of rFVIIa was decided based on the institutional practice at each centre. The rFVIIa dosing regimen and adverse events were recorded, and the clinical efficacy evaluated according to three criteria: (i) blood loss (during a procedure) compared with that expected from previous surgeries in haemophilia patients; (ii) effectiveness of bleeding control (during a few days post-procedure), and (iii) maintenance of bleeding control (during a few weeks post-procedure until removing stitches). Patient follow-up was for 6 months to evaluate other complications.

Results: Twenty-two patients with congenital haemophilia A underwent 38 surgical/invasive procedures (orthopaedic: 43%, central venous access device: 16%, dental/oral: 16%, gastrointestinal: 11%, small incision/suture: 11%, thoracic: 5%). Of these 38 procedures, 92% were evaluated as 'blood loss as expected or less' and 89% as 'effective in bleeding control, i.e. bleeding was stopped completely or reduced considerably'. Bleeding control was maintained in 95% procedures after wound closure. Seven congenital haemophilia B patients underwent 13 surgical/invasive procedures (orthopaedic: 62%, central venous access device: 15%, dental/oral: 8%, gastrointestinal: 8%, small incision/suture: 8%). Of these, 69% were evaluated as 'blood loss as expected or less' and 77% as 'effective in bleeding control'. Bleeding control was maintained in 95% of procedures. Five patients with AH underwent five surgical/invasive procedures (gastrointestinal: 40%, dental/oral: 20%, obstetrics: 20%, central nervous system: 20%). Of these, 60% were evaluated as 'blood loss as expected or less' and 80% as 'effective in bleeding control'. In 60% of procedures, the bleeding control was maintained. Activated prothrombin complex concentrate was used when bleeds were not controlled with rFVIIa. Thrombophlebitis occurred in an 11-year-old male with congenital haemophilia A with an inhibitor. He underwent elbow surgery with continuous infusion of rFVIIa, and a mild thrombophlebitis of a superficial vein was observed in the arm in which the catheter was placed. The thrombophlebitis resolved naturally. The case was determined to be related to rFVIIa administration.

Conclusions: rFVIIa provided adequate haemostasis for the majority of surgical/invasive procedures in patients with CHwI and AH, without serious adverse reactions. The efficacy and safety profile for Japanese patients was similar to the published data from other countries.

PB 4.39-4

The first review of global spontaneous adverse event reports for a third generation recombinant factor VIII concentrate (Octocog Alfa): 10 years of safety experience

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Background: The low number of spontaneous Adverse Event (AE) reports in rare disorders and the variety of reporter sources such as physicians or patients require a thorough understanding of reporting trends to evaluate safety data. Spontaneous reporting represents an important tool to identify Adverse Drug Reactions previously unrecognized in the clinical development process. Regional or local databases, e.g. FDAs FAERS, EUHASS, are subject to different reporting cultures or research questions that hold a potential for biased perspective. Pharmacovigilance activities of Marketing Authorization Holders provide a unique, global picture allowing to identify and thereby minimize potential bias.

Aims: The a priori assumption that all biologics of one class have a similar safety profile can be hazardous: e.g., manufacturing processes may result in different safety profiles. Herein we provide a comprehensive 10-year overview of Octocog Alfa safety profile since its first marketing authorization as determined by the longest and largest dataset of spontaneous AE reports published for a 3rd generation rFVIII.
Methods: The global safety database for Octocog Alfa was reviewed for all spontaneously reported AEs (excluding solicited reports) received between July 2003 and September 2012. Reporting trends were analyzed per reporting sources and regions. Important AEs such as inhibitor formation are further discussed.
Results: More than 13 billion international units of Octocog Alfa were distributed during the review period, corresponding to an estimated 87,000 patient-years of exposure. A total of 648 spontaneous AE reports were received. Of these, 311 AE reports included information on the development of factor VIII inhibitors. The reporting rate of FVIII inhibitors for both PUPs and PTPs remained stable over time suggesting the absence of safety signals. Five spontaneous AE reports included the event of an anaphylactic reaction. Significant differences were observed for reporting trends in individual countries with respect to reporting rates as well as per reporting source. For the US, total numbers of AE reports from patients and healthcare professionals such as nurses and pharmacists by far exceed the number of AE reports received from physicians. This is in sharp contrast to other regions, such as Japan, where AE reports from physicians dominate spontaneous reporting. Globally, consumer reports included a higher percentage of non-serious events compared to reports from physicians. In addition consumers and healthcare professionals were focused on different types of AEs. The often cited Weber-effect (the increase of the absolute number of AE reports during the first 2 years of product marketing with a subsequent reporting decrease thereafter despite increasing sales) could not be observed.
Summary/Conclusions: Despite the complexity of AE reporting trends, the 10-year review of spontaneous AE reports received globally for Octocog Alfa did not result in the detection of previously unrecognized risks or product quality issues. This data is usually not disclosed publicly but is important information to confirm the premarketing safety profile given the limited sample size needed for licensing FVIII therapeutics. Data derived from spontaneous reporting is an integral part of the overall safety profile of a product and needs to be taken into account for optimal treatment decisions.

PB 4.39-5

National Haemophilia Registry of China – a developing registry

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Background: In Jaunary 2010, as a result of continuous efforts to raise the awareness at authority level, the ministry of health of china launched the Haemophilia data management system project.
Aims: The aim of this summary is to describe the development of the China National Haemophilia registry.
Methods: National hemophilia registry of China is a registry to collect the information of patients with hemophilia or other inherited bleeding disorders. It's a government authorized, medical centres participated registry. One national registry centre and 31 provincial centres were established. National hemophilia registry centre(Blood disease hospital, PUMC) is responsible for merging, analyzing the data centralized from all provincial centre. All the provincial registry centres are medical centres and responsible for collecting and transfer the data of whole province using both stand-alone entry software and web-based entry programme protected by password. The National identification number is used as the unique ID and marker for routine duplicate management.
The system will generate a general data and presented on web-site for public. The privacy of patients entered would be fully preserved during registered.
Result: The data analyzed here are from January 2010 to December 2012, total 10,652 patients entered in the registry, including 9055(85%) hemophilia A(HA) patients, 1298(12.2%) hemophilia B (HB) patients, 54(0.5%) vWD patients and 245(2.3%) patients with rare bleeding disorders.
There are 6600(72.9%) severe, 1691(18.7%) moderate, 668(7.4%) mild and 96(1%) unknown type HA and 874(67.3%) severe, 290 (22.3%) moderate, 124(9.6%) mild and 10(0.8%) unknown type HB patients enrolled in the registry. Age distribution of patients <13-year old is 27% for HA and 24% for HB, between 14 and 18-year old is 11% for both HA and HB, between 19 and 60-year old is 56% for HA and 58% for HB, more than 60- year old is 2% for HA and 4% for HB. At last, 4% HA patients and 3% HB patients do not have age data.
Among 1345(15%) HA and 243(19%) HB patients have HBs-Ag data, 80(5.9%) HA and 17(7%) HB patients have positive result. Among 1273(14%) HA and 235(18%) HB patients have HCV-Ab data, 171 (13.4%) HA and 31(13.2%) HB patients have positive result. There are 31 HA and 1 HB patients detected with HIV-Ab, constitute 2% and 0.4% of patients who have HIV infection data.
Refer to the first bleeding age, 6437(71.1%) HA and 246(19.0%) HB patients presented before 2-year old, 1718(20.0%) HA and 277(21.3%) HB between 2 and 7-year old, 654(7.2%) HA and 134(10.3%) HB between 7 and 18-year old, 246(2.7%) HA and 72(5.6%) HB older than 19-year old.
Summary/Conclusions: The national hemophilia registry comprises a great deal of data on variant aspect of patients. However it also has some shortcomings need to be updated or modified in future to obtain more precise and dynamic information of patients. For example, because the stand-alone software cannot be allied with electronic chart used by variant centres, the data cannot be updated in time. We will continue update the system to make it more useful in improving healthcare condition of China.

PB 4.39-6

Comparison of a ELISA FVIII inhibitor assay with the Nijmegen Modified Bethesda assay in patients with inherited and acquired haemophilia A

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Backgound: FVIII inhibitors are usually detected and quantified using Nijmegen modified Bethesda (NMB) assays or enzyme-linked immunosorbent assays (ELISAs). Current UKHCDO guidelines recommend testing patients with severe haemophilia after a wash out period of 24 h and in those patients with mild or moderate haemophilia a heat inactivation step of patient plasma can be used prior to testing.
Aims & Methods: To compare NMB assay in heat inactivated plasma samples (90 min @ 56 °C) with ELISA method (FVIII Antibody Screen – GTI Diagnostics, USA) using normal plasma. Patients were included even if the trough factor VIII level, either due to endogenous or treatment related FVIII was >1 IU/dL.
Results: In total 160 samples were tested {including patient samples with severe HA (n = 82), Moderate HA (n = 7), mild HA (n = 18), and acquired HA (n = 51)}. Of 160 samples, 103 were negative by NMB assay and of these seven were positive by the ELISA inhibitor assay (two with severe HA, three with mild HA and one with acquired HA). 57/160 patient samples were NMB positive of which 10 samples were negative by the ELISA inhibitor assay. All 10 samples were from four patients with acquired HA and had NMB levels ranging from 0.7 to 2.2 BU and FVIII:C levels ranging from 40 to 109 iu/dL. The 51 samples with acquired HA came from 12 patients in whom FVIII levels ranged from <1 to 118 iu/dL and NMB unit levels that ranged from 1 to 73,000 BU.

Discussion: 4% of samples in this study showed a discrepancy with the NMB assay with higher positivity by ELISA. Low titre inhibitors are not uncommon in severe haemophilia A and it is possible that the ELISA detects FVIII inhibitors at an earlier point than the NMB assay or that they are non-neutralising FVIII antibodies and not significant. The negative ELISA results in the acquired HA samples but positive with NMB assays is hard to explain, and it may be related to epitopes not recognised by the ELISA. Heat inactivation of plasma from patients with acquired HA for use in the NMB assay is useful especially when FVIII levels are within the reference range (>50 IU/dL), as it allows detection of low titre FVIII antibodies and therefore it may help decide on the duration of immunosuppression before tapering and preventing relapses. In conclusion the value of both these assays should be explored with correlation to clinical endpoints.

PB4.40 – Heparin-induced thrombocytopenia (HIT): Clinical – III

PB 4.40-1

Frequent off-label use of fondaparinux in patients with suspected acute heparin-induced thrombocytopenia (HIT) – final results from the GerHIT multi-centre registry study

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Background: In life-threatening HIT, alternative anticoagulation with a non-heparin anticoagulant is strictly recommended. However, off-label use with fondaparinux has been reported in the literature.
Aim: The aim was to collect data on the 'real-life' management of patients with suspected acute HIT.
Methods: In a national multi-centre registry study, the 4T's scores for the clinical probability of HIT were determined retrospectively in 261 patients diagnosed with HIT between 01/2005 and 10/2009. Only patients with a score of ≥4 points and treatment with at least one dose of argatroban (A), lepirudin (L), danaparoid (D), or fondaparinux (F) were included for evaluation of laboratory diagnostic strategies and prescription practices of anticoagulants.
Results: 195 patients were included. The 4T's scores were 4/5/6/7/8 points in 46 (23.6%)/50 (25.6%)/74 (38.0%)/13 (6.7%)/7 (3.6%) patients, respectively. The median treatment duration before HIT was 6.0 (unfractionated heparin [UFH]), 7.0 (low-molecular weight heparin [LMWH]), and 10.0 days (UFH/LMWH). During heparin therapy, 53 (27.2%) thromboembolic events, 5 (2.6%) skin lesions, 1 (0.5%) amputations, 24 (12.3%) Hb-relevant bleedings, and 2 (1.0%) deaths occurred. A functional heparin-induced platelet activation assay was performed in 96.9%, an immunogenic PF4/heparin-dependent ELISA was performed in 89.2%, a particle gel immunoassay was used in 12.3%, and a serotonin-release assay none of the patients. The alternative monotherapy strata were: argatroban in 16.4%, lepirudin in 2.1%, danaparoid in 23.6%, fondaparinux in 40.0% of the patients; the sequential therapy strata were: AF in 5.6%, DA in 5.6%, DL in 2.1%, and DFL in 0.5% of the patients.
Summary/Conclusion: The laboratory diagnostic strategy for HIT in Germany is mostly (>96%) based on the recommended 2-step strategy (immunogenic plus functional assay). Regarding therapeutic approaches, fondaparinux is widely applied off-label in up to 50.3% of the patients with acute suspected HIT and a high clinical pretest probability (median 4T's score: 6 points).

PB 4.40-2

Apixaban as an alternate anticoagulant for the management of patients with heparin-induced thrombocytopenia

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Background: Heparin-induced thrombocytopenia (HIT), an immune mediated disorder due to antibodies generated against platelet factor 4 (PF4) complexed with heparin, is associated with a pronounced hypercoagulable state, thrombin generation, endothelial cell damage, and upregulation of an inflammatory state. Anticoagulant alternatives to heparin that do not interact with HIT antibodies are needed for anticoagulant management. Intravenous use direct thrombin inhibitors have been proven effective but their use is associated with a bleeding risk and drug-specific limitations. For prevention and long-term anticoagulation, oral warfarin is used despite its shortcomings including protein C reduction and skin necrosis. Fondaparinux is used off-label by some. Apixaban is a new small molecule, direct acting oral FXa inhibitor that may be considered for the anticoagulant management of patients with HIT.
Methods: In order to determine if there is a lack of functional platelet activation for apixaban in the presence of HIT antibodies, the two traditional and widely used clinical laboratory tests for the diagnosis of HIT were utilized: the ^{14}C-Serotonin Release Assay (^{14}C-SRA; using washed platelets) and the heparin-induced platelet aggregation assay (PA-HIT; using platelet rich plasma). HIT antibodies from multiple patients and platelets from different donors were employed to assure the robustness of the data. The response to apixaban concentrations covering the clinical dose range (0.05–50 mg/mL) was compared to the response obtained with unfractionated heparin (UFH; 0.1 and 100 U/mL).
Results: In the ^{14}C-SRA (n = 35) and in the PA-HIT (n = 37), only baseline negative platelet activation and aggregation responses with all HIT specimens were observed at all apixaban concentrations (average across all concentrations: 11 ± 4% serotonin release and 8 ± 3% aggregation, respectively; mean ± SEM; positive responses are >20%). In comparison, UFH gave strong positive responses to each of the same HIT antibody specimen/platelet donor combinations (82 ± 3% release and 78 ± 6% aggregation at 0.1 U/mL; $P < 0.01$ vs. apixaban). Comparative studies demonstrated strong responses for enoxaparin (n = 10; 73 ± 5% release and 62 ± 7% aggregation at 10 mg/mL) equal to UFH and no positive response for fondaparinux (n = 20) in both test systems.
Conclusions: This study demonstrated a consistent absence of platelet activation with apixaban in the presence of HIT antibodies using two different methodologies. Based on the inert response of apixaban in these *in vitro* studies and because this drug is structurally unrelated to heparin, apixaban is not expected to contribute to the propagation of the HIT syndrome. Since apixaban is an inhibitor of thrombin generation it is expected to have an additional benefit in blunting the hypercoagulable state observed in HIT. Apixaban may provide an option for oral anticoagulation in patients with HIT, particularly for the extended management and prevention of HIT. Clinical trials to determine safe and effective anticoagulation regimens during the various clinical phases of HIT are warranted.

PB 4.40-3

Rapid exclusion of the diagnosis of immune HIT by AcuStar HIT and heparin-induced multiple electrode aggregometry: a prospective monocenter study

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Background: Immune heparin-induced thrombocytopenia (HIT) is a severe adverse reaction of heparin treatment, potentially leading to life-threatening thrombosis. Early and accurate diagnosis is essential but remains challenging. The combination of an immunological and a functional assay improves HIT diagnosis. We have recently shown, in a retrospective study on 106 inpatients in CHU UCL-Mont-Godinne-Dinant, Belgium, that the combination of 4T's rule, AcuStar HIT (immunological assay) and heparin-induced multiple electrode aggregometry (HIMEA) (functional assay) with optimized cut-offs is useful for rapid and accurate diagnosis of immune HIT. This led to a proposal of diagnostic algorithm for immune HIT based on 4T's rule, AcuStar HIT, and HIMEA. Briefly, when 4T's score is ≤3, no additional laboratory test is required because HIT is considered very unlikely. When 4T's test score is >3, AcuStar HIT is performed: (i) when positive, HIMEA is performed; (ii) when negative, HIT is considered very unlikely. In case of negative Acustar HIT and 4T's test score is >6, HIMEA is performed.

Aims: The primary objective of this study was to explore prospectively the performances of our optimized diagnostic algorithm on inpatients suspected of HIT. The secondary objective was to evaluate performances of 4T's rule, AcuStar HIT and HIMEA in comparison to the clinical outcome.

Methods: This study initially included 99 consecutive unselected inpatients with clinically suspected immune HIT. 4T's rule was calculated. AcuStar HIT-Ab (PF4-H), AcuStar HIT-IgG (PF4-H), and HIMEA tests were performed, and clinical outcome collected. AcuStar HIT-IgG (PF4-H) and HIMEA were performed in case of positive AcuStar HIT-Ab (PF4-H) or 4T's rule >6. Sensitivity and specificity of 4T's rule, AcuStar HIT-Ab at manufacturer's cut-off and at our cut-off as well as the overall diagnostic strategy were calculated using clinical outcome as the reference.

Results: One patient had the diagnosis of immune HIT. Among the 99 patients, 58 presented a low pre-test probability (≤3/8). None of these 58 patients had immune HIT. AcuStar HIT-IgT (cut-off >9.41) presented one false positive, also positive with AcuStar HIT-Ab (cut-off >2.89). The negative HIMEA allowed diagnostic exclusion. Among the 41 patients presenting a medium-high pre-test probability (>3/8), AcuStar HIT-IgT was 100.0% concordant to the clinical outcome. The only patient affected by immune HIT was positive on AcuStar HIT-IgT, AcuStar HIT-Ab and HIMEA. AcuStar HIT-IgT at our cut-off (>9.41) and at manufacturer's cut-off (>1.00) both showed sensitivity of 100.0% with a specificity of 99.0% and 89.8%, respectively. Sensitivity and specificity of 4T's rule are 50.0% and 58.8%. Overall, our optimized diagnostic algorithm showed sensitivity and specificity of 100.0% in this patient cohort.

Conclusion: We demonstrated in this prospective study that our optimized algorithm is a rapid and accurate diagnostic tool to exclude the diagnosis of immune HIT.

PB 4.40-4

Prospective comparison of the HIT expert probability (HEP) score vs. the Warkentin's 4T's score in a quaternary care center

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Background: Heparin-induced thrombocytopenia (HIT) can be an elusive, difficult diagnosis when multiple comorbidities exist; a common issue in patients of tertiary and quaternary care centers. Currently, clinicians' routinely employ the Warkentin's 4T's score to assess and quantify the pre-test probability of actual HIT. More recently, the novel Heparin Expert Probability (HEP) score has been developed in hopes of supplanting the 4T's score as a model that is easier to use and more predictive of HIT.

Aim: To prospectively evaluate and compare Warkentin's 4T score vs. the HEP score and their correlation with (i) PF4 optical density and (ii) serotonin release assay (SRA).

Methods: From May 2011 to February 2012, we evaluated 453 consecutive HIT lab tests. We further evaluated 49 patients (86 cases) that exhibited positive polyspecific PF4 assays (OD >0.40) with 4T's score, HEP score, and serotonin release assays (SRA). Residents rotating on the coagulation service determined the 4T's/HEP scores, which were later confirmed by one of two coagulation consultants.

Results: For PF4 OD = 0.41–0.99, the mean and 95% confidence interval (CI) for 4T's/HEP scores were 3.2 ± 0.7 and 2.8 ± 1.1, respectively. For PF4 OD = 1.0–1.99, the mean and 95% CI for 4T's/HEP scores were 3.5 ± 1.2 and 2.5 ± 2.2, respectively. For PF4 OD ≥2.0, the mean and 95% CI for 4T's/HEP scores were 4.3 ± 2.0 and 4.6 ± 4.7, respectively.

For SRA-negative patients, the average 4T's/HEP scores were 3.3 and 2.2, respectively. For SRA-positive patients, the average 4T's/HEP scores were 4.4 and 5.0, respectively. The p-values for the comparison of the mean score between SRA-positive and SRA-negative individuals for 4T's/HEP scores were 0.13 and 0.088, respectively.

Discussion: The average 4T's score demonstrated an incremental increase with increasing PF4 optical densities. In contrast, the average HEP scores did not demonstrate an incremental increase with increasing PF4 optical densities (i.e. the average HEP score was lower for OD 1.0–1.99 than for OD 0.4–0.99 and did not demonstrate a positive correlation).

The HEP score did not distinguish between SRA-positive and SRA-negative patients (mean HEP score of 5.0 vs. 2.6, $P = 0.08$). Therefore, the HEP score is not more predictive of HIT than the 4T's score. This is in contrast to a previously published, retrospective study comparing HEP vs. 4T's as they relate to SRA results.

Since no differences were found between the HEP and 4T's score in their ability to correlate with SRA and because 4T better correlated with OD, we advocate for the use of the 4Ts score. Furthermore, the 4T score is easier to recall and relatively straightforward than the HEP score which can be complex and cumbersome.

PB 4.40-5

Cost-effective HIT diagnosis: utilizing IgG-specific PF4 assays reduces the number of confirmatory serotonin release assays without missing true HIT

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Background: Heparin-induced thrombocytopenia is a pro-thrombotic, potentially fatal entity requiring integration of clinical and laboratory data for diagnosis. No widely standardized laboratory approaches for diagnosis exist and there is debate as to whether a polyspecific or IgG-specific PF4 assay is superior for screening. The polyspecific assay has a low threshold to call 'positive' (OD >0.40) and may result in high false positive HIT diagnoses that can produce costly outcomes, includ-

ing confirmatory serotonin release assay (SRA) testing and/or treating non-HIT, thrombocytopenic patients with expensive direct thrombin inhibitors (DTIs) that carry a bleeding risk.

Aim: Compare polyspecific vs. IgG-specific anti-PF4/heparin ELISAs (PF4) to determine whether the IgG assay can reduce confirmatory SRA testing (expensive send-out with long turn around time), without missing true HIT, and/or unnecessary treatment with DTIs.

Methods: 453 HIT work-ups (4T's score, polyspecific PF4) were reviewed (05/2011–02/2012), including 86 work-ups on 49 patients with polyspecific OD ≥0.4. The manufacturer recommends reporting results as 'positive' (OD ≥0.4) or 'negative'. In our lab, we additionally report the numerical OD values: 'negative' 0–0.39; 'borderline' 0.4–0.84; 'positive' 0.85–2.79; 'strong positive' ≥2.80. For patients with OD >0.4, SRA testing and an IgG-specific PF4 was performed (GTI, PF4 kits).

Results: 29 patients had 'borderline' polyspecific PF4 (OD 0.4–0.84); none were SRA-positive; corresponding IgG PF4 was 0.07–0.73. Nineteen patients had 'positive' polyspecific PF4 (OD 0.85–2.79); 7 of 19 were SRA-positive; corresponding IgG PF4 was 0.29 to >3.0. One patient had 'strong positive' polyspecific PF4 (OD ≥2.80); one of one was SRA-positive; corresponding IgG PF4 was 1.86. HIT work-up was requested more than once in 15/49 patients; 1/15 converted from borderline to positive SRA.

Discussion: Laboratories performing PF4s should report the numerical OD values. Since most U.S.laboratories use the same kits (according to CAP surveys), we propose the following interpretive ranges for the IgG-specific PF4 correlating with the degree of suspicion for true HIT:

Negative: OD <0.4
Intermediate positive: 0.4 ≤ OD <1.0
Positive: OD ≥1.0

Previous literature suggested that an IgG-specific assay could miss rare cases of non-IgG-mediated HIT; however, in this study, cases with PF4 ≥ 1.0 by the polyspecific assay were also positive by the IgG assay. Therefore, the IgG assay did not miss any true HIT.

True clinicopathologic HIT diagnoses confirmed by SRA were made in 8 of 20 patients (40%) with positive polyspecific PF4. DTI was or could have been stopped after negative SRA results were obtained on three patients with strongly positive PF4 (by either polyspecific or IgG assay). Therefore, since PF4 is a screening assay (sensitive but not specific), an SRA is required to confirm HIT in such cases.

The use of IgG PF4 reduced 'borderlines' by 75% (22 of 29 borderline polyspecific PF4 were negative by IgG PF4). Had the IgG assay been used, these cases would not have required SRAs and 6 of 14 patients (43%) who had received DTI would not have received the drug.

In conclusion, utilization of the IgG-specific PF4 assay can help reduce the number of confirmatory SRAs performed and also reduce DTI utilization.

PB 4.40-6

Incidence of Heparin-induced thrombocytopenia in sick children who used unfractioned heparin for prophylaxis in intensive care unit

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Heparin is the standard therapy for the prevention of venous thromboembolism in children. Heparin-induced thrombocytopenia (HIT) is a well-known serious adverse effect related with heparin therapy or prophylaxis. Although HIT affects up to%5 of adult patients treated with unfractionated heparin (UFH), prospective studies are so rare in children in respect of incidence.

Aim of this study was to determine the incidence of HIT in UFH-exposed children for prohylaxis. Patients who used treatment doses of UFH were excluded. Only ICU patients were included. We performed a prospective single center phase-IV study in Ege University Children's Hospital after approved by Ethics Commitee and Ministry of Health. Children who exposed to UFH for any time in ICU service were eligible for study. Study group consists of 28 ICU patients. Mean age was 52 month (range: 1–204). Thirteen of patients were female and others were male. All patients used diluted UFH for flushing to maintain patency of central lines in the ICU.

Blood samples were collected for platelet counts (CELL DYN 3700 counter, Abbott), anti-Heparin/PF4 antibody (ELISA)(Asserachrom HPIA-Ig G, Diagnostica Stago) and Heparin-induced platelet agregation tests (APACT-4004, Tokra) before and after 10 days (median) (range: 7–15) of first UFH exposure.

After 2 years of prospective observation, we have not found any single patient diagnosed as HIT by clinical findings and confirmed by laboratory analysis. Significant dropping (>50%) of platelet count after 10 days of heparin exposure was observed in only four ICU patients. Only one patient had below <150.000/mm^3. However, ELISA and agregation tests were negative for these patients. Reasons for thrombocytopenia were evaluated for underlying medical conditions as trauma, pneumonia and sepsis. They were not evaluated as HIT. In one another ICU patient, ELISA test was found border-line positive (O.D: 1.374) (positive controls: 1.35 ± 0.34). However, no thrombocytopenia and no activation in agregation related heparin studies were found. This result was evaluated as false-positivity for ELISA. However, mean OD values for ELISA test was found significantly elevated after heparin exposure (before heparin: 0.119 ± 0.061 vs. after heparin: 0.258 ± 0.262)(Wilcoxon test; $P < 0.0001$). Elevated heparin-induced agregation response (more than >20% after heparin exposure) was not determined in any ICU patient.

In routine clinical practise, infants with requiring admission to ICU are particularly at risk of venous thromboembolism and likely to be exposed to heparins for prevention and treatment. However, in this single center phase-IV study, we have not found prospectively any HIT positive patient among 28 ICU patients. Reasons for low incidence may be dilution of heparin, lower and prophylactic doses, short exposure time and medical vs. surgical intervention. UFH Heparin seems to be safe agent for children with ICU for prophylaxis.

PB4.41 – Rare bleeding disorders – V

PB 4.41-1

Variant Bernard Soulier syndrome with ambiguous mutation data: a diagnostic dilemma

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Bernard-Soulier syndrome (BSS) is a rare autosomal recessive bleeding disorder, with an estimated frequency of 1:1,000,000, characterized clinically by mucocutaneous bleeding, post traumatic haemorrhage, and menorraghia in females. The classic laboratory findings are macrothrombocytopenia, absent ristocetin-induced platelet aggregation and absent or markedly reduced expression of the Glycoprotein Ib-IX-V complex on platelet surfaces. Genetic abnormalities have been identified in three of the genes encoding the sub units of the GP Ib-IX-V complex, *GP1BA*, *GP1BB* and *GP9*. No mutations causative of BSS have been reported in *GP5*, and this is consistent with a lack of requirement for GPV expression for expression of the other subunits of the GPIb-IX-V complex.

We present a young patient with a complex clinical history, in whom non classical laboratory findings complicate the diagnosis of BSS.

A 20 year old Zimbabwean female, with no family history of bleeding, had a long personal history of recurrent epistaxis, haematemesis and menorraghia. She also suffers from migraine, epilepsy and asthma. Initial investigations resulted in a presumptive diagnosis of ITP, and she received treatment on that basis.

She recently presented with severe acute abdominal pain, haematuria and haematemesis necessitating blood transfusion. Further investiga-

tive work-up revealed a macrothrombocytopenia and a markedly reduced platelet aggregation in response to ristocetin alone. A full panel of platelet glycoprotein expression studies showed normal levels of glycoproteins Iba and IX. This prompted molecular analysis of the *GP1BA, GP1BB & GP9* genes to confirm the diagnosis of BSS. The exons and splice junctions of the three genes were PCR amplified and sequenced, and the only variant detected was a homozygous c.488C>A substitution within exon 3 of *GP9*. This base change predicts the replacement of the native Alanine at codon 163 with an Aspartic Acid (pAla.163Asp). This change has not been previously reported as causative of BSS, but results of 5/6 *in silico* software algorithms support this change being pathogenic. It is, however, reported as a very low frequency polymorphism in the African population, with a Minor Allele Frequency of 0.0018. This frequency results from seven heterozygous individuals from a total of 2624: No individuals homozygous for c.488A were recorded.

The c.488C>A variant may represent the first pathogenic *GP9* substitution which produces mutant complexes that appear on the cell surface essentially at normal levels, in a similar manner to the *GP1BA* variant Bolzano (c.515C>T). Our findings demonstrate that, in cases of macrothrombocytopenia, reduced ristocetin induced platelet aggregation may be sufficient for a diagnosis of BSS to be made even in the absence of a reduction in glycoprotein expression. The vast amount of SNP data, generated by genome projects will increasingly present interpretive challenges in assessing variants of unknown clinical significance in specific patient populations. Further, the curation of SNP and locus specific mutation databases does not always favour the distinction between pathogenic and benign variants. In BSS, as in other autosomal recessive disorders, the finding of a small number of heterozygous individuals should not preclude the variant from being considered potentially pathogenic in the homozygous state.

PB 4.41-2

Mild bleeding diathesis in 10 patients due to functional deficiency of plasminogen activator inhibitor type 1 (PAI-1)

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Background: Plasminogen activator inhibitor type 1 (PAI-1) down-regulates fibrinolysis and plays an important role in maintaining homeostasis of the coagulation system. PAI-1 deficiency is one of the rarest bleeding disorders, described for the first time in 1989. Quantitative or qualitative PAI-1 deficiency causes hyperfibrinolysis and mild to moderate bleeding tendency, mainly mucocutaneous. The disorder is difficult to diagnose as available PAI-1 activity assays lack sensitivity in the lowest range. The true prevalence of this condition is not known.

Aims: The aim of this study was to identify cases of PAI-1 deficiency among patients referred to our clinic due to bleeding symptoms.

Methods: In every patient with a history of bleeding (spontaneous/excessive bleeds from at least two anatomical localizations or bleeds from one localization with family history of bleeds) standard coagulation test were performed to exclude common coagulation disorders: platelet count, APTT, PT, thrombin time, fibrinogen activity, von Willebrand factor activity (at least twice), platelet aggregation tests, factor VIII, IX and XI activity. If no abnormality was detected, PAI-1 activity was measured with Berichrom PAI (Siemens, Germany). In patients with decreased PAI-1 activity PAI-1 antigen level was measured with ELITEST PAI-1 (Hyphen BioMed, France). Alpha2-antiplasmin deficiency was excluded. Blood samples were drawn between 8 a.m. and 10 a.m. as PAI-1 plasma level shows a diurnal variation and reaches highest levels in the early morning.

Results: PAI-1 activity was measured in 93 patients and was decreased in 10 females (0–1.5 U/mL, mean 0.6 U/mL, normal range 2.0–7.0). Repeated measurement from separate plasma samples showed concordant results (0.2–1.4 U/mL, mean 0.8 U/mL). PAI-1 antigen level in all 10 patients was normal or moderately decreased (22–62 ng/mL, mean 39.5, normal range 30–60 ng/mL). All 10 patients suffered from menorrhagia, nose and/or gum bleeds and excessive bleeding post surgery, delivery or tooth extraction. Eight suffered from mild iron deficiency anaemia. Four patients required blood transfusion at least once in their life. Nine patients had positive family history for bleeds. Tranexamic acid treatment was tried in six patients with good results.

Summary/Conclusions: PAI-1 deficiency should be taken into consideration in the differential diagnosis of bleeding patients. Results must be interpreted with caution, however, until standardized diagnostic assays will be available. We can suspect, that bleeding symptoms in our patients are caused by PAI-1 functional defect. Good response to antifibrinolytic treatment supports this hypothesis.

PB 4.41-3

Congenital Factor XIII deficiency in women; a systematic review of literature

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Background: Factor XIII deficiency is a rare congenital bleeding disorder. There is a paucity of data in the literature about obstetrics and gynecological problems in women affected with Factor XIII deficiency.

Aims: To examine gynecological problems and obstetrical complications and outcome in women with congenital FXIII deficiency

Methods: An electronic search was performed to identify the published literature on PUBMED, EMBASE, BIOSIS Previews, Journals @OVID and CINAHL Plus databases using the following keywords: 'congenital factor XIII deficiency' AND 'women OR Pregnancy'. A total of 34 relevant articles were found and included in this systematic review; 23 case reports and 11 case series dating from 1964 to 2012.

Results: A total of 120 women were identified. Menorrhagia (28%) was the second most common bleeding symptom. Ovulation bleeding was reported in 10% of women. Among 60 women, 189 pregnancies were reported; of these, 125(66.2%) resulted in a miscarriage with history of recurrent miscarriage observed in 16(27%) women. Among the 129 pregnancies without prophylactic therapy, 122 resulted in a miscarriage and only seven progressed to viability stage. Out of 64 pregnancies reaching viability status, ante partum hemorrhage occurred in four pregnancies while postpartum hemorrhage was seen in 15 (23%) of cases.

Conclusion: Women with congenital Factor XIII deficiency suffer significant bleeding complications. Menorrhagia and ovulation bleeding are common gynecological problems and possibly more prevalent than reported. Pregnancies in women with Factor XIII deficiency have a significant risk of miscarriage, placental abruption and postpartum hemorrhage if not on prophylaxis treatment.

PB 4.41-4

Solvent-detergent plasma abolished severe bleeding tendency and normalized procoagulant activity in Calibrated Automated Thrombogram both in plasma and platelets of a FV deficient patient

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Background: Congenital FV deficiency is a rare (1:1,000,000) bleeding disorder with varying bleeding tendency. Our patient, a 43-year-old

woman got diagnosis of FV deficiency at the age of 6 months. She has suffered from severe bleeding symptoms, including mucosal, intra-abdominal and muscular bleeds and hemarthrosis. The bleeds have occurred cyclically about every 10–14 days. For the last 4 years, we have treated her with prophylactic solvent-detergent (S/D) plasma (Octaplas®, 2 units) infused every 2 weeks. The treatment has not shown any adverse effects and clinical efficacy has been excellent. She has not had any clinically relevant bleeds during prophylactic plasma replacement.

Aims: We studied coagulation variables and platelet functions before and after adminstration of S/D plasma.

Methods: Citrated blood samples were collected to assess, APTT, prothrombin time (PT, with Owren and Quick methods), thrombin time, antithrombin, FV, FVIII, fibrinogen, protein C and S, von Willebrand factor (VWF) (VWF:RCo and antigen). Thrombin generation was measured with calibrated automated thrombogram (CAT®, Thermo Labsystems) in platelet-poor (PPP) (1 pM or 5 pM TF) and platelet-rich plasma (PRP) (1 pM TF) without adding corn trypsin inhibitor. Agonist (collagen, ADP, arachidonic acid, TRAP) -induced platelet aggregation (Multiplate®) and platelet function analyses (PFA-100®) were performed in whole blood. The PFA® was cross-supplemented with normal PPP.

Results: At baseline 2 weeks after plasma infusion the patient had undetectable FV activity, APTT >100 s, normal PT (131%) with Owren but highly elevated (INR 8.0) with Quick method (Coagucheck®). All other coagulation test results and Multiplate® were normal. In PFA collagen/ADP-triggered closure time (CT) (mean 131 s, SD ± 19, $n = 8$) was prolonged while collagen/EPI-triggered CT (mean 135 s, SD ± 24, $n = 9$) was normal. Thrombin generation was completely undetectable in CAT® both in PPP and PRP. After S/D plasma infusion (at 2 h) FV inclined to 12–18%, but this was enough to normalize APTT (33 s). In whole blood INR measured with Coagucheck (with Quick method) decreased from 8.0 to 1.7–1.9. Collagen/ADP-triggered CT in PFA® remained somewhat elevated and unchanged after plasma infusion. Supplementation of patient plasma with normal PPP corrected the prolonged collagen/ADP-triggered CT in PFA. Intriguingly, simultaneously thrombin generation increased above that of normal control both in PPP and PRP. Specifically, in PPP the thrombin peak increased 1.6-fold and in PRP time to peak shortened.

Conclusions: After administration of regular prophylactic S/D plasma replacement therapy in every 2 weeks abolished the severe bleeding tendency without any adverse effects. The patient did not experience any bleeds. Although plasma infusion increased FV activity only to 12–18%, less than claimed for the threshold for proper hemostasis, it normalized APTT and in CAT® reversed the FV-associated deficiency in plasma and PRP of the patient. Mere factor V coagulation activity assessment failed to associate with clinical efficacy of the regular S/D plasma supplementation.

PB 4.41-5

An international systematic study for the assessment of bleeding phenotype in platelet type von Willebrand disease

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Background: Rare bleeding disorders such as platelet type von Willebrand disease (PT-VWD) pose diagnostic challenges and remain under-reported. Definitive information about the presenting bleeding symptoms and the need for haemostatic support during times of haemostatic challenge such as pregnancy, childbirth and surgery is lacking. Specific guidelines regarding the diagnosis and treatment together with appropriate patient monitoring are compromised by the limited understanding of the clinical phenotype. Quantitation of bleeding symptoms using a standardized bleeding questionnaire that produces a valid bleeding score (BS) has proven to be useful in the diagnosis of mild bleeding disorders. So far, there have been no systematic studies to evaluate the bleeding phenotype in PT-VWD.

Aim: To highlight the importance of bleeding score in assessing bleeding phenotype and its variability in PT-VWD patients together with systematic assessment of the laboratory phenotype. The study also Aims to investigate the value of an electronic bleeding questionnaire (eBQ) in international studies on rare bleeding disorders.

Methods: An electronic version of the condensed MCMDM-1VWD questionnaire was developed as a web application using the PHP scripting language and MySQL relational database platform. Security was ensured by using password protection, database encryption and encrypted transfer protocols. Electronic questionnaire responses collected via the application were validated to ensure congruency with the MCMDM1-VWD scoring key and thus maintain the same specificity and sensitivity as the original questionnaire. Internal validation and archiving of data at the point of entry eliminated transcription/interpretation error and allowed automatic computation of the BS upon completion of the questionnaire. A call for study participation was made available on ISTH website and individual participation was invited by emails sent to potential collaborators. Each investigator was provided with a unique ID and passcode to fill the online eBQ during a patient's clinic visit (http://pt-vwd.org/ebq/). Full systematic laboratory analysis will be performed on plasma obtained from PT-VWD patients worldwide including conventional haemostatic tests, specific VWF related tests as well as thromboelastography.

Results: To date, five PT-VWD patients (1 male and 4 females) from four countries representing different platelet GP1BA mutations: M239V (1 patient), G233V (2 patients) and D235Y (2 patients) have participated. The average age of participants was 46.4 (range: 34–67). The bleeding scores were variable (mean = 9.6, range: 3–17). Variability was seen in BS even in patients with similar mutations and appeared independent of patient age. Post-operative bleeding occurred in 3 (60%) patients. Menorrhagia was not the most common symptom in female patients. One patient experienced gastrointestinal bleeding without additional mucocutaneous bleeding symptoms. The eBQ was tested in 50 normal females, three of which showed abnormally high scores and are currently under investigation for VWD type 1.

Conclusions: The eBQ helps standardize recording of phenotypic data related to PT-VWD patients and objectively assesses the bleeding phenotype. This is not only of value in PT-VWD, but also in patients with other suspected rare bleeding disorders. Automatic computation of the BS improves efficiency in terms of time and resources for international studies.

PB 4.41-6

Long-termed secondary prophylaxis in rare factor deficiencies of children in eight cases

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Rare factor deficiencies (RFD) cover the deficiencies of fibrinogen, Factor II, Factor V, Factor V + VIII, Factor VII, Factor X, Factor XI, Factor XIII, and vitamin K associated factors. Many national and international study groups conducted studies regarding the diagnosis and treatment of rare factor deficiencies in 2000s. The existence a mortality and morbidity risk associated with severe bleedings has brought the search for prophylaxes related to these disorders into the agenda. In addition, the good results obtained with primary and secondary prophylaxes in haemophilia cases have attracted the attention to the possibility that the same consideration could also be applied to rare factor deficiencies. Prophylaxis is recommended in these patients if such severe bleedings as central nervous system bleedings, gastrointestinal bleedings, and articular hemorrhages recur. Our prophylaxis application related to eight congenital rare

factor deficiency cases being followed at our clinic comprising of five patients with Factor VII deficiency, one patient with afibrinogenemia, one patient with FV deficiency, and one patient with Factor X deficiency were evaluated in our article. Application ages of our patients ranged between 2 weeks and 7 years of age. Six of them were boys and two were girls. Prophylaxis was applied due to intracranial bleeding to three of our Factor VII deficiency patients, for gastrointestinal system bleeding to one of them, and for the development of chronic hemarthrosis and hemarthrosis that requires the application of radioisotope synovectomy to one patient. 20 mcg/kg active recombinant FVII was used once a week in the prophylaxis of our FVII deficiency cases. Transition to two doses a week was required for one patient. Prophylaxis is being applied with 30 IU/kg prothrombin complex concentrate 2 days a week to our patient with Factor X deficiency for recurring central nervous system bleedings and with 50 mg/kg fibrinogen every 2 weeks to our patient with afibrinogenemia for recurring intramuscular bleedings causing severe pain and difficulty of walking. Our patient with FV deficiency to whom prophylaxis is applied due to intracranial bleeding, on the other hand, receives 20 mL/kg fresh frozen plasma once a week. The prophylaxis periods of our patients range between 10 months and 7 years. The observation as well as clinical and laboratory findings of our patients together with the details related to their prophylaxis processes were presented in our proceedings accompanied with the relevant literature.

PB4.42 – Rare bleeding disorders – V

PB 4.42-1

Health-related quality of life and Caregivers' burden in partners of persons with haemophilia

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Background: Assessment of HRQoL in haemophilia is important on one hand to provide information for clinical decision making and on the other hand to verify which impact has haemophilia on patients but also on their partners. Advances in haemophilia care and prophylactic treatment throughout life have improved HRQoL for patients and the older haemophilia population has different needs compared to the younger population. In countries with a long tradition of prophylaxis, patients with haemophilia have nearly reached the same life expectancy as the general population and have to face the same problems related to co-morbidities and cognitive impairments as the general population. These co-morbidities may influence the HRQoL of haemophilia patients; on the other hand these problems are supposed to have a further impact on the burden of partners of haemophilia patients in terms of financial, physical and psychological aspects.
Aim: A cross-sectional single-centre cohort study in partners of adult haemophilia patients was performed in Malmö, Sweden aiming to assess the burden of the disease on partners of haemophilia patients. The study was approved by the Regional Ethical Review Board in Lund, Sweden.
Method: Study subject-rated outcomes were filled in by partners (SF-36, Visual Analogue Scale of Interference, Caregivers' Burden Scale) and haemophilia patients (SF-36, VAS of Interference, Haemo-QoL-A, HEP-Test-Q).
Results: A total of 108/150 eligible partners of haemophilia patients (72%) participated. Mean age for partners was 44.7 years and for patients 47.1 years. The majority of couples were married (65.7%). In average couples were together since 19.8 years and had in average 1.7 children. Patients and their partners reported in general a quite similar HRQoL; by contrast partners reported significant better HRQoL in the domains 'physical functioning' ($P < .001$) and 'general health' ($P < .031$). No significant difference compared to the General Swedish population for the respective age group 45–54 years was found.
Partners of haemophilia patients across different severities reported lower HRQoL in the domain 'emotional role' of the SF-36 ($P = <.041$), with highest impairments for partners of moderately affected patients.
Interference of haemophilia with daily life measured by means of the VAS showed significant difference between partners and patients, ($P < .001$); partners reported less interference with their daily life (Mean = 12.32) compared to patients (Mean = 23.10).
Partners reported in general low burden of haemophilia in the Caregivers Burden Scale. No differences across haemophilia severity groups were found. 'Emotional involvement' was the biggest burden in the mild and moderate group, while in the severe group 'general strain' was the biggest burden. In addition 'isolation' was a big burden in moderate partners.
Conclusion: Partners of haemophilia patients reported in general good HRQoL and a low burden of the disease except for partners of moderate patients where a decreased HRQoL and higher burden was reported. In Sweden patients start prophylaxis at an early age with lifelong treatment which is probably the reason for low impact of the disease in partners seen in this study. For partners to a PWH with severe joint problems, a higher impact of the disease in partners may be foreseen.

PB 4.42-2

Congenital FXI deficiency: evaluation of bleeding phenotype and correlation with FXI activity (FXI:Act)

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Background: Bleeding phenotype in FXI deficiency is variable and generally related to surgery/trauma. Moreover, there is a poor correlation between bleeding and baseline FXI:Act.
Aim: To retrospectively describe the hemorrhagic phenotype of our FXI deficient population and to relate the phenotype with FXI:Act.
Patients and Methods: Since 1973, we have been following 94 FXI deficient patients from 65 different families: 43 F, 51 M; diagnosis median age: 28.7 years (0.9–83.9); median follow-up: 0.9 years (0.1–36.2); median FXI:Act of all patients: 38% (range 0.5–69%; normal values: 70–140); FXI:Act ≤1% in five patients, >1 ≤ 5% in 12, >5 ≤ 10 in 3, >10 ≤ 20 in 6, >20 ≤ 70 in 68. Excessive bleeding is reported as described in medical records.
Results: Fifty six patients experienced bleeding episodes not surgery-related: ecchimoses in 27, hematomas in 2, epistaxes in 23, gastrointestinal hemorrhages in 14, meno-methrorrhagia in 2, hematuria in 4, post-traumatic intracranial hemorrhage in 1, gum bleeding in 1, pulmonary bleeding in 1.
Prior to diagnosis, 64 patients underwent 133 surgeries (93 major, 40 minor). Prophylactic treatment was administered in 3/133 procedures: tranexamic acid (TA) in 1, fresh frozen plasma (FFP) in 2. Twenty eight/133 (21%) post-surgery hemorrhages were reported in 19 patients; in 12/28 cases, transfusional therapy (FFP and/or red blood cells units) was needed. Median FXI:Act of bleeder patients was 28% (0.5–53%). Twenty nine spontaneous deliveries (SD) and eight caesarian sections (CS) were performed without prophylaxis: 4 post-partum hemorrhages occurred (patients FXI:Act: 2, 6, 27, 52.3% respectively). In three cases transfusional therapy was necessary. Forty nine patients underwent dental surgeries without prophylaxis: 17 experienced haemorrhages at least after one procedure (bleeders median FXI:Act: 11%, [1–57%]). In one case FFP was necessary to stop the bleeding.
After diagnosis, 23 patients underwent 34 surgeries (14 major, 20 minor). Prophylactic treatment was administered in 23/34 procedures: TA in 7, FFP in 2, desmopressin in 4, FFP + TA in 8, desmopressin + TA in 2. The only bleeding reported (1/23, 4%) was after an

emergency appendectomy performed under TA administration, in a patient whose FXI:Act was 2.8%. In 2/11 surgeries performed without prophylaxis, an excessive bleeding was reported but transfusional therapy was not necessary; FXI:Act was 29% for both bleeder patients. Four SD and five CS were performed with prophylaxis: FFP in 4, TA in 2, desmopressin in 3. No post-partum hemorrhages occurred. Sixteen patients underwent 20 dental surgeries; prophylactic treatment was administered in 15/20 procedures without any bleeding complications: TA in 6, FFP + TA in 4, FFP in 4, desmopressin + TA in 1. During two dental procedures performed without prophylaxis, patients bled excessively; their FXI:Act was 6% and 45% respectively.
Conclusions: We confirm the wide variability in bleeding phenotype in FXI deficient patients, not related to the FXI:Act levels. Moreover we highlight that a good management of prophylaxis treatment dramatically reduces the percentage of bleedings in case of surgery (21% vs 4%), deliveries and dental procedures. Because of the low correlation between FXI:Act and the phenotype, we highlight the need of laboratory-based prognostic factors for a better management of these patients.

PB 4.42-3
A novel mutation in downstream half of wasp gene correlates with worse clinical presentation of the Wiskott-Aldrich syndrome

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Background: Wiskott-Aldrich syndrome (WAS) is a rare recessive disease characterized by thrombocytopenia, eczema, recurrent infections, microthrombocytopenia and an increased risk of autoimmunity and malignancy. In this syndrome the gene of Wiskott-Aldrich protein (WASP) is affected; it is located on chromosome X-p11.22-p11.23, encomprises 12 exons and encodes for a protein of 502 amino acids. WASP is selectively expressed in hematopoietic stem cells and is involved in cell signaling and reorganization of the cytoskeleton. Mutations in the WASP gene may also be associated with other mild manifestations such as X-linked thrombocytopenia (XLT) and X-linked neutropenia (XLN).
Aim: To correlate the mutational status of the WASP gene with clinical manifestations in Wiskott-Aldrich syndrome.
Patients and Methods: In this study, we collected peripheral blood samples from three patients and their mothers, performed genomic DNA extraction from the leukocyte pool and sequenced WASP gene.
Results: Case 1. JCM, male, 4 years old, with mild hemorrhagic manifestations since 9 months old, with no family history of bleeding or thrombocytopenia. He had platelet counts ranging from 23 to 208×10^9/L and low mean platelet volume (MPV), ranging from 5.6 to 6.5 fL (lower normal limit: 7 fL). He did not show any evidence of immunodeficiency or eczema (WAS clinical score: 1). Molecular analysis of WASP gene identified a missense mutation in exon 2, resulting in an amino acid change (p.T45M). Both the clinical presentation and the location of the found mutation (exon 2) are compatible with the diagnosis of X-linked thrombocytopenia. Case 2. JCF, male, 18 months old, with severe thrombocytopenia ($<20\times 10^9$/L) and severe hemorrhagic history, frequently requiring platelet transfusions. He also displays serious immunodeficiency and extensive eczema. Upon molecular investigation, it was detected the presence of p.E133K mutation in exon 4 of WASP gene. Case 3. LSMN, male, 6 months old, who has had scattered petechiae and recurrent gastrointestinal bleeding since birth with severe microthrombocytopenia. Since 4 months old he has eczema and recurrent infections. This child has a family history of a brother who died at 6 months old with similar bleedings and recurrent infections. Molecular assessment of WASP gene revealed a mutation that has not been previously described, with an insertion in intron 11, leading to involvement of exon 11.
Summary/Conclusion: WAS implicates a spectrum of clinical manifestations ranging from mild thrombocytopenia or neutropenia to severe cases of microthrombocytopenia, eczema, immunodeficiency, and increased susceptibility to autoimmune diseases and cancer. Generally lighter manifestations of the syndrome are associated with mutations in exons 1 and 2 of WASP gene, as observed in the first case described. More severe manifestations, such as those observed in cases 2 and 3, are caused by mutations affecting other regions of the gene, occurring predominantly in its downstream half. Thus, this new mutation found in case 3 and the clinical presentation of this patient are in agreement with previous data, confirming that the clinical phenotype of WAS is strongly influenced by the effect of the mutation in WASP gene.

PB 4.42-4
Changes in FXIII levels during various stages of pregnancy

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Background: Coagulation factors show significant changes during pregnancy, most factors showing a progressive increase until the end of gestation. There is only limited data on factor XIII level, its reference range and its variation during pregnancy.
Aims: To assess changes in factor XIII and establish the reference range for factor XIII activity during normal pregnancy.
Methods: A cross sectional study was performed on 376 women who were at various stages of normal non-eventful pregnancies. Blood samples were collected during first trimester (gestational age 0–12 weeks, $n = 116$), second trimester (gestational age 13–28 weeks, $n = 132$) or third trimester (gestational age 29–42 weeks, $n = 128$). Factor XIII was assayed on a CS-5100 analyser (Sysmex UK Ltd, Milton Keynes, UK) using a chromogenic reagent (Berichrom FXIII chromogenic assay kit. Siemens Healthcare Diagnostics, Marburg, Germany). The manufacturers declared normal reference range for this reagent is 70–140 μ/dL. Data from the clinical samples were found to be normally distributed, therefore, reference ranges were calculated using the mean and two standard deviations.
Results: The mean +/− SD (observed range) Factor XIII activity was 111.7 ± 29(57–186) IU during first trimester, 96 ± 26 (38–162) IU during second trimester, and 83.4 ± 21(27–142) IU during third trimester. 95% Reference intervals of Factor XIII activity were measured during the first (54–169 IU; six results <70 IU), second (45–147 IU; 15 results <70 IU), and third (42–125 IU; 34 results <70 IU) trimester of pregnancy. There was a statistically significant reduction in the FXIII activity between each trimester of pregnancy ($P < 0.0001$; ANOVA).
Conclusion: Relative to the manufacturer's reference range, this study has shown that some women have a significantly decreased level of factor XIII activity during a normal uneventful pregnancy. However, further investigation of those women with apparently decreased FXIII levels is required before the reference ranges of FXIII in normal pregnancy can be properly established.

PB 4.42-5
Inhibitor in congenital factor VII deficiency, report of two cases

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Factor VII deficiency (FVIID) is a congenital rare autosomal recessive bleeding disorder with the incidence of 1/300,000 to1 in 500,000. We

present a two cases of congenital FVIID, who had received intermittent intravenous recombinant activated factor VII (rFVIIa) that has experienced factor VII inhibitor production. Factor VII deficiency is a life threatening bleeding disorder with prevalence of one symptomatic case out of half a million individuals. Fresh frozen plasma, prothrombin complex concentrates, factor VII concentrates and (or) recombinant activated factor VII (rFVIIa) are considered for treatment of factor VII deficiency.

We present two boys (4 and 8 years old) with congenital FVII deficiency. Both of them were diagnosed after intracranial bleeding during infancy and rFVIIa was used for their treatment. After a period of unresponsiveness to rFVIIa, inhibitor was found in the level of 191 and 170 BU respectively.

We used Feiba for the older case and controlled his musculoskeletal bleedings and the titer of inhibitor gradually decreased to <20 BU. Furthermore we performed full genetic analysis and found their mutations.

To the best of our knowledge this is the first report of inhibitor against factor VII among patients with congenital FVII deficiency. Moreover we suggest a successful therapeutic management in these cases.

PB 4.42-6

Laboratory assessment of Hermansky-Pudlak Syndrome: a case report

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Hermansky-Pudlak Syndrome (HPS) is a rare, autosomal recessively inherited disorder that is divided into seven subtypes (HPS 1–7) that share the following three symptoms: oculocutaneous tyrosine positive albinism, ceroid lipofuscinosis and a platelet disorder due to a δ-Storage-Pool deficiency leading to spontaneous bleeding episodes, especially epistaxis and mucocutaneous bleedings at a normal platelet count. Pulmonary fibrosis is a major complication in some types of HPS.

An 11-year old Russian girl presented with facial haematoma, oculocutaneous albinism and severely impaired sight. The parents reported recurrent epistaxis and haematoma.

Routine parameters showed normal PTT, normal FVIII- and von-Willebrand-Factor (vWF) levels and a normal *in vitro* bleeding time. Platelet analysis showed a rather normal platelet aggregation with a slight desaggregation in response to collagen and no aggregation in response to TRAP.

Fibrinogen binding was normal in response to ADP but pathological in response to TRAP, Mepacrine staining was reduced and *in vivo* bleeding time was longer than 15 min.

FACS analysis of platelet granules using mabs CD62P and CD63 showed a total lack of dense bodies but normal alpha granule content. ATP determination test showed a total lack of ATP after platelet activation confirming the absence of dense bodies. Thrombin generation in platelet rich plasma showed a reduced ETP and Peak.

A DDAVP challenge test was conducted resulting in a normalized *in vivo* bleeding time. FVIII and vWF-levels were elevated approximately threefold.

We conclude that not all laboratory methods are able to reveal a complex haemorrhagic disorder like HPS. For example *in vitro* bleeding time is not a possible screening parameter in case of HPS. Here, it is necessary to evaluate platelet function using specialized tests such as granules determination, fibrinogen binding in response to various agonists or ATP-determination in order to reveal the platelet dysfunction.

PB4.43 – Von Willebrand disease: Clinical – V

PB 4.43-1

The effectiveness of screening of exon 28 in patients with Type 3 Von Willebrand disease

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Type 3 Von Willebrand disease is a severe autosomal recessive bleeding disorder with almost undetectable levels of VWF. Its molecular diagnosis is particularly complex due to the large size of the VWF gene, with 52 exons and the existence of a highly homologous (>96% homology) pseudogene. Presently, 1157 VWD patients are registered in this centre's database, of whom 508 (43.9%) have been tentatively diagnosed as type 3 or severe VWD. The high prevalence of this type of VWD is due to consanguineous marriages practiced in Iran. Although molecular diagnosis of type 3 VWD by direct sequencing of the entire VWF gene is now carried out in developed countries, in developing countries with limited resources such as Iran, this is not as yet feasible.

In this study we report the results of the investigation of 155 unrelated patients from the type 3 VWD patients on our database, sequencing only exon 28 of the VWF gene, as this exon is the first step to genetically investigate any patient with severe VWD.

In this group, we have identified 15 (9.6%) p.Arg1659X nonsense mutations, 10 of whom are of Arab ethnicity. Pseudogene conversion was found in 7 (4.5%) patients having a p.Gln1311X nonsense mutation. Finally, five previously unreported mutations, including, one c.4710C>A substitution leading to p.Tyr1570X; three frameshift mutations in position 4029 due to a T deletion; one A insertion in position 3968, one A deletion in position 4308 and the entire deletion of exon 28, causing VWF inhibitor formation in one patient.

With this limited approach, we have managed to identify 27(17.4%) genetic defects in our type 3 VWD patients. In addition to facilitating genetic counselling and carrier detection in these families, these findings could also help in better understanding of the correlation between genetic defects and bleeding symptoms.

PB 4.43-2

Four novel von Willebrand factor mutations in mild von Willebrand disease

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Background: The likelihood to detect a causative von Willebrand factor (VWF) gene mutation in patients with von Willebrand disease (VWD) decreases significantly with increasing VWF levels >30 U/dL (Goodeve et al, 2007).

Aim of the study. We identified four novel VWF mutations in patients with mild VWF deficiency and a disproportionately significant bleeding tendency (bleeding score ³ 5).

Methods: The entire coding sequence and promoter region of VWF was screened by direct sequencing.

Results: One mutation occurred in the propeptide region (c. 430 G>A in exon 4 leading to G144S) in a patient with a type 2 N Normandy phenotype (FVIII:C 16 U/dL, VWF:Ag 47 and VWF:RCo 44 U/dL). Interestingly, desmopressin administration corrected VWF deficiency completely (VWF:Ag and VWF:RCo >130 U/dL), while FVIII:C remained below 50 U/dL, suggesting impaired VWF-FVIII binding. A second mutation (c.2878 C>T in exon 22 leading to R960W) occurred in the FVIII binding region of VWF. Basal FVIII:C was slightly

higher than VWF (FVIII:C 54 U/dL, VWF:Ag 40 and VWF:RCo 42 U/dL), but its survival after desmopressin was shorter. A third mutation (c.3583 G>T in exon 27 leading to E1195Y) occurred in the D3 domain and was associated with a type 2A/2M phenotype (FVIII: C 44 U/dL, VWF:Ag 55 and VWF:RCo 20 U/dL). The latter (c.5827 C>T in exon 34 leading to R1943C) occurred in the D4 domain and was associated with a true type 1 VWD phenotype (FVIII:C 54 U/dL, VWF:Ag 48 and VWF:RCo 44 U/dL), although the loss of cysteine could be implicated in abnormal multimerization.

Conclusions: This study confirms the wide heterogeneity of phenotypes in VWD accounted for by the importance of the domain in which the mutation is located. Furthermore, a causative VWF gene mutation could be often detected even in milder VWF deficiency with a significant bleeding history

PB 4.43-3

Atorvastatin ameliorates angiodysplasia-associated bleeding in von Willebrand Disease

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Background: The association between angiodysplasia and congenital/acquired von Willebrand Disease (vWD) has been well described and appears to be related to a loss of high molecular weight multimers or von Willebrand Factor (VWF)-mediated regulation of angiogenesis[1,2]. Management of angiodysplasia-associated bleeding in these patients consists of various therapies, including VWF replacement therapy, octreotide, hormone therapy, beta-blockers and anti-fibrinolytics. Recent case reports describe the successful use of anti-angiogenic therapies such as thalidomide or atorvastatin in these patients. Statins exhibit cholesterol-independent vascular effects and inhibit angiogenesis in high doses[3]. The availability and side effect profile of atorvastatin make it an attractive option to manage angiodysplasia-associated bleeding in patients with VWD.

Aim/Methods: Retrospective chart review and case report describing successful management of angiodysplasia-associated bleeding in two patients with VWD

Case 1: 73 year old female with Type 2B VWD (previously described as Montreal Platelet Syndrome) with recurrent angiodysplasia-associated bleeding and iron-deficiency anemia since 2008. Bleeding was controlled with VWF replacement therapy twice weekly (60 IU VWF:RCo/kg), octreotide, combination estrogen/progestin therapy and intermittent tranexamic acid. While on therapy in 2011, she had three hospital admissions for acute gastrointestinal bleeding requiring red blood cell (RBC) transfusions. Endoscopy confirmed bleeding from angiodysplasia in the small bowel. Despite alternate day VWF replacement therapy and addition of proton pump inhibitor, patient continued to have melena and needed intravenous iron replacement for chronic anemia.

Patient has had no GI bleeding since beginning atorvastatin in December 2011. Began 10 mg daily with dose titration every 4 weeks to maximum of 80 mg. No adverse side effects were experienced clinically or on monthly laboratory monitoring. Patient discontinued use of octreotide in February 2012 and hormone therapy in September 2012. Has not required tranexamic acid or iron infusions. Laboratory parameters have improved (December 2011: hemoglobin 111, MCV 79, ferritin 16 compared to December 2012: hemoglobin 144, MCV 90, ferritin 77). VWF replacement therapy was decreased to three times weekly in January 2013.

Case 2: 71 year old female with Type 2M VWD and multiple comorbidities including angiodysplasia-associated bleeding from the cecum confirmed on colonoscopy, dialysis-dependent renal failure and myelofibrosis – receiving VWF replacement therapy post-dialysis three times weekly (60 IU VWF:RCo/kg). Began to have hematochezia in May 2012 requiring RBC transfusions every 3–4 weeks. Started atorvastatin 10 mg daily with no further hematochezia and reduction in RBC transfusion requirements (every 6–8 weeks) after 6 months of therapy.

Conclusions: These cases illustrate that atorvastatin may be used to successfully control angiodysplasia-associated bleeding in patients with VWD, especially in those who have failed standard therapies. It may also result in an improvement in iron deficiency anemia with reduced iron and RBC transfusion needs. Atorvastatin is preferred because it is easily accessible and has a better side effect profile than thalidomide which is associated with venous thromboembolism.

References:

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PB 4.43-4

Predictors of von Willebrand disease diagnosis in individuals with borderline von Willebrand factor plasma levels

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Background: Individuals with borderline von Willebrand factor (VWF) plasma levels represent a diagnostic challenge. Second level tests are required to confirm or exclude the diagnosis of von Willebrand disease (VWD). These tests are time-consuming and expensive. Advance knowledge which individual parameters can predict the diagnosis of VWD would allow performing second level tests only in a subset of individuals with borderline VWF.

Aims: To assess which individuals with borderline VWF plasma levels are likely to have VWD and should undergo second level tests.

Methods: Between 2007 and 2011, 950 individuals referred to our center after bleeding episodes or detection of abnormal coagulation tests were investigated with first screening tests (blood count, PT, APTT, factor VIII:C, VWF:RCo and VWF:Ag). In 95 of these patients [64 females and 31 males; median age: 28 years (IQR: 15–44)] borderline VWF plasma levels (i.e., VWF:RCo between 30 and 60 IU/dL) were found. All 95 patients underwent second level tests (VWF collagen binding, VWF multimeric analysis, ristocetin-induced platelet agglutination, and VWF intra-platelet analysis) to diagnose or exclude (and, if diagnosed, to characterize) VWD. In all of them the bleeding diathesis was evaluated by calculating the bleeding severity score (BSS). A positive family history was defined as having at least one first- or second-degree family member with hemorrhagic diathesis. Data of all the 95 individuals with borderline VWF were used to build a multivariable logistic regression model in which VWD (confirmed or excluded with second level tests) was the outcome and individual characteristics (e.g., sex, age, BSS, family history, VWF:RCo plasma levels, ABO blood group) were considered as predictors. This model was used to assess which variables were predictive of VWD. The predictive capability of the logistic model was measured as the area under the ROC curve (AUC).

Results: In 47 of the 95 individuals with borderline VWF (49%) the diagnosis of VWD was established with second level tests. Of them, 38 (81%) were type 1, 7 (15%) type 2 and 2 (4%) had an acquired von Willebrand syndrome. In the multivariable logistic model, a negative linear relationship between VWF:RCo plasma levels and risk of having VWD was present, most evident for individuals with non-0 blood group, in whom a 7-fold increased risk of a positive diagnosis was found for every 5 IU/dL decrease in VWF:RCo [adjusted odds ratio: 7.00 (95% CI: 1.48–33.11)]. The other variable clearly associated with VWD diagnosis was female sex [adjusted odds ratio: 5.86 (95% CI: 1.49–19.37)]. BSS, positive family history and age were poorly

associated with VWD diagnosis. The full logistic model explained about 60% of the outcome variance (R^2: 0.596) and its AUC was 0.89 (95% CI: 0.83–0.95).
Conclusion: Low VWF:RCo plasma levels and female sex are the two strongest predictors of VWD diagnosis in individuals with borderline VWF. The role of low VWF:RCo levels is mainly evident in individuals with non-0 blood group. This predictive model has a promising discriminative capability to identify patients with VWF borderline levels who are likely to have VWD.

PB 4.43-5
Significant improvement of live birth rate in VWD patients with repetitive reproductive failure treated with VWF concentrate

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Background: Women with von Willebrand Disease (VWD) are at risk of a variety of complications including miscarriage. Pregancy loss may occur pre-, peri- or post-implantation. Currently, there are limited data defining the exact risk of miscarriage and preventive therapeutic options.
Aim: The aim of this study was to improve the live birth rate in patients with von Willebrand disease and recurrent pregnancy losses. Here, we report data on their treatment with VWF concentrate.
Method: In total 55 pregnant patients (mean age: 31.4; 22–42) were included in this open-label, single-centre study (52 patients with VWD type 1 and 3 with type 2A). They received VWF concentrate (VWF-C) because of previous miscarriage associated with bleeding (78%), bleeding during pregnancy (13%) or other reason (9%). Prior pregnancy losses (in total 101 of 135 pregnancies, 75%) occurred in trimester 1 (t1: $n = 86$), t2 ($n = 10$) and in t3 ($n = 5$). Historical live birth rate (LBR) was 25.4%. Blood samples were collected up to 279 days of pregnancy to analyse FVIII:C, VWF antigen (VWF:Ag) and ristocetin cofactor activity (VWF:RCo). Furthermore, the median doses of factor concentrates/kg BW and the median total number of infusions were calculated. A total of 109 healthy pregnant women served as a control to establish pregnancy adopted reference ranges.
Results: Our data show an increase of mean FVIII:C (106–163 IU/dL), VWF:Ag (124–239 IU/dL) and VWF:RCo (108–183 IU/dL), resp., under replacement therapy throughout the whole pregnancy. In spite of replacement therapy FVIII:C, VWF:Ag and VWF:RCo stayed close to the lower limit of the controls. Mean total VWF concentrate administered per pregnancy was 163644 IU during 43.2 treatment days (range: 1–107) and life birth rate increased to 80% ($P < 0.0001$) with a relative risk reduction to 0.19 to develop pregnancy loss.
Conclusion: In bleeding patients with VWD and recurrent miscarriages we could demonstrate a dramatic increase of LBR from 25.4 to 80% and a risk reduction of miscarriage of 80% in women receiving VWF concentrate. The optimal treatment strategy remains to be clarified.

PB 4.43-6
Analysis of clinical phenotype and genotype in patients with VWD2B, in a single institution of Argentina

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Background: Type 2B von Willebrand disease (VWD2B) is characterized by gain-of-function mutations in the A1 domain of von Willebrand factor (VWF) inducing a greater affinity for platelet GPIb, associated with the disappearance of large forms of multimers (HMWM), thrombocytopenia, positive ristocetin induced platelet aggregation (RIPA) at low concentrations, and VWF:RCo/VWF:Ag <0.6. An atypical form of VWD2B was also described, characterized by both normal platelet count (PC) and multimers, but positive RIPA at low concentrations (Casonato A, 2010). It is extremely important to determine the degree of severity of the disease, correlating in each patient the phenotype with genotype. This could help to decide for example, the input to the patient prophylactic treatment, prophylaxis of bleeding in a challenge as surgery, childbirth, etc.
Aims: We tried to find a relationship between the mutations identified, the BSS, PC, multimeric pattern, lower minimal aggregating ristocetin concentration, and propeptide/VWF:Ag ratio (VWFpp ratio) in 19 patients (pts) with VWD2B with candidate mutations.
Methods: The exon 28 was amplified and sequenced (ABI Prism310). BSS was calculated in each pts.
Pts were grouped according to their mutation.
Results: The following mutations were described in our pts:
p.R1304V ($n = 3$; females = 2); BSS = 4.3 ± 0.9; all pts with both normal PC, and multimers, and positive RIPA at 0.5 mg/mL. VWFpp ratio (normal value = 1.5 ± 0.5) = 2.5 ± 0.3.
p.R1306W ($n = 4$; females = 2); BSS = 6.3 ± 1.9; low PC and absence HMWM = all of pts; positive RIPA at 0.3 mg/mL = 25% of pts, at 0.5 mg/mL = 75% of pts. VWFpp ratio = 2.21 ± 0.4.
p.R1308C ($n = 4$; females = 4), BSS = 4.3 ± 1.8; low PC = 50% of pts; absence of HMWM = all pts; positive RIPA at 0.3 mg/mL = 25% of pts, at 0.7 mg/mL = 75% of pts. VWFpp ratio = 2.1 ± 0.5.
p.V1316M ($n = 8$; females = 5) was the most frequent mutation in our pts. BSS = 4.6 ± 1.0; low PC = 87.5% of pts; absence of HMWM = all of pts; positive RIPA at 0.3 mg/mL = 25% of pts, at 0.5 mg/mL = 62.5% of pts, at 0.6 mg/mL = 12.5% of pts. VWFpp ratio = 2.5 ± 0.4.
Conclusion: Phenotypic profile of p.R1304V revealed atypical VWD2B, given the presence of both normal PC and multimers in those pts.
Considering the other 16 pts with typical VWD 2B, all of them had absence of HMWM, and 81.25% had low PC.
Pts with p.R1306W had BSS higher than the other pts, although the difference was not statistically significant ($P = 0.158$); all those pts also had low PC and absence of HMWM. These findings could indicate a higher tendency to more severe clinical symptoms associated with this mutation. No differences were observed in the BSS with the other pts.
It is important to take into account that only 15.8% of our 19 pts showed positive RIPA at 0.7 mg/mL. In contrast, a 75% of pts with p.R1308C had positive RIPA at 0.7 mg/mL.
According to these results, we agree with the recommendations of using 0.5–0.7 mg/mL RIPA for diagnosing VWD2B (SSC, ISTH 2010).
VWFpp ratio was slightly high in all our pts with typical and atypical VWD2B, but unrelated to the different mutations, the BSS, the PC, and the lower aggregating concentrations of RIPA.

PB4.44 – Von Willebrand disease: Clinical – VI

PB 4.44-1

Individually tailored prophylaxis in Type 3 von Willebrand Disease patients: efficacy and safety of a von Willebrand Factor concentrate with a low factor VIII content

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Background: Patients with von Willebrand disease (VWD) have highly variable bleeding tendency including life-threatening and spontaneous/trauma-related bleeds. As two bleeding patterns mucosal and non-mucosal are identified for VWD, individualized prophylactic therapy was introduced in a clinical protocol in order to provide optimal treatment.

Aims: To evaluate, in a prospective multicentre study, the efficacy and safety of WILFACTIN (von Willebrand factor concentrate with low factor VIII content) in patients on long-term prophylaxis.

Methods: Prophylaxis regimen was defined according to the type of bleeding episodes to be prevented (mucosal or non-mucosal) and the patient's response to WILFACTIN (plasma levels at 48 h post-infusion of 60 IU VWF:RCo/kg). In patients with predisposition to mucosal bleeds and post-infusion VWF:RCo levels >10 IU/dL, the regimen was 60 IU/kg twice a week (if <10 IU/dL, three times a week). In patients with predisposition to non-mucosal bleeds and post-infusion FVIII:C levels >20 IU/dL, the regimen was 40 IU/kg twice a week (if FVIII:C < 20 IU/dL, three times a week).

The primary efficacy endpoints were the number of spontaneous bleeds and the annual frequency of bleeding episodes. Both treated and non-treated bleeds were taken into account. The secondary endpoint was the analysis of all bleeding symptoms.

Results: Analysis included seven patients with type 3 VWD. Patient median age at prophylaxis start was 16 years (11–45). The follow-up was at least 1 year. They had a history of recurrent bleeding episodes from musculoskeletal (3), gastrointestinal (GI) (1) or other mucosal (3) origin.

Median annual bleed rate was lower during prophylactic treatment compared to during the year prior to enrolment: reducing from 33 to 1.1 in 'musculoskeletal' group and from 52 to 4.2 in 'mucosal' group, excluding GI. No episode of GI bleeding occurred during the 31 months of follow-up in the patient included for this prevention. Individually, the annualized bleeding rate was reduced by more than 67% in six of the seven patients. Due to defective dentition at study entry, the seventh patient had an average of 58.2/year during prophylactic treatment. This patient had repeated traumatic gingivorrhagia (not treated) until his dental condition was corrected. Across all patients, the median reduction in bleeding rates was 81.9%. Two patients had no spontaneous bleeds during the first year. When analysing all bleeding symptoms, the patients experienced a total of 109 bleeding episodes over 206 patient-months or 4.35 bleeds per year per patient. Only 23 of 109 bleeds (21%) required a curative treatment and most of them (86%) were controlled by a single injection of WILFACTIN. No factor VIII concentrate was used. Five patients developed trauma-induced bleeds for a total of 85 (78%) bleeds. No patient presented thrombotic complications nor developed a neutralizing inhibitor to VWF.

Conclusion: Prophylactic treatment of VWD resulted in an obvious reduction in annual bleeding rate compared to episodic treatment. This study shows that individually tailored prophylaxis with WILFACTIN is safe and efficacious.

PB 4.44-2

Analysis of clinical severity of VWD2A and VWD2M patients, according to their candidate mutations, in a single institution of Argentina

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Background: Type 2A and 2M Von Willebrand disease (VWD2A and VWD2M) are characterized by VWF:RCo/VWF:Ag <0.6, absent 1.2 mg/mL ristocetin induced platelet aggregation (RIPA). Type 2A shows absence of high and intermediate molecular weight multimers, whereas type 2M shows normal multimers. Given the variable bleeding tendency, it is extremely important to determine the degree of severity of the disease, correlating in each patient the phenotype with genotype. This could help to decide for example, the input to the patient prophylactic treatment, prophylaxis of bleeding in a challenge as surgery, childbirth, etc. At least two different pathophysiological mechanisms are known within VWD2A: mutations that impair multimerization (group I) and those with increased susceptibility of ADAMTS13 (group II), predominantly in the A2 domain.

Aims: We wanted to look for clinical parameters that would be significant to establish 'a priori' differences in clinical behavior of VWD2A and 2M pts. We analyzed the bleeding score system (BSS, ISTH) and the propeptide/VWF:Ag (VWFpp ratio: normal value = 1.5 ± 0.5) between VWD2A patients (pts) (n = 11), and VWD2M pts (n = 33) trying to find (if any) some differences in the severity of clinical symptoms in type 2A and 2M variants with candidate mutations.

Methods: The exon 28 was amplified and sequenced (ABI Prism310) in VWD2A and VWD2M pts previously diagnosed. Pts were grouped according to the VWD variant and their mutation. BSS, VWFpp ratio and mutation were analyzed in each VWD variant

Results: VWD2A:
All (n = 11): BSS = 6.18 ± 2.7; VWFpp ratio = 2.46 ± 0.76.
In the A1 domain: p.P1266Q (n = 1): BSS = 5; VWFpp ratio = 2.13;
p.C1272F (n = 2): BSS=8.0 ± 2.0; VWFpp ratio = 3.26 ± 0.12;
In the A2 domain: p.L1503P (n = 1): BSS = 4; VWFpp ratio = 1.6;
p.G1505R (n = 1): BSS = 10.0; VWFpp ratio = 2.56;
p.Y1542D (n = 1): BSS = 2; VWFpp ratio = 1.27;
p.R1597W (n = 3): BSS = 5.3 ± 1.9; VWFpp ratio = 2.8 ± 0.4;
p.I1628T (n = 1): BSS = 5; VWFpp ratio = 2.08;
VWD2M
All (n = 33): BSS = 4.5 ± 2.5; VWFpp ratio = 2.2 ± 0.74;
In the A1 domain: p.F1293C (n = 2); BSS = 4.5 ± 1.5; VWFpp ratio = 2.9 ± 0.6
p.R1315C (n = 6); BSS = 3.8 ± 1.7; VWFpp ratio = 2.0 ± 0.6
p.G1324S (n = 1): BSS=13; VWFpp ratio = 1.22
p.R1334Q (n = 1); BSS = 3; VWFpp ratio = 1.09
p.R1374C (n = 7); BSS = 3.0 ± 1.4; VWFpp ratio = 2.9 ± 0.56
p.A1437T (n = 2); BSS = 4.5 ± 0.5; VWFpp ratio = 2.76 ± 0.19
In the A2 domain: p.E1549K (n = 10); BSS = 4.1 ± 1.8; VWFpp ratio = 2.06 ± 0.4
p.R1564W (n = 1); BSS = 8; VWFpp ratio = 1.12
p.R1583W (n = 1); BSS = 4; VWFpp ratio = not done
p.I1628T (n = 3); BSS = 4.0 ± 2.9; VWFpp ratio = 1.79 ± 0.17

Conclusion: Globally considered, BSS were higher in VWD2A than in VWD2M variant, although the difference was not statistically significant (P = 0.065). There was no difference with the VWFpp ratio between VWD2A and 2M variants. No differences could be established on BSS and VWFpp ratio and the candidate mutations in each VWD variant; however, the number of pts with mutations is too small to draw conclusions. Given these results, it is possible to consider that

the presence of all multimers might be associated with a better clinical profile of VWD2M pts, despite the VWF:RCo/VWF:Ag <0.6 seen in these pts. Some other unknown conditions could be influencing the severity of the clinical profile observed in patients VWD2A.

PB 4.44-3

Evaluation of a commercial von Willebrands factor: factor VIII binding assay for the identification of von Willebrands disease Normandy variant

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Background: von Willebrands disease (VWD) Normandy variant (type 2N) is a rare autosomal recessive sub-type of VWD resulting from a missence mutation that impairs the ability of von Willebrands factor (VWF) to bind factor VIII (FVIII) resulting in a reduced FVIII concentration and half-life in the circulation. If there is no informative family history and limited clinical features, patients with type 2N VWD can potentially be diagnosed with a mild or moderate haemophilia A phenotype in the case of males and possible haemophilia A carrier in the case of females, due to low levels of factor VIII and usually normal levels of VWF. Until recently only 'in-house' assays have been available for the assessment of VWF binding to FVIII. There is now a commercial assay available designed to identified patients homozygous or compound heterozygous for type 2N VWD which offers standardisation of reagents and calibration.

Aims: To evaluate the enzyme immunoassay (ELISA) based Stago Asserachrom VWF:FVIIIB assay in the differential diagnosis of suspected patients with type 2N VWD, mild or moderate haemophilia A or haemophilia A carrier and the suitability of assay method for automation.

Methods: VWF binding to FVIII using the Stago Asserachrom VWF:FVIIIB was measured using frozen citrated plasma samples from 40 patients with known type 2N VWD, type 1 VWD, mild or moderate haemophilia A and normal subjects. The assay was performed manually according to the manufactures instructions and an automated method established on the Triturus plate reader (Grifols). The patients VWF antigen (VWFAg) level must be known and for both methods the initial dilution of plasma samples to a standardised 10.0 IU/dL VWFAg concentration was performed manually.

Results: Patient and normal VWF:FVIIIB levels ranged from 10.0 to 145.0% and results correlated (r = 1.0) with patient clinical diagnosis based on phenotype and genotype results. VWF:FVIIIB levels <20.0% correlated known type 2N VWD patients. There was good correlation (r = 0.98) and no significant difference (P = 0.2) between manual and automated performance of assay.

Summary/Conclusions: The evaluation has demonstrated that the assay is suitable for manual or automated performance following primary dilution of sample and provides a standardised approach as an alternative to 'in-house' assays. However, the determination of individual patients VWFAg level and subsequent dilution of sample is critical to the accurate final measurement of VWF:FVIIIB levels. The Stago Asserachrom VWF:VIIIB assay was found to correlate 100% with patients previously diagnosed with homozygous or compound heterozygous for type 2N VWD or excluded. The availability of a commercial assay for VWF:FVIIIB should assist in the diagnosis and clinical management of patients presenting with reduced levels of circulating FVIII.

PB 4.44-4

Genetic analyses of two patients with von Willebrand disease (VWD) type2B: implication of type 2B in the differential diagnosis for thrombocytopenic patients

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Background/Aim: Von Willebrand disease (VWD) is one of the most common inherited bleeding disorder characterized by quantitative and/or qualitative von Willebrand factor (VWF) abnormalities. In this study, we performed genetic analyses of the VWF gene from four Japanese patients from two unrelated families with thrombocytopenia, who had been diagnosed as idiopathic thrombocytopenic purpura (ITP).

Case 1: a 14-year-old man. His abdominal purpura was found at 1 year and 10 months old. He presented unexplained thrombocytopenia (17×10^9/L) with increased bone marrow megakaryopoiesis and had been diagnosed as ITP. He had no family history of bleeding tendency. Pulsed corticosteroid therapy or high dose intravenous γ-globulin administration had no effect on thrombocytopenia. Sustained easy bruising and epistaxis continued until he was referred to our institution. Factor VIII procoagulant activity (FVIII:C) was found to be decreased to 52% of normal and the plasma ristocetin cofactor activity of VWF (VWF:RCo) and the antigen (VWF:Ag) was 25% and 69%, respectively.

Case 2: The propositus is a 41 years old father of a family pedigree and had been diagnosed as chronic ITP. He and his two sons had moderate thrombocytopenia (57, 60, 32×10^9/L, respectively) and showed moderate bleeding symptoms of easy bruising and epistaxis. When they lived in US, he was diagnosed as VWD and his plasma VWF lacked higher molecular weight large multimers. FVIII:C, VWF:RCo, VWF:Ag was 30% and 13%, 28%, respectively.

In both patients, ristocetin-induced platelet aggregation was normal or enhanced in the presence of lower concentrations of ristocetin. In the peripheral blood smear of the both patients, giant platelets and large platelet aggregates were visible, suggesting that thrombocytopenia was due to uncontrolled platelet aggregation in the circulation.

Methods: We amplified the exon 28 including the exon/intron boundaries of the VWF gene by polymerase chain reaction using allele-specific primers, and analyzed DNA sequences of the patients.

Results: Direct sequencing showed that case 1 patient was heterozygous for a A1461D (c.4382C > A) substitution. Case 2 and his sons were heterozygous for a V1316M (c.3946G > A) substitution. These mutations are located in the VWF A1 domain and have been found in VWD type2B patients.

Conclusion: Patients presenting muco-cutaneous bleeding and thrombocytopenia are usually diagnosed as ITP when the bone marrow megakaryocytes are normal/increased. Especially case 1 had no relatives of VWD and the diagnosis of type 2B VWD was not performed unless plasma VWF concentrations were tested. We conclude and propose that type 2B VWD should be included in the differential diagnosis of thrombocytopenia and VWF:RCo or VWF:Ag is tested when other conditions are excluded causing thrombocytopenia.

PB 4.44-5

Analysis of von Willebrand Disease in the South Moravian population (Czech Republic): The BRNO-vWD Study; an update

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Von Willebrand Disease (VWD) is an autosomally inherited bleeding disorder caused by a quantitative or qualitative defect of von Willebrand factor (VWF). The current ISTH classification is based on measurement of VWF:Ag, VWF:RCo, Ristocetin Induced Platelet Aggregation (RIPA) and VWF multimers.

In a collaboration between the University Hospital Brno (Czech Republic) and the Antwerp University Hospital (Belgium) a family-based analysis of VWD in the Moravia area of the Czech Republic was performed. Blood samples were collected from patients with suspected or known VWD with one of the following characteristics: VWF:Ag <35%, VWF:RCo/VWF:Ag <0.7, VWF:CB/VWF:Ag <0.7, FVIII:c/VWF:A < 0.5, or positive low concentration RIPA, and grouped in families. From each family the proband was included in the study together with a at least one affected sibling or parent.

Blood was collected from 205 patients from 95 families with suspected VWD. FVIII:c, VWF:Ag, VWF:RCo, VWF:CB, VWFpp, VWF-FVIII binding (if indicated), VWF multimers and molecular analysis were performed at Antwerp University Hospital. Platelet Function Analyzer and RIPA testing were done locally.

Based on laboratory tests and VWF multimers distribution of different subtypes of VWD was as follows: VWD type 1 in 60/95 families (incl. 1 type 1 Vicenza family), and type 2 in 29/95 families (type 2A in 14/95, type 2B in 4/95, and type 2M in 8/95), with 6/95 still unconfirmed/unclassified. All cases of type 3 (6/205 patients) were based on the presence of (often asymptomatic) type 1 VWD in both parents. Diagnosis of VWD type 1 Vicenza was suggested by a high VWFpp/VWF:Ag ratio (4.87) and confirmed by the presence of the R1205H mutation. Molecular analysis is still ongoing with currently 41/95 families fully analysed and the remainder partially. Sofar, 28 Mutations in the VWF gene have been found in 55/95 families, of which 2 are new to the ISTH VWD database (http://www.vwf.group.shef.ac.uk/), and awaiting gene expression studies. In 12/95 families heterozygous type 2N mutations were detected.

This study is the first characterisation of VWD in the (South) Moravian population of the Czech Republic. These data are important for understanding of phenotype-genotype relationship in VWD. The full laboratory and multimeric analysis for all samples are finished. Further molecular analysis is ungoing.

PB 4.44-6

DDAVP use and cardiovascular outcomes in patients with bleeding disorders undergoing invasive procedures

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Background: Desmopressin (1-deamino-8-D-arginine vasopressin, DDAVP) is commonly administered for surgical prophylaxis or treatment of acute bleeding in patients with hemophilia A, von Willebrand's disease (vWD) and various platelet disorders. There are anecdotal reports of myocardial infarction in adult patients treated with desmopressin. For this reason, it is used with caution in patients with cardiovascular disease and is considered contraindicated in patients with unstable coronary artery disease and poorly controlled hypertension. The Southern Alberta Rare Blood and Bleeding Disorders Clinic (RBBDC) in Calgary have used DDAVP regularly for surgical prophylaxis for patients with mild hemophilia A and von Willebrand disease who have been proven to have an increase in von Willebrand and Factor VIII levels after administration of DDAVP.

Aim: The objective of this study was to determine if DDAVP use in our patients with mild hemophilia A and von Willebrand disease, increases the risk of adverse cardiovascular events, including myocardial infarction, angina or atrial fibrillation. Secondary outcome of interest was the incidence of hyponatremia after DDAVP.

Study Design: Retrospective database and chart review.

Methods: Retrospective chart review and RBBDC database review was conducted on all patients with bleeding disorders who underwent surgical procedures requiring DDAVP prophylaxis/treatment between Jan 2008 and Feb 2012.

Results: A total of 289 patients with bleeding disorders underwent surgical procedures between Jan 2008 and Feb 2012. Out of these, DDAVP prophylaxis was used for 119 procedures (38 minor, 81 major) in 78 patients (M:F = 25:53, mean age 45 (range 17–71). Diagnosis included: 55(70%) VWD [6(10%) type 2, 49(89%) type 1], 16 (20%) mild hemophilia A and other bleeding disorders 7(9%). Cardiovascular comorbidities included: coronary artery disease 10 (13%), hypertension 19 (24%), diabetes 7(9%) and congestive heart failure 1 (1%). All the patients received DDAVP at mean dose of 19.8 ± 3.4 mcg. The mean number of treatments was 2.2 ± .5 (range 1–5) for major and 1.2 ± 0.7 (range 1–5) for minor procedures. Adjunctive therapies used included tranexamic acid in 21(27%), Kogenate FS® in 4(5%) and Humate P in 1(2%). Sixteen patients aged ≥60 years (mean age 66.4 ± 4.4) underwent a total of 24 procedures (major:15.62%; minor 9.37%). Of these, 8(50%) had comorbid coronary artery disease, 8(50%) had hypertension and 3(19%) had diabetes. The mean number of treatments for this group was 1.9 ± 1.3 (range 1–4) for major and 1 (range 1–3) for minor procedures. Adverse events were described in 2 (2%) patients within 1 month of DDAVP administration. A 60 year old female with undiagnosed bleeding disorder, hypertension, and coronary heart disease developed atrial fibrillation on Day 20 post DDAVP for transjugular liver biopsy. Postoperative hyponatremia noted in a 66 year old male with mild hemophilia A undergoing colonoscopy. There were no cases of myocardial infarction, angina or congestive heart failure reported.

Conclusion: Surgical prophylaxis/treatment with DDAVP was generally safe and well tolerated. There were few adverse cardiovascular outcomes in patients above 60 years of age

PB4.45 – Anticoagulant agents – XVIII

PB 4.45-1

Factors predicting choice of anticoagulant treatment in elderly patients with VTE – findings from the RIETE registry

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Background: The incidence of venous thromboembolism (VTE) increases significantly with age, and clinicians face multiple age-specific challenges in real life management of VTE.

Aims: We investigated factors influencing the choice of acute and long term anticoagulant treatment in an elderly, non-selected population diagnosed with VTE, with the aim of clarifying what treatment decisions clinicians are currently making for this population.

Methods: Using data collected in the RIETE registry (an international registry of consecutively enrolled patients with VTE), we analyzed clinical characteristics and therapy choices in 11,403 patients ≥75 years old. Acute treatment is defined as treatment administered in the first days (<10 days). Long term treatment is all anticoagulant treatment administered afterwards. Patients were treated according to standard practices. All patients were followed up for at least 3 months.
Results: Low molecular weight heparins (LMWH) were the initial treatment in 93.5% of patients over 75. Presence of severe renal insufficiency (estimated GFR <30 mL/min/m^2) was significantly associated with clinicians' use of unfractionated heparin (UFH) in the acute phase of treatment (OR 2.05, 95% CI 1.5–2.6).
For long term treatment, clinicians followed two major strategies: vitamin K antagonists (VKAs) were used in 8124 (71.2%) patients and LMWH used in 2879 (25.2%) patients. In long term treatment, the major determinant in choosing long term LMWH therapy over VKAs was the presence or not of cancer (OR 2.52, CI 2.2–2.76). Clinicians favoured long term LMWH treatment when anaemia was present (OR 2.07, 95% CI 1.9–2.27). Severe renal insufficiency (estimated GFR <30 mL/min/m^2) did not discourage practitioners from choosing long term LMWH therapy, as 405 patients (14.07%) treated with long term LMWH had a creatinine clearance <30 mL/min/m^2. Age, weight and laboratory abnormalities such as thrombopenia were other major influences on treatment choice.
A direct comparison between patients treated with VKA and those treated with long term LMWH revealed that the latter patients were more fragile, had lower body weights, had significantly more comorbidities (such as chronic lung disease, chronic renal disease, anaemia, and thrombopenia), were more prone to immobility, and had shorter durations of anticoagulant treatment. Amongst these patients, 946 (32.86%) had a concomitant diagnosis of cancer.
Conclusion: Real life assessment of VTE management shows that choosing anticoagulant treatment remains difficult in elderly patients. Overall, this choice was determined by parameters classically associated with the risk of bleeding. Unexpectedly, clinicians seemed to consider LMWH to be safer for chronic use in extremely fragile elderly patients, perhaps due to an overestimation of bleeding risks associated with VKA treatment. Our study highlights the need for controlled randomised studies with the aim of determining the best anticoagulation strategy to adopt in patients over 75 years old.

PB 4.45-2

Association between bleeding risk and persistence on warfarin therapy in patients with VTE in clinical practice

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Background: It is unclear how healthcare providers weigh bleeding risk in patients with venous thromboembolism (VTE) and various levels of VTE recurrence risk who are treated with warfarin.
Aim: To examine the association between bleeding risk level and length of warfarin therapy among patients following a first observed VTE event.
Methods: Data from the HealthCore Integrated Research Database, representing 45 million members in 14 US commercial health plans, were used in this retrospective observational study. Patients meeting the following criteria between June 2007 and September 2011 were identified: (i) had a first observed VTE, designated as the index event; (ii) had no prior use of anticoagulants; (iii) were aged ≥18 years old; (iv) had ≥12 months continuous health plan eligibility before and after the index event; (v) had ≥1 anticoagulant prescription fill within 7 days after the index event and ≥1 warfarin fill during the post-index period; and (vi) had no mechanical valve replacements, atrial fibrillation, or rivaroxaban/dabigatran use during 12 months before the index event. Patients were categorized by bleeding risk level based on the Computerized Registry of Patients with Venous Thromboembolism bleeding risk scheme: high (score >4), intermediate (score 1–4), and low bleeding risk (score 0). Length of therapy was measured by continuous warfarin treatment, based on consecutive fills of warfarin with <90 days of allowable gap, unless there was an international normalized ratio test every 42 days. Survival analyses (Kaplan-Meier curves and Cox proportional hazard model) were used to estimate the effects of bleeding risk levels on length of therapy and were adjusted for baseline demographics and VTE recurrence risk levels (provoked VTE, cancer-related VTE, and unprovoked VTE).
Results: Of the 1868 patients who met inclusion criteria (mean age 57 ± 15 years, 47% female), 45% had low, 54% had intermediate, and 1% had high bleeding risk. The average length of warfarin therapy was 314 ± 266 days (mean follow-up period 733 ± 245 days). Patients with low bleeding risk had a mean of 277 ± 254 days of therapy, while patients with intermediate/high bleeding risk had a mean of 344 ± 273 days. Stratifying by VTE recurrence risk (provoked, cancer-related, and unprovoked VTE) showed that patients with intermediate/high bleeding risk were less likely to discontinue warfarin therapy than were those with low bleeding risk (all P values <0.001). After adjusting for demographics and VTE recurrence risk, patients with intermediate/high bleeding risk remained 25% less likely to discontinue warfarin therapy than those with low bleeding risk (hazard ratio 0.75, 95% CI: 0.64–0.87, $P < 0.001$).
Summary/Conclusion: VTE patients with intermediate/high bleeding risk were less likely to discontinue warfarin therapy than were those with low bleeding risk, which seems counterintuitive. Patients with high bleeding risk may also be perceived to have higher VTE recurrence risk; however, these patients were still less likely to stop warfarin therapy after statistical adjustment for VTE recurrence risk. Additional studies are needed to investigate whether clinical prediction rules for bleeding and recurrent VTE are associated with duration of warfarin therapy in real-world clinical practice.

PB 4.45-3

Differential effects of dabigatran etexilate and ticagrelor on bleeding as assessed by washed blood and shed blood tests in healthy subjects

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Background & Aim: The most frequent side effect associated with long term anticoagulant and antiplatelet therapy is bleeding. It has been attempted with different methods to assess blood loss in healthy volunteers (HV). The purpose of this study was to examine the feasibility of two alternative methods for the assessment of bleeding due to anticoagulation with dabigatran etexilate (DE) vs. antiplatelet therapy with ticagrelor.
Methods: Washed blood and shed blood methods were tested in two groups of 12 HVs. DE (Pradaxa®, 220 mg, $n = 8$) or ticagrelor (Brilinta®180 mg, $n = 4$) was given as a single dose and bleeding methods were done at 0, 2, (3 only washed blood) and 6 h. Washed blood: a forearm skin incision with junior Surgicutt® was made and the wound area was washed with saline. Wash solution was collected and measured photometrically. AUC was recorded as blood loss, bleeding time as the first well with optical density (OD) <0.05. Shed blood: two incisions in the forearm using an adult Surgicutt® were made and blood emerging from the wound was collected over 4 min and placed in a protease inhibitor solution. Plasma was assayed by ELISA for ß-thromboglobulin (ß-TG), thrombin-antithrombin (TAT), prothrombin fragment

(F1 + 2), fibrinopeptide A (FPA). Venous blood was taken at the same time points.

Results: Study drugs were well tolerated. Blood loss (AUC) at predose was 70 ± 42 OD*sec and bleeding time was 3.6 ± 0.6 min. DE increased AUC 2–3-fold over baseline at 2, 3, and 6 h in washed blood test, but had no effect on bleeding time prolongation vs. control. Ticagrelor increased blood loss (AUC) >10-fold and prolonged bleeding time from 5.4 to >20 min. DE reduced ß-TG, FPA and slightly reduced TAT, but had no effect on F1 + 2 in shed blood. Ticagrelor reduced ß-TG, but had no effect on other parameters in shed blood. Parameters were unchanged in venous blood.

Conclusions: This study shows that quantification of bleeding with DE and ticagrelor is possible using the washed blood technique. Shed blood increases sensitivity of detecting the pharmacological activity of these compounds compared to venous blood. These two methods could serve as experimental models to sensitively assess blood loss and to also allow for the investigation of reversal agents.

PB 4.45-4

A randomized clinical trial for the effects of an anticoagulation clinic in heart disease patients at a Brazilian public hospital

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Background: Warfarin sodium is the oral anticoagulant most commonly prescribed in Brazil. The management of patients under warfarin therapy is challenging due to the drug's narrow therapeutic index, wide variability in dose response, and the need for frequent monitoring of the international normalized ratio (INR). Clinical evidence shows that better outcomes are achieved when anticoagulation therapy is managed by anticoagulation clinics rather than by the usual medical practice.

Aims: To determine the efficacy and safety of an anticoagulation clinic in heart disease patients.

Methods: A randomized clinical trial was performed including a calculated sample size of 280 heart disease outpatients recruited from 2009 to 2010 at a public hospital in Belo Horizonte, Brazil. The inclusion criteria were age of >18 years, definitive cardiopathy diagnosis and at least one indication for long-term oral anticoagulation. The exclusion criterion was treatment with warfarin for <30 days prior to recruitment. Data collection was performed during the patient admission process in an anticoagulation clinic recently established in this hospital. Of the 280 patients enrolled, 151 were randomly assigned to the control group and followed-up for 6 months while assisted by the usual medical practice and for additional 6 months after the intervention represented by the anticoagulation clinic. The variables analyzed were: sex, age, time in therapeutic range (TTR) and number of thromboembolic and bleeding events. Any type and severity of hemorrhage were considered as bleeding event (eg. hematoma, epistaxis, gastrointestinal or intracerebral hemorrhage). Thromboembolic events included arterial and venous thrombosis. INR results were used to calculate TTR by linear interpolation. Measurements of central tendency and variability were calculated. The comparison of TTR was performed by paired *t*-test. The analysis of complications was performed by nonparametric Willcoxon. The database was created by double entry in Epidata and statistical analyses were performed with SPSS, v. 18.0. The Ethics Committee of the Universidade Federal de Minas Gerais approved this research. All participating patients signed a written informed consent. The study was registered in ClinicalTrials.gov under the code NCT01006486.

Results: The mean age was 56.3 ± 13.9 years and 80 (53.0%) patients were female. In the first semester, the TTR was 55.5 ± 28.5% and there were 182 complications related to the use of warfarin (171 hemorrhagic and 11 thromboembolic events). In the second semester, the TTR was 62.1 ± 22.6% and there were 95 complications (93 hemorrhagic and 2 thromboembolic events). There was statistically significant difference in TTR between the two semesters ($P = 0.006$). Additionally, there was a statistically significant difference of adverse events between the two semesters ($P = 0.003$).

Summary/Conclusion: These results demonstrated that the anticoagulation clinic was associated with the improvement of TTR which is an important parameter of quality of the oral anticoagulation. There were fewer complications after assistance in the anticoagulation clinic. These findings suggest that the availability of this service as a supportive specialized care may represent a useful strategy to manage the risks associated with warfarin therapy. Moreover, it can help to improve the quality of care focused on heart disease patients assisted by the Brazilian Public Health System.

PB 4.45-5

Correctness of the identification by patients of colour of urine samples obtained a point of care test for dabigatran and rivaroxaban

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Background: In certain clinical situation such as for fibrinolytic therapy it may be necessary to know weather patients have a new oral anticoagulants (NOAC) on board or not. Otherwise it makes the decision for a medical intervention difficult. Also, it is important that patients are fully compliant with NOACs, more so than with warfarin, which has a longer half-life. NOACs are excreted between 25% and 80% into the urine and can be determined by point of care (POC) methods. The precision of the POC test is close to 100% for dabigatran (D) and 97% for rivaroxaban (R) when performed by trained medical personal.

Aim: We determined the precision of the reading of the colour of the POC tests by the naked eye of patients because the test will be performed by patients themselves.

Methods: Urine was obtained from 15 patients each during therapy with 110 mg bid or 150 mg bid D or 10 mg od R or no anticoagulation. The presence of NOACs were analysed by the POC tests. These are based on the development of specific colours within 15 min in the presence (positive, blue or clear colour) and absence (negative, green or yellow colour) of D and R, respectively. The colour of the total of 60 samples was identified by the naked eye of 30–32 patients.

Results: A total of 480 and 465 naked eye readings of the colours were available for D and R samples obtained by the POC tests were available for analysis. The results are given as mean and 95% confidence interval (CI), sensitivity, specificity, accuracy, positive (PPI) and negative predictive index (NPI, all%), and the Youden-Index (sensitivity + sensitivity – 100) for D and R. The sensitivity was 100% (99.2–100) and 96.6% (94.5–98.0) for D and R; the specificity was 99.2% (97.9–99.8) and 98.0% (96.1–99.1) for D and R; the accuracy was 99.6% (98.9–100) and 97.2% (95.9–98.2) for D and R; the PPI was 99.2% (97.9–99.8) and 98.3% (96.6–99.2) for D and R; the NPI was 100% (99.2–100) and 96.1% (94.2–98.0) for D and R; and the Youden Index was 0.99 for D and 0.95 for R, respectively.

Conclusion: Patients identify the colours of urine samples obtained by a POC method specific for D and R in urine from patients on treatment with high precision. Patients have to learn testing and identification of colour by expert personal. During this exercise patients with therapy of D with so far unknown very rare amblyopia for red-green colour are identified. For those few patients another person has to perform the POC test. Patients currently perform the test themselves using a prototype test system.

PB 4.45-6

Analysis of the preference of patients to choose conventional or new oral anticoagulants based on a short questionaire

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Background: Heparins and vitamin K antagonists (VKA) are treatments of chose for prevention of embolism in atrial fibrillation, for prophylaxis of postoperative deep vein thrombosis following total hip- and knee replacement surgery and treatment of acute venous thromboembolism and of their recurrent events. Several new oral anticoagulants (NOAC) are available for these indications. Certain factors are now identified which patients with atrial fibrillation would benefit from NOAC compared to VKA. Personality traits are known to influence patient's decision to choose specific treatment options.

Aim: Aim of the project is to identify personality traits which influence the decision of patients to choose treatment with conventional or new oral anticoagulants.

Methods: In the first step, patients (n = 110) on stable treatment VKA for at least 3 months completed the validated personality inventory (Freiburger Persönlichkeitsinventar FPI-R) and a self-developed questionnaire on general attitudes regarding anticoagulant therapy with about 47 items. For evaluation of the results, patients were divided in two groups according to the reply to the question weather they would be willing to switch from VKA to a NOAC. Six items of the self developed questionaire and the introversion/extraversion scale of the FPI were identified by means of a logistic regression to discriminate patients willing or not willing to change VKA to NOAC therapy. This short questionnaire was completed by the same patients (n = 85) after 6 months to confirm the reproducibility of the regression analysis.

Results: Logistic regression analysis defined the following seven items of the long questionaire and of the FPI for their willingness to change the medication from VKA to NOAC with 98% probability: extraversion – introversion scale on the FPI-R, hope for a better quality of life with a NOAC, no scepticism for new drugs, wish of no routine monitoring for dose adjustment, relevance of the practitioner's opinion, thoughts in the past of alternatives for anticoagulation, and difficulty to adjust the prothrombin time. The short questionnaire identified with a probability of 85% of the same patients willing to change the anticoagulant therapy (chi square test $P < 0.0001$).

Conclusion: Seven items were identified for the identification of the preference of patients to perform oral anticoagulant with VKA or NOAC. These questions are now available in the internet to substantiate the results www.blutverduennung.uni-hd.de. The study compares the results of the replies of to the questions for patients on treatment with VKA, patients who changed anticoagulation from VKA to NOAC and patients without experience of VKA treatment and being on anticoagulant therapy with one of the NOACs from initiation of therapy.

PB4.46 – Anticoagulant agents – XIX

PB 4.46-1

Asian patients with splanchnic vein thrombosis: a sub-analysis from an international registry

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Background: Splanchnic vein thrombosis (SVT) is a manifestation of unusual site venous thromboembolism (VTE). Studies on patients with deep vein thrombosis and pulmonary embolism suggest that epidemiology, pathogenesis and management in Asian countries might be different from Western regions. However, whether these differences pertain also to SVT is unknown.

Aims: The aim of this analysis was to compare the characteristics of SVT in Asian and Western countries.

Methods: We enrolled in a prospective multicenter international registry, 613 consecutive patients with an objectively diagnosed SVT between May 2008 and January 2012. Information on clinical presentations, risk factors and therapeutic strategies was collected and analysed separately for Asian and non-Asian patients and then compared.

Results: 137 Asian patients were included. Asian patients were younger than non-Asian patients (51 vs. 54 years, P = 0.032), most commonly of male gender (70.1% vs. 60.5%, P = 0.052) and less likely to have a personal or family history of VTE (3.7% vs. 13.6%, P = 0.002, and 0.7% vs. 10.6%, P = 0.001, respectively).

Computed tomography was more frequently performed in Asian patients (96.4% vs. 63.9%, $P < 0.001$), while ultrasonography was more commonly used in non-Asian patients (2.2% vs. 28.1%, $P < 0.001$). According to the site of thrombosis, Budd-Chiari syndrome (BCS) was predominantly found in Asian patients (19.0% vs. 5.8%, $P < 0.001$), whereas portal vein thrombosis was more often diagnosed in non-Asian patients (67.9% vs. 79.7%, P = 0.005).

Among the 46 patients with BCS, Asian patients presented more frequently liver cirrhosis (45.8% vs. 14.3%, P = 0.050), but less haematological cancer (0% vs. 28.6%, P = 0.007). BCS was incidentally detected in more Asian patients (50.0% vs. 14.3%, P = 0.027); while abdominal pain (25.0% vs. 76.2%, P = 0.002), anorexia (0% vs. 23.8%, P = 0.017) and fever (0% vs. 19.0%, P = 0.040) were more commonly reported in non-Asian patients.

Among the 552 patients with spleno-porto-mesenteric (SPM) vein thrombosis, Asian patients were more likely to have underlying solid cancer (31.5% vs. 19.4%, P = 0.008) and less likely haematological cancer (1.8% vs. 10.3%, P = 0.008) or ongoing hormonal therapy (0% vs. 5.3%, P = 0.007). SPM thrombosis was more frequently unprovoked in Asian patients (36.9% vs. 24.7%, P = 0.013). No differences between the two groups emerged in the proportion of patients with incidentally detected SPM thrombosis.

Anticoagulant treatment was less utilized in Asian patients, since during the acute phase only 51.1% patients were started on anticoagulation, compared with 84.8% non-Asian patients ($P < 0.001$). Asian patients received more frequently unfractionated heparin (15.3% vs. 8.8%, P = 0.039) and less frequently low molecular weight heparin or fondaparinux (32.1% vs. 77.4%, $P < 0.001$), compared to non-Asian patients. No difference was found in the prescription of vitamin K

antagonists. Thrombolysis was more commonly performed in Asian patients (3.6% vs. 0.9%, $P = 0.032$).

Conclusions: The results of our analysis suggest that Asian patients with SVT may have specific characteristics. Differences in pathogenesis and clinical manifestations are possibly explained by ethnicity, while the diverse management approaches may also be due to the observational nature of our study.

PB 4.46-2

Vitamin K in oral solution or tablets: a cross-over study and randomized controlled trials to compare effects

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Background: Vitamin K1 (VK1) is used to reverse effects of coumarins. The current formulation in the Netherlands is an oral solution in oil, but recently tablets have become available. Tablets have a longer shelf life and are easier to ingest. However, bioavailability of VK1 is higher in the presence of fatty substances, possibly resulting in different effects of the oily solution compared with the tablets.

Aims : To compare the bioavailability and the effect on the international normalized ratio (INR) of tablets and solutions of VK1 in healthy volunteers and anticoagulated patients.

Methods: The study consisted of three parts, all carried out with 5 mg of VK1. First, the bioavailability of the tablets and solution was assessed in a crossover study with healthy volunteers. The volunteers took the tablets or oral solution and crossed over to the other formulation after a wash-out period of 2 weeks. VK1 plasma levels were measured at $t = 0,2,4,6$ and 24 h. Primary outcomes were VK1 concentrations after 4 h and the area under the curve (AUC). Second, patients on phenprocoumon who had to undergo an invasive procedure and had to take VK1 were asked to participate. INRs were measured at $t = 0.24$ and 48 h to compare effects. These were considered similar when the percentage of patients with and INR <2.0 at 48 h did not differ more than 10% between both formulation groups. Third, patients using phenprocoumon with an indication for VK1 due to a high INR were asked to participate. INRs were measured at $t = 0.24$ and 48 h and VK1 levels were measured at $t = 0$ and 24 h. The primary outcome was the decrease in INR after 24 h and results of this part will be available at the ISTH. Informed consent was obtained from all subjects and the study was approved by the medical ethics committee of the LUMC.

Results: In the crossover study, concentrations of VK1 at 4 h were 96 nM for the tablet and 83 nM for the solution group, and were not different (mean difference 13 nM, 95% confidence interval [CI] = −44–69). The AUCs were 301 nmol/mL·h for the tablet and 282 nmol/mL·h for the solution group, and were not different (mean difference 19 nmol/mL·h, 95% CI = −126–164). In the second part, clinical characteristics of the two patient groups were similar. We excluded one patient because he was previously shown to be resistant to VK1. Two patients dropped out of the tablet group at day three (due to earlier admission to the hospital and a too high INR at 24 h [INR = 2.3]). The average INR decreased from 2.52 (95% CI 2.42–2.62) to 1.59 (95% CI 1.55–1.63) to 1.39 (95% CI 1.36–1.42) for the solution, and from 2.55 (95% CI 2.48–2.62) to 1.65 (95% CI 1.61–1.68) to 1.41 (95% CI 1.39–1.43) for the tablets at 0, 24 and 48 hours respectively. The patients with INRs under 2.0 were 36 (100%) in the solution compared to 33 (100%) in the tablet group.

Conclusions: Bioavailability and effects on the INRs of VK1 solution and tablets are similar.

PB 4.46-3

Efficacy and safety of dabigatran in over 75 years old patients with atrial fibrillation

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Background: The use of dabigatran, an oral direct thrombin inhibitor, for the prevention of stroke is increasing in patients with atrial fibrillation and is being applied also to the elderly, who might be exposed to higher bleeding risk.

Aim: To assess efficacy and safety of dabigatran in patients with atrial fibrillation aged more than 75 years.

Methods: Patients attending our Anticoagulation clinic that started dabigatran, after its release in our country in September 2012, were included. Before starting the treatment renal and liver function and hemoglobin and platelets count were assessed and all patients completed an educational course on anticoagulant treatment.

Results: 290 patients on dabigatran were followed for mean 91 ± 58 days, 136 were aged >75 (80.1 ± 3.9) years and 154 < 75 (64.8 ± 8.6) years. The older group had higher $CHADS_2$ (2.7 ± 1.1 vs. 1.5 ± 1.1, $P < 0.001$) and HASBLED scores (1.3 ± 0.5 vs. 0.8 ± 0.7, $P < 0.001$). Majority of the elderly patients (93%) received 110 mg bid dose (D110), while the majority of the younger patients (75%) received 150 mg bid dose (D150).

There was no major bleeding in the older group. In the younger group two patients suffered major gastrointestinal bleeding, both on D150, and treatment was discontinued. There were 19 (14.0%) minor bleeding in the older and 26 (16.9%) in the younger group (RR = 0.83; 95% CI 0.48–1.43; $P = 0.59$), predominantly epistaxis, subconjunctival, gum bleeding, bruises and minor haematochezia, gynecological and urological bleeding. Only in nine cases of minor bleeding treatment was interrupted for a few days. There was no statistically significant difference in age, $CHADS_2$ or HASBLED score between patients that experienced minor bleeding and those that had no bleeding events, neither in the older, nor in the younger group. Due to gastrointestinal side effects treatment was discontinued in 10 patients (3.5%), in seven patients in the older (all on D110) and in three patients in the younger group (all on D150). None of the patients suffered a thromboembolic event.

Conclusion: In our population of patients older than 75 years, treatment with dabigatran was safe and effective using lower dose of dabigatran in majority of the old population.

PB 4.46-4

Assessment of apixaban on automated analyzers using a Liquid Anti-Xa assay

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Background: Apixaban (ELIQUIS) is a direct factor Xa inhibitor anticoagulant indicated to prevent venous thromboembolism after elective hip or knee replacement and to reduce the risk of stroke and systemic embolism with nonvalvular atrial fibrillation. Although apixaban does not require regular monitoring, its assessment in certain situations is helpful.

Aims: HemosIL Liquid Anti-Xa assay (Instrumentation Laboratory, Bedford, MA, USA) is an automated chromogenic assay intended for the quantitative determination of unfractionated heparin (UFH) and low molecular weight heparin (LMWH) in human citrated plasma on IL Coagulation Systems. Here, we report its performance on ACL TOP Family analyzers to measure apixaban in plasma samples.

Methods: HemosIL Apixaban calibrators and controls were used to calibrate and quality control, respectively the HemosIL Liquid Anti-Xa assay on ACL TOP analyzers. Two levels of calibrators (0 and 500 ng/mL apixaban) and two levels of controls (150 and 300 ng/mL

apixaban) were prepared with known concentrations of apixaban spiked into normal plasma. The lyophilized calibrators and controls were reconstituted with 1 mL of water. A 5-point calibration curve was created and apixaban plasma values were assessed using the stored calibration curve. A similar method can be applied to other automated analyzers.

Results: The auto-dilution mode on the ACL TOP Family analyzers with the bilevel apixaban calibrators was used to create a 5-point calibration curve (0, 100, 200, 350 and 500 ng/mL apixaban). The test's nominal linearity range of 10–500 ng/mL can be extended to 1000 ng/mL with reflex testing. After reconstitution, the controls and calibrators have on-board stability of at least 8 h, closed-vial 2–8 °C storage stability of 10 days, and closed-vial −20 °C storage stability of at least 2 months. Precision studies showed overall CV% of 1.42, 2.07 and 2.24 for the 500 ng/mL calibrator, 150 ng/mL low control and 300 ng/mL high control, respectively. The test is interference free with bilirubin up to 25 mg/dL, hemoglobin up to 500 mg/dL and triglyceride up to 1000 mg/dL.

Summary/Conclusions: HemosIL Liquid Anti-Xa assay has been proven to be a robust assay for the detection of antithrombin-dependent anticoagulants. Here, we report its application for testing of the direct factor Xa inhibitor anticoagulant, apixaban. The apixaban test with HemosIL Liquid Anti-Xa can be easily implemented on ACL TOP analyzers. The test has good sensitivity in the low range to verify clearance of apixaban prior to surgery, as well as sensitivity in the clinical range and high range. The test has satisfactory calibrator/control stability, good sample precision, and minimum interference from pre-analytical variables.

PB 4.46-5

The influence of novel oral anticoagulants (NOAC) on routine coagulation assays

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Background: Novel oral anticoagulants licensed for use in Europe include the direct thrombin inhibitors (DTI) Dabigatran (Pradaxa®) and Argatroban (Argatra®, Arganova® or Novostan®) and the factor Xa inhibitor, Rivaroxaban (Xarelto®). These drugs are increasingly being prescribed as an alternative anticoagulant to Warfarin or Heparin. Evaluation of the effects of these anticoagulants on local coagulation assays is recommended.

Aims: The aim of this project was to assess the effect of the novel anticoagulants on routine coagulation assays including the Prothrombin Time (PT), Activated Partial Thromboplastin Time (APTT), Thrombin Time (TT) and the Fibrinogen assay.

Method: A series of reference plasmas were prepared with increasing doses of the relevant anticoagulant. The direct thrombin inhibitor plasma concentrations were assayed by a dilute thrombin time (Hemoclot Thrombin-Inhibitor assay, Hyphen BioMed). The Rivaroxaban® plasma concentrations were measured using an anti-Xa assay (HemosIL Test^{a} Heparin, Instrumentation Laboratory) with calibrators from Hyphen BioMed. The PT and APTT were measured using HemosIL® RecombiPlasTin 2G and SynthASil respectively, the TT using an in-house dilution of DIAGEN Bovine Thrombin and Fibrinogen by the Clauss method using two reagents, HemosIL® Fibrinogen-C and TriniCLOT[TRADEMARK] Fibrinogen. Plasma levels of anticoagulant were correlated with the PT, APTT, TT and Fibrinogen level.

Results: The Thrombin Time proved most sensitive to the DTIs showing prolongation above the reportable range of 60secs at 0.03 μg/mL Dabigatran and 0.27 μg/mL. Argatroban. However, the rapid prolongation of the TT means that while a normal TT indicates very low levels of DTI, a prolonged TT does not give an indication of drug concentrations. The PT and APTT showed a dose dependent increase for the DTIs ($R^2 > 0.98$) with the APTT effect being more pronounced. The most notable effect of the DTIs on routine assays was on the Fibrinogen assay using the HemosIL® Fibrinogen-C reagent. A decrease in measured Fibrinogen levels occurred with increasing concentration of both Argatroban and Dabigatran. This decrease was less apparent with TriniCLOT[TRADEMARK] reagent which has a higher thrombin concentration of approximately 75 NIH U/mL vs. 35 NIH U/mL for the HemosIL® Fibrinogen-C reagent. The reagent discrepancy for measured fibrinogen increased with increasing concentrations of DTI. The PT and APTT showed a dose dependant increase for Rivaroxaban and again the APTT effect was more pronounced.

Conclusion: Argatroban, Dabigatran and Rivaroxaban cause prolongation of the PT and APTT. Argatrobanand Dabigatran cause marked prolongation of the TT. Clauss Fibrinogen assays may give falsely reduced measured levels in the presence of a DTI but a more accurate estimation of Fibrinogen concentration is obtained if the concentration of thrombin is increased. The effect of the novel anticoagulants on routine coagulation assays may be difficult to interpret in the acutely ill or bleeding patient who may have concomitant dilutional or consumptive coagulopathy. In these situations, a specific assay is recommended for analysis of plasma drug concentrations but knowledge of the effects of the novel anticoagulants on local coagulation assays is also useful for clinicians.

PB 4.46-6

Moderate-intensity warfarin therapy in extended treatment of venous thromboembolic disease improve patient comfort, decreasing bleeding risk without increasing risk of relapse: a matched-paired study

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Background: Extended anticoagulant treatment is recommended for patients with venous thromboembolic disease (VTD) and cancer activity or a proximal non-provoked deep venous thrombosis with low bleeding risk. Low-intensity warfarin therapy (INR target range between 1.5 and 2) has been used for this situation; however a significant number of relapses occurred.

Aims: We suggest treating these patients with moderate-intensity of Vitamin-K antagonists (VKA) (INR target range 1.8–2.5).

Methods: All patients were followed in one Centre. Consecutive patients with VTD intended to be in extended anticoagulant treatment, taking VKA for more than 3 months in standard regimen (INR range 2–3) were switched to a new control range (INR range 1.8–2.5) after patient's consent. Intervals for INR testing were programmed following a previously designed algorithm. Time in Therapeutic Range (TTR) was computed by Rosendaal method. Annual Risk of Intracerebral Haemorrhage (ARICH) was computed by weighting each INR value-day for adjusted annually incidence of intracerebral haemorrhage reported by Hylek (2003) in warfarin treated patients. Paired-*t* test for small samples and Wilcoxon signed rank tests were used to evaluate differences. Discriminant and data analyses were performed using STATA12 statistical software.

Results: Starting in September 2003 this study was conducted to October 2012. In this period 22 patients were recruited mean age 64.6 ± 18.9 years (59% of men), treated previously with VKA at INR 2–3 therapeutic range for a median of 1.02 years, and then were treated for a median of 2.04 years with VKA at the new therapeutic range. No recurrences appear in a follow-up of 78.1 patients-years, two major bleeding episodes (one post-traumatism) were found, two patients died for an unrelated VTD episode. A significant reduction in therapeutic cost was attained (from 78–113 €/month to 52–63 €/months) consequence of reduction in monitoring frequency (from 17.9 to 12.8 of median tests/year, $P < 0.001$). From 1591 INR measurements, median INR value's was significantly decreased in patients treated with the proposed regimen (INR 2.42–2.27 $P < 0.005$). No statistically differences between both series were found in TTR (67.2 ± 11.2% in the INR standard range and 62.7 ± 15.4% in the new range), nor in

Percentage of Time Elapsed (PTE) at INR <1.5, but a significant difference is found between both series in PTE at INR >3.0, but this significance disappear when INR >3.5 was analyzed. The TTR computed between the INR extended to 1.8 and 3.2, was 83.06% and 89.45 for each series respectively. ARICH (0.4%) was significantly reduced with the proposed regimen ($P = 0.04$). But despite using this therapeutic regimen we identified 13.6% of unstable or/and unacceptable treatments, predisposing these patients to a bleeding and/or thrombotic risk and making them candidates to stop therapy or to switch to the news orals anticoagulants.
Conclusion: With moderate-intensity VKA treatments there are significant tendencies to ameliorate patient comfort, decreasing INR monitoring frequency and costs, without increasing adverse effects, supporting safety to reduce the lower INR limit to 1.8. But a more extensive study would avoid a type II error.

PB4.47 – Anticoagulant agents – XX

PB 4.47-1

The effect of venous thromboembolism treatment patterns and clinical outcomes in a real world population: the Q-VTE study cohort

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Background: Few studies have assessed treatment patterns of acute venous thromboembolism (VTE) and their effect on clinical outcomes in a real world population.
Aim: We aimed to identify determinants of anticoagulant treatment patterns for acute VTE, and the effects of these patterns on VTE death, recurrence, and bleeding in a real world setting.
Methods: We used the linked administrative healthcare databases of the province of Québec, Canada, including the hospitalization, universal healthcare services, out-patient prescription, and vital statistics databases. We identified all beneficiaries with an incident DVT or PE between 2000 and 2009 using diagnostic algorithms based on ICD-9-CM or ICD-10-CA diagnosis codes. Subjects who were not eligible for drug insurance coverage at the time of diagnosis were excluded. Subjects were followed until VTE recurrence, death, major bleeding, or end of study (December 31, 2009). Anticoagulant use was characterized by anticoagulant type and duration of treatment. Hazards ratio models were used to estimate the effect of treatment patterns as a function of patient characteristics, and to determine their effect on clinical outcomes.
Results: Among the 40,776 cases with VTE, 29,598 cases (73%) were dispensed a prescription for an anticoagulant, and most ($n = 26,247$; 89%) received a vitamin K antagonist (VKA). In all, 58% of cases discontinued VKA within 90 days of initiating warfarin. In a multivariable analysis, predictors of VKA initiation over time included a diagnosis of PE (Hazard Ratio (HR) 1.62, 95% Confidence Interval (CI) 1.58–1.66), older age groups, and patients hospitalized for VTE. Predictors of VKA discontinuation included a diagnosis of DVT (HR 1.18, 95% CI 1.15–1.22), younger age groups, and out-patient diagnosis and management of VTE. Short-term (<30 days) and long-term (>30 days) VKA use were associated with improved mortality compared to no VKA use (Rate Ratio (RR) 0.63, 95% CI 0.60–0.68 and RR 0.59, 95% CI 0.59–0.67; respectively). The mortality benefit was no longer observed following VKA discontinuation and there was excess mortality 15–70 days after discontinuation in the short-term (RR 1.26, 95% CI 1.15–1.37) and long-term (RR 1.53, 95% CI 1.36–1.73) users. Compared to no VKA use, short-term and long-term use were associated with a decreased recurrence rate (RR 0.71; 95% CI 0.62–0.82 and RR 0.60; 95% CI 0.50–0.71; respectively). The rate of recurrence increased after 15 days of discontinuation and at 1 year remained elevated in short-term (RR 2.10; 95% CI 1.64–2.68) and long-term (RR 2.49; 95% CI 1.87–3.33) users. The rate of major bleeding was increased with short-term (RR 1.34, 95% CI 1.22–1.48) and long-term VKA use (RR 1.50, 95% CI 1.36–1.66) and remained elevated until 70 days following discontinuation.
Conclusions: Our study provides useful information regarding predictors of treatment patterns for VTE in a real world setting. Moreover, irrespective of duration, anticoagulation therapy with VKA is associated with improved mortality, a decreased risk of recurrence, and an increased risk of major bleeding, and that following discontinuation, the mortality benefit is no longer seen, the rate of recurrence is significantly higher, and the risk of bleeding may persist.

PB 4.47-2

Alterations induced by Rivaroxaban on hemostasis can be reversed by different coagulation factor concentrates: *in vitro* experimental studies with steady and circulating human blood

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Background: Rivaroxaban is a new oral anticoagulant with inhibitory action on FXa with a good efficacy and safety profile, but with no specific antidotes that could rapidly reverse its prohemorrhagic potential.
Aims: We explored the effects of rivaroxaban on hemostasis with special emphasis on possible interferences with platelet and coagulation mechanisms under flow conditions. We also investigated the potential action of different factor concentrates at reversing the antihemostatic action of rivaroxaban.
Methods: Concentrations of rivaroxaban equivalent to the Cmax reported at steady state after 20 mg rivaroxaban (230 ng/mL) were added *in vitro* to blood alliquots from healthy donors. A series of laboratory approaches were applied to explore modifications in hemostastic mechanism using technologies implying steady and flow conditions. Modifications in platelet reactivity towards surfaces were measured at elevated shear rates in a cone and plate analyzer (Impact R®). Effects on thrombin generation (TG) and on thromboelastometry parameters (TEM) were assessed. Modifications in platelet adhesive, aggregating and procoagulant activities were evaluated in studies with flowing blood maintained at a shear rate of 600-1. The potential reversal action of rFVIIa at final concentrations in blood equivalent to 270 μg/kg, activated prothrombin complex concentrates (aPCCs) at 75 IU/kg or non activated prothrombin complexes (PCCs) at 50 IU/kg, were assessed.
Results: Rivaroxaban did not affect the reactivity of platelets to a polymeric surface in the Impact R®. Rivaroxaban altered TG parameters as confirmed by a prolongation in the lag-phase and a reduction in the maximal thrombin peak. The previous alterations were significantly improved by rFVIIa, aPCCs and PCCs. The intensity of the effects of rivaroxaban and the efficacy of the concentrates to improve TG were highly dependent on the composition of the reagents used to trigger TG. Rivaroxaban prolonged clotting time (CT), clot formation time (CFT) and reduced maximal clot firmness (MCF) in TEM studies. Alterations in these parameters were corrected and occasionally overcompensated (MCF) by rFVIIa and aPCCs. Rivaroxaban quantitatively reduced platelet and fibrin interactions ($P < 0.05$ and $P < 0.01$, respectively) with damaged vascular surfaces in studies with flowing blood. These alterations were counteracted by the different concentrates, with platelet interactions showing a more pronounced correction with rFVIIa and aPCCs, and fibrin formation being variably compensated with efficacies following this quantitative order rFVIIa>aPCC>PCCs.
Conclusions: Rivaroxaban at 230 ng/mL added to blood from healthy volunteers caused evident alterations in hemostasis parameters related to its recognized anticoagulant action. Alterations in hemostasis induced by rivaroxaban were variably compensated or even reversed by the different factor concentrates. Studies with flowing blood under

conditions that more closely relate to the bleeding situation reveal that relatively high doses of rFVIIa, aPCCs or PCCs could be useful in the restoration of hemostasis in patients subjected to treatment with this new oral anticoagulant. Further studies should help establishing doses of these concentrates that can effectively and safely restore the alterations on hemostasis in to the different clinical settings. Grants: EC10-070, SAF2009-10365, SAF2011-28214, RedHERACLES RD06/0009/1003, FIS(CP04-00112, PS09/00664).

PB 4.47-3

Adherence to anticoagulant treatment with dabigatran in a real-world setting

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Background: In clinical trials the adherence to prescribed regimen with dabigatran was enhanced by frequent follow-up visits and pill counts. There is concern that in clinical practice without laboratory monitoring or pill counts the adherence to a twice-daily regimen will be reduced and the risk of thromboembolic events is thereby higher than in the trials.

Aims and Methods: In a cross-sectional cohort study we interviewed During July–September 2012 all patients who had been started on dabigatran by us or referred to our clinic, had remained on treatment for at least 3 months and are followed by our anticoagulant clinic. As part of routine clinical care all patients receive an initial teaching session, explaining the rationale for anticoagulation and careful instructions regarding the treatment. After a visit at 3 months we only see the patients every 12 months. We obtained information on the number of capsules of dabigatran dispensed by the pharmacy of each patient covering the entire treatment period, not including RE-LY and RELY-ABLE participation time. Adherence was calculated as daily doses dispensed divided by number of days between first and last refill minus days off dabigatran for invasive procedures or hospitalization, and was expressed as percent. In addition, information on bleeding, thromboembolic events and other adverse events, specifically dyspepsia, was captured from the interviews and medical records.

Results: We interviewed 103 patients and could not reach another four patients by telephone. Seventeen patients had participated in the RE-LY and RELY-ABLE trials and had been treated with dabigatran for a total of 62.8 (±15.6) months, of which 14.4 (±2.0) months after conclusion of those studies, and 86 patients were study-naïve with treatment for 13.0 (±5.5) months. The mean age was 75.5 (±8.5) years, 45% were females, and mean CHADS2 score was 2.5. Dispensation data was obtained for 99 patients and adherence was 99.7% (median; interquartile range 94.6% to 100%) with 10 patients showing <80% adherence. On the interview 40 patients (39%) acknowledged that they sometimes had missed taking the medication, ranging from 'twice in 6 years' to 'every day'. Eighteen patients (17%) reported bleeding complications, four of which required hospitalization; one patient had an ischemic stroke and 28 (27%) reported some degree of dyspepsia. There were no significant differences in the results between study-experienced and study-naïve patients.

Conclusion: In our clinical practice, adherence to the twice-daily dabigatran regimen was generally good, although 10% of the patients had a low adherence of <80%. Routine feedback from the pharmacies could inform the physician to improve the anticoagulant management. A limitation of our study is that we cannot validate that the patients actually ingested the capsules.

PB 4.47-4

Thrombelastometry monitoring of the anticoagulant effect of dabigatran etexilate

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Introduction: Dabigatran etexilate is an oral, reversible, competitive direct thrombin inhibitor, affecting both thrombin generation and activity. While coagulation monitoring is not required with dabigatran etexilate in routine clinical practice, effects on various coagulation assays have been studied. Thrombin clotting time (TT), ecarin clotting time (ECT), TT determined by Hemocloth® thrombin inhibitor assay and plasma diluted thrombin time (PDTT) have been used to evaluate the anticoagulant effects of dabigatran. Lack of readily available method to determine the degree of dabigatran induced anticoagulation activity creates a major challenge.

Aim: Thrombelastometry assess and interpretation of the anticoagulant effects of direct thrombin inhibitor on patients undergoing dabigatran therapy.

Methods: The study includes 35 patients receiving dabigatran (110 mg twice daily), five of them undergoing elective hip replacement surgery and 26 patients with atrial fibrillation. Rotation Thrombelastometry analyses were performed on ROTEM® analyzer (Pentapharm GmbH, Munich, Germany) in citrated blood, based on reagent activation of the haemostatic processes with thromboplastin tissue factor (TF) for EXTEM test, and aprotinin for APTEM test. STA Compact (Diagnostica Stago-Roche) was used to determine TT. The assays were performed before starting dabigatran therapy, on the first and on the seventh day after the beginning of the therapy. Two patients passed sight bleeding esophageal incidents during this period. The same ROTEM® parameters were also assayed in citrated blood assays with consecutive increasing plasma concentrations of dabigatran.

Results: ROTEM® parameters EXTEM/CT (x = 160s ± 25s; ref. r. 38–79s) и INTEM/CT (x = 310s ±42s; ref. r. 100–240s) are significantly increased in patients with fixed therapeutic doses of dabigatran. These parameters express high linearity in consecutively increasing dabigatran plasma concentration (from 15 mkg/mL to 44 mkg/mL). The rest ROTEM® parameters providing information for clot formation function (EXTEM/CFT and INTEM/CFT), fibrin polymerization (FIBTEM), firmness of the clot (EXTEM/MCF and INTEM/MCF) and clot elasticity (MCE) remain in reference ranges and show no significant changes in consecutively increasing dabigatran plasma concentrations. The two patients with clinical manifestation of dabigatran overdoses expressed very high values of EXTEM/CT (230s; ref.r.38–79s) и INTEM/CT (448s ref.r. 100–240s).

Conclusion: Our investigations suggest that Rotation thrombelastomethry (ROTEM®)could be used as a possible method for monitoring the anticoagulant effect of dabigatran therapy .

PB 4.47-5

Large inter-individual variability of the response to new oral anticoagulants

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Background: New, direct inhibitors of thrombin and factor Xa (FXa) show more stable pharmacokinetics than vitamin K antagonists do. Before the conclusion can be drawn that this warrants standard dose treatment it should be ascertained that the pharmacodynamic characteristics of the agents are predictable as well.

Aims: To investigate the response of the plasma from healthy subjects and patients with atrial fibrillation (AF) to a fixed dose of conventional and new anticoagulants.

Methods: Thrombin generation (TG) was determined by calibrated automated thrombinography (CAT).
Platelet poor plasma (PPP) of 44 healthy subjects was spiked with otamixaban (direct FXa inhibitor), melagatran (direct thrombin inhibitor), unfractionated heparin, dermatan sulfate and pentasaccharide at concentrations around IC50. Thrombin generation was measured at 5 pM tissue factor and 4 μM phospholipids. The effect on TG of rivaroxaban (direct FXa inhibitor) in five patients with AF was assessed in whole blood, platelet rich plasma (PRP) and PPP before therapy and in the 1st and 2nd week of therapy, 3 h after ingestion of the drug.
Results: In the PPP of 44 healthy subjects the coefficients of variation (CV's) were 18% for the uninhibited endogenous thrombin potential (ETP) and 16% for the uninhibited peak height. A dose-dependent inhibition of all anticoagulants was observed in both ETP and peak height. The concentration of anticoagulant that inhibited ETP and peak around 50% was added to the individual plasmas. After inhibition the variation increased to 20–24% (ETP) and 24–43% (peak) for conventional and modern anticoagulants alike.
In the patients, both after 1 and 2 weeks of therapy, the ETP in PPP, PRP and whole blood decreased to a highly variable extent. The least variation was seen in PPP. The individual values of the response to rivaroxaban of the ETP were (1st week [%]/2nd week [%]): 21.9/29.3; 33.4/38.9; 9.1/11.3; 50.4/40.8 and 34.6/0.5. The values are to disparate and the number of patients too small to draw any quantitative conclusion, but it is obvious that the inter-individual variation is enormous. The differences in inhibition between the first and the second week in the same individual appear more similar than the inter-individual differences, which suggests that there may exist high and low responders.
Conclusions: This study indicates that the new anticoagulants cause quantitative changes in thrombin generation, that are as variable as those brought about by administration of vitamin K antagonists or by heparin treatment. The first results in patients suggest that, before relying on the effect of a fixed dose, the dose response in a patient should be determined.

PB 4.47-6

A single-centre prospective study on fluctuations in INR control for Singapore's Muslim patients on oral anticoagulation therapy with stable dosing over the months of Ramadan and Hari Raya Aidilfitri

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Background: Oral Vitamin K Antagonist (OVKA) is known to have its anticoagulant effects augmented by changes in dietary vitamin K intake and lifestyles. During the Muslim fasting month of Ramadan, where dietary habits and lifestyles are altered, such effects may surface.
Aims: To detect and study INR deviations and percentage time within therapeutic range (%TTR) before, during and after Ramadan in stable warfarinized patients, whom otherwise have steady INR control.
Methods: Potential study subjects were screened from hospital anticoagulation clinic (ACC) database, and pharmacy's warfarin dispensing records. Only stable warfarinized patients who met the inclusion criteria were shortlisted and contacted for recruitment. All recruited patients underwent weekly INR monitoring, with four readings per subject per month, using Coaguchek XS Point-of-care coagulometer throughout a 3-month study period from a month before Ramadan to a month after it. The warfarin doses for these patients were kept constant, and they were instructed to keep to their usual religious practices, eating habits and lifestyle as previous years. All readings were blinded to the subjects until the end of the study to minimize confounding factors.
Results: In the intention-to-treat analysis population (n = 32), the mean difference in INR during the Pre-Ramadan month and the Ramadan month was significant at 0.207 [P = 0.032, 95% CI 0.013–0.401]. In contrast, a greater mean difference in INR of −0.309 [P = 0.001, 95% CI −0.506-(−0.112)] was observed between the Ramadan and the Post-Ramadan months. No significant changes in INR were detected between the non-Ramadan months [P = 0.629, 95% CI 0.93-(−0.298)], thereby suggesting that these increases in INR during Ramadan could be attributed to fasting. In the per-protocol analysis population (n = 30), where study drop-outs and confounding results were excluded to increase sensitivity, the difference in INR between the Pre-Ramadan and Ramadan periods was 0.228 [P = 0.002, 95% CI 0.86–0.369]; and −0.256 [P = 0.002, −0.399] for Ramadan and post-Ramadan periods. Mean difference across non-Ramadan months was insignificant [P = 0.692, 95% CI −1.172–0.114]. These show that the INR readings had increased significantly during the fasting month in line with our postulation that a reduced vitamin K intake and changes in lifestyle could lead to an increase in INR. The percentage time within therapeutic range (TTR) also showed a dip to 69.56% compared to 80.99% before Ramadan and 70.80% after Ramadan. The secondary outcome of the study focused on the time taken to return to baseline INR after a fluctuation is observed during the Ramadan period. From the Kaplan-Meier survival analysis, on average, the first out-of-range INR can be seen 12.1 days [95% CI 9.0–15.1] after the start of fasting. Subsequently, an average of 10.8 days [95% CI 7.9–13.7] is required for the INR to return back to its therapeutic range after Ramadan ends.
Conclusion: The results of this study suggests possibility of significant INR fluctuation during Ramadan fasting. Additional caution or even pre-emptive dose reduction may be warranted especially for patients maintained at higher end of their target ranges, or for patients prescribed with narrower target ranges.

PB4.48 – Anticoagulant agents – XXI

PB 4.48-1

Aptamer based thrombin inhibitors: efficacy and functional stability *in vitro*

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Background: Thromboembolic diseases are a major cause of human mortality worldwide. Any strategy of anticoagulant therapy is essentially aimed at neutralizing excess thrombin production. A new approach in this field is development of enzyme inhibitors on the basis of single- or two-stranded DNA or RNA fragments (aptamers). The advantage of such type of inhibitor is possibility to create antidotes to them. Thrombin was the first protease to which an inhibitory aptamer (15TBA) was produced. Subsequent research with computer modeling provided a basis for engineering other aptamers (31TBA etc.). However the life span of these inhibitors in circulation *in vivo* was limited by a few minutes. It was assumed that the reason for such effect was destructive action of 3'-exonuclease.
Aims: Investigation *in vitro* of anticoagulant properties by a new global coagulation test of thrombodynamics and measurement of functional stability in human blood of two single-stranded DNA thrombin-inhibiting aptamers (15TBA and 31TBA).
Methods: Anticoagulant activity of 15TBA (5'-GGTTGGTGTGGTTGG-3') and 31TBA (5'-CACTGGTAGGTTGGTGTGGTTGGGGCCAGTG-3') was investigated by a new coagulation test of thrombodynamics, measuring the spatial clot growth rate in nonstirred plasma. The functional stability of aptamers during incubation in human blood was investigated *in vitro* by measuring APTT. Samples for analysis of coagulation were withdrawn after 1 h, 2 h, 3 h, 4 h of incubation.
Results: Both aptamers reduced the spatial clot growth rate (IC_{50} for clot growth rate decreasing are 9.5 мМ and 4.0 мМ for 15TBA and

31TBA, correspondingly). Thrombin-inhibiting aptamersretained their anticoagulant activity during incubation in human whole blood *in vitro* for at least 4 h.
Conclusions: Thrombodynamic test perfectly monitors anticoagulant conditions in human plasma. It was shown that 15TBA and 31TBA decrease clot growth rate, with 31TBA being a more active anticoagulant. The anticoagulant activity of both aptamers was stable in human whole blood *in vitro* for hours. So, the 3'-exonuclease could not be the reason for fast decrease of aptamers' functional activity *in vivo*. The main role in the removal of oligonucleotides from the circulation is played obviously by the liver.
The work was supported by RFBR grant N11-04-01530.

PB 4.48-2
Laboratory monitoring of novel oral anticoagulants rivaroxaban and dabigatran

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Background: Rivaroxaban and dabigatran are new oral anticoagulants that both have been licensed worldwide for the treatment of atrial fibrillation and rivaroxaban also for venous thrombosis. Both drugs specifically inhibit one coagulation factor, factor Xa and thrombin, respectively, and both compounds have stable pharmacologic profiles, making regular monitoring as rewuired for Vitamin K antagonists unnecessary. However, in specific circumstances, such as in renal insufficiency, patients using these anticoagulant drugs may need laboratory monitoring and perhaps dose adjustments.
Aims : The aim of this study was to assess a proper monitoring method for both drugs.
Methods and Results: Samples were used from a cross-over study in healthy volunteers, originally designed to find a method of reversal for both anticoagulants (Eerenberg et al, Circulation, 2011). Twelve healthy male volunteers received rivaroxaban 20 mg twice daily, and dabigatran 150 mg twice daily for two and a half days, with a washout period of 11 days. Blood was drawn at the end of each treatment period. Following rivaroxaban treatment, the median plasma concentration was 273 μg/L, with an almost three fold difference in the interquartile range (158–393). Rivaroxaban significantly prolonged the anti-FXa assay and prothrombin time (PT), and both assays showed the best correlation with plasma concentrations (rho 0.63 for the anti-FXa assay and 0.47 for the PT). Dabigatran administration led to a median plasma concentration of 88 μg/L, with also a nearly three fold variability in the interquartile range (54–133). Dabigatran increased the activated partial thromboplastin time (aPTT), the Hemoclot and ecarin clotting time (ECT) significantly, and all assays correlated well with plasma concentrations (rho 0.99, 0.99 and 0.97, respectively).
Conclusions: Plasma concentrations of rivaroxaban and dabigatran in healthy volunteers show considerable variability. Coagulation assays that corresponded well with plasma concentrations are the anti-FXa and PT for rivaroxaban, and the aPTT, Hemoclot and ECT for dabigatran.

PB 4.48-3
Thrombin generation assay to monitor the reversal of anticoagulants by FEIBA

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Background: Rivaroxaban and dabigatran, direct inhibitors of factor Xa and factor IIa (thrombin) respectively, are new oral anticoagulants (NOACs) used in a large number of patients to prevent thromboembolic disease following orthopedic surgery and non-valvular atrial fibrillation. In the event of a major bleed, however, there is no specific way to reverse their anticoagulant effect.
Aims: The objective of the study was to evaluate and compare the effect of rivaroxaban and dabigatran on the thrombin generation (TG) potential of human normal plasma and to determine the potential of FEIBA, a marketed and clinically used activated prothrombin complex concentrate, to reverse the anticoagulant effect of these NOACs.
Methods: The effect of rivaroxaban and dabigatran on the TG of human normal plasma was assessed using the Technothrombin TG assay. TG was triggered by a tissue factor reagent containing a low concentration of phospholipid micelles. Plasma was spiked with up to 1900 ng/mL of NOACs and TG determined with or without addition of a concentration series of FEIBA. Prothromplex total, a marketed and clinically used 4-factor PCC (prothrombin complex concentrate), and Prothromplex, a marketed and clinically used 3-factor PCC, served as active control items.
Results: Both rivaroxaban and dabigatran inhibited TG in a concentration-dependent manner. In contrast to dabigatran which mainly delayed the initiation phase (lag time), rivaroxaban inhibited TG with respect to lag time and peak thrombin level even at low concentrations. FEIBA corrected the impaired TG parameters induced by both NOACs to the level of normal human plasma. 0.5 U/mL FEIBA completely restored TG of plasma spiked with 60 ng/mL rivaroxaban, and had a similar effect on dabigatran. The potential to reverse the anticoagulant effect of NOACs *in vitro* was less pronounced for Prothromplex and nonexistent for Prothromplex total. This lack of efficacy turned out to be associated with the presence of heparin in the formulation buffer of these drugs. While FEIBA showed a concentration-dependent increase of TG in human normal plasma, Prothromplex had no effect and Prothromplex total even had an anticoagulant effect on TG. Neutralization of heparin in Prothromplex total increased TG to a level similar to that obtained with FEIBA when added to human normal plasma.
Summary/Conclusions: Thrombin generation assay was demonstrated to be suitable for monitoring the effect of NOACs and determining the potential of FEIBA as a reversal agent. The mechanisms of rivaroxaban and dabigatran to inhibit TG in human normal plasma were shown to differ. Nonetheless, FEIBA effectively corrected the anticoagulant effect of each NOAC. By contrast, other coagulation factor complex concentrates containing heparin were not effective, and in case of Prothromplex total, even showed an anticoagulant effect.

PB 4.48-4
Small-molecule thrombin inhibitors based on derivatives of N-arylbenzamidines

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Thrombin is an enzyme which belongs to the class of hydrolases from the group of serine proteases catalyzing the conversion of fibrinogen to fibrin in blood clotting. Searching for substances that can control the process of blood coagulation, particularly, by means of inhibition of thrombin is one of the main areas of medicinal chemistry.

At present argatroban is the only synthetic direct inhibitor of thrombin, used for intravenous introduction. Another medicine is dabigatran etexilate, a low molecular weight prodrug, which has been recently approved by the US Food and Drug Administration (FDA) for oral use. Thus, the development of new direct thrombin inhibitors is an urgent task.

In the present work on developing an oral drug with anticoagulant activity a molecular design of a series of *N*-arylbenzamidines containing strongly basic amidine functional group in their structure was conducted. Such groups are typical for many serine protease inhibitors.

Virtual screening was performed using the original docking program FlexX that enables docking of a low molecular weight organic compounds and calculating the energy of inhibitor-enzyme interaction.

The crystal structure of thrombin was used as a target for screening and docking (PDB code 1O2G; http://www.rcsb.org/pdb). Virtual screening was performed using our own library of ligands. The criteria for selection of potential thrombin inhibitors were the highest value scoring function (predicted binding energy, kcal/mol).

After computer simulation several compounds were selected for further chemical synthesis and experimental verification of their activity. Among the selected compounds *N*-phenylbenzamidine (**I**) and *N*-4-nitro-phenylbenzamidine (**II**) demonstrated similar values of binding energy.

However, despite the similarity of compounds' **I** and **II** chemical structure, the conformation and location of these compounds regarding the active site of the enzyme differed significantly.

Compound **II** showed a similar interaction with the active site of the enzyme due to binding to the hydrophobic side chains of residues Trp 60A, Trp 60D, and also forms hydrogen binding with Gly 216 of thrombin active center. In contrast, the interaction diagram of compound **I** with the enzyme did not contain all of the interactions for the known inhibitors. Analysis of the results allowed us to assume that compound **II**, in contrast to **I,** may demonstrate anticoagulant activity.

To confirm this, compounds **I** and **II** were synthesized and studied for potential anticoagulant activity.

The results of biological studies had demonstrated that only compound **II** exhibited anticoagulant activity. Moreover this compound is less toxic than the ligand **I**.

Thus, the virtual screening allowed the accurate identification of the anticoagulant effect of compound **II**. This effect has been manifested in increasing of the prothrombin time value among intact laboratory rats after repeated (5 days) administration of this compound.

Compound **II** exhibits anticoagulant activity in the intact animals, low toxicity (LD_{50} more than 2000 mg/kg intraperitoneal injection of laboratory mice), and is of practical interest. The patent on the structure of the corresponding potential inhibitor of coagulation is pending.

PB 4.48-5

Purification and characterization of fibrinogenolytic serine protease from Agkistrodon brevicadus venom

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Complex mixtures of toxins from over 300 poisonous snakes found throughout the world have been widely used as sources for discovering drugs to dissolve blood clots and prevent strokes. Snake venoms contain a number of serine and metalloproteinases, included among these are the fibrinogenolytic serine proteanases[5]. Fibrinogenolytic enzymes act on fibrinogen, leading to defibrinogenation of blood and a consequent decrease in blood viscosity[6]. A Fibrinogenolytic serine protease was purified from the venom of *Agkistrodon brevicaduss*.

The purpose of our study was identification, purification and characterization of new fibrinogenolytic substance that can be a candidate for antithrombotic agent.

Fibrinogenolytic enzyme was purified by HPLC 3-columns procedure Blue Sepharose FF, Phenyl Sepharose HP and Source 15S column. Homogeneity, molecular weight, iso-electric focus, fibrinogenolytic activity of the purified protein was determined by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis with reducing and nonreducing condition. Modification on Bigs method was used to thrombin time determination. 0.2 mL of diluted plasma and sample were incubated for 3 min and added 1NIH thrombin and clotting time was recorded on MC1 coagulometer. Platelet-rich plasma (PRP) was prepared freshly from rabbit blood containing 3.8% sodium citrate by gentle centrifugation (100 rpm for 10 min). Platelet poor plasma (PPP) was prepared from remaining platelet solution by centrifugation. PRP was diluted to 200,000–250,000 platelets per microliter with PPP. PRP (350 μL) plus 100 μL of sample was incubated for 20–30 s in a Ap2110 'SOLAR' aggregometer and collagen (0.2%) was added to initiate platelet aggregation. The maximum aggregation response obtained from addition of inducer and working buffer instead of purified enzyme.

Two types of fibrinogenolytic enzyme (27 and 36 kDa) are contained in *Agkistrodon blomhoffii ussuriensis* venom. Purified fibrinogenolytic enzyme with 12 and 24 kDa molecular weight included in 36 kDa dimer serine protease. Fibrinogenolytic enzyme cleaved quickly β-chain and produced X-fragment. Fibrinogenolytic enzyme inhibited platelet aggregation in 25–50% induced by collagen.

The purified enzyme is postulated that the enzyme is capable of functioning in a cooperative manner to effectively remove fibrinogen and consequently to reduce the blood viscosity. Also some developments and more characterization studies need to be done with this enzyme.

PB 4.48-6

Interaction of defibrotide with dabigatran, rivaroxaban and apixaban in the whole blood, platelet rich plasma and platelet poor plasma studies

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Introduction: Defibrotide represents a single stranded mammalian DNA derived agent originally developed for anti-thrombotic and anti-ischemic indications. Defibrotide is currently used to treat or prevent a failure of normal blood flow (Veno-occlusive disease, VOD) in the liver of patients having had bone marrow transplants or received certain drugs such as oral estrogens and mercatopurine. Defibrotide is a polypharmocologic agent with multiple sites of actions, which include anti-inflammatory and vaso-facilitatory effects. The purpose of this investigation is to determine potential interactions of defibrotide and some of the newer oral anticoagulant drugs, such as dabigatran, apixaban and rivaroxaban.

Materials & Methods: Defibrotide (lot no. DV0601) was obtained in powder form from Gentium s.p.A (Villa Guardia, Italy). The active form of dabigatran was purchased from Selleckcham (Houston, TX). Apixaban and rivaroxaban were obtained commercially and were of synthetic origin. The effect of defibrotide and the new oral anticoagulant drugs, such as dabigatran, apixaban and rivaroxaban, on agonists (epinephrine, ADP, collagen, arachidonic acid and 2.5 U thrombin) was measured using platelet aggregation technique. The platelet rich plasma collected from normal healthy donors (n = 15) were also supplemented with each of the individual anticoagulant drugs, alone in a range of 0–1000 ng/mL and in combination with defibrotide at 100 μg/mL. Such clotting times as the PT, aPTT and Heptest and thrombin generation studies were carried out. In addition, whole blood activated clotting time (celite) studies were carried out by supplementing each of the individual oral anticoagulant agents at 1 μg/mL, alone and with defibrotide at 100 μg/mL.

Results: Neither defibrotide nor the newer anticoagulants produced any effects on the epinephrine, ADP, collagen and arachidonic acid induced aggregation of PRP ($P > 0.05$). However, dabigatran at concentrations of >62.5 ng/mL produced inhibition of thrombin induced aggregation, all of the other agents did not have any effect on thrombin. Defibrotide at a concentration 100 μg/mL did not alter the aggregation profile in anticoagulant supplemented PRP. In both the plasma and whole blood anticoagulant assays, dabigatran produced stronger anticoagulant effects (306 + 30 s) than both apixaban (145 + 12 s) and rivaroxaban(160 + 15 s). Defibrotide exhibited minimal effects (135 + 10 s). Defibrotide in combination with dabigatran produced modest augmentation of the anticoagulant responses (360 + 42 s), however, it had much weaker effects on rivaroxaban (168 + 18 s) and apixaban(152 + 14 s). All of the anticoagulants in the TGA produced varying degrees of inhibition of thrombin generation in the following order; dabigatran > rivaroxaban > apixaban. Defibrotide did not produce any effects at concentrations of 100 μg/mL. When combined with oral anticoagulants, it did not show any augmentation of rivaroxaban and apixaban; however, it enhanced dabigatrans inhibitory effects.
Conclusions: These results suggest that defibrotide itself has weak or negligible anticoagulant effects in the plasma and whole blood assays. It does not produce any inhibitory effects on platelet function, moreover, it does not have any modulatory effects on the new anticoagulant mediated platelet aggregation responses. While defibrotide does not show any interactions with apixaban and rivaroxaban, it has some effect on the anticoagulant responses of dabigatran. These studies demonstrate that unlike heparins, defibrotide exhibits minimal interactions with newer oral anticoagulant drugs.

PB4.49 – Intrinsic pathway of coagulation

PB 4.49-1

The Alzheimer's disease-related peptide beta-amyloid accelerates thrombin generation and clot formation in plasma in a factor XII-dependent manner

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Background: There is strong evidence that the beta-amyloid peptide (Aβ) plays a key role in Alzheimer's disease (AD). This disease is characterized by cerebrovascular abnormalities, and numerous vascular risk factors have been linked to AD development and progression. Epidemiological and experimental data also suggest the existence of a prothrombotic state in AD, manifested as elevated plasma levels of thrombin generation markers, increased levels of activated platelets, and improvement in memory and pathology following anticoagulant therapy. Such a hypercoagulable state could contribute to cognitive decline by causing cerebral hypoperfusion and inflammation, leading to neuronal dysfunction. Aβ, which is cleared from the brain into the circulation, may contribute to the prothrombotic state in AD by enhancing the activity of coagulation factors.
Aims: Here, we investigated whether Aβ can potentiate coagulation factor activity and promote thrombin generation and clot formation in plasma.
Methods and Results: Clot formation in human plasma, monitored by turbidity assay, was accelerated in the presence of Aβ. To determine if accelerated clotting was a result of Aβ's enhancement of one or more coagulation factors, thrombin generation in platelet rich and platelet poor plasma was monitored by calibrated automated thrombogram. Lag time to thrombin generation was decreased and thrombin peak height was increased by Aβ in a dose-dependent manner. This effect was abolished when coagulation factor XII (FXII) was inhibited and when $FXII^{-/-}$ mouse plasma was used, but not when the extrinsic coagulation pathway was blocked. Chromogenic substrate assays and Western blotting showed that Aβ can activate FXII and lead to factor XI activation, suggesting that Aβ may accelerate thrombin generation via activation of the intrinsic coagulation pathway.
Conclusion: These findings, combined with our previous results demonstrating Aβ-mediated impaired fibrinolysis, provide a possible mechanistic role for Aβ in the procoagulant state observed in AD.

PB 4.49-2

Activation of the intrinsic pathway of coagulation without kinin generation in factor XII-deficient chicken plasma

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Background: After binding to negatively charged materials *in vitro*, mammalian activated factor XII (FXIIa) triggers both the intrinsic pathway of coagulation and plasma kallikrein-kinin system (KKS), respectively through its natural substrates factor XI (FXI) and plasma prekallikrein (PPK). However, it is unclear how these two systems are activated *in vivo* and whether they are activated simultaneously. In mammals, high molecular weight dextran sulfate (DXS, ~500 kDa) can activate factor XII (FXII), leading to plasma kallikrein formation, and generation of bradykinin, inducing to a transient depressor response. A single gene corresponding to the predecessor of both FXI and PPK is present in chicken genome, but that corresponding to FXII is missing. The contact system activator ellagic acid-based reagent (EA) can reduce the clotting time (CT) of recalcified chicken whole blood (WB) samples despite the lack of FXII.
Aims: We investigated in chickens whether both fibrin formation and KKS can be activated by negatively charged materials *in vitro* and *in vivo*, respectively.
Methods: WB samplesfrom rats and chickens ($n = 6$ each) collected in the presence of sodium citrate (0.38%)were recalcified after pretreatment with EA (¼ of the dose used in aPTT assay)and theCT measured by rotational thrombelastometry (final volume 340 μL). Possible activation of the KKS was tested by measuring mean arterial pressure (MAP) changes after i.v. injection of DXS (150 μg/kg) into the jugular veins of anesthetized, non-heparinized, rats and chickens ($n = 6$ each) (sodium pentobarbital, 60 mg/kg, i.p., and 100 mg/kg, i.m., respectively). The responses to DXS injection were measured either in the presence or absence of the bradykinin-potentiating peptide Captopril (0.1 mg/kg).
Results: Exposure to EA reduced CT of chicken and rat WB samples from 1013 ± 156 to 375 ± 62 s, and from 248 ± 32 to 98 ± 07 s, respectively (mean ± SEM). The FXII inhibitor, corn trypsin inhibitor (CTI, 100 μg/mL), did not prevent the stimulation of clotting by EA in chicken WB (375 ± 62 s), although it completely blocked this effect of EA in rat WB (235 ± 29 s). However, the stimulation of clotting by EA in chicken WB was blocked by the FXI inhibitor, aprotinin (500 UI/mL) (1097 ± 187 s). Chicken WB clotting induced by the factor X activator, Russel's viper venom (10 ng/mL) (456 ± 56 s) was not inhibited in the presence of the same dose of aprotinin (472 ± 47 s). In rats DXS caused a transient fall in MAP (from 85 ± 5 to 42 ± 4 mmHg, performing 120 s to return to basal level) andduration of the hypotension was prolongedby Captopril (from 92 ± 8 to 48 ± 6 mmHg, performing316 s to return to basal level). The same dose of DXS failed to affect basal values of MAP (88 ± 9 mmHg) in chickens either in the presence or absence of Captopril.
Summary/Conclusions: Our data suggest that the chicken intrinsic pathway can be activated in the absence of FXII, leading to fibrin formation through activation of FXI, without activation of the KKS. We propose this clotting system as useful for studying regulatory molecular mechanisms for FXI and KKS activation *in vivo*.

PB 4.49-3

Inhibiting the activation of the intrinsic pathway with a FXII-targeting RNA aptamer

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Background: Understanding the differences between thrombosis and hemostasis in the vascular system is critical to developing safe and effective anticoagulants, as this depends on striking the correct balance between inhibiting thrombus formation (efficacy) and reducing the risk of severe bleeding (safety). To this end, constituents of the intrinsic pathway of coagulation, namely factor XII, appear to be involved in pathological thrombus formation, but are not required for hemostasis. Exposure of the plasma protein factor XII to an anionic surface generates activated factor XII that not only triggers the intrinsic pathway of blood coagulation through the activation of factor XI, but also mediates various other vascular responses through activation of the plasma contact system. While deficiencies of factor XII are not associated with excessive bleeding, thrombosis models in animals deficient in factor XII have suggested that this protease contributes to stable clot formation. Therefore, factor XII has emerged as an attractive therapeutic target to treat or prevent pathological thrombosis formation without increasing the risk for hemorrhage.
Aims: Using an *in vitro* directed evolution and chemical biology approach, we sought to isolate a nuclease resistant RNA aptamer that binds specifically to factor XII and directly inhibits factor XII coagulant function.
Methods and Results: Utilizing convergent systematic evolution of ligands by exponential enrichment (SELEX), we first selected for an enriched pool of aptamers that bound to the plasma proteome. We then used this 'focused' pool to isolate a nuclease resistant RNA aptamer that binds to FXII and FXIIa with high affinity and specificity. This aptamer dose dependently prolongs fibrin clot formation in an aPTT, while having no effect in a PT. Biochemical assays employed to determine the method of anticoagulation found that while this aptamer does not inhibit the active site of FXIIa, it does inhibit the autoactivation of factor XII by various substances. In addition, this aptamer inhibits factor XI activation, while not affecting the ability of FXIIa to activate prekallikrein.
Conclusions: Thus, using the aptamer platform, we have characterized a potent and effective anticoagulant, which offers targeted inhibition of discrete macromolecular interactions involved in the activation of the intrinsic pathway of blood coagulation. We believe that we have isolated a powerful tool which can be utilized to further elucidate the role of FXII in hemostasis and thrombus formation, as well as identified a promising therapeutic agent that might prove to be an effective and safe means of controlling coagulation.

PB 4.49-4

Cycling induces a hypercoagulable state via contact activation

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Background: Although a clear association between exercise and activation of coagulation has been demonstrated, evidence is fragmented and the trigger remains unknown. Given the protease activated receptor (PAR) activation by coagulation proteases and the subsequent cellular effects such as inflammation, migration and apoptosis, haemostatic changes during exercise may contribute to the development of endofibrosis in cyclists. This pathology is characterized by intimal thickening of the iliacal artery, with reduced blood flow as a consequence.
Aim: In this proof-of-principle study the overall changes in the haemostatic and haematological profile of young (semi)-professional cyclists after long-term strenuous exercise was investigated.
Methods: Venous blood was collected from 17 male cyclists before and after 4 h of cycling (≈120 km). The coagulation system was assessed by thromboelastometry (ROTEM; NaTem), thrombin generation (TG: CAT method), FXIa concentration using a modified CAT based assay, and Thrombin-Antithrombin (TAT) levels. Platelet reactivity was measured by impedance aggregometry (Multiplate). Von Willebrand factor (vWf) levels, tissue plasminogen activator (tPA) activity, and D-dimer levels were measured for evaluation of endothelial function and fibrinolytic activity.
Results: Hematocrit and hemoglobin levels remained constant during exercise. Compared to baseline, white blood cell count was significantly elevated, predominantly caused by a 1.2 fold increase in monocytes and a 2.7 fold increase in neutrophils, which were related to a significant increase in cortisol (375 ± 81 vs. 440 ± 106 nM). Likewise, the vWf level was increased (110 ± 44 vs. 193 ± 72%). An increased coagulation activity after exercise was shown by a significant elevation in TAT levels (1.9 ± 0.6 vs. 2.5 ± 0.8 ng/mL). Furthermore, non-triggered ROTEM clot formation was significantly accelerated (CT 1109 ± 188 vs. 918 ± 82 s) with increased clot strength (MCF 44.0 ± 3.6 vs. 49.1 ± 5.3), suggesting the presence of an endogenous coagulation trigger. These results were supported by a two fold increased thrombin generating potential in the absence of a tissue factor trigger, as well as by 1 pM tissue factor triggered TG. The enhancement of TG was not influenced by inhibition of the extrinsic pathway through active site inhibited factor VII, suggesting the contribution of the intrinsic pathway of coagulation. This finding was supported by a 2 fold increase in FXIa level. The procoagulant state was accompanied by enhanced platelet aggregation in response to ADP, arachidonic acid and TRAP. The fibrinolytic activity was increased, as indicated by elevated tPA activity (0.6 ± 0.3 vs. 1.9 ± 1.1 U/mL) and D-dimer levels (210 ± 66 vs. 246 ± 119 ng/mL).
Conclusions: Cycling induces a hypercoagulable state through both increased platelet reactivity and contact dependent activation of coagulation. Furthermore, cycling leads to increased fibrinolytic activity and endothelial dysfunction. Although this procoagulant response in cyclists may primarily reflect a physiological response to physical stress, it may eventually contribute to abnormal vascular responses including the development of endofibrosis.

PB 4.49-5

Genetic characterization of seven patients affected by FXI deficiency

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Background: FXI deficiency is a rare bleeding autosomal disorder characterized by a heterogeneous bleeding tendency, often unrelated to the levels of circulating FXI. More than 150 mutations have been reported in FXI gene and two are most frequent in the Jewish Ashkenazi population (Glu117X and Phe283Leu).
Aim: In this study we report the genetic analysis of nine Italian patients (five with severe and four with mild FXI deficiency).
Results: One asymptomatic patient (FXI:C = 0.3%) has two known heterozygous mutations (Glu117X/c.908DelG). The other four patients with a severe deficiency (FIX:C = 1–5%) have four different homozygous mutations: two novel, Gln-13X (in the propeptide) and Trp407Arg (in the active site), one known splice site mutation in intron 6 (c.595 + 3A>G) and one Gly578Cys (in the active site), already described by our group. The latter two patients have a mild bleeding

history. Both missense variations are in the Apple 3 domain of the protein. In position 407 a substitution of the amin oacid Tryptophan with a Cysteine has been already described. The substitution of an aromatic and hydrophobic amino acid (Trp) with a linear and basic one (Arg) possibly interferes with the polar bond with the Ile403 and Gly404 residues causing protein instability.
Two patients with mild deficiency but with a significant bleeding history show the heterozygous variation Trp228Cys. Another mild patient with post-surgery bleeding has a heterozygous Cys122Tyr substitution. Finally, the last asymptomatic patient with mild FXI deficiency is heterozygous for a Arg184Gly mutation.
Conclusions: This study confirms the wide genetic heterogeneity in Italian patients of non-Jewish origin affected by FXI deficiency. Furthermore we confirm the lack of correlation between levels of circulating FXI and the manifestation of bleeding symptoms.

PB 4.49-6
Novel coumarins with improved solubility as FXIIa inhibitors

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Background: Thrombotic diseases remain a major cause of death in industrialised countries. Anticoagulants have proven their efficacy to address these disorders. However severe bleeding complications are still reported even with the use of recently marketed drugs. Novel and safe antithrombotics are thus required.
In this perspective, coagulation factor XIIa (FXIIa), a serine protease implicated in the contact phase of coagulation cascade, recently emerged as a promising target in the development of such agents. Indeed, it was demonstrated that FXII deficiency or inhibition protects against thrombosis without causing spontaneous bleeding in mice.
Aim: Based on these considerations, the goal of our project is to develop novel selective FXIIa inhibitors to detail the exact role of this enzyme in thrombotic diseases. These compounds could also be a good starting point for the development of new antithrombotic drugs.
The 3-carboxamide coumarins are to date the only nonpetidic and selective inhibitors of FXIIa described in literature (COU294). However, their low solubility and poor pharmacokinetics resulted in a lack of activity in *in vivo* models of thrombosis. As a consequence, we aim to improve these characteristics while keeping the selectivity and potency towards FXIIa.
Methods: Twenty-five compounds were synthesized. Their solubility was investigated in pH 7.9 TRIS-imidazole. Inhibition potency on FXIIa was screened at several concentrations according to the maximum of solubility.
Results: A new series of coumarins with an amino group at position 3 or 6 of the carboxamide coumarin scaffold was first synthesized. While solubility was largely increased the inhibition potency on FXIIa remained relatively low.
Conclusion: A new series of 3-carboxamide derivatives was synthesized to selectively inhibit FXIIa and with the aim of improving their solubility. Pharmacomodulations will be fully discussed.

PB4.50 – Blood coagulation system – V

PB 4.50-1
Protein C inhibitor and the ambivalent regulation of apoptotic cell removal

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Background: Protein C inhibitor (PCI) is a secreted serine protease inhibitor, first ascribed to inhibit activated protein C, but it also inactivates other clotting factors and fibrinolytic enzymes, suggesting a regulatory role in hemostasis. Glycosaminoglycans and certain negatively charged phospholipids, like phosphatidylserine (PS) and phosphatidylethanolamine (PE), bind to PCI and stimulate its activity. PS is best known as a phagocytosis marker, exposed on the surface of apoptotic cells, serving as phagocytosis signal. Deranged apoptotic cell removal by phagocytes can lead to tissue damage and chronic inflammation, which is a risk factor for the development of vascular disease.
Aims: It is the aim of this study to determine binding of PCI to cell membranes with different lipid surface exposure (e.g.: live and apoptotic cells) and to determine the influence of PCI on the process of apoptotic cell removal.
Methods: To investigate the influence of PCI on phagocytosis we used a dual color Flow Cytometry based study design, and apoptotic Jurkat cells as 'meal' for phorbol ester differentiated U937 cells, that show macrophage like functions. To study the binding of PCI to different cell membranes PCI was directly coupled with a fluorophore. Different cell types and -stages were incubated with PCI-Cy3 together with the apoptosis marker Annexin V-FITC, and analysed by confocal microscopy, fluorescence resonance energy transfer (FRET) microscopy, and Flow Cytometry .
Results: Our results indicate that exogenously added PCI inhibits the phagocytosis of apoptotic cells. PCI bound primarily to apoptotic cells and only to a small extent to U937 cells. The amount of PCI-binding U937 cells increased with differentiation, probably due to a higher degree of PS exposure on the surface of macrophages involved in phagocytosis. Interestingly, the expression of PCI increased with the time of phorbol ester induced differentiation, resulting in enhanced phagocytosis. Data obtained by FRET analysis suggest an interaction of PCI and PS on the surface of apoptotic cells, probably leading to coverage of the phagocytosis marker PS, preventing recognition by phagocytes.
Conclusion: On the one hand this study underlines the importance of PCI for the function of mature macrophages, but on the other hand uncovering secreted PCI as a negative regulator of apoptotic cell removal. Further studies are needed to clarify this ambivalence of one protein performing differently in the process of apoptotic cell phagocytosis and its relevance for vascular disease.

PB 4.50-2
Hemostasis in acute respiratory viral infections in often sick children with gene's polymorphism (asp299gly) toll-4 and (ser 249 pro) toll-6 receptors

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We were interested in hemostasis in acute respiratory viral infections in often ill children, which were carriers of genetic defects in the Toll-4 (Asp299Gly) and Toll-6 (Ser249Pro) receptors. Purpose: To examine the state of the hemostatic system with SARS in sick children in the presence of polymorphism of Toll-4 (Asp299Gly) and Toll-6 (Ser249-Pro) receptors.
Materials and Methods: 190 children from 1 to 3 years often ill with acute respiratory infections were examined. The control group consisted of healthy children from 1 to 3 years with the number of episodes of SARS not more than four cases per year. Of 190 patients 90 children were carriers of the gene polymorphism (Asp299Gly) Toll-4 receptor, and 100-were carriers of mutations in the gene (Ser249Pro) Toll-6 receptor. Blood sampling was performed at admission to hospital; coagulation and fibrinolytic activity was determined by conventional methods in children with ARI.
Results: It was established that acute respiratory viral infection was developed during the following hypercoagulability in often ill children. It was expressed by the shortening of plasma recalcification time, increasing concentration of the fibrinogen and the number of fibrin monomer complexes. Fibrin was depressed by fibrinolysis due to rapid formation. The main cause of hypercoagulability in ARI was antigenic

activation of immune cells and destruction of affected tissues. The expression occurred on the cell surface of tissue factor, which was initiated by the formation of prothrombinase on the external mechanism by activation of cells involved in inflammation. The degree of expression was higher in sick children than in healthy ones, which could be one of the causes of hypercoagulability in blood flow in respiratory infections. The expression of tissue factor was marked greater in genetic defects in membrane receptors (Toll-4) than in similar cells with normal structure of signaling receptors. Besides, the level of fibrin monomer complexes was higher in holders of polymorphic variants of the gene Toll-4 receptor concentration of fibrinogen than that of similar ill children with normal allele 299 Asp. Fibrinolysis was slowed due to rapid formation of fibrin especially in carriers of mutant heterozygote (Asp299Gly) and homozygote with the replacement of allele (299Gly). Such changes in findings of hemostasis were revealed in sick children with polymorphisms Toll-6 (Ser249Pro) receptor. They had high blood concentrations of fibrinogen, fibrin monomer complexes. Euglobulin fibrinolysis was lengthened in heterozygotes (Ser249Pro) to 12.5% in comparison with carriers of the normal homozygotes and by 32% for the holders of the mutant homozygotes with allele 249Pro. Tissue factor expression on cells with the genetic defect in the Toll-6 receptor was more marked than in healthy children.

Conclusion: Consequently, hypercoagulability in children with ARI was more marked in the polymorphism of genes Toll-4 and Toll-6 receptor than those of the same children, but without the genetic mutations in the signaling receptor.

PB 4.50-3

Developmental haemostasis: the quantity of haemostatic proteins change with age

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Background: Developmental haemostasis recognises the physiological differences in the haemostatic system of neonates and children compared to adults. Compared to the knowledge of haemostatic system physiology in adults, our understanding in neonates and children remains inadequate.

Routine clinical coagulation testing most commonly measures functional parameters of the haemostatic system once it has been activated. While many studies have demonstrated age-specific differences in functional haemostatic assays, very few studies have measured age-specific quantitative levels of haemostatic proteins.

The majority of studies in the paediatric setting use the term 'quantity' when in fact they are referring to a functional measurement rather than the direct measurement of a specific protein.

An understanding of the normal fluctuations in the quantity of haemostatic proteins is vital in the prevention, diagnosis, and treatment of haemostatic problems during infancy and childhood. This is especially important in the setting of novel anticoagulants that target specific proteins of the coagulation system.

Aim: To develop age-specific, quantitative reference ranges for a comprehensive number of haemostatic proteins.

Methods: Plasma samples were obtained from 120 healthy individuals from the following age groups: neonates (Day 1 and Day 3), 28 days to 1 year, 1–5 years, 6–10 years, 11–16 years and adults. Each age-group consisted of 20 individuals.

Factors II, V, VII, VIII, IX, X, XI, XII, and XIII, Plasminogen, Protein C as well as total and free Protein S were quantified using commercially available sandwich ELISA assays (Diagnositica Stago and Affinity Biologicals).

Reference ranges were determined for each protein and age-group by encompassing 95% (between the 2.5th and 97.5th centiles) of the population. Neonatal and paediatric results were compared to adult results using an independent *t*-test, where a P value of <0.05 was considered statistically significant. The Levene's Test for Equality of Variance was used to determine the variance. Where unequal variance was detected, an Aspin-Welch test was used. Correction for multiple testing was conducted using the Monte-Carlo approach based on 10,000 samples. All analyses were performed using NCSS 2007, Version 07.1.12.

Results: The concentration of the majority of coagulation proteins was found to vary significantly with age. Of the proteins measured, 10 were significantly different between neonates and adults and these differences persisted throughout childhood for most of these proteins. Factor XIII and Factor VIII were the only proteins that did not change with age.

Conclusion: The results of this study confirm for the first time that the quantity of most coagulation proteins changes significantly with age. Further research is required to investigate how the quantity of haemostatic proteins influences functional assays. Furthermore, it is imperative that we investigate how the differences in the quantity of these proteins contributes to the effect of anticoagulants, especially when considering the use of novel anticoagulants that target specific proteins of the coagulation system.

PB 4.50-4

Effects of rVIIa in thrombocytopenic cord blood derived plasma

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Background: Primary haemostasis of newborns is excellent in normal conditions, although neonatal platelets show impaired function in aggregation measurements. Thrombocytopenia is a well known problem in neonatal care, and poses a significant risk for bleeding, particularly intracranial haemorrhage.

RVIIa, developed and licensed for bleedings of haemophiliacs with inhibitors, has almost become an universal haemostatic agent in different bleeding events including thrombocytopenic bleeding. In cases of life-threatening bleedings, rVIIa also has been applied for newborns.

Aim: Aim of our study was to assess *in vitro* data about the effects of rVIIa on thrombin generation in normal and thrombocytopenic neonatal plasma in comparison to adults.

Methods: Cord blood and adult blood was collected (n = 5 each), PRP was prepared by centrifugation at 250 ***g*** and different concentrations of platelets (100,000/μL, 75,000/μL, 50,000/μL, 10,000/μL and PPP) were created by diluting with autologous PPP. Thrombin generation with PRP reagent was measured using Calibrated Automated Thrombography (CAT) with rVIIa (1.5 μg/mL and 3 μg/mL final concentration) and without.

Results: Addition of rVIIa leads to a shortening of lag time. This effect is more pronounced in cord blood. Time-to-peak is slightly shortened in cord blood, but not in adult blood, velocity index is decreased only in cord blood. Total ETP and peak levels are not markedly influenced by rVIIa. These effects are comparable in all tested platelet concentrations. There is no difference in the effects of 3.0 μg/mL or 1.5 μg/mL.

Conclusion: Data show that platelets in cord blood derived plasma can create enough surface for rVIIa to accelerate initiation phase of coagulation even in thrombocytopenic plasma. This implicates that rVIIa has similar effects in neonatal plasma even under conditions of thrombocytopenia compared to adult controls. As controlled studies on use of rFVII in neonatal patients are rare, our in-vitro measurements shed light into the procoagulatory efficacy in this particular patient group. The right dosage in neonates remains a point of discussion.

PB 4.50-5

Thrombin generation testing to monitor warfarin anticoagulation in thrombotic antiphospholipid syndrome patients

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Background: Warfarin monitoring in patients with antiphospholipid antibodies (aPL) can be complicated due to the variable responsiveness of thromboplastin reagents to lupus anticoagulants (LA), which may in turn potentially influence the validity of the prothrombin time (PT)-International Normalised Ratio (INR) in monitoring anticoagulation treatment in thrombotic antiphospholipid syndrome (APS).
Aim: To evaluate thrombin generation potential in thrombotic patients with and without APS treated with warfarin anticoagulation and to compare the INR values obtained using two commonly used PT/INR reagents, which have differing sensitivity to LA.
Methods: Eighty thrombotic patients with (n = 35) and without (n = 45) APS receiving warfarin anticoagulation were studied (32 out of 35 of the APS patients were LA positive). Thrombin generation test (TGT) was performed using the calibrated automated thrombinogram with the PPP reagent (5 pM tissue factor, 4 μM phospholipid, Diagnostica Stago). Three parameters were derived from the thrombin generation curves: lag time, peak, and endogenous thrombin potential (ETP). PT in all samples was measured with the Innovin (Siemens) and the PT Fib HS Plus (IL) thromboplastin reagents using the Sysmex CS-5100i and the IL TOP500, respectively. INR was calculated using instrument-specific assigned ISI values. Thrombin generation parameters were correlated with INR values and compared between the patient groups. Amidolytic Factor X (FX) levels were measured using a commercial kit (Biophen, Hyphen). Unless otherwise indicated, results are expressed as median and observed range.
Results: INR with Innovin and PT Fib HS Plus were 2.93 (1.55–5.93) and 3.03 (I.58–5.17) respectively for the APS patients and 2.48 (0.98–5.85) and 2.45 (0.95–4.79) respectively for the non-APS patients. Seven patients (4 APS) showed >0.5 INR unit difference between reagents; five of these seven patients had an INR >4.5. There was an inverse correlation between the INR and the normalised endogenous thrombin potential (ETP%) in both patient groups: Innovin and PT Fib HS Plus vs. ETP in APS patients: rs = −0.84 and rs = −0.80 respectively ($P < 0.0001$) (ETP% 20.7 [8.9–49.2]); and in non-APS patients: rs = −0.83 and rs = −0.73 respectively ($P < 0.0001$) (ETP% 29.9 [5.8–95.6]). Similar relationships were observed with normalised peak thrombin values. The normalised ETP and peak thrombin were significantly decreased and lag times significantly increased in the APS compared with the non-APS patients ($P < 0.001$, 0.007 and 0.0001 respectively), which may reflect the higher INR values in the APS patients. Amidolytic FX levels (19.7 (12.7–34) APS, 20.9 (12.7–131.5) non-APS) correlated well with the INR with both thromboplastins and with the ETP.
Conclusion: These data suggest that anticoagulant intensity on warfarin determined using Innovin and PT Fib HS Plus, with instrument-specific ISI values, concurs with that measured by the TGT which provides a good assessment of anticoagulant intensity in APS as well as non-APS patients. ETP evaluation combined with PT-INR may give a better indication of the intensity of anticoagulation in both thrombotic APS and non APS patients.

PB 4.50-6

Evaluation of a new chromogenic protein C assay (DG-Chrom PC) on the Q Hemostasis Analyzer (Grifols)

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Background: Protein C (PC) assays need to demonstrate strong precision, linearity, and specificity to be suitable techniques for PC estimation. Grifols (Spain) have developed a new chromogenic PC (DG-Chrom PC) assay for their Q Hemostasis Analyzer.
Aim: Evaluate the performance characteristics of Grifols DG-Chrom PC assay with comparison to an established PC technique.
Method: All blood samples were taken into 0.109 M citrate tubes and centrifuged at 2000 ***g*** for 10 min prior to freezing aliquots at −70 °C. The DG-Chrom PC (Q-PC) assay on the Q Hemostasis Analyzer utilises reconstituted lyophilised snake venom PC activator (Protac) and chromogenic substrate. The reference PC method was an in house assay performed on ACL TOP analyser (Instrumentation laboratory, UK) utilising Protac (Pentapharm, Switzerland) and chromogenic substrate, S-2366 (Quadratech, UK).
Results: All calibration curves (n = 11) generated r^2 values >0.999 with Q-PC assay. Three levels of precision (n = 40) were performed for Q-PC; normal pool (99 IU/dL) returned CV of 1.7%, abnormal pool (48 IU/dL), CV 2.5% and low abnormal pool (12 IU/dL), CV 5.3%. PC assays can suffer from cleavage of chromogenic substrate by targets other than PC e.g. kallikrein. Replacing Protac activator with buffer and testing normal (n = 10) and abnormal plasmas (n = 10) established that no non-specific cleavage of chromogenic substrate was detected. Q-PC estimations performed on PC deficient plasma (Technoclone, UK) generated undetectable PC levels (n = 10).
Pooled plasma with PC of 130 IU/dL demonstrated good linearity between 0 and 130 IU/dL (n = 5), mean r^2 of 0.998 with Q-PC assay. Accuracy of Q-PC assay was evaluated (n = 5) using 3rd SSC analysed at four dilutions. Undiluted plasma (89 IU/dL) generated mean estimate of PC 90.1 IU/dL, at 44.5 IU/dL dilution, mean PC of 46.4 IU/dL, at 22.2 IU/dL dilution, mean PC of 24.1 IU/dL, and at 11.1 IU/dL dilution, mean PC of 11.3 IU/dL. The limit of sensitivity of this assay was evaluated using 3rd SSC diluted in buffer (n = 5). Q-PC accurately measured PC level of a 5.6 IU/dL dilution, estimates of PC level ranged from 4.3 to 5.2 IU/dL.
For method comparison residual plasmas stored at −70 °C were utilised. Study groups included low PC (congenital and acquired, n = 54), healthy normals (n = 57), heparinised (n = 11) and liver disease (n = 13) samples. Linear regression analysis of all samples calculated weighted r value of 0.984 (n = 143), within test groups correlation (r^2) ranged between 0.782 and 0.977. There were statistically significant differences between PC assay results obtained in individual test groups and in the combined group, however the differences were small and in no group was this difference large enough to be considered clinically relevant.
Conclusion: The new Grifols PC assay (DG-Chrom PC) demonstrates good precision and excellent linearity. Q-PC limit of sensitivity was measured at 5.6 IU/dL using 3rd SSC and the assay appears suitable for diagnosis of hereditary homozygous PC deficiency. No significant optical density was observed for Q-PC assay when blank assessment was performed, suggests PC blank estimation is not routinely required. Q-PC compared well with the established in house assay when pathological and healthy normal patient samples were tested.

PB4.51 – Blood coagulation tests – XV

PB 4.51-1

Evidence that low protein C is a crucial determinant of the pro-coagulant imbalance in cirrhosis

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Background & Aims: Cirrhosis is associated with a plasmatic pro-coagulant imbalance detected by thrombin generation tests performed in the presence-vs.-absence of such activators of protein C as

thrombomodulin or Protac®. This imbalance is thought to be due to decreased protein C and increased factor VIII. To test this hypothesis we analyzed plasma from 50 patients with cirrhosis before and after *in vitro* addition of purified protein C meant to restore normal levels.

Methods: Results for two thrombin generation assays were expressed as ratios of endogenous thrombin potential (ETP) with-to-without-thrombomodulin or as Protac®-induced coagulation inhibition (PICI %). By definition, high ETP ratios or low PICI% reflect a pro-coagulant imbalance.

Results: The median (range) protein C level before addition was 40% (4–101%) and increased to 156% (110–305) after addition ($P < 0.001$). The pro-coagulant imbalance, which was high before protein C addition [ETP-ratio = 0.83 (0.44–1.00)] was reduced after addition [ETP-ratio = 0.60 (0.14–0.84)], $P < 0.001$. ETP-ratios were inversely correlated with protein C activity (rho = −0.46, $P < 0.001$). Similar results were obtained with the Protac®-assay.

Conclusions: The results provide direct evidence that the pro-coagulant imbalance observed in plasma from patients with cirrhosis is due to reduced protein C and can be reversed by increasing its levels. The findings may have clinical implications for the treatment or prophylaxis of thrombosis in these patients.

PB 4.51-2

Effekt of recombinant Factor VIIa BAY 86-6150 on clot formation kinetics

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Background: BAY 86-6150 is a new recombinant FVIIa preparation with increased half-life and improved potency by enhanced binding to activated platelet surfaces. In hemophilia A and B the onset of blood coagulation is delayed and the clot formation is diminished. Clot waveform analysis is a fast and affordable method to investigate fibrin generation by analyzing the kinetic properties of fibrin formation after activation of the intrinsic coagulation cascade.

Aim: Clot waveform analysis is used to demonstrate the capability of BAY 86-6150 to correct a hypocoagulable state and is compared to thrombin generation (TG) and thrombelastography.

Method: All patients were from the centre of blood coagulation and had a history of bleeding disorders. Healthy subjects from our lab served as controls. Blood drawings and subsequent experiments where performed after informed consents were obtained in accordance to the Declaration of Helsinki. The measurements were performed in whole blood, in platelet-rich and platelet-poor plasma. BAY 86-6150 was added *in vitro* and coagulation was tested using clot waveform analysis (ACL TOP, activator Synthasil, Instrumentation Laboratory, Kirchheim, Germany), ROTEM (Tem International, Munich, Germany), CAT (Thrombinoscope, Maastricht, The Netherlands) and Technothrombin TGA (Technoclone, Vienna, Austria). For clot waveform analysis the time-dependent change of absorbance was analyzed as well the first three derivatives of the curve.

Result: All used methods showed a dose-dependent correction of delayed and diminished coagulation in hemophilia by BAY 86-6150. TG showed a complete normalization as well as thrombelastography. In clot waveform analysis, independent of tissue factor activation, BAY 86-6150 normalized the delay and total fibrin formation; fibrin formation velocity remained slightly slowed down. The procoagulant effect of BAY 86-6150 was strong in severe hemophilia and diminished in healthy controls.

Conclusion: Recombinant FVIIa BAY 86-6150 showed a dose-dependent normalization of coagulation in severe hemophilia. Onset and velocity of clot formation was improved significantly. BAY 86-6150 did not lead to a procoagulant state in healthy controls. Clot waveform analysis is an affordable, extremely fast and convenient method to investigate different aspects of clot formation and may pose as an alternative to thrombin generation and thrombelastography.

PB 4.51-3

Design and establishment of a biobank for a multicenter and interdisciplinary prospective cohort enrolling elderly patients with venous thromboembolism (SWITCO65+)

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Background: In the field of haemostasis, many preanalytical variables influence the results of coagulation assays and may affect the quality of the subsequent results. Therefore, methods to limit these results variations should be foreseen when building a biobank in the field of haemostasis. A biobank of biological material from venous thromboembolism (VTE) patients is essential for translational research aiming to better characterize VTE at several levels ranging from diagnostic and prognostic classification to prediction of response to therapy.

Aim: Our detailed description of the biobank of the Swiss Cohort of Elderly Patients with Venous Thromboembolism (SWITCO65+) is thus intended to facilitate set-up of other biobanks in the field of hemostasis and to contribute substantively to the discussion of Good Practice and Standard Operating Procedure.

Methods: SWITCO65+ is a multicentre interdisciplinary cohort that prospectively enrolled consecutive patients aged ≥65 years with VTE at nine Swiss hospitals from September 2009 to March 2012. The patients will be followed up until December 2013. The cohort includes a large biobank with biological material from each participant taken at baseline and after 12 months of follow-up. Whole blood from all participants was assayed with a standard haematology panel, for which fresh samples are required. Two buffy coat cryovials, one PAXgene Blood RNA System tube and one whole blood sample were also collected at baseline and will be used for RNA/DNA extraction. Blood samples were processed and cryovialed within 1 h of collection for special assays, and transported in batches by commercial courier to a central laboratory where they were stored in ultra-low temperature archives. Use of barcoding and electronic databases ensured efficient management of the biobank. All analyses of the same type were performed in the same laboratory in batches. Using multiple core laboratories increased the speed of sample analyses and reduced storage time.

Results: Among the 1003 patients who agreed to participate in the cohort study, 905/1003 (90.2%) had a blood collection at baseline. The SWITCO65+ DNA and RNA depository was designed to permit both genome wide association and-replication studies, with the single limitation that 99.8% of the study patients were Caucasians. Only a small minority of those 905 patients refused to provide blood samples for genetic analyses (6.5%). By October 16th, 2012, after an average follow-up time of 512 days, 799 (80.1%) patients were still actively participating, 150 (15.0%) had died, and 1 (0.1%) was lost to follow-up. Forty-seven out of 1003 (4.7%) patients had withdrawn their consent, but only 6 (0.6%) did not allow us the use of their data and blood samples for analysis. By November 2012, 20,802 vials were stored in the central SWITCO65+ biobank and 7852 vials had already been sent for subsequent analyses in one of the four participating laboratories (Lausanne, Zürich, Bern, Geneva).

Discussion: After recruiting, processing and analyzing the blood of 905 study participants, we determined that the adopted methods and technologies were fit-for-purpose and robust.

PB 4.51-4

A simple screening method for the identification of the presence of FVIII and FIX inhibitors by determining the steepness of the routine aPTT reaction curves

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Background: The majority of modern coagulation analyzers have the feature to continously monitor the changes in light transmittance or absorbance during the performance of routine aPTT. Consequently, every aPTT measurement is recorded as a reaction curve characterized by the specific shape and slope that depends on coagulation acceleration associated with the formation of fibrin. Besides the routine evaluation mode, some analyzers check the plausibility of a kinetic curve by using additional checking methods in order to protect from false aPTT results. If kinetics does not meet the criteria, the result is flagged with a question mark, meaning that the result is questionable and that the kinetic curve should be visualy inspected. We have observed that in plasma samples with FVIII and FIX inhibitors flagged aPTT results were obtained due to the doubtful onset of coagulation because kinetic curve did not reach the predefined angle.

Aims: This prompted us to investigate the possibility to detect the presence of FVIII and FIX inhibitors by analyzing flagged aPTT results after visual inspection and manual measurement of angle that determines the steepness of reaction curve.

Methods: The study included 61 patient plasma samples with request for the determination of FVIII or FIX inhibitors. Routine aPTT measurement was performed using Actin FS on the BCS-XP coagulation analyzer (Siemens Medical Solutions Diagnostics, Germany) at 405 nm with the manufacturer's proposed evaluation method DRIFTING BASELINE (DB) and checking method ANGLE DB with predefined minimal angle of 30 degrees. Each aPTT result with angle lower than 30 degrees was flagged and the measurement continued at 570 nm (checking method ANGLE DB with predefined minimal angle of 15 degrees), followed by the visual inspection of the kinetic curve. Additionally, for each aPTT result manual measurement of reaction curve angle at 405 nm was performed. FVIII and FIX activities were measured by one-stage clotting assay. Inhibitor screens were performed and inhibitor titers determined by the Nijmegen modified Bethesda assay. Patients were divided into two groups according to the absence (group I, $n = 38$) or presence (group II, $n = 23$) of FVIII ($n = 21$) and FIX ($n = 2$) inhibitors.

Results: A flagged aPTT result at 405 nm was recorded in all patients with inhibitors, and in three patients without inhibitors. When measurement continued at 570 nm, no flagged aPTT result was obtained. For all tested samples an excellent correlation between FVIII activities (range: 1–91 U/dL; median: 7.0 U/dL) and measured angle (range: 25.5–78.0 degrees; mean angle ±SD: 49.2 ± 19.4 degrees) was obtained ($r = 0.928$; $P < 0.001$). Statistically significant difference ($P < 0.001$) for measured angle was identified between group I (range: 29.5–78.0 degrees; mean angle ±SD: 62.1 ± 12.6 degrees) and group II (range: 25.5–29.5 degrees; mean angle ±SD: 28.0 ± 1.3 degrees). ROC curve analysis revealed that the predefined minimal angle of 30 degrees distinguishes between patients with and without inhibitors with 100% sensitivity and 92.1% specificity.

Summary/Conclusions: Based on obtained results, ANGLE DB checking method together with visual inspection of aPTT reaction curve and angle measurement, provides a simple and reliable tool as a screening method for the presence of FVIII and FIX inhibitors.

PB 4.51-5

Laboratory estimation of expanded uncertainty of D dimer measurement by ELISA.

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D dimer (DD) is a useful biomarker of thrombin and plasmin effect that is widely used by Haemostasis and Thrombosis laboratories. There is a lot of discrepancy of results obtained by different methods, but ELISA one has been recognized as the most sensitive with the higher negative predictive value for the exclusion of venous thromboembolism. The cut off value for exclussion of venous thromboembolism widely used by this method is 500 ng/mL FEU. The aim of the present study was to estimate the uncertainty of the measurement of DD in our laboratory. We did it by using the data of the Internal Quality Control provided by the manufacturer and those from the results of the last five CGL Surveys of College of American Pathologist. We calculated the extended uncertainty of DD measured by an ELISA (Vidas D-Dimer Exclusion) following the Nordtest Handbook formula: Internal Quality Control + Interlaboratory comparison results.

Formulas used were:

Combined standard uncertainty (μ_c) $= \sqrt{(cv^2 + m\ bias^2)}$

cv = cv Internal Quality Control

m bias $= \sqrt{(RMS\ b)^2 + \mu\ (Cref)^2}$

RMS b: $\sqrt{\Sigma\ bias^2/n}$ (total DD surveys results)

μ (Cref): RMS of cv interlaboratory/$\sqrt{N}$ (number of participant laboratories of CGL)

Expanded uncertainty (U) $= 2 \times \mu_c$ (95% of confidence Interval)

We calculated U for the two levels of control provided by the manufacturer: one around 500 ng/mL FEU and the other around 5000 ng/mL FEU. Each lot of the reagent provides different lot of both controls (low and high), we performed an ANOVA analysis to make sure that their values were statistically comparable.

U results obtained were 15.57% for the lower level of control (500 ng/mL FEU) and 16.76% for the higher (5000 ng/mL FEU). The results were similar for the two levels of control, and both were acceptable considering that U should not be >⅔ of the allowed total error (TEA), Gella Tomás et al, Documents of SEQC 2009: 27–29. In our laboratory we chose TEA of 30%, requirement informed by The American Association of Bioanalysts.

U values expressed as quantity of analyte were 78 and 838 ng/mL FEU for the lower and the higher level, respectively, being the possible report for patients' plasma results 500 ± 78 and 5000 ± 838 ng/mL FEU.

Conclusion: The expanded uncertainty estimation is a useful tool to assess the quality of results produced by the laboratory. The U of DD measurement by ELISA estimated in our laboratory was acceptable, but its value should be considered and it could be very important when clinical decisions are taken, particularly at 500 ng/mL FEU level.

PB 4.51-6

Validation of rivaroxaban anti-Xa activity measurement

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Background: New oral anticoagulants are increasingly being used. Specifically, rivaroxaban has been approved for use without routine monitoring, but the lack of a predictable drug level measurement may complicate management of anticoagulated patients. Drug level measurement is instrumental in monitoring compliance, potential medication failure, safety for interventional procedures, and for the care of the chronically anticoagulated patient and pediatric, pregnant, renal disease patients. Activated partial thromboplastin time, and

prothrombin time have been evaluated as surrogate measurements for rivaroxaban and found to have poor correlation. Alternatively, high-performance liquid chromatography-tandem mass spectrometry [HPLC-MS/MS] can be used to measure rivaroxaban levels between 0.50 and 500 μg/L with excellent inter-assay accuracy, but the timely clinical availability is limited. To overcome these limitations, an anti-factor Xa based assay using rivaroxaban calibrators is currently under investigation as a method to monitor rivaroxaban.

Aims: The aims of the project are to correlate the STA®-Liquid Anti-Xa using the STA®-Rivaroxaban Calibrators (Diagnositica Stago) with the serum drug levels via HPLC-MS/MS in patients currently receiving rivaroxaban, and secondly, to correlate the PT/PTT, thrombin generation (CAT assay) and Thromboelastograph (TEG) with the anti-Xa activity and HPLC-MS/MS rivaroxaban level.

Methods: This is a prospective validation cohort study including 20 adult patients receiving rivaroxaban and 10 healthy control subjects. Recruited patients receiving rivaroxaban have a total of three blood samples taken at specific time points at least 2 h apart. Plasma is divided into four aliquots of 0.5 mL for measurement of PT/PTT, anti-factor Xa rivaroxaban assay, rivaroxaban HPCL-MS/MS, and thrombin generation. TEG activity is measured at one random time point for each patient. Correlation and linear regression evaluations are performed on each of the clotting based tests including both TEG and LC/MS/MS values. All tests are centrally measured except for the independent measurements of HPLC-MS/MS. A rho2 of >0.9 will be considered acceptable for clinical use.

Results: To date, all 10 healthy controls and 4 of 20 identified patients receiving rivaroxaban have been completed including TEG analysis. Upon completion of sample collection, statistical and clinical analysis will be completed. Based on our preliminary evaluation, the anti-factor Xa assay with rivaroxaban calibrators and controls will have a positive correlation with serum measurements by HPLC-MS/MS and a good correlation with the PT/PTT. To date, the TEG results are intriguing in their response to clot formation and structure. The thrombin generation assay will provide additional information regarding the level of anticoagulation and rivaroxaban induced variation.

Summary: We anticipate that the results of this study will validate a viable rivaroxaban-specific quantitative assay using rivaroxaban calibrators in a standard anti-factor Xa activity for the measurement of rivaroxaban that is applicable for patient monitoring when medication compliance or peri-procedural evaluation is required.

PB 4.52-1

Dilute Russell Viper Venom Time: a useful assay for the monitoring of direct oral anticoagulants in patients?

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Background: Direct oral anticoagulants (DOACs) include anti-IIa agent (dabigatran etexilate: DE) and anti-Xa agents (rivaroxaban, apixaban and edoxaban). DOACs do not require monitoring nor frequent dose adjustment. However, searching for the optimal response at the individual patient level may be useful in some situations. Activated partial thromboplastin time, Hemoclot Thrombin Inhibitor® (HTI) and ecarin clotting time have been proposed to monitor dabigatran whereas anti-Xa chromogenic assays are preferable to monitor anti-Xa agents. However, there is still a need for a global test easily implementable and widely available that may be used for all DOACs. Recent studies showed that the dilute Russell Viper Venom Time (DRVV-T) could be used for the monitoring of DOACs but these results have not been performed on clinical samples.

Aim: To analyse and compare the results obtained with STA®-DRVV Screen and Confirm with the clinical plasma drug levels estimated with Hemoclot Thrombin Inhibitor® (HTI) for DE samples and Biophen Direct FXa Inhibitor® (DiXaI) for rivaroxaban samples.

Materials: Three patients under DE (150 mg *bid*) and three patients under rivaroxaban (20 mg *od*) were included in this study. Blood samples were taken at 2 h and 3 h after drug administration, and just before the next scheduled intake of the drug (C_{trough}: 12 h for DE and 24 h for rivaroxaban). The following tests were performed at each time-point:

Estimation of plasma drug concentrations: The HTI® (Hyphen Biomed) assays and the Biophen DiXaI® (Hyphen Biomed) were performed to estimate plasma drug concentration for DE and rivaroxaban samples, respectively. These tests have been shown to highly correlate with the HPLC-MS/MS reference measurement.

Dilute Russell Viper Venom Time: STA®-DRVV Screen and Confirm (Stago Diagnostica) were performed according with the recommendations of the manufacturer. Normal pooled plasma used as reference for the ratio was obtained from 42 healthy individuals.

Results: The estimated plasma range concentration was from 0 to 308 ng/mL for DE and 0 to 388 ng/mL for rivaroxaban. STA®-DRVV and the estimated plasma dabigatran concentration highly correlates (r^2 = 0.90 and 0.95 for Confirm and Screen, respectively) but showed a plateau at 100 and 200 ng/mL for Confirm and Screen, respectively. The correlation between STA®-DRVV and the estimated rivaroxaban concentrations depends on the reagent (r^2 = 0.95 and 0.78 for Confirm and Screen, respectively). The relation was linear on the range of concentration encountered in this study. The use of ratio did not improve the correlation parameters.

Conclusion: STA®-DRVV Confirm and Screen should not be used to evaluate DE therapy since concentrations above 100 and 200 ng/mL could not correctly be discriminated. STA®-DRVV Confirm could be used as a screening test to estimate rivaroxaban plasma levels thanks to the high correlation and the good sensitivity on the whole therapeutic range. Further investigations are required to propose normal range value at different sampling time.

PB 4.52-2

Validation of an assay for determination of anti-xa activity of Rivaroxaban

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Aim: Rivaroxaban (Xarelto®) is a new oral direct factor Xa inhibitor. The purpose of this study was to evaluate the performance of STA® – Liquid anti-Xa reagent (Diagnostica Stago), for the ex vivo measurement of rivaroxaban anti-Xa activity. Accuracy of this assay was evaluated by comparison of results with HPLC/MS method, across a wide range of concentrations.

Method: Samples were tested with STA® – Liquid Anti-Xa reagent used with a dedicated set-up on STA-R® analyzer in combination with STA® – Rivaraxaban Calibrator and STA® – Rivaroxaban Control. A second aliquot of each plasma sample was tested in HPLC/MS following a protocol previously validated.

109 samples from two different studies were included in the method comparison.

- 74 plasma samples obtained from patients receiving rivaroxaban therapy (10 mg OD) for prevention of venous thromboembolic risk in hip/knee replacement.
- 35 plasmas obtained from seven healthy young male volunteers drawn: 2 h, 3 h, 4 h, 6 h and 10 h after ingestion of a 80 mg single dose of rivaroxaban. Besides, samples withdrawn at T0 h were tested only with STA® – Liquid Anti-Xa to verify the residual anti-Xa activity (in the absence of the molecule).

Both studies were conducted in accordance to ethical rules and had received ethical committee and health agency approvals.

Correlation was analyzed through linear regression and differences were plotted in Bland & Altman graph.

Results: Analysis of QC results shows good inter-day precision: CV = 2% with STA® – Liquid Anti-Xa based on results in ng/mL, for both levels of concentration.
Results obtained in ex vivo plasma samples ranged from 25 to 370 ng/mL.
The correlations between STA® – Liquid Anti-Xa and HPLC/MS demonstrated results equivalence.
Linear regression equation: y = 1.09x + 13.10 (r = 0.990)
Results obtained for samples withdrawn at T0 h were all inferior to the detection limit of the assay (25 ng/mL).
Conclusion: The combination of STA® – Rivaroxaban Calibrator and Control with STA® – Liquid Anti-Xa gives consistent results with the reference method, the HPLC/MS, and is suitable for the daily activity of any laboratory.

PB 4.52-3

Point of care heparin monitoring: combined use of clot rate and clot time

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Background: The Sonoclot® analyser (Sienco Inc., Denver, Colorado) assesses the viscoelastic properties of whole blood using resistance to motion imparted on a vertically oscillating tubular probe allows assessment of plasma coagulation, platelet function, clot retraction and fibrinolysis, in combination and independently.
Heparin dosing with the kaolin activated clotting time (ACT) is used in Cardiopulmonary bypass and cardiology interventions but not in ICU where aPTT is preferred. There is an unmet need for a point of care analyser that allows reliable heparin dosing between 0.3 and 1.0 U/mL in ICU patients.
The Sonoclot ® allows the measurement of an ACT equivalent reaction time to clot formation alongside kinetic measurements of clot formation, the clot rate (CR).
We investigated the Sonoclot to determine whether the values generated by this technology could give a more accurate and therefore clinically useful heparin estimate in patients receiving heparin therapy.
Aims: To assess whether a combination of Activated Clotting Time (ACT) and Clot Rate (CR) provides a better prediction of Heparin Anti-Xa level than each parameter separately using the Sonoclot analyser.
Methods: Samples from nine volunteers were collected into 3 mL S-Monvette collection tubes containing 0.106 mol trisodium citrate. These were pooled within 15 min of collection and were spiked to heparin spike levels of 0, 0.2, 0.4, 0.6 and 0.8 U/mL using unfractionated heparin (LEO Laboratories Ltd, Risborough, UK). Actual Anti-Xa levels were determined using the HaemosiL liquid heparin assay (IL UK, Ltd.)
Routine ACT measurements (Haemochron) were compared to the aPTT assay on the Sonoclot analyser. Results for ACT and CR were assessed for utility in predicting heparin level.
Statistical analysis: Data summaries based on comparative plots were constructed to assess the relationship between heparin levels and viscoelastic parameters provided by the Sonoclot analyser. Thereafter, equations were made to model the relationships in order to calculate the dose of heparin from individual and combined parameters.
Results: Using the Sonoclot analyser, there is a strong linear relationship between heparin Anti-Xa level and ACT, and strong non-linear relationship with clot rate; using log (CR) this relationship was linear. ACT alone explains 91% of the variation in heparin, log(CR) 95% and a combination of the two 96% of the variation.
Summary/Conclusions: Statistically, ACT and CR are very highly correlated and when combined in a single model compete against each other to explain the effect of heparin rather that contributing separately to the anticoagulant effect. However, when ACT and log(CR) are combined the predicted heparin level is expected to be within ±0.18 of the true heparin value 95% of the time compared to within ±0.30 (ACT alone) and ±0.22 (log(CR) alone).
These results suggest a model incorporating both the heparin dependant increase in clot formation time as well as the decreased rate of clot formation will provide a more accurate estimate of the true level of heparin in a patient than traditional time based heparin estimates as is the case for aPTT and ACT.

PB 4.52-4

Effects of rFVIIa and vatreptacog alfa on clot formation and thrombin generation in blood from patients with haemophilia

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Background: Monitoring the effectiveness of bypassing agents remains a challenging issue in the treatment of haemophilia patients with inhibitors as no validated assays are yet available. Thromboelastography (TEG) and thrombin generation test (TGT) are currently being evaluated to assess the clinical efficacy of by-passing agent such as activated recombinant factor VII (rFVIIa). These analytical procedures are subjected to large variability and no standardized protocol exists which makes clinical application difficult. In order to obtain more insight into the mechanism of action of rFVIIa and vatreptacog alfa (a potent rFVIIa analogue), and to define linking of TEG/TGT variables to clinical outcome after treatment with these agents, we evaluated the effect of both drugs on TEG and TGT.
Aims: To study the effect of vatreptacog alfa and rFVIIa on TEG and TGT throughout application of several assay conditions.
Methods: Whole blood from 11 patients with severe haemophilia (one patient with inhibitor against FIX) and 15 healthy donors was collected in citrate tube and citrate tube containing 50 μg/mL corn trypsin inhibitor (CTI)(HTI). Whole blood, platelet poor plasma (PPP) and platelet rich plasma (PRP; 100,000 platelets/μL) from patients with haemophilia were spiked with concentrations of rFVIIa and vatreptacog alfa representing the expected *in vivo* concentration after i.v. administration of 90 μg/kg (rFVIIa) and 80 μg/kg (vatreptacog alfa). Normal range for TEG and TGT was established based on healthy controls with no addition of drugs. Conditions for TEG analysis included addition of three final concentrations of tissue plasminogen activator (tPA, 1.2–1.5–1.8 nM) and two final dilutions of Innovin (1:30,000 or 1:50,000) as source of tissue factor (TF). Conditions for TGT included coagulant activation by addition of Reagent PPP-Low (Thrombinoscope) for PPP, and preactivation with convulxin/TRAP (Bachem) for PRP samples.
Results: In PPP-TGT tests, CTI produce an anticoagulant effect in all TGT variables in both controls and untreated plasma of patients with haemophilia. Both rFVIIa and vatreptacog alfa shortened lagtime and time-to-peak significantly. Both compounds showed significant difference from untreated patient samples. The response of vatreptacog alfa was, as expected, more pronounced on time-to peak, ETP and peak. Influence of CTI in PRP-TGT tests was stronger than observed in the PPP-tests. In the TEG test, final dilution 1:50,000 of Innovin (0.1 pM TF) produced the largest difference between rFVIIa and vatreptacog alfa for all parameters, in particular in the MA. Inclusion of CTI induced a strong anticoagulant effect in all parameters but improved differentiation between the effect of rFVIIa and vatreptacog alfa.
Conclusions: Addition of CTI to blood prior analysis by TGT and TEG dampens the response in both haemophilia and normal blood. TGT in PRP without CTI was the best condition to evaluate effect of rFVIIa and vatreptacog alfa. In the TEG test, a dilution of 1:50,000 Innovin is useful for monitoring effect of rFVIIa and vatreptacog alfa. Addition of 1.5 nM tPA further allows to study effect on clot strength (MA). All results are based on experimental *in vitro* assays, the clinical response requires further studies in the future.

PB 4.52-5

Evaluation of the Silica Clotting time (SCT) test as a secondary test for the detection of Lupus Anticoagulant

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Background: Anti-phospholipid antibodies are a large heterogenous family that are commonly detected as Lupus Anticoagulant (LA), anti-cardiolipin antibodies (aCL) or anti-ß2 Glycoprotein1 antibodies. LAs are antibodies which inhibit in-vitro phospholipid dependent coagulation assays and are not specific to any coagulation factor. Published guidelines for lupus testing require laboratories to use two screening tests of different principles to test for LA. The latest guidelines published by Keeling *et al*. 2012 specify that the first test should be the DRVVT and the second 'will usually be an aPTT using a reagent with proven LA sensitivity or a modified aPTT' with the addition of a confirmatory step to demonstrate phospholipid dependence.
Aims: Our current practice involves performing a DRVVT and aPTT 50:50 mix. Our aim was to evaluate the Instrumentation Laboratory (IL) SCT assay to be used as a secondary screening test for LA. The SCT is a lupus sensitive aPTT based test which includes a confirmatory step to demonstrate phospholipid dependence.
Methods: Initial screening tests including PT, INR, aPTT, TT and aPTT 50:50 mix were analysed using IL reagents, DRVVT was analysed using American Diagnostica Lupus reagents and SCT was analysed using the IL SCT kits on an ACL TOP (IL UK). In total, double spun plasma from149 patients were analysed. aCL and anti-ß2 Glycoprotein1 levels were included in the evaluation. 35 normal plasma controls were also tested for the SCT to establish a local reference range.
Results: Both the SCT-Screen and SCT-Confirm assays showed good intra and inter assay coefficient of variation (CV) of 2.9% and 2.8% respectively and the establishment of the 99th percentile (Pengo *et al*., SSC 2009) from 35 normal controls indicated a cut-off value of 1.16. Both the DRVVT and SCT ratio demonstrated good agreement in 79% of patient samples. A total of 30 (20%) positive LA's were picked up using both tests. The DRVVT test alone detected 18/30 (60%) and the SCT alone detected 28/30 (93%). Of the samples positive for LA, 12/30 (40%) were not detected by the DRVVT test. Anti-ß2 Glycoprotein1 antibodies were requested in only 61/149 patients in which four were positive. Three out of four tested positive with both DRVVT and SCT and one was negative with both. aCL antibodies were requested in 104/149 patients with eight having a raised IgG level. Three were positive with DRVVT and SCT and a further two were only positive with the SCT. Three patients tested negative with both.
Conclusion: Anti-phospholipid antibodies are very heterogenous. There is no single test or reagent available to detect all positive LA's. Our results demonstrate the value of investing in a secondary test which can make testing for LA more sensitive. Being an aPTT based test, the SCT will also show a prolongation with both the screen and confirmatory steps in presence of clotting factor deficiencies. SCT is a very useful secondary test that compliments the DRVVT and meets the current guidelines.

PB 4.52-6

Hemocompatibility of manufactured nanoparticles: guidelines proposal

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Background: Nanosciences and nanotechnologies are in constant evolution. Development of new therapeutic and diagnostic agents using nanotechnologies for reach their pharmaceutical target require the knowledge of biocompatibility of nanoparticles with the blood compounds. Hemostasis is the ensemble of physiological phenomena which prevent and lead to stop bleeding; it maintains the vascular integrity. A dysfunction of the hemostasis can lead to slow down or even to completely stop the circulation of the blood. It is therefore primordial to know what NPs can have an effect on coagulation.
Aims: The aim of this study is the evaluation of the biocompatibility of manufactured nanoparticles (NPs) on erythrocytes integrity, on platelets aggregation and on coagulation cascade.
Methods: Five types of NPs were studied: carbon nanotubes, fullerenes, silicon dioxide, copper oxide and silicon carbide. Various techniques assessing activation and aggregation of the platelets or the impact of NPs on coagulation cascade were investigated. An approach in transmission and in scanning electron microscopy was also accomplished.
Results: The Impact-R® with scanning electron microscopy support and the calibration thrombin generation tests were the reference method to investigate the potential impact of NPs on platelet function and the procoagulant activity of NPs, respectively.
Conclusions: Guidelines for testing NP hemocompatibility were suggested which responds to a request of scientific community due to lack of recommendations for the evaluation of nanomaterial hemocompatibility.

PB4.53 – Blood coagulation tests – XVII

PB 4.53-1

Clot waveform analysis in patients with thrombophilia

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Background: Clot waveform analysis derives additional information from the fibrin formation process in an aPTT or PT measurement beyond the usual clotting time. Using an optical detection system, alteration of absorbance during clot formation is mathematically processed to obtain data regarding fibrin formation (measured curve), thrombin activity (first derivative), prothrombinase activity (second derivative) and tenase activity (third derivative).
The only available global assay to detect hypercoagulability is the cost- and labor-intensive thrombin generation assay; an actual hypercoagulable state can be detected by the determination of D-dimer or prothrombin fragments f1 + 2.
Aim: The majority of known procoagulant alterations of the coagulation cascade are located in the vicinity of thrombin and factor Xa formation. Therefore, using clot waveform analysis is a promising tool to investigate patients with thrombophilia. The study is aimed to show the feasibility of clot waveform analysis for screening and monitoring patients with inherited or acquired thrombophilia. The results are compared to established thrombophilia markers, including thrombin generation, D-dimer and prothrombin fragments f1 + 2.
Methods: Clot waveform analysis is measured using an ACL TOP (Instrumentation Laboratory, Kirchheim, Germany), using a Synthasil-activated aPTT assay (Instrumentation Laboratory). Measured optical data were processed using the Savitzky-Golay algorithm. D-dimer and prothrombin fragments f1 + 2 (Siemens Healthcare Diagnostics, Marburg, Germany) were determined, as well as thrombin generation using the ETP assay (Siemens Healthcare Diagnostics) or Technothrombin TGA (Technoclone, Vienna, Austria).
Only patients with a clearly defined diagnosis and a complete thrombophilia screening were included. Pregnant women were observed from the first trimester till 6 weeks post partum. From each subject an informed consent was obtained.
Results: Patients with thrombophilia showed significantly higher thrombin generation than healthy controls or patients without thrombophilia. Clot waveform analysis showed significant differences between these groups in all investigated parameters.

D-dimer, prothrombin fragments f1 + 2 and thrombin generation increase through pregnancy from first to third trimester and normalize post partum. The same tendency was observed with all parameters calculated from clot waveform analysis. Pregnant women with LMWH (measured at trough level) have higher prothrombotic markers than patients without therapy.

Conclusions: Clot waveform analysis is an inexpensive, easy and fast method to screen and monitor patients with thrombophilia and can be done without any additional measurements beside a standard aPTT. Clot waveform analysis also reflects the physiological and unphysiological changes occurring in uncomplicated or complicated pregnancy, respectively. The method can be easily adapted to other optical detection systems.

PB 4.53-2

Measurement of dabigatran concentrations by calibrated thrombin clotting time in comparison to LC-MS/MS in human volunteers on dialysis

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Background: Dabigatran etexilate is an oral direct thrombin inhibitor approved for prophylaxis of venous thromboembolism following surgical total knee or hip replacement and for stroke prevention in patients with non-valvular atrial fibrillation in more than 80 countries worldwide. Although clinical monitoring of dabigatran etexilate is not required, a simple and precise laboratory method to measure dabigatran concentrations in patient plasma may be useful in certain clinical circumstances, e.g. emergency situations.

Aims: In a PK/PD study in generally healthy end stage renal disease (ESRD) human volunteers, dabigatran pharmacokinetics and -dynamics were investigated. Ethics committee approval and patients' informed consent were obtained. Serial plasma samples were collected for analysis of dabigatran plasma concentrations by LC-MS/MS, and by the quantitative Hemoclot® direct thrombin inhibitors (dTT) assay. Assay precision and accuracy, and comparability of dabigatran concentrations determined by the dTT assay with those determined by an LC-MS/MS reference method, were investigated.

Methods: Samples were obtained from seven ESRD human volunteers without atrial fibrillation in steady state conditions of dabigatran in a Phase I clinical trial for measurement of dabigatran by dTT (citrated plasma samples) and by a validated LC-MS/MS reference method (EDTA plasma samples). Plasma concentrations were not corrected for dilution by citrate solution in sampling tubes. The dTT assay was calibrated with commercially available dabigatran standards (Hyphen BioMed, France). Agreement of dabigatran concentrations determined by the dTT assay and the reference LC-MS/MS method was assessed by regression analysis and Altman-Bland difference plots. Mean bias and limits of agreement were calculated.

Results: Prolongation of dTT paralleled the dabigatran plasma concentration-time profile in these healthy volunteers and displayed a linear correlation with dabigatran plasma concentrations. The mean R^2, slope and intercept of the regression line were 0.93, 0.146 s/(ng/mL) and 32 s (baseline dTT), respectively. The Hemoclot assay showed excellent assay precision with coefficients of variation (CV) below 5% for total, within-day and between-day imprecision. Accuracy determined by analysis of quality control samples (QC) at 122 and 297 ng/mL dabigatran was 90% and 111%, respectively. Between-day imprecision (CV) of QC analyses was 5% at 297 ng/mL and 9% CV at 122 ng/mL dabigatran. Dabigatran concentrations in study samples were between 7 and 440 ng/mL. In total, 304 paired data points were included in the method comparability study. Correlation of dabigatran concentrations determined by dTT and LC-MS/MS were assessed by regression analysis. The R^2 of the linear regression line was 0.96 with a slope of 1.013 which is within the acceptance limits of 0.85–1.15. The mean concentration ratio of the LC-MS/MS and dTT was 0.955.

Conclusion: The Hemoclot® thrombin inhibitor assay is a simple method to measure dabigatran plasma concentrations in otherwise healthy human volunteers undergoing regular haemodialysis. Agreement between dabigatran concentrations determined by the dTT assay and the LC-MS/MS reference method met pre-specified acceptance criteria.

PB 4.53-3

Thromboelastometry in liver transplantation of end-stage liver disease and familial amyloidotic polyneuropathy patients

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Background: Bleeding is a serious intraoperative complication of orthotopic liver transplantation (OLT) compromising graft and patient survival. Thromboelastometry (TE) is a point-of-care test that monitors haemostasis in a cell-based model, providing different information from plasmatic standard laboratory tests (SLT), namely platelet function and fibrinolysis. In our center TE was used to diagnose and manage haemostasis disorders in OLT, performed at surgery theater by hematologists. In contrast to most candidates to OLT with end-stage liver disease (ELD), familial amyloidotic polyneuropathy (FAP) patients have normal liver function (1). Clinical data results are contradictory concerning TE cost/effectiveness analysis. We evaluated the consumption of blood and blood products in OLT performed in ELD and FAP groups of patients.

Methods: From 59 OLT, 42 were performed in ELD and 17 in FAP groups. SLT data [fibrinogen concentration, prothrombin time (PT), international normalized ratio (INR), platelet count (PC) and activated partial thromboplastin time (APTT)], hematocrit, haemoglobin, and TE using ROTEM® technology have been recorded at basal, hepatectomy, anhepatic, reperfusion and final phases of OLT surgery. The severity of disease has been evaluated by the Model for End-Stage Liver Disease (MELD) *United Network for Organ Sharing* modification score for patients with hepatic failure. We compared the number of units of blood components [red blood cells (RBC), plasma and platelets)] transfused between groups of patients who underwent OLT with TE (+TE) with the control group without TE monitoring (-TE) presenting similar severity of liver disease [ELD + TE (n = 23) vs. ELD-TE (n = 19) and FAP + TE (n = 10) vs. FAP-TE (n = 7)]. This evaluation included the blood and blood components transfused during surgery till 48 h after OLT. Student-t test was used to assess differences between groups ($P < 0.05$).

Results: There were no differences in MELD score between ELD + TE and ELD-TE groups (15.1 ± 1.6 vs. 12.9 ± 1.6; P = 0.36). Basal hematocrit (%), hemoglobin level (g/dL) and PC (x10^3/mL) were not different between ELD + TE and ELD-TE groups [(32.8 ± 1.1 vs. 30.3 ± 1.5; P = 0.24); (10.9 ± 0.4 vs. 10.4 ± 0.6; P = 0.48); (128.7 ± 8.9 vs. 119.5 ± 16.3; P = 0.61) respectively]. We did not find significant differences in the number of RBC, platelets nor plasma between ELD groups (+TE vs -TE) [(5.9 ± 1.3 vs. 4.3 ± 1.2; P = 0.39); (1.0 ± 0.6 vs. 1.6 ± 0.8; P = 0.52); (4.0 ± 1.1 vs. 2.1 ± 0.9; P = 0.19) respectively]. In FAP group, 48 h after surgery the TE+ group showed higher RBC consumption (1.22 ± 0.4 vs. 0.29 ± 0.2; P = 0.02) despite higher basal PC (246.7 × 10^3/mL ± 16.3 vs. 181.8 × 10^3/μL ±25.7; P = 0.04) and lower PT and INR at hepatectomy (12.4 ± 0.36 vs. 13.8 ± 0.26; P = 0.02 and 1.1 ± 0.03 vs. 1.3 ± 0.03; P = 0.045, respectively). No significant differences were found in the other parameters.

Conclusions: In our center, thromboelastometry use in liver transplantation was not associated with lower blood transfusion in patients with or without hepatic failure. This result may be due to the fact that a

hematologist in the operating theater made transfusion decisions putting together TE and SLT results with clinical judgment in accordance with validated transfusion algorithms.

(1)-LismanT, PittauG, LeiteF *etal*. (2012) The circulating platelet count is not dictated by the liver, but may be determined in part by the bone marrow: analyses from human liver and stem cell transplantations. *J Thromb Haemost*; 10:1624–30.

PB 4.53-4

Impact of apixaban on haemostasis diagnosis assays: practical recommendations

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Background: As other anticoagulant drugs, apixaban may affect the results of a series of coagulation assays routinely used in case of thrombophilia or in the exploration of a haemorrhagic event. There is a need for the clinician and the biologist to know whether these tests are influenced by apixaban.

Aim: We aim at providing good laboratory recommendations for the accurate interpretation of haemostasis routine laboratory assays that may be influenced in patients treated with apixaban.

Materials: Apixaban is spiked at increasing concentration (0–500 ng/mL) in normal citrated human platelet-poor plasma to assess the Thrombin Time (TT), Ecarin Clotting Time (ECT), Reptilase Time (RT), testing for lupus anticoagulant (dilute Russell Viper Venom Time (dRVVT), PTT-LA® and Staclot-LA®), Activated Protein C Resistance (APC-R), measurement of clotting factors (XII; XI; IX; VIII; VII; V; X and II), antithrombin measurement (with thrombin and factor-Xa based assay), fibrinogen measurement (Clauss and PT-derived method) and Protein-C and free Protein-S (immunological and clotting method). All methodologies were performed according to the recommendations of the manufacturer.

Results: *Thrombin time, ecarin clotting time and reptilase time*

Both of these tests are not influenced by apixaban.

Determination of lupus anticoagulant

The PTT-LA® and the Staclot-LA® are both prolonged in a concentration dependent manner in presence of apixaban. DRVV-T (screen and confirm) is also prolonged dose dependently. For PTT-LA® and Staclot-LA® (with buffer or phospholipids)the relation is exponential and the concentration needed to double the clotting time (2xCT) is 52 ng/mL, 23 ng/mL and 32 ng/mL, respectively. For DRVV-T (screen and confirm) the relation is linear and the 2xCT is 205 ng/mL and 230 ng/mL, respectively.

Activated protein C resistance, clotting factor measurement, protein-C and protein-S measurement

Apixaban mainly influences the aPTT-based clotting factor measurement (FVIII, FIX, FXI and FXII). This may be explained by the relatively low sensitivity of Innovin® (the PT-reagent used for the PT-based determination of clotting factor II, V, VII, X) in comparison with the CKPrest®. The protein-C measurement is not affected by apixaban as well as the measurement of free protein-S using the immunological method. The measurement of free protein-S using the chronometric method is affected by apixaban (increase of 13% by 100 ng/mL of apixaban). The APC-R ratio is only affected at high concentration (447 ng/mL) of apixaban.

Antithrombin measurement

Apixaban influences the measurement of antithrombin using factor Xa-based chromogenic assay (12% per 100 ng/mL). Thrombin-based chromogenic assay is not affected by the presence of apixaban.

Fibrinogen measurement (Clauss and PT-derived methods)

The measurement of fibrinogen is not influenced by apixaban using the Clauss method. For the PT-derived method, the variability is greater with an overestimation of the fibrinogen rate at higher concentrations (>200 ng/mL) in apixaban.

Conclusions: We show that immunological assays are not influenced by apixaban. When applicable, these tests should be recommended. Apixaban mainly interferes with chronometric or chromogenic assays that involve FXa or upstream coagulation factors. When these tests are used, it is mandatory to take into account the inter-reagent variability as well as the delay between the last intake of the drug and the blood sampling.

PB 4.53-5

Multimodal microscopy for real time imaging of thrombus formation

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Background: The use of multimodal optical diagnostic approach has a great potential in biomedical research and It can significantly expand the capabilities of vascular diagnostics.

Objective: To evaluate a multimodal approach for simultaneous *in vivo* imaging of laser induced thrombus formation.

Methods: In the present study we combined time domain Laser Speckle Imaging (tLSI) technique with Digital Fluorescence Intravital Microscopy (dFIM).

Results: tLSI approach has been used for rendering blood vessels structure and monitoring blood flow in the mouse ear *in vivo*, with high spatial resolution, whereas dFIM provides visualization of thrombus formation and changes in the vascular permeability, assisted by application of various fluorescent contrast agents (Fluoroscein, FITC, Rhodamin 6G).

Conclusion: The combined application of tLSI and dFIM approaches provides efficient *in vivo* imaging method of laser induced thrombus formation along with changes in blood flow velocity within all vessels in the region of interest.

PB 4.53-6

Evaluation of a new liquid recombinant human thromboplastin reagent for the determination of prothrombin time (PT) in an optical coagulometer

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Background: A new liquid recombinant human thromboplastin reagent (DG-PT L Rec) for the determination of prothrombin time (PT) was evaluated by Grifols prior to its launch. This reagent is intended mainly for screening of the extrinsic coagulation pathway and monitoring oral anticoagulant therapy (OAT).

Aims: The aim of this study was to evaluate the performance of the reagent in Q Hemostasis Analyzer (Grifols) with normal and OAT samples, by comparing it to an established similar reagent, as well as assessing its precision with commercial control plasma.

Additionally, performance of the reagent in PT-derived fibrinogen assay (PT-DF) was evaluated by comparing it to fibrinogen Clauss assay, and assessing its precision with commercial control plasma.

Methods: Precision was determined according to CLSI EP5 guidelines in normal (DG-C1) and abnormal (DG-C2) lyophilised control plasma and in a commercial pool of normal plasma (PNP).

DG-PT L Rec performance was compared to an established recombinant human thromboplastin (Innovin, Siemens). Method comparison was performed by analysing 105 fresh platelet poor plasma (PPP) from patients under OAT therapy and apparently healthy individuals.

Comparison of PT-DF assay and fibrinogen Clauss assay (DG-FIB L Human, Grifols) was performed by analyzing 35 fresh PPP from apparently healthy individuals.

Results: Within-run precision (CV_{wr}) in seconds and ratio was lower than 3% in normal and abnormal level. Within-device precision

(CV_{wd}) in seconds and ratio was within 4% in normal and abnormal level.
Least-square linear regression of the results obtained by DG-PT L Rec and Innovin achieved a correlation coefficient (r) higher than 0.990. DG-PT L Rec obtained longer clotting times in OAT samples, being thus highly sensitive for these kind of samples. There was no clinically significant difference neither in ratio for normal samples nor in INR for OAT samples in the therapeutic range.
For PT-DF assay, CV_{wr} and CV_{wd} in mg/dL were lower than 6% and 8%, respectively, in normal and abnormal level.
Least-square linear regression of the results obtained by PT-DF assay and DG-FIB L Human achieved a correlation coefficient (r) higher than 0.970. There was no clinically significant difference in normal fibrinogen values.
Conclusion: DG-PT L Rec is a liquid recombinant human thromboplastin reagent suitable for screening of the extrinsic coagulation pathway and monitoring oral anticoagulant therapy in Q Hemostasis Analyzer, obtaining precise and reliable results.
DG-PT L Rec is adequate for PT-DF assay in Q Hemostasis Analyzer, achieving precise results and being comparable to Clauss fibrinogen assay results for normal samples.

PB4.54 – Blood coagulation tests – XVIII

PB 4.54-1
Influence of FIX and FVIII PEGYLATION on FIX and FVIII activity based on APTT assays

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Background : N9-GP is a 40kD PEGylated recombinant human FIX derivate and N8-GP is a 40kD PEGylated recombinant human FVIII derivate in development at NOVO NORDISK. It is a new class of molecules for the treatment of haemophilia, characterized by a prolonged half-life. It has been shown that PEGylation of proteins could influence the clotting times in certain APTT based assays.
Aim : The aim of this study is to evaluate the impact of PEGylation of FIX (N9-GP) and FVIII (N8-GP) on respectively FIX and FVIII activity using the one stage clotting assay with a wide range of APTT reagents (Diagnostica Stago and TCoag) on the STA-R® analyser (mechanical clot detection, Diagnostica Stago) and chromogenic assays (Hyphen).
Methods : FIX deficient plasmas were spiked with FIX preparations: BeneFIX® (Pfizer) or N9-GP (Novo Nordisk). FVIII deficient plasmas were spiked with FVIII preparations: Advate® (Baxter) or N8-GP (Novo Nordisk). These preparations were tested both in one-stage clotting assays using different APTT reagents from Stago and TCoag, on the STA-R®, and with chromogenic assays. WHO International Standards, BeneFIX® or Advate® and N9-GP or N8-GP preparations were used as calibrators.
Results : The results of this study for N9-GP showed a wide variability of the FIX activity using non-PEGylated molecules as calibrators, depending on the APTT reagent used. Levels of FIX were underestimated with a Kaolin based APTT reagent (STA® - CK Prest®), overestimated with Silica based APTT reagents (STA® - PTT A, TriniCLOT Automated aPTT, TriniCLOT aPTT HS and TriniCLOT aPTT S), slightly underestimated with an Ellagic Acid based APTT reagent (STA® - Cephascreen ®) and in the normal range with a FIX chromogenic assay.
Using the N9-GP molecule as calibrator, FIX levels were all in the normal range, whatever the APTT reagents.
Concerning FVIII activity, using non-PEGylated molecules as calibrators, levels of FVIII were underestimated with Silica based APTT reagents (STA® - PTT A, TriniCLOT Automated aPTT, TriniCLOT aPTT HS and TriniCLOT aPTT S) and in the normal range with a Kaolin based APTT reagent (STA® - CK Prest®), with an ellagic acid based APTT reagent (STA® - Cephascreen®) and with a FVIII chromogenic assay.
Using the N8-GP molecule as calibrator, FVIII levels were all in the normal range, whatever the APTT reagents.
Conclusions : The study confirmed the impact of PEGylation of FIX and FVIII on FIX and FVIII activity APTT based assays, respectively. Some reagents provided activity in the normal range while other APTT reagents showed a wide variability. Further investigations are needed to understand the biochemical mechanisms involved in these interactions and to try to normalise the results by different means such as the use of a specific calibrator or assigning an assay specific conversion factor to obtain results comparable to those obtained with chromogenic assays.

PB 4.54-2
Influence of time delays in sample processing on protein S assays

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Background: There is increasing centralisation of coagulation services with requirement to transport samples to central sites for testing. Plasma may be frozen for transport but the cheaper option, transportation of blood samples at ambient temperature may sometimes be considered. Preanalytical variables such as time delay in sample processing can adversely influence coagulation tests.
Aims: To investigate the effect of storing citrated whole blood at room temperature for 24 h, on different activity and antigenic assays of free protein S (FPS).
Method: Five samples were drawn from healthy donors (n = 10) into Becton Dickinson (BD) citrated (0.109 M) vacutainer tubes. One sample was centrifuged (2000 g for 10 mins) and plasma aliquots frozen at −70 °C within 30 min of venepuncture (time 0). The remaining samples were stored at 20–25 °C for 4, 8, 12 and 24 h and plasma frozen at −70 °C. FPS antigen was measured by; automated immunoassay (FPS-IL) (Instrumentation Laboratory, UK), Liatest FPS (Stago, France), Innovance FPS (Siemens, UK), and ELISA (Woodhams method). Samples were also tested with PS activity assays; Clot S (Precision Biologic, Canada), Pro S (IL, UK), Protein S Ac (Siemens, UK) and StaClot (Stago, France).
Results: There was no statistically significant change over 24 h in FPS levels when tested by Liatest, Innovance or ELISA assay ($P \geq 0.05$) The Liatest and Innovance FPS antigen assays showed a mean 3% fall in FPS at 24 h and the FPS ELISA, a mean 2% fall in FPS at 24 h. With FPS-IL statistically significant change was observed at 8 h ($P \leq 0.05$), with a mean 11% fall (range −8 to −14%) in FPS at 24 h. Statistically significant change in StaClot PS activity occurred at 12 h ($P \leq 0.01$), however diagnostically significant changes were observed in individual samples by 4 h. The other activity assays showed statistically and diagnostically significant change in PS activity at 8 h ($P \leq 0.05$). Overall falls in PS activity at 24 h; Clot C (mean −15%, range −9% to −29%), Pro S (mean −17%, range −12 to −26%), Protein S Ac (mean −14%, range −7 to −24%), StaClot (mean −14%, range +10 to −32%).
Conclusion: FPS antigen demonstrates differing stability in samples stored as whole blood depending on methodology employed for analysis. Liatest and Innovance FPS methods employ two monoclonal antibody and share similar stability to ELISA technique utilising polyclonal antibody. Increasing loss of measurable FPS is observed in the FPS-IL assay which incorporates an initial stage of binding FPS to a C4b-binding protein latex reagent prior to the addition of a monoclonal antibody latex reagent. Whole blood samples stored at room temperature are stable for FPS measurement for 24 h if analysed using Liatest, Innovance or ELISA FPS assays, but <8 h if using FPS-IL.

We have previously shown that FPS can be reliably measured by ELISA (polyclonal antibodies) up to 72 h, whilst FPS-IL demonstrated increasing instability with statistically significant change at 48 and 72 h ($P \leq 0.001$).

PB 4.54-3

Time-and space-resolved imaging of coagulation as a method for monitoring the effectiveness of LMWH therapy after total hip replacement

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Background: In the postoperative period after total hip replacement patients assigned LMWH. Anticoagulant therapy in some patients can be ineffective. The aim of this study was to investigate the possibility of a new global coagulation assay to control effect of enoxaparin.
Methods: *In vitro* study – plasma samples frome nine patients after total hip replacement with the addition of increasing concentrations of enoxaparin 0.0, 0.03, 0.06, 0.12, 0.25, 0.5, 1.0, 2.5 anti-Xa IU/mL. The clinical study included 44 patients before and after total hip replacement. Enoxaparin 40 mg opd, 1–10 day after surgery. Plasma samples were collected before surgery, after surgery before the start of enoxaparin, after 5 days of enoxaparine treatment, after 10 days of enoxaparine treatment, after 30 days postop, after 90 days postop. Time-and space-resolved imaging of coagulation (Thrombodynamics, HemaCore LLC, Russia) – a new global coagulation assay based on videomicroscopy of a fibrin clot growing from an imitated damaged vessel wall (surfaces with immobilized tissue factor), the growth rate of the fibrin clot in space V was measured (norm 20–30 μm/min).
Data are presented as Median (25th percentile; 75th percentile), * - $P < 0.05$.
Results : *In vitro* experiments V were: 0.0 IU/mL – 34.7(33.7;35.8), 0.03 IU/mL – 30.9(27.1;31.0)($P = 0.008$ vs. 0.0 IU/mL), 0.06 IU/mL – 23.(21.6;24.2) ($P = 0.008$ vs. 0.03 IU/mL), 0.12 IU/mL – 14.1 (13.8;15.5)($P = 0.008$ vs. 0.06 IU/mL), 0.25 IU/mL – 10.0 (9.5;10.1) ($P = 0.008$ vs. 0.12 IU/mL), 0.5 IU/mL – 5.5(5.0;8.3)($P = 0.008$ vs. 0.25 IU/mL), 1 IU/mL – 4.9(3.0;5.3($P = 0.01$ vs. 0.5 IU/mL), 2.5 IU/mL – 2.0(1.6;2.5($P = 0.008$ vs. 1.0 IU/mL).
In clinical study V in patients plasma samples were: before surgery – 28.1(26.1; 31.1), after surgery – 41.9 (34.6; 46.2)(* vs. preop.), on the 5th day – 26.9(20.6;30.8)(* vs. postop.), 10 day – 26.5(21.3;29.7), 30 day – 28.5(26.0;33.4)(* vs. 10 day), day 90 – 36.0(32.3;42.3)(* vs. 30 day).
Conclusion: Growth rate of the fibrin clot in space V can be use for detecting of enoxaparin anticoagulant effect. In pts after HIP replacement V can detect enoxaparin anticoagulant effect and can detect some pts with return postop. condition after end of enoxaparin treatment

PB 4.54-4

Evaluation of TEG® and ROTEM® inter-changeability

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Background: The principle behind Viscoelastic Haemostatic Assays is based on the tendency of blood to increase its viscosity and elasticity through the process of coagulation. The clot changes over time are visualized as an evolving trace where the time to initiation of clot formation, the rate of clot formation and the maximum strength of the clot are among the most commonly utilized variables. The greatest use has been the application of VHAs-guided transfusion of blood components in hepatic and more widely in cardiac surgery. Recent years have seen a renewed interest in this technology with applications for both pharmaceutical monitoring and patient screening. Two frequently used VHA devices are TEG® and ROTEM®. They differ markedly in terms of function and user interface, and it is not clear whether this affects their performance in a clinical setting. Only a few studies have previously compared the interchangeability of TEG® and ROTEM®. In particular, studies performed within cardiac surgery and liver transplantation procedures concluded that the assays may be inter-changeable. Conversely, inter-changeability seemed to be limited in the trauma setting.
Aim: The aim of our study was to compare the results of the TEG® and ROTEM® analyses in a cohort of patients with different pro-hemorrhage conditions and to assess the inter-changeability of the two devices.
Methods: We measured blood samples from 18 consecutive patients referred to our Unit for a pro-hemorrhagic condition. The TEG® and ROTEM® assays were performed simultaneously. In the TEG® system, we performed three measurements: the kaolin TEG® (kaoTEG), the rapid TEG® (r-TEG), and the functional fibrinogen (FF-TEG). In the ROTEM® system three measurements were also performed: INTEM, EXTEM and FIBTEM. We compared three widely used variables from the VHA trace: the reaction time from initiation of the assay to the first detectable coagulation, denoted R-time for TEG® and clotting time (CT) for ROTEM®; the time from start of coagulation to clot amplitude of 20 mm, called the K-time in TEG® and clot formation time (CFT) in ROTEM®; lastly, the maximum amplitude (MA) for TEG®, which corresponds to the maximum clot firmness (MCF) for the ROTEM® device. Correlations were calculated using Sperman's rank correlation coefficient.
Results: Patients enrolled were bleeding during bi-ventricular assistance (4), during liver transplantation (3), cardiovascular surgery (5), trauma (2), pediatric patients under L-Asparagenase induction therapy for acute lymphatic leukemia (4). We found a good correlation between: (a) MCF and MA in the three tests considered (INTEM vs. kaoTEG $r = 0.77$, $P < 0.01$; EXTEM vs. r-TEG 0.76, $P = 0.01$; FIBTEM vs. FF-TEG $r = 0.69$, $P < 0.01$); (b) CT and R-time in INTEM vs. kaoTEG ($r = 0.57$, $P = 0.002$) and in EXTEM vs. r-TEG ($r = 0.51$, $P = 0.01$); (c) CFT and K-time in INTEM vs. kaoTEG ($r = 0.27$, $P = 0.04$) and in EXTEM vs. r-TEG ($r = 0.31$, $P = 0.03$).
Conclusions: According to these observations inter-changeability between TEG® and ROTEM® is confirmed in different clinical settings. The search for a 'global' assay of haemostasis continues and the TEG®/ROTEM® or a derivative of this technology may provide the answer, or part of the answer, to that question.

PB 4.54-5

Unravelling the thrombin generation assay

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Background: The enzyme thrombin is central to blood coagulation, being the end product of a complicated protein cascade with multiple feedbacks that operates over multiple time scales to ensure thrombin is delivered to the right place at the right time. The propensity for a patient's blood to clot may be determined by the thrombin generation assay. A typical profile of thrombin generation over time is characterised by an initial lag phase followed by a rapid increase that is subsequently inhibited. Recent improvements in, and commercialisation of, the thrombin generation assay mean that there are an expanding number of studies utilising it.
Aims: By modelling and mathematical analysis of the reactions within the thrombin generation assay we hope to enhance our understanding of the sequence of reactions within this complicated network providing simplified approximations and network diagrams that clearly indicate which reactions dominate at each point in time. Comparison of model

simulations to experimental data in principle allows us to determine if current biological knowledge of the thrombin generation cascade is sufficient to explain experimental results.
Method: We present a mathematical model for the early steps in the generation of thrombin. We have analysed this model using the method of matched asymptotic expansions to derive a sequence of simplified models in time that characterise the key reactions within the cascade.
We have then extend our initial model to represent the coagulation cascade as it would occur in the environment of the thrombin generation assay. The resulting system of ordinary differential equations is used to generate numerical simulations that we compare to some of the experimental data now being generated from the commercial assay under a variety of disease conditions.
Results: The mathematical analysis breaks down the complicated network of reactions into a series of much simpler mathematical approximations in time. Two of the simplified models found provide an excellent substitute for the full model, capturing the exposive growth in thrombin generated and then its decay. We also derive simple mathematical expressions for the key parameters of time-lag, peak concentration and ETP (area under the curve) that describe the thrombin generation assays trace in time.
Numerical simulations of our full model, without any modification in parameter values obtained from literature, show a close fit to published experimental data.
Conclusion/Summary: By applying the method of matched asymptotic expansions to this complicated network of reactions we have produceda series of simple mathematical snapshots at distinct points in the time scale over which thrombin is generated. This allows us to understand the dependenceof eachtime-point on protein levels and rate parameters. Fromthis we are able to derive simple mathematical expressions for the key markers of thrombin generation(e.g. peak concentration and ETP) allowingamuch simpler interpretation of them in terms of the kinetic parameters. Approximations such as these could be used to connect experimentally derived biological markers to the underlying mechanisms that cause them to vary.

PB 4.54-6
Validation of a new liquid fibrinogen assay

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Background: The Clauss method for fibrinogen testing appears to be the most reliable method for general use in clinical laboratories.
Aim: STA® – Liquid Fib is a new liquid fibrinogen assay for the quantitative determination of fibrinogen levels in plasma which is based on the Clauss clotting method.
The aim of this study was to evaluate STA® – Liquid Fib performances through a method comparison conducted in three different laboratories: Le Puy en Velay, Montfermeil and Percy;France.
Method: Patients were tested with STA® – Liquid Fib and the reference method: STA® – Fibrinogen 5 on STA-R® or STA® Compact analyzers using precalibrated test setups.
673 patients referred to the laboratory for fibrinogen testing and were included in the study.
45 samples had fibrinogen levels under 1.5 g/L and 47 samples had fibrinogen levels above 8 g/L. Samples were spread along the working range (0.4–12 g/L).
Correlations were analyzed through linear regressions (STA® – Liquid Fib vs. STA® – Fibrinogen 5) and Bland Altman Graph.
Results: Results obtained with the new reagent STA® – Liquid Fib vs. the current reagent STA® – Fibrinogen 5 are well correlated:
- Puy en Velay: y = 1.00x – 0.13 (r = 0.99)
- Montfermeil: y = 1.00x −0.01 (r = 1.00)
- Percy: y = 1.03x −0.12 (r = 0.99)

A Bland Altman Graph shows that more than 95% of samples are within the acceptable ranges.
Conclusion: Results show good consistency between STA® – Liquid Fib and STA® – Fibrinogen 5. In addition, STA® – Liquid Fib improves workability by its ready to use liquid format.

PB4.55 – Coagulation factor VIII

PB 4.55-1
Detection of non-human sialic acid *N*-glycolylneuraminic acid in Factor VIII products by ultra-performance liquid chromatography with fluorescent labeling

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Background: Glycosylation is a common posttranslational modification found on mammalian proteins, and these glycan structures often terminate with a sialic acid. Human glycans terminate with a specific form, *N*-acetylneuraminic acid (NANA). However, recombinant glycoprotein therapeutics produced in animal cell lines and/or with animal serum often contain *N*-glycolylneuraminic acid (NGNA), a derivative of NANA, which is not normally found in humans. It has been reported that all humans possess anti-NGNA antibodies and sometimes at high level approaching 0.1–0.2% of circulating IgG. As a result, NGNA is potentially immunogenic and needs to be monitored.
Aims: The aim of the study is to determine NGNA levels in recombinant FVIII Fc fusion protein (rFVIIIFc), which is expressed in a human cell line, HEK 293, and compare to NGNA levels in other recombinant FVIII products that are produced in animal cell lines, by using a sensitive ultra-performance liquid chromatography (UPLC) method with fluorescence detection after 1,2-diamino-4,5-methylenedioxybenzene (DMB) labeling.
Methods: FVIII samples were incubated with 50 mM sulfuric acid at 80 °C for an hour, and the released NGNA was then derivatized with DMB at 50 °C for 3 h covered by aluminium foil. The DMB derivatized sample was analyzed by UPLC fluorometric detection using a C18 column (1.7 μm particle size). The NGNA standards were prepared the same way as protein samples to obtain the calibration curve. The NGNA levels in FVIII molecules were determined based on the obtained calibration curve. The percentage mole NGNA per mole of protein was calculated for all the tested FVIII products.
Results: The method showed very good linearity for NGNA from 2.5 fmol to 6.2 pmol. The limit of detection (LOD) and limit of quantitation (LOQ) for NGNA were estimated to be 2.5 fmol and 4.5 fmol, respectively. No detectable NGNA was found in rFVIIIFc that was produced with human cell line HEK293, whereas different levels of NGNA (mole percentage) were detected in the marketed recombinant B-domain deleted FVIII (20.31 ± 0.73%) and two marketed full length recombinant FVIII (1.33 ± 0.14% and 5.99 ± 0.32%, respectively) which are all produced with animal cell lines, CHO or BHK.
Conclusions: A very sensitive and robust UPLC method with fluorescence detection was developed for the detection of NGNA after DMB labeling. No NGNA was found in rFVIIIFc by this method as expected, whereas all the other recombinant FVIII products tested contained levels of NGNA significantly above the LOD of the assay. Although the impact of the presence of these potentially immunogenic non-human modifications are not known for FVIII, recombinant expression using a human cell line grown in chemically defined media ensures that the glycan structures are fully human.

PB 4.55-2

Molecular characterization of nine F8 splicing mutations in RNA isolated from patient's leukocytes. Evaluation of in silico prediction tools accuracy

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Background: The inherited deficiency Haemophilia A (HA) is caused by a broad spectrum of defects in the FVIII gene (*F8*). Mutations affecting mRNA splicing comprise approximately 8% of the reported HA cases in the *F8* mutation database. However, only a few of them have been fully characterized by mRNA analysis.

It is known that correct mRNA splicing involves four different signals to recognize exon-intron boundaries: 5' and 3' splice junctions of the intron, branch sites and a polypyrimidine tract. In addition, exon recognition can be affected by several cis-acting elements within the exons and introns. *In silico* prediction analysis can provide a valuable means to predict the molecular effect of splice site mutations, however it only displays score variations, which difficult the interpretation regarding the exact biological consequences.

Aim: To systematically characterize the phenotypic effect of potential splice site mutations (PSSM) and to understand the correlation with FVIII levels and disease severity.

Methods: Eight pairs of primers were designed for full-length *F8* cDNA amplification by single-step RT-PCR in RNA isolated from leukocytes. This procedure was used to study nine (six of them hitherto unreported) PSSM, previously identified at genomic level in the routine molecular diagnostic of HA patients. The effect of PSSM in the *F8* mRNA expression and processing was studied using samples from healthy donors as a control. The concordance of *in silico* predictions and experimental data was then assessed.

Results: The mRNA analysis in the HA index cases demonstrate diverse molecular mechanism: c.601 + 1delG produces either the exon 4 or 4 plus 5 skipping; c.602-11T>G results in the exon 5 skipping; c.671-3C>G where either exon 6 or 5 and 6 were lost; c.6115 + 9C>G that skipped exon 19; and c.6016-1G>A where the exon 20 was skipped. Both c.1009 + 1G>A and c.1009 + 3A>C mutations resulted in the activation of a cryptic donor splicing site skipping the last 121 bp and 135 bp respectively of exon 7; and finally, intron retention was showed in cases: c266-3delC that result in the activation of a cryptic acceptor splicing site (ASS) 57 bp within the intron 2 and c.5587-1G>A that presented a cryptic ASS 44 bp within intron 16. The experimental results obtained were compatible with observed phenotypes. Although, *in silico* analyses were unable to predict the splicing effect of several mutations, combination of distinct *in silico* tools running distinct algorithms is sensitive and accurate predicting in many cases whether an effect on splicing is expected and can be used prudently as a decision-making guide.

Conclusion: The method developed can be extended to study the pathogenic effects of any PSSM along *F8*. Our results also demonstrated the presence of ectopic aberrant transcripts in leukocytes from HA patients and from control samples, that could hinder the interpretation of results. Hence, the study of *F8* mRNA from leukocytes has to be considered carefully but it is still a useful tool to determine the mechanisms by which PSSM contributes to the phenotype in HA patients.

Funding: HEMO-iPSC (FIS PI11/03024-PI11/03029)

PB 4.55-3

Heat-sensitization allows the detection of anti-FVIII antibodies in patients with acquired hemophilia, who have a negative Bethesda assay

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Background: The Bethesda assay is the standard way to detect anti-FVIII antibodies (Abs) in both hereditary hemophilia A (HA) with inhibitors, and acquired hemophilia A (AHA). Unfortunately, the Bethesda assay is insensitive. A recent modification reported that heat treatment sensitized the traditional Bethesda assay in patients with HA who had presumed inhibitors based on the fact that they had shortened *in vivo* FVIII survival. There is no available data so far with the modified assay in AHA. AHA antibodies are different from the inhibitors found in hereditary HA in many ways. Thus, it is not trivial that the modification should also work in AHA. Also, the setting of treated AHA offers a much more convenient way of detecting the *in vivo* effect of the inhibitor, because endogenous FVIII levels rise gradually with treatment and there is no need to measure exogenous FVIII half life.

Aims: To prove the concept of heat-sensitization for the classic Bethesda assay, and to see if heat treatment will also render the Bethesda assay more sensitive in detecting antibodies in AHA patients.

Methods: To prove the concept, we tested the sensitized assay in a cohort of 15 AHA patients followed at our institution. We identified samples stored between October 2011 and January 2013. We identified 14 samples with FVIII <70%, and a traditional Bethesda assay result of <5 BU for testing with the heat sensitized method1. As a negative control, we also tested 24 samples from the same patients with FVIII>70% (i.e. samples from patients in complete remission).

Results: Of 15 consecutive patients with AHA, we identified seven patients who had samples with FVIII levels <70%. Of these seven patients, five had at least one sample that tested negative with the traditional Bethesda assay, while positive with the modified heat-sensitized assay. Of 14 samples with FVIII <70% and negative traditional Bethesda assay, eight tested positive with the modified heat-sensitized assay. In four patients, where the false-negative FVIII zone with the traditional Bethesda assay could be determined (i.e. the zone where only the sensitized assay detects anti-FVIII ABs), the zone fell between 42% and 67% FVIII. All samples that tested positive with the classic assay were also positive with the modified assay. None of the 24 patient samples with >70% FVIII levels had positive antibody tests with either of the assays.

Conclusion: We conclude that the heat-sensitized modified Bethesda assay allows detection of previously undetected anti-FVIII Abs in a large proportion of acquired hemophilia A patients. This proves the concept of the heat treatment making the assay more sensitive while preserving specificity.

Reference:

1. Dardikh M et al., *J Throm b Haemost* 2012, **10**: 706.

PB 4.55-4

Identification of structurally permissive regions in coagulation factor VIII suitable for the insertion of exogenous peptidyl elements

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Background: Several strategies for extending the half-life of human factor VIII (FVIII) have resulted in the development of variants that achieve up to a 1.6-fold half-life increase in humans compared to unmodified FVIII. The intra-domain insertion of unstructured hydrophilic polypeptides of defined amino acid composition and low immunogenic potential (XTENs) is a promising alternative approach for generating FVIII variants with improved pharmacokinetic properties. The impact on the clearance and function of FVIII may be optimized by varying the location, composition, length and number of XTEN insertions by entirely recombinant methods. This work provides the first example of a systematic effort to identify sites within FVIII that can structurally accommodate the insertion of exogenous peptidyl elements, including XTEN, without the loss of FVIII activity.

Aim: To evaluate the amenability of FVIII to intra-domain polypeptide insertion and to lay the groundwork for the development of multiple XTEN-containing FVIII molecules with improved pharmacokinetic properties.

Methods: Insertion sites within FVIII were selected on the basis of the following criteria: (i) significant inter-species protein sequence variability, (ii) high calculated accessible surface area, (iii) exclusion of sites within defined secondary structural elements, (iv) moderate to high intrinsic flexibility predicted by molecular dynamics simulation, and (v) exclusion of sites proximal to known hemophilia A missense mutations. A 42 residue XTEN element (XTEN-42) was inserted at sites that satisfied these criteria, as well as at selected sites that did not, and the resulting FVIII activity in conditioned medium of transfected HEK293 cells was measured. Next, XTEN-144 was substituted at sites that had yielded activity with XTEN-42 insertion and inserted at several additional sites, including those determined to be structurally analogous by spatial alignment of FVIII domains, and activity was similarly measured. Several different non-XTEN peptidyl elements were also substituted at positions determined to be permissive for insertion.

Results: Based on the criteria described above, a total of 59 sites in FVIII were evaluated with XTEN-42 and/or XTEN-144 insertion. Of these, 37 sites, designated 'permissive sites', accommodated the insertion of either XTEN-42 or XTEN-144, or both, as determined by FVIII chromogenic assay. No permissive sites for XTEN-144 insertion were identified in the C domains of FVIII. Rather, permissive sites clustered within two distinct surface loops, designated 'permissive loops', in each of the three A domains. These permissive loops occupy structurally analogous positions in the A domains of FVIII and correspond spatially to sodium binding elements on the surface of ceruloplasmin, a copper-binding paralog of FVIII and FV. Tolerability to insertion was not XTEN-specific, as FVIII accommodated diverse polypeptides including GFP, a 27 kDa protein with a defined tertiary structure, at these sites without abrogation of cofactor activity.

Conclusions: The permissive sites identified in FVIII are located within particular regions that tolerate insertion of peptidyl elements without loss of FVIII activity. These results set the stage for combinatorial approaches to evaluate the effect of multiple XTEN insertions on the activity and pharmacokinetic properties of FVIII.

PB 4.55-5

Factor VIII assessment using one-stage clot and chromogenic assay in trials investigating pharmacokinetics of different FVIII products

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Background: Two assays are widely used for assessment of FVIII:C activity in plasma, the one-stage clot assay (OS) and the two-stage chromogenic substrate (CS) assay. The CS assay is mainly used for potency labelling of FVIII concentrates, in particular inEuropeaccording to EMA guidelines. Outside Europe, FVIII concentrates are often labelled according to the OS assay, which is also more widely used to monitor treatment in clinical practice. Discrepancies between these two assays have been reported and attributed to specific products, assay conditions, or the standard used.

Aims: To assess the impact of OS vs. CS assay discrepancy on pharmacokinetic parameters that are typically reported in clinical trials.

Methods: Clinical samples: Samples were derived from clinical trials in adult previously treated patients (ED>150) with severe haemophilia A (FVIII <1%) after a washout period of at least 4 days. A single intravenous dose of different FVIII products (recombinant or plasma-derived) was administered at a dose of 25, 50 or 75 IU/kg. Blood samples were collected before dosing, (15 min), 30 min, 1, 4, 8, 12, 24, 30 and 48 h post dose. Spiked plasma: Haemophilia A plasma was spiked with FVIII concentrates (target concentration: 0.03, 0.2, 0.6 and 0.9 IU/mL). Products: Three recombinant products (Advate® Baxter, Kogenate® Bayer, and turoctocog alfa, Novo Nordisk) and three plasma-derived FVIII products (Hemofil M® Baxter, Emoclot® Kedrion, and Fandhi® Grifols). Assays: OS assay using the SynthASil (Instrumentation Laboratory) aPTT reagent and CS assay based on the Coamatic® kit (Chromogenix). All measurements were done against commercial standard human plasma (Siemens) for the clinical samples and the 6th WHO FVIII plasma standard for the spiked samples.

Results: All recombinant products showed a discrepancy between results from OS and CS assay. The FVIII activity measured with the CS assay was higher than when measured with the OS assay (mean CS/OS ratios: Kogenate 1.65, Advate 1.22, turoctocog alfa 1.26). Plasma-derived products showed either no or very low discrepancies (mean CS/OS ratios: Hemophil 1.11, Emoclot 0.97, Fandhi 1.11). Similar results were obtained with spiked plasma samples. The discrepancy was consistently observed across the whole range of activities tested in clinical samples (0.01–1.5 IU/mL) and spiked plasma (0.03–0.9 IU/mL). The assay discrepancy affected most PK parameters (AUC, CL and $C_{30\ min}$), but had less impact on the terminal half-life.

Conclusion: The discrepancy between the two types of FVIII assay affects all recombinant products regardless of the length of the B-domain. The discrepancy affects parameters reported in clinical trials as well as routine monitoring. This should be taken into account when comparing trials and products. The consistent use of CS assay for labelling and monitoring of products or the use of product specific standards may eliminate discrepancies.

PB 4.55-6

Pre-clinical pharmacokinetic and pharmacodynamic (PK/PD) characteristics of rVIII-SingleChain, a novel recombinant single-chain FVIII

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Background: rVIII-SingleChain, a novel recombinant coagulation factor VIII, produced and formulated without added animal- or human-derived materials, is under development at CSL Behring in the clinical trial program AFFINITY.

Aim: The present nonclinical studies were conducted in animals to gather knowledge about the PK/PD characteristics and as a result may enable a more precise estimate of the haemodynamic efficacy of rVIII-SingleChain for future clinical use in haemophilia A patients.

Methods: The PK behaviour of rVIII-SingleChain was assessed in different preclinical animal species. Doses ranging between 50 and 250 IU/kg of rVIII-SingleChain or licensed full-length FVIII concentrates were given to animals as a single intravenous administration. Plasma samples were drawn at various time-points following drug administration and systemic FVIII levels were determined by means of FVIII activity or FVIII antigen measurements. For investigation of the PD activity, escalating doses of rVIII-SingleChain and comparators, comprising either B domain deleted or full length FVIII, were administered to haemophilia A mice. Following drug treatment total blood loss was monitored as a primary endpoint after tail clip and a subsequent 30 min observation period. Furthermore, procoagulant activity was measured as occlusion rate in a $FeCl_3$-induced arterial thrombosis model *in vivo* and by recording thrombin generation activity *ex vivo*, both after administration of rVIII-SingleChain or full-length FVIII to haemophilia A mice at dose levels of 200–250 IU/kg.

Results: Overall, treatment with rVIII-SingleChain resulted in slightly, but consistently improved pharmacokinetic properties compared to full-length rFVIII when measuring plasmatic FVIII concentrations. In all animal species systemic availability, mean residence time and terminal half life were increased between 1.6 and 2 fold, while clearance rates were decreased two fold for rVIII-SingleChain compared to full-length rFVIII. Whereas, other variables like *in vivo* recovery and volume of distribution of rVIII-SingleChain were comparable to full-length rFVIII. Under acute bleeding conditions as present in the tail clip model, the pharmacodynamic efficacy of rVIII-SingleChain was found to be indistinguishable from all licensed rFVIII products tested. Furthermore, consistent with the favourable pharmacokinetic characteristics, equal doses of rVIII-SingleChain showed increased thrombin generation vs. full-length rFVIII: For advanced time points between 2 and 6 days thrombin peak levels of 50–250 nM were sustained after treatment with rVIII-SingleChain indicating an average extension of PD effects by 20 h. Similarly, prolonged procoagulant activity of rVIII-SingleChain compared to full-length rFVIII was observed in haemophilic mice over 64 h after treatment following $FeCl_3$-induced arterial thrombosis as a consequence of endothelial damage.

Summary/Conclusion: In conclusion, the nonclinical studies presented interesting PK/PD properties of rVIII-SingleChain in animals, since pivotal characteristics required for sustained haemostatic efficacy appear favourable. Results obtained from this investigations support the evidence necessary for proceeding with clinical trials and to explore whether such favourable nonclinical properties of rVIII-SingleChain may translate into clinical benefits for the treatment of haemophilia A patients.

PB4.56 – Angiogenesis and Arterial Vascular Disorders

PB 4.56-1

A Novel Technetate-99 m labeled anti-intergin peptide dimer useful for tumor imaging

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Background: Integrin a_vb_3 plays a significant role in tumor angiogenesis and metastasis, is expressed at very low levels on quiescent epithelial cells and mature endothelial cells, but it is overexpressed on activated endothelial cells of neovasculature and some tumor cells. Therefore, integrin a_vb_3 is considered as an interesting molecular target for early cancer detection. Cyclic RGD peptides are potent integrin a_vb_3 receptor antagonists.

Aims: Evaluation of ^{99m}Tc-HYNIC-6D-RGD_2as a new radiotracer for tumor imaging.

Methods: HYNIC-6D-RGD_2 was evaluated for its integrin $\alpha_v\beta_3$ binding affinity against ^{125}I-echistatin bound to U87MG cells in a whole-cell displacement assay. After radiolabeling, ^{99m}Tc-HYNIC-6D-$RGD_2$was analyzed by radio-HPLC. Female athymic *nu*/*nu* mice (4–5 weeks) were inoculated subcutaneously with 5×10^6 of U87MG cells into the shoulder flank of each animal. Three weeks after inoculation, the animals were used for biodistribution and imaging studies. The tumor-bearing mice (20–25 g) were randomly selected, and each animal was administered with ~0.1 MBq of ^{99m}Tc-6D-RGD_2 by tail vein injection. Animals (5–8) were sacrificed by sodium pentobarbital overdose (~200 mg/kg) at 5, 30, 60 and 120 min post-injection (p.i.). Blood, tumors and normal organs (brain, eyes, heart, spleen, lungs, liver, kidneys, muscle and intestine) were harvested, weighed, and counted on a Perkin Elmer Wizard – 1480 g-counter. The organ uptake was calculated as the percentage of injected dose per gram of wet tissue (%ID/g). The blocking experiment was performed using RGD_2 as the blocking agent in six animals.

Results: The IC_{50} value for HYNIC-6D-RGD_2 was calculated to be 66.68 ± 0.19 nM. ^{99m}Tc-6D-RGD_2 was prepared in very high specific activity and high radiochemical purity (>95%), and was able to remain stable in solution for >24 h post-labeling. The tumor uptake of ^{99m}Tc-6D-RGD_2 was 4.20 ± 0.42, 3.13 ± 0.47, 2.45 ± 0.90 and 0.30 ± 0.01%ID/g at 5, 30, 60 and 120 min post-injection, respectively. The blocking experiment clearly showed that the tumor uptake of ^{99m}Tc-6D-RGD_2 is integrin a_vb_3-specific.

Conclusion: Considering its high uptake in the xenografted U87MG glioma tumors, fast clearance from normal organs and high metabolic stability, it was concluded that ^{99m}Tc-6D-RGD_2 is may be a useful tool for imaging integrin a_vb_3-positive tumors.

PB 4.56-2

Optimal antithrombotic strategy in patients undergoing carotid endarterectomy

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Background: Over the last decades carotid endarterectomy (CEA) has become one of the most common surgical procedures. The aim of CEA is to prevent stroke or transient ischemic attack (TIA) in patients with carotid artery diseas (CAS). Although protective, this procedure may be associated with minor and major neurological complications including stroke and TIA. Some of these complications are due to

improper surgical technique, inadequate neurological control or underestimation of collateral blood flow at the time of surgery. However, improper regimen of antithrombotic treatment during and after carotid endarterectomy is known to be important factor contributing to the development of neurological deficits in this group of patients.
International guidelines on the surgical management of carotid artery disease advocate for the routine use of low-dose aspirin (50–325 mg daily) before, during and after surgical procedure in order to prevent intraoperative and postoperative arterial thromboembolic complications. However, no algorithm has been proposed with regard to patients with aspirin-resistance as well as those, who are at a high risk for venous thromboembolism (VTE).
Aim: To optimize existing guidelines on the surgical management of carotid artery disease by suggesting the differentiated antithrombotic strategy for patients with aspirin resistance and those at high risk of venous thromboembolism.
Methods: 60 patients with carotid artery disease have been consecutively operated with conventional endarterectomy technique involving synthetic patch angioplasty. All patients were split in two groups according to their aspirin-resistance test. Group A (n = 12) was formed from the patients with aspirin-resistance and Group B (n = 48) without it. All patients received standard regimen of preoperative antiplatelet treatment with aspirin 75–150 mg daily. However, patients from group A (n = 12), have been put on clopidogrel 75 mg daily immediately after surgery provided that they didn't have documented clopidogrel-resistance. Patients from group B (n = 48) have been left on aspirin after surgical procedure. Those patients with high venous thromboembolism risk score (four patients in all, one from group A and three from group B) have been managed with prophylactic doses of low-molecular-weight heparins in addition to their antiplatelet treatment.
Results: Mean age of the study individuals was 68.5 ± 5.4 years for all enrolled subjects and did not vary considerably between two groups. Both groups were uniformly sex-matched. There have been no strokes or transient ischemic attacks in either group after surgery. Postoperative wound hematoma was diagnosed in two patients (4.2%) from group B, one of which required repeated surgical intervention.
Conclusions: In light of commonly encountered aspirin resistance, differentiated antithrombotic strategy is essential when considering optimal antithrombotic treatment in patients undergoing CEA. However, larger studies will be needed to confirm clinical efficacy of this approach.

PB 4.56-3
VEGF-A, sVEGFR-1, sVEGFR-2 in myeloproliferative neoplasms

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Background: The most important and effective angiogenic factor is vascular endothelial growth factor (VEGF), responsible for differentiation, migration and survival of endothelial cells. VEGF works by binding with specific tyrosine kinase receptors: VEGFR1, VEGFR-2 and VEGFR-3. Currently, there are known two soluble forms of the VEGF receptors (sVEGFR): sVEGFR-1 and sVEGFR-2, which may act as inhibitors of angiogenesis. The results of studies conducted in many diseases and pathological conditions indicate a significant role of VEGF in tumor angiogenesis. There is an ongoing search for non-invasive, effective research methods to assess the intensity of angiogenesis in the course of many diseases, including those in myeloproliferative neoplasms.
Aims: The aim of this study was to evaluate the concentration of VEGF-A, sVEGFR-1 and sVEGFR-2 in patients with MPNs.
Methods: The study involved 79 patients with myeloproliferative neoplasms (mean age 61.42), hospitalized and diagnosed at the Clinical Ward of Hematology of the Dr. J. Biziel University Hospital No. 2 in Bydgoszcz, Poland. These patients were enrolled in the study at the time of the diagnosis of MPNs and prior to the implementation of appropriate treatment. The study group included 46 patients with essential thrombocythaemia (ET), 19 with polycythemia vera (PV), seven with chronic myeloid leukemia (CML) and seven with the primary myelofibrosis (PMF). The control group consisted of 39 healthy volunteers to correspond to the age (the mean age of 60) and gender to the study group. In blood samples were determined VEGF-A, sVEGFR-1 and sVEGFR-2 using ELISA tests (R&D Systems, USA). The study was approved by the Bioethics Committee of Collegium Medicum in Bydgoszcz, the Nicolaus Copernicus University in Torun, Poland.
Results: In the present study, there was a significantly higher concentration of VEGF-A in all patients with MPNs, as compared to the control group (Me = 96.00 pg/mL vs. 18.82 pg/mL, $P < 0.000001$). There were also demonstrated significantly lower levels of sVEGFR-1 and sVEGFR-2 in patients with myeloproliferative neoplasms compared to the control group ($P = 0.004001$ and $P < 0.000001$, respectively). Furthermore, we made a detailed analysis of the results in respective subgroups of patients with myeloproliferative neoplasms, as compared to the control group. The concentration of VEGF-A was significantly higher in all subgroups: ET (Me = 105.74 pg/mL), PV (Me = 71.72 pg/mL), PMF (Me = 64.80 pg/mL) and CML (Me = 553.84 pg/mL) compared to the control group (Me = 18.82 pg/mL). It was also found significantly lower levels of sVEGFR-1 in patients with PV (Me = 87.66 pg/mL) and ET (Me = 87.44 pg/mL), as compared to the control group (Me = 133.70 pg/mL) ($P = 0.021815$ and $P = 0.009522$, respectively). SVEGFR-2 concentration was significantly lower in all subgroups of patients with MPNs, as compared to the control group.
Conclusion: Increased levels of VEGF-A indicate the increased angiogenesis in patients with myeloproliferative neoplasms.

PB 4.56-4
Evaluation of arterial stiffness in β-thalassemia/Hb E patients

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Background: The common disease-related complications of thalassemia intermedia (TI) are venous thromboembolism and pulmonary arterial hypertension leading to right-sided heart failure. β-thalassemia/Hb E has TI phenotypes and is more prevalent in South East Asia. However, systemic arterial complications have been less studied. Non-invasive and blood pressure independent arterial stiffness measurement of Cardio-Ankle Vascular Index (CAVI) has been used and able to predict outcomes in general population.
Aims: To assess arterial stiffness using CAVI method in β-thalassemia/Hb E compared to normal population.
Methods: Age and sex matched cases of 43 β-thalassemia/Hb E and 43 normal subjects were examined with CAVI, echocardiography, ferritin, CBC and blood chemistry.
Results: CAVI values in β-thelassemia/Hb E patients were significantly higher than controls (7.79 vs. 7.26, $P = 0.002$), and significantly correlated with pulmonary pressure, PASP ($r = 0.39$, $P = 0.012$) and PADP ($r = 0.47$, $P = 0.002$), heart rate ($r = 0.36$, $P = 0.02$), MCV ($r = 0.31$, $P = 0.045$) and MCH ($r = 0.32$, $P = 0.039$). In addition, β-thalassemia/Hb E patients had significantly higher heart rate, pulmonary pressure, BUN, triglyceride levels and peak tricuspid annular systolic velocity (RV S') but significantly lower BMI, diastolic blood pressure, Hb, MCV, MCH, Cholesterol, HDL and LDL levels than controls. Subgroup analysis revealed post splenectomy has higher PADP than controls but has no correlation with CAVI values.

Conclusions: Systemic arterial stiffness using CAVI method was significantly increased in β-thalassemia/Hb E patients and correlated with increased pulmonary arterial pressure. Therefore, CAVI might be considered as a surrogate marker of atherosclerosis as well as cardiovascular morbidities in TI patients. A prospective study in a large population of patients is warranted.

PB 4.56-5

HDL cholesterol, apolipoprotein A-I, and HDL subfractions predict severity of coronary artery disease

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Background: Observational studies demonstrated an inverse relation between HDL cholesterol and risk for developing coronary artery disease (CAD) and its clinical sequelae including MI, death, and need for revascularization. HDL2 (large) is protective, HDL3 (small) is associated with increased risk for disease. More recent studies suggest that HDL2 and HDL3 are equally beneficial. We evaluated the relationship between HDL-C, apo A-I, and HDL subfractions and severity of CAD.

Methods: Patients (n = 353) with suspected CAD with or without statins therapy, were enrolled. Lipids and lipoproteins were measured by a vertical density gradient, ultracentrifugation technique in blood samples collected prior to coronary angiography (CA). The severity of CAD was defined as minimal stenosis (<25%), intermediate stenosis (25–75%), and severe stenosis (>75%) of any major coronary vessel as indicated by CA.

Results: The severity of atherosclerotic lesions inversely correlated with HDL-C and apo A-I irrespective of statin therapy. Among patients not receiving statin therapy, both HDL 2 and 3 correlated inversely with severity of atherosclerotic disease. For patients on statin therapy, the relationship between disease severity and HDL3 was significant.

Conclusions: In the MAGMA study, serum levels of HDL-C, apoA-I, and HDL3 have an inverse and significant relationship to severity of CAD. These results support accruing evidence that higher levels of HDL2 and HDL3 are associated with less severe CAD.

PB 4.56-6

Releasate of PAR1-activated platelets enhances capillary formation of endothelial progenitor cells

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Background: Endothelial progenitor cells (EPCs) contribute importantly to endothelial regeneration and new vessel formation. Platelets contain both pro- and anti-angiogenic regulators, and release them differently upon different stimuli. Thrombin receptor PAR1 stimulation is known to induce selective release of pro-angiogenic regulators.

Aims: To investigate if the releasates of PAR1-activating peptide (PAR1-AP) stimulated platelets can enhance the angiogenic properties of EPCs.

Methods: Venous blood was collected from healthy subjects for Ficoll isolation of mononuclear cells. The cells were cultured in an endothelial conditioning medium (ECM: EBM-2 Single Quots medium plus 10% FBS) during 4 weeks, with culture media changed every third day. Autologuous washed platelets (2×10^9/mL) were stimulated by PAR1-AP (10 μM), and the releasate was collected after centrifugation. EPC proliferation was monitored using a [^{3}H]-thymidine incorporation assay. EPC migration was assessed using a modified Boyden chamber, and an *in vitro* tube formation assay was performed on Matrigel-covered culture plates.

Results: EPC colonies usually appeared after 3 weeks culture, with 1–2 colonies per 60 mL blood. EPC colonies generated from adherent peripheral mononuclear cells displayed a cobblestone morphology. Supplementation with PAR1-AP releasate (10% ECM volume) approximately doubled the migration of EPCs in modified Boyden chambers (n = 3). Moreover, PAR1-AP releasate enhanced EPC tube formation on Matrigel[TRADEMARK] Matrix. Thus, as compared to ECM alone, EPC cultures with PAR1-AP releasates developed denser capillary network, with increased numbers of branch points (by approximately 55%). Surprisingly, the releasate did not promote but rather attenuated EPC proliferation as evidenced by a 30% reduction of thymidine incorporation in EPC cultures with PAR1-AP releasates.

Conclusions: Releasates from PAR1-AP-stimulated platelets enhance the capillary forming capacity of EPCs. Inhibitory effect of the releasate on EPC proliferation is probably due to the fact that PAR1 stimulation can still induce a considerable release of anti-angiogenic factors.

PB4.57 – Fibrinogen/Fibrin – V

PB 4.57-1

Combination of haemostatic agents reduces blood loss and enhances survival in a two-hit model of blunt liver injury

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Background: In the treatment of trauma-induced coagulopathy there has been a shift from an empirical treatment with FFP to a targeted therapy with coagulation factors concentrates and other haemostatic agents in recent years. Tranexamic acid (TX) is increasingly used as first line therapy for traumatised patients. Prospective studies investigating the combination of TX with other haemostatic agents are missing.

Aims: In a two-hit pig model with two blunt liver injuries and haemorrhagic shock we explored the effects of a combined therapy with TX, fibrinogen, and prothrombin complex concentrate (PCC) on blood loss and coagulation variables.

Methods: After local ethical approval trauma was induced in 36 anaesthetised pigs. A second hit standardised blunt liver injury grade III was induced using a custom made instrument. After the first injury phase with severe haemorrhagic shock over 30 min, animals were resuscitated with crystalloids to maintain nadir blood pressure. Ten minutes after the following second liver injury animals were re-transfused with washed erythrocytes and randomised to receive either normal saline (control), TX (15 mg/kg, TX group), TX and fibrinogen (90 mg/kg, TXF group) or TX, fibrinogen and PCC (20 U/kg, TXFP group).

Thromboelastometry (ROTEM), including a modified method for fibrinolysis ('tPA-ROTEM') and global coagulation parameters were monitored over 4 h and blood loss was measured. Statistical analysis was performed using ANOVA with Tukey *post hoc*. The significance was defined as $P < 0.05$.

Results: Coagulation was severely impaired after the infliction of injuries and haemorrhagic shock. Prothrombin time (PT: 15 ± 2 s) and clot formation (CFT: 81 ± 14 s) prolonged over time. Correspondingly, concentrations of fibrinogen (49 ± 10 mg/dL) and clot strength (MCF: 53 ± 4 mm) were severely reduced. Infusion of fibrinogen restored CFT, MCF and increased concentrations of fibrinogen significantly. An additional impact on global coagulation variables after the infusion of PCC could not be observed. In contrast, coagulation in the control group was increasingly impaired over time. TX-treated animals showed an inhibition of tPA induced fibrinolysis with no impact neither on MCF nor CFT.

The total blood loss as the primary endpoint of this study was lowest in the TXF (1012 ± 86 mL) and TXFP (1037 ± 118 mL) groups, followed by the TX (1579 ± 306 mL) and control group (2376 ± 478 mL; $P < 0.05$. Accordingly, all animals of TXF and TXFP groups survived, whereas five of nine animals (55%) and two out of nine animals (22%) died in the control and TX groups, respectively ($P < 0.05$). Clinically and macroscopically no adverse events were observed.

Conclusions: The early application of TX and fibrinogen significantly reduced blood loss and enhanced coagulation variables in a highly lethal model with blunt liver injuries and prolonged haemorrhagic shock. The additional use of PCC showed no further effects in this specific animal model, which is most likely attributed to a lack of impaired thrombin generation. Thus, the early use of a combined treatment with coagulation factors and TX is a reasonable and safe approach to reduce the need for the transfusion of FFP and red blood cells.

PB 4.57-2
Detection, localisation and quantification of intracellular phosphorylated human fibrinogen by proximity ligation: a novel approach for the characterisation of fibrinogen phosphorylation

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Background: Human fibrinogen is secreted from hepatocytes in its phosphorylated form, with 20–25% of circulating fibrinogen phosphorylated exclusively at Aα chain Serine 3 (Ser3) and Serine 345 (Ser345). Elevated levels of phosphorylated fibrinogen have been observed in foetal fibrinogen, ovarian cancer patients, venous thromboembolism and acute phase responses; however, the cause of this increase and the effect of fibrinogen phosphorylation on clot structure and function remain undetermined. The latter has previously been investigated *in vitro* using several potential kinases but the results have proved inconsistent, failing to clarify the role of these phosphorylation sites. Similarly, this post-translational modification has yet to be explored at a cellular level.

Aims: The aim of the current investigation was to develop a method for the detection of intracellular phosphorylated human fibrinogen Aα chains and determine the subcellular location of fibrinogen phosphorylation.

Methods: We have carried out a proximity ligation assay (PLA) using commercially available reagents to detect and quantify phosphorylated fibrinogen in two cell lines: i) human hepatocytes (HepG2 cells), and ii) Chinese hamster ovary cells expressing recombinant human fibrinogen. The cells were fixed, permeabilised and blocked for non-specific binding before probing with anti-fibrinogen Aα chain and anti-phosphoserine primary antibodies. Hybridisation of secondary PLA probes in close proximity results in the generation of a DNA template, which is amplified and detected with fluorescently-labelled oligonucleotides. Markers for endoplasmic reticulum, Golgi apparatus and nucleus detection were incorporated to reveal the site of fibrinogen phosphorylation. Finally, cells were mounted and imaged using confocal microscopy.

Results: Using this technique we have visualised, for the first time, phosphorylated fibrinogen within HepG2 and CHO cells. Preliminary data suggests phosphorylation of fibrinogen takes place in the endoplasmic reticulum, as indicated by the distribution of signal and minimal co-localisation with the Golgi apparatus. In contrast, control cells expressing the Bβ and γ chains of fibrinogen contained negligible signal of random distribution, highlighting the specificity of this method.

Conclusion: This novel approach will facilitate identification of the protein kinase(s) responsible for this modification *in vivo*, providing a valuable tool for the characterisation of Ser3 and Ser345 phosphorylation. Furthermore, it will be used to determine whether elevated levels of fibrinogen phosphorylation accompanying the acute phase response result from increased synthesis of high molecular weight fibrinogen, or an up-regulation of fibrinogen kinase activity in response to extracellular stimuli. Elucidating the mechanism of cellular fibrinogen phosphorylation and its contribution to haemostatic and thrombotic processes could aid the diagnosis and management of associated pathologies.

PB 4.57-3
Functional aspects of platelets and factor XIII in hereditary afibrinogenaemia

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The clinical phenotype of hereditary afibrinogenaemia (HAF) is heterogeneous. Thus, even profound deficiency of fibronogen (Fg) and, consequently, in fibrin (Fn) formation may not be associated with a hemorrhagic phenotype. We now studied a patient with HAF and examined platelet and FXIII function with regard to Fg/Fn parameters.

Patient: The proposita is a 30-year-old woman in whom afibrinogenaemia resulting from a mutation in the *FGA* gene (c.510 + 1_510 + 4delGTAAinsCCAAAA) was diagnosed after gastrointestinal hemorrhage at birth. During childhood and adolescence, the patient suffered from easy bruising only. In 2012, ankle joint bleeding for the first time necessitated replacement therapy with a fibrinogen concentrate (FgC, Haemocomplettan® CSL Behring).

Methods: Coagulation screening was performed by prothrombin time (PT)(Thromborel S®, Siemens), activated partial thromboplastin time (aPTT) (Pathrombin SL®, Siemens) and thrombin time (TT)(BC Thrombin®, Siemens). For fibrinogen assessment, the Clauss method (Multifibren®, Siemens) and nephelometric determination of the antigen concentration (Sigma®) were used. FXIII activity was measured with the Berichrom FXIII® test (Siemens), FXIII antigen concentration was assessed using an ELISA test (Haemochrom®). Platelet function was screened with PFA-100® (Siemens) and platelet aggregation studies were performed by light transmission with a DiaSysGreiner® instrument. For thrombelastometry ROTEM®(tem international) was used. To assess dysfunctional fibrinogen, the ratio of Fg (Clauss) and Fg concentration was used (Fg-R, normal >0.9). Likewise, FXIII dysfunction was estimated by the ratio of FXIII activity and FXIII antigen concentration (FXIII-R, normal >0.9). All tests were performed before as well as after hemotherapy with FgC.

Results: Prior to substitution, none of the coagulation tests (PT, PTT, TT, TEM) revealed any detectable clot formation. Platelet function appeared intact upon PFA screening and aggregation testing in response to high-dose arachidonic acid and collagen. By contrast, platelet aggregation with 'weak agonists' was strongly reduced (epinephrine 5%, normal >70%; ADP 46%, normal >70%). FXIII levels were reduced to 22% in activity (normal >80%) and to 58% in concentration (normal >70%). Hemotherapy with FgC led to an increase of Fg levels (Clauss) in a dose- and time-dependent manner. Consequently, the results of the hemostasis tests, including TEM, improved or normalized, which was also true for platelet aggregation in response to weak agonists. Moreover, FXIII activity increased. This increase was strongly correlated to Fg levels (Clauss) ($r = 0.98$). Of note, despite the uniform increases both in Fg and FXIII activity, the Fg ratio (0.62) as well as the FXIII ratio (0.54) remained abnormal.

Conclusion: This case report illustrates that impaired platelet function in HAF may be restricted to reduced aggregation upon stimulation with weak agonists. Substitution of fibrinogen can restore normal platelet aggregation. FXIII function in HAF appears to be compromised due to reduced concentration and reduced function. Even though fibrinogen substitution leads to strongly correlated increases of Fg levels (Clauss) and FXIII activity, functional deficiencies of Fg/Fn and FXIII may persist. Our observations warrant extended assessment of platelet function and FXIII function in patients with fibrinogen disorders.

PB 4.57-4

Fibrinogen clottable protein assays (Clauss assay vs. CLOTr methods): the effects of fibrinogen & thrombin concentrations on clot formation, structure and clot turbidity

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Background: Following the establishment of the WHO 3rd International Standard (IS) for Fibrinogen Plasma (09/264) and more recently the WHO 2nd IS Fibrinogen Concentrate (09/242), it was evident that, although Clauss assays were found to be suitable for assaying fibrinogen clottable protein in plasma [low inter-laboratory variability, GCV = 3.2%; $n = 22$ with good agreement (<4% difference) with CLOTr (assays following clot removal) methods], they were not found to be suitable for assaying fibrinogen concentrates [high inter-laboratory variability, GCV = 17.2–26.8%; $n = 17$–24 with poor agreement (18–81% higher potency) with CLOTr methods]. Studies were carried out to investigate this discrepancy.

Aim: To investigate the influence of fibrinogen and thrombin concentrations on assays used to determine thrombin clottable protein in fibrinogen concentrates.

Methods: Clot turbidity (as measured by absorbance at 405 nm) measurements were carried out at varying fibrinogen and thrombin concentrations to assess their effects on clot structure and function. SDS-PAGE analysis and Transmission Electron Microscopy (TEM) were also carried out to assess the structure of the clot, and fibre & protofibril formation respectively.

Results: At physiological concentrations of fibrinogen (1–4 mg/mL), an increase in low levels of thrombin concentration (from 0.03 to 0.6 IU/mL) leads to a proportional increase in turbidity (absorbance) of the clot. However, higher thrombin levels (from 0.6 to 20 IU/mL) lead to a reduction in turbidity. Furthermore, at supra-physiological concentrations of fibrinogen (e.g. @ 10 mg/mL), this reduction in turbidity is observed at a much lower thrombin concentrations (from 0.3 to 5 IU/mL). At low thrombin concentrations, it is thought that fibrinopeptide cleavage is slower (as observed by slower rate of absorbance) so that protofibrils are formed more slowly and shorter protofibrils aggregate as they are formed, resulting in thicker fibres. But at higher thrombin concentrations, the maximum rate of assembly is greater and the maximum turbidity is consequently lower. The lower turbidity with an increase in fibrinogen concentration can be similarly explained. These results are further confirmed by SDS-Page gels and TEM scans.

Conclusions: Results from these studies indicate that clotting can be influenced by thrombin and fibrinogen concentrations. Small differences between the concentrations of these analytes can have a significant effect on the turbidity & opacity of fibrinogen clot being formed and this may explain the high variability observed in Clauss assays, where both mechanical and photometric clot detection systems are employed and where significantly higher thrombin concentrations are used (100–200 IU/mL). These differences are less apparent with the CLOTr method where clot is initially formed, removed and washed prior to protein analysis. For assays of thrombin clottable protein in fibrinogen concentrates, optimal concentrations of fibrinogen and thrombin concentrations are found to be 1–4 mg/mL and 1–5 IU/mL respectively. For Clauss assays further validation is likely to be required. These finding are to be proposed for the EP monograph revision for this method.

PB 4.57-5

New liquid quality controls for D-Dimer assays with extended stability

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Background: Due to evolutions in quality assessment programs undertaken by medical laboratories, Stago has developed the STA® – D-Di Control. The kit comprises of two levels of liquid controls, which are dedicated for use with D-Dimer assays, when performed on Stago analyzers. These controls can be used in conjunction with STA® – Liatest® D-Di or STA® – Liatest® D-Di PLUS. The levels of these controls are targeted to have a D-Dimer concentration of around 0.75 μg/mL and 2.30 μg/mL.

Aim: The aim of this study is to demonstrate the performance of the STA® – D-Di Control kit, in terms of analyzer on board stability and after opening.

Methods: STA® – D-Di Control 1 and 2 were opened and placed on board:- the STA-R® and STA Compact® analyzers for 72 h- the STA Satellite® for 48 h. The controls were then assayed with STA® – Liatest® D-Di. The controls were also assayed after the opening of the vials, which were then recapped and stored at 2–8 °C for 30 days.

Results: The on board stability results show that the absolute variations tested on a lot

- after 72 h on the STA-R® and the STA Compact® is 0.05 μg/mL and 0.02 μg/mL for Control 1 and 0.13 μg/mL and 0.04 μg/mL for Control 2, respectively
- after 48 h on the STA Satellite® is 0.00 μg/mL for Control 1 and 0.08 μg/mL for Control 2.

Stability after the opening of the vial and storage at 2–8 °C for 30 days shows that the absolute variations observed for a lot does not exceed 0.00 μg/mL for Control 1 and 0.05 μg/mL for Control 2.

Conclusion: STA® – DDi Control is stable over time, allowing the use of Quality Controls on board the analyzer over a period of 72 h on the STA-R® and STA Compact® or 48 h on the STA Satellite®. In addition, STA® – DDi Control when stored at 2–8 °C in its original recapped vial, is stable for 30 days. With this extended stability, the STA® – D-Di Control allows laboratories to adapt the frequency of quality control in an optimized way. The liquid formulation also allows better practicality and the reduction in handling errorsSTA® – D-Di Control can be used with the STA® – Liatest® D-Di or STA® – Liatest® D-Di PLUS for D-Dimer Assays.

PB 4.57-6

Purification and characterization of a new fibrinogen concentrate: results at laboratory scale

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Background: Fibrinogen (Fg) replacement therapy is mandatory during the hemorrhagic episodes in congenital and acquired fibrinogen deficiencies, the latter being associated with liver failure, disseminated intravascular coagulation, massive transfusion and cardiac surgery. At present, only a few Fg concentrates are available worldwide.

Aims: The objective of this study is to show the results achieved, in a laboratory scale, of a new Fg concentrate obtained from discarded material produced during the process of purification of Factor VIII.

Methods: After the dissolution of the cryoprecipitate, aluminum hydroxide treatment, viral inactivation process, adjustment of pH and ionic strength, this protein mixture is loaded on an ion exchange column. Under these conditions, the unbound protein material eluted is used as a source for isolation of Fg. After adjusting the pH and conductivity, this eluate is mixed with heparin-Sepharose in a ratio near 1:1 and washed with buffer of equilibrium (sodium citrate, sodium chloride, glycine and lysine) until absorbance near to 0. Fg is eluted with the same buffer but containing more high concentration of

glycine and lysine in a sharp peak. Next, the product obtained is concentrated for ultra-filtration and lyophilized.
Results: In total 20 chromatographic runs were made starting from different eluates which contained an average of 246 mg/dL of immunologic Fg (range 180–306 mg/dL). The concentration of functional and immunological Fg obtained in the peaks after elution were 319 ± 48 mg/dL and 340 ± 58 mg/dL, respectively and the total protein concentration was 351 ± 65 mg/dL. The ratio between Fg functional/immunologic was 0.94 and Fg functional/protein concentration was 0.91. The electrophoresis in polyacrylamide gels and Western blots analysis revealed the presence of a single band representing >90% of total protein content in the zone of Fg.
Summary: The results allow us to conclude that using a fast and inexpensive method starting from an eluate of proteins, which in our working conditions were discarded; we can isolate and purify a concentrate of Fg with a high degree of purity and coagulability judging by the protein analysis.

PB4.58 – Other coagulation factors – IV

PB 4.58-1
Spectroscopic evidence of intrinsic disorder in the activation peptide of coagulation factor X

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Background: The vitamin K-dependent coagulation factors (F) VII, IX, X and protein C share the same domain organisation, with the exception of an activation peptide missing in FVII. FX possesses the longest half-life and activation peptide, where two N-linked glycans (N39 and N49) are key determinants. Upon activation of the zymogens, the activation peptide is released and the circulatory half-life drops dramatically. The half-life of the zymogen and activated forms of FVII are similar. A half-life prolonging effect of the activation peptides has been shown when these were inserted in FVII. Altogether, this indicates that the activation peptides play a fundamental role in the retention and clearance mechanisms of the coagulation factors. Recently, the N-linked glycans in the activation peptide of FX were found to be important for localisation to macrophages protecting it from rapid clearance. The influence of the N-linked glycans on the structure of FX and that of the activation peptide is unknown. In order to gain more knowledge about the life cycle of vitamin K-dependent coagulation factors structural information about the activation peptides would be valuable.
Aims: To investigate the structure of the activation peptide of FX alone and in the context of zymogen FX and the influence of the N-linked glycans on the structure.
Methods: Structural characterisation of the human FX activation peptide, expressed in *E. coli*, was performed using multidimensional heteronuclear nuclear magnetic resonance (NMR) spectroscopy. Plasma-derived human FX, N-deglycosylated FX and FXa were studied using circular dichroism (CD) and fluorescence spectroscopy.
Results: The NMR studies of the glycan-free FX activation peptide showed that it is intrinsically disordered without a defined fold. However, determination of residual secondary structure, from backbone carbon chemical shifts using a urea-denatured sample as the internal reference, revealed a transient α-helix located between residues 32 and 39 in the activation peptide. Far-UV CD spectroscopy of FX, N-deglycosylated FX and FXa indicated that FX and N-deglycosylated FX have indistinguishable secondary structure contents, whereas FXa appeared to be less disordered. Near-UV CD studies indicated only minor differences between the tertiary structures of FX, N-deglycosylated FX and FXa. Fluorescence spectroscopy experiments revealed no differences in the emission intensity or in wavelength of emission maximum between FX and N-deglycosylated FX, whereas the emission maximum for FXa was blue-shifted indicating a lesser degree of structural disorder.
Summary/Conclusions: The isolated activation peptide of FX is intrinsically disordered with a transient, low-populated α-helix. Our data strongly indicates that the structural disorder persists when the activation peptide is in the context of the parent protein, and that the N-linked glycans do not influence the structure of FX.

PB 4.58-2
Pseudonaja textilis venom FXa is poorly inhibited by human antithrombin

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The venom of the Common Brown Snake (*Pseudonaja textilis*) contains an analogue of the mammalian prothrombinase complex consisting of factor Va (fVa) and factor Xa (fXa) orthologues with identity to the human versions of 54% and 49% respectively. Evolutionary modifications of its own plasma fX and fV, such as the removal of the membrane dependency of the complex and improved affinity of venom fVa for fXa, led to the venom having powerful systemic procoagulant activity resulting in the conversion of its prey's prothrombin to thrombin. This leads to consumptive coagulopathy of the victim, causing severe fibrinogen depletion, early hypotension and spontaneous bleeding and pulmonary thromboembolism. The use of heparin to prevent the catastrophic coagulation as a result of envenomation by *P. textilis* has been investigated, however one study suggested that pre-treatment with heparin can prevent the coagulant effects of the venom, while another showed that treatment with heparin 15 min after envenomation had no effect.
Here our aim is to investigate the inhibition of *P. textilis* fXa by human antithrombin and to determine if inhibition is affected by heparin pentasaccharide. Rates of inhibition of recombinant *P. textilis* fXa were measured in the presence and absence of pentasaccharide by measuring depletion of uncomplexed fXa over time on SDS PAGE. In the absence of pentasaccharide human antithrombin inhibited *P. textilis* fXa poorly with a k_{app} of 7.1 M/s, at least 500 fold slower than human fXa. Inhibition was enhanced by pentasaccharide by around 1000 fold (similar to the enhancement seen for human fXa). The poor inhibition rates, both in the presence and absence of pentasaccharide, can be explained by differences in the 36 loop and the autolysis loop that contribute to recognition of antithrombin, including the critical arginine150 (chymotrypsin numbering) residue in human fXa which is a proline in *P. textilis*. Evolutionary changes in *P. textilis* venom fXa have therefore resulted in a molecule that is poorly inhibited in mammalian blood, thus enhancing the potency of the venom.

PB 4.58-3
Real-life use of activated recombinant Factor VII (rFVIIa) in elderly patients with haemophilia with inhibitors – data from the UK National Haemophilia Database

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Background: Improved management has led to greater life expectancy and a growing population of elderly patients with haemophilia. Activated recombinant Factor VII (rFVIIa) has been approved globally for the treatment of bleeds in patients with haemophilia with inhibitors. It is important to assess the safety of rFVIIa from post-marketing reports in elderly patients, who are at increased risk of thromboembolism, and for whom data have previously been scarce.

Aims: To assess the real-world use and safety of rFVIIa in elderly patients (≥65 years old) in a post-marketing surveillance study requested by EMA when licensing the higher (270 μg/kg) rFVIIa dose, and conducted on behalf of Novo Nordisk by the UK National Haemophilia Database between 1/1/08 and 30/6/11.
Methods: The National Haemophilia Database (NHD) collects data from all UK haemophilia centres on all patients with bleeding disorders, collecting treatment data quarterly and adverse event death and diagnostic data as it arises. Data on rFVIIa dosing and safety, including adverse drug reactions, were collected prospectively. The study was conducted in accordance with the Data Protection Act and the Declaration of Helsinki.
Results: Overall, 139 patients were treated with rFVIIa for a total of 1356 bleeding episodes (episodes/patient: median 3.0; mean 9.76; range 1–124). Of these, 29 (20.9%) were elderly patients (>65 years old) (median age 70.6 years; mean 73.1 years, range 65.2–94.9 years), who were treated for 130 treatment episodes (episodes/patient: median 2.0; mean 4.48; range 1–59). Most (79/130 [61%]) of these episodes were in patients with haemophilia A, with lower proportions in acquired haemophilia (33%) and FVII-deficiency (6%). Elderly patients received a lower initial rFVIIa dose compared with the rest (mean 106.8 vs. 152.7 μg/kg; median 90.2 vs. 112.2 μg/kg), a higher number of rFVIIa doses (mean 5.7 vs. 4.4; median 3.0 vs. 1.0) and had a longer median duration of treatment (7.0 vs. 2.6 h). In addition, elderly patients received a higher accumulated rFVIIa dose than the total population (mean 590.0 vs. 548.8 μg/kg; median 273.0 vs. 253.4 μg/kg). Initial rFVIIa dose administered was between 10.6 and 285.7 μg/kg and 24-h dose range was 35.1–938.7 μg/kg in elderly patients. Single-dose regimens (i.e., a dose spaced >26 h from previous and following doses) were administered to 48.3% of elderly patients for treatment of 21/130 (16.2%) episodes; this is substantially lower than the proportion of patients and episodes treated with single-dose regimens in the total population (71.9% and 51.3%, respectively). No adverse drug reactions, including thromboembolic events, disseminated intravascular coagulation or anti-rFVIIa antibody formation occurred in patients of any age.
Conclusions: This study documents the extensive use of rFVIIa in elderly patients with haemophilia with inhibitors. Importantly, no safety concerns have been raised for this population, including no reports of thromboembolic events. Single-dose regimens were less frequent in the elderly population and the total accumulated dose per treatment episodes was higher. The data suggest that higher initial dosing may shorten treatment duration in the elderly, but this requires further study.

PB 4.58-4

Ribavirin effects on expression of coagulation factors in HepG2 cells

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Background and Aims: Previously, Yamamoto et al. reported significant decreases in doses of clotting factors used for hemostatic therapy and increased activities of clotting FVII in henophiliacs during administration of anti-HCV agent, ribavirin. They also showed that ribavirin upregulated FVII mRNA in cultured human normal hepatocytes as well as hepatoma cell line (HepG2). Although the action of ribavirin was evaluated, its complete effects were not yet elucidated. In this study, we investigated molecular mechanisms of FVII upregulation by ribavirin. Furthermore, we also focused on the other coagulation factors and analyzed ribavirin effects on their mRNA expressions in HepG2 cells.
Methods: We treated HepG2 cells with ribavirin (100 μg/mL) or mycophenolic acid (MPA; 100 μM) for 24 h, and added guanosine at a final concentration of 100 μM if necessary. We isolated total RNA from the cells and subjected to quantitative RT-PCR to determine mRNA levels of prothrombin, FV, FVII, FX, FXI, FXII, and HPRT1. HPRT1 was used as ribavirin-insensitive control.
Results: First, we verified that FVII mRNA was increased by ribavirin treatment. Ribavirin metabolite, ribavirin-monophosphate, is an inhibitor of IMP dehydrogenase (IMPDH), which is a rate-limiting enzyme for the *de novo* purine nucleoside synthesis. We tested another IMPDH inhibitor, mycophenolic acid (MPA), and found MPA treatment also upregulated FVII gene expression. Thus, we hypothesized IMPDH inhibition was critical for FVII mRNA increase. We used guanosine together with ribavirin to confirm our hypothesis, because intracellular GTP depletion induced by IMPDH inhibition could be reversed by addition of guanosine. As a result, we found that the FVII upregulation was completely diminished by guanosine supplementation. Meanwhile, we analyzed expression levels of the other coagulation factors, and found that ribavirin upregulated expression levels of several coagulation factors such as prothrombin, FV, FX, FXI, and FXII. While the increase levels differed individually, prothrombin, FX, and FXII mRNAs were increased only slightly; FV mRNA was upregulated to the same extent as FVII mRNA and FXI mRNA much more abundantly. We also analyzed FIX mRNA; however, HepG2 cells did not express FIX mRNA in a detectable level. MPA also upregulated these coagulation factors similarly. All of these upregulations were retracted by addition of guanosine.
Summary/Conclusions: Herein, we demonstrated that FVII was upregulated by ribavirin treatment. The FVII mRNA increase was induced in response to the intracellular GTP depletion following IMPDH inhibition. We also observed that ribavirin as well as MPA upregulated the other coagulation factor expressions, which were cancelled by addition of guanosine. Taken together, intracellular GTP reduction induced by ribavirin or MPA resulted in the significant mRNA upregulations of FV, FVII, and FXI. Although not all coagulation factor expressions were evaluated, the effect of ribavirin on these genes might attenuate hemorrhagic episodes *in vivo*.

PB 4.58-5

Elimination capacity of transmissible spongiform encephalopathy (TSE) model agents by the production process of therapeutic antithrombin

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Background: The variant Creutzfeldt-Jakob disease (vCJD) is a transmissible spongiform encephalopathy (TSE) mainly present in UK (UK) that peaked in the year 2000 with 28 cases. From 1995 (1st case) to December 2012, 227 cases have been reported across the world, 176 of them in the UK and 27 in France. Worldwide, it can be observed a downward trend in deaths and new cases, and no new cases of vCJD have been reported in UK since October 2011. The vCJD agent is mainly located in the Central Nervous System. However it can be found in the lymphatic system and non-neural tissues at much lower concentration. It has never been biochemically detected in human plasma suggesting that the concentration may be lower than in the above mentioned tissues. Nevertheless, as a precautionary measure, UK plasma is not used for the production of plasma derivates. Furthermore, even in the current absence of vCJD prevalence in the general population, Grifols studies the manufacturing processes to evaluate their capacity of removing prions in case they were present. In that sense, spiking and endogenous infectivity experiments have shown that different plasma protein manufacturing steps are very effective removing TSE model agents. Aims:Laboratory studies were conducted to establish the capacity of Grifols' antithrombin (Anbinex®) production process, to remove a spiked TSE-model agent.

Methods: For this purpose, two different brain-derived materials of Scrapie affected hamsters (strain 263K), clarified brain homogenate and detergent treated brain homogenate, were deliberately spiked into intermediate process solutions. Prion protein (PrP^{Sc}) was detected by Western Blot assay (WB) for protease-resistant prion protein. TSE infectivity was measured using a hamster bioassay. Three production steps were assessed: Fraction I and Fraction II + III precipitations and Nanofiltration through 15 nm filters (Planova™ 15N).
Results: The studied manufacturing steps showed a high capacity to remove the TSE model agent. Reduction Factors for the different TSE preparations (clarified brain and detergent treated brain homogenates, respectively) were 1.02 and 1.32 $\log_{10}$ WBU for Fraction I precipitation step, 3.83 and 2.39 $\log_{10}$ WBU for Fraction II + III precipitation step and ≥3.59 and ≥3.76 $\log_{10}$ WBU for the nanofiltration step. These results obtained by Western blot were in good agreement with bioassay data (Reduction Factors of 1.02 and 3.33 $\log_{10}$ ID_{50} for Fraction I and Fraction II + III precipitations).
Conclusion: Anbinex® manufacturing process effectively removes TSE model agents used as a model of vCJD, indicating that the production process has a high potential to eliminate TSE agents, in the unlikely event of a donor with vCJD.

PB 4.58-6

A prospective, open-label, randomized, parallel study with AICC to evaluate the efficacy and safety of prophylactic vs. on-demand treatment in hemophilia A or B subjects with inhibitors

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Background: Treatment with FEIBA NF (AICC) is a key therapeutic option for controlling acute and difficult to treat hemorrhages in hemophilia A or B patients with inhibitors. Severe and recurrent episodes of joint, muscle, and soft tissue bleeds lead to significant joint damage and morbidity. Prevention of hemorrhages with prophylactic therapy reduces morbidity and improves quality of life. This clinical study evaluated the safety and efficacy of prophylaxis with AICC compared to on-demand treatment.
Aims: To demonstrate reduction of annualized bleed rate (ABR) in subjects using AICC prophylactically compared to on-demand therapy
To evaluate the safety of prophylactic AICC treatment compared to on-demand treatment.
Methods: This prospective, randomized study was designed with two arms: prophylaxis and on-demand regimens over 12 months ± 14 days. Thirty-six hemophilia A or B subjects with inhibitors refractory to FVIII or FIX treatment were enrolled across 17 sites globally. Seventeen subjects randomized to the prophylaxis arm, received AICC at a dose of 85 ± 15 U/kg every other day. Nineteen subjects randomized to the on-demand arm received AICC for control of acute bleeding episodes at dosages per their treating physician. Median age of the subjects was 23.5 years. The study was approved by each institutional ethics committee and all subjects signed informed consent.
Results: The median ABR for subjects in the prophylaxis arm (7.9) was significantly lower than subjects in the on-demand arm (28.7). For both intent-to-treat (ITT) and per-protocol (PP) efficacy analysis sets, the differences in mean transformed ABRs between prophylaxis and on-demand were statistically significant ($P = 0.0003$ and $P = 0.0006$, respectively). Occurrence of new target joints was substantially lower in the prophylaxis arm (7 in 5/17 subjects) compared to the on-demand arm (23 in 11/19 subjects). Median ABR for new target joints was higher in the on-demand arm (5.9) than in the prophylaxis arm (0); the difference is statistically significant ($P = 0.027$). A majority of the bleeding episodes were treated with a single infusion (both regimens 56.5%) and rated as excellent or good (on-demand 90.2%; prophylaxis 75.7%).
Of the 104 AEs reported, 30 were serious and 74 non-serious. Twenty-seven AEs (26.0%) in 8 (22.2%) subjects were deemed related to FEIBA; of these three were serious. Thirteen of the 36 subjects treated with AICC did not report any AEs. There was one unrelated death and one subject discontinued the study due to a non-serious hypersensitivity reaction. There were no thromboembolic events reported.
Conclusion: AICC was safe and efficacious in controlling acute and difficult to treat bleeding episodes in both prophylaxis and on-demand regimens
Significant reduction (72.5%) in ABRs for all bleeds was achieved with prophylactic use of AICC compared to on-demand therapy
Zero bleeding episodes was achieved on prophylaxis in 3/17 (17.6%) and 1/13 (7.7%) subjects in ITT and PP analysis sets respectively
Prophylaxis with recurrent dosing of AICC was safe without any clinical thromboembolic events or laboratory signals of thrombogenicity

PB4.59 – Regulation of coagulation and fibrinolysis – IV

PB 4.59-1

Applying phage display to screen a library of a1-protease inhibitor mutants for thrombin inhibitory activity

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Background: Proteases of the coagulation system are primarily regulated by serine protease inhibitors (serpins) such as antithrombin (AT) and heparin cofactor II (HCII). Alpha-1-proteinase inhibitor (α_1-PI) is the most abundant serpin in plasma. The α_1-PI M358R ('Pittsburgh') mutant exhibits greatly increased rates of thrombin inhibition compared to wild-type α_1-PI, which predominantly regulates neutrophil elastase. M358 (P1) lies at the reactive centre (P1-P1') bond of the reactive centre loop (RCL) of α_1-PI, cleaved by cognate proteases as they become trapped in the serpin-type inhibitory complex. The relationship between RCL structure and serpin inhibitor function is incompletely understood and has not been subjected to saturation mutagenesis. α_1-PI M358R is a less potent inhibitor of thrombin than AT or HCII maximally activated by glycosaminoglycans, suggesting that it could be further engineered into a more potent thrombin-inhibitory serpin.
Aims: To apply phage display as a tool for the directed evolution of more active and/or more specific thrombin inhibitors based on the α_1-PI scaffold.
Methods: The T7Select 10-3b (Novagen) phage display system was used to express α_1-PI M358R on the bacteriophage surface, fused C-terminally to the first 348 residues of the T7 10B capsid protein. The T7Select 10-3b system was then used to express a library of α_1-PI mutant proteins with all possible codon combinations at positions P2 (P357) and P1 (M358) (441 possible mutants). The library was biopanned using a biotinylated sheep anti-human thrombin affinity-purified IgG and streptavidin- coated magnetic beads, in order to select the α_1-PI P2P1 mutants capable of forming stable complexes with thrombin. All sequences retrieved after five rounds of biopanning were inserted into arabinose-inducible *E. coli* expression vectors, and the recombinant α_1-PI P2P1 mutants were expressed in soluble form and screened for thrombin inhibitory activity.
Results: Following incubation of purified phage displaying only α_1-PI M358R with thrombin, denaturation-resistant α_1-PI M358R fusion

protein-thrombin complexes were detected by immunoblotting of solubilized phage, validating the approach. After biopanning of the α_1-PI P2P1 mutant phage library, the α_1-PI P357/M358R sequence appeared with a 50% frequency in randomly selected phage plaques, with the remaining candidates expressing a variety of novel mutant sequences. The α_1-PI P357/M358R sequence did not appear in phage plaques from the sample library mock-biopanned for five rounds without the addition of thrombin. When expressed in *E. coli*, the α_1-PI P357/M358R protein was the only P2P1 mutant capable of forming a stable complex with thrombin.

Summary/Conclusions: α_1-PI serpin maintained its inhibitory function when fused to T7 10B capsid protein on the surface of bacteriophage. Only one other serpin (plasminogen activator inhibitor 1) has been previously expressed in this manner. The number of mutants generated and screened in this study was over five times greater than the total number of α_1-PI mutants previously described in the literature. These findings suggest that the α_1-PI M358R protein has the optimal P2P1 sequence for thrombin inhibition, and constitute proof-of-principle for the application of this system to screen libraries of up to 10 million mutants in order to discover novel thrombin inhibitors of potential therapeutic value.

PB 4.59-2

Factors IXa and XIa promote uncontrolled clot growth under static conditions

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Background: The fibrin clot stabilizes aggregated platelets and contributes to thrombogenesis by recruiting cells and proteins from circulating blood. However, the role of coagulation cascade reactions in the growth of the thrombus edge remains poorly understood.

Aims: In this study we evaluated three pathways of fibrin clot elongation under stagnant plasma conditions: passive diffusion of activated coagulation factors (F) IIa, IXa, Xa and XIa from the clot base to its edge, generation of activated factors inside the clot, and activation of coagulation by pre-activated FIXa and FXIa that may be present in circulation outside the growing clot.

Methods: A thin layer of recalcified human plasma was embedded in a low temperature melting point agarose gel and brought in contact with the immobilized tissue factor (TF) or a chamber with mixtures of activated coagulation factors and TF. Videomicroscopy of clot growth – thrombodynamics – was used to continuously monitor propagation of the clot edge into unclotted plasma. Small molecule coagulation factor inhibitors, inhibitory antibodies and single or double factor deficient plasmas (DP) were used to block individual steps of the coagulation cascade.

Results: In FIX-DP, immobilized TF, liquid TF and diffusion of FXa and FIXa induced dose-dependent but short (2–10 min) initial phases of rapid (up to 100 μm/min) growth rates resulting in small dense clots (0.2–2 mm in length) at the plasma/activator interface. These clots continued to grow further at a slow but almost steady rate (5–20 μm/min) for the duration of experiment (up to 120 min).

Addition of FIX in the absence of FXI(a) produced an intermediate phase of moderate clot growth rates for up to 40 min, at which time the clot growth rate declined steadily. Addition of FXI in the absence of activation by FXIIa reversed the decline, i.e., the rate of clot growth became quasi-steady. The quasi-steady clot growth was independent of FXI activation by FXIa; however, it was regulated by levels of FIX and FXI, and it could be reduced by blocking activation of FXI by thrombin and activation of FIX by FXIa. This quasi-steady clot growth could be induced by contact with TF, beta-thrombin or FXIa. Premixing FIX-DP with picomolar amounts of FIXa or FXI-DP with sub-picomolar FXIa (i.e., at concentrations that do not activate clotting independently of TF) resulted in fast and steady clot growth that was independent of the starting concentration of immobilized TF but proportional to FIXa or FXIa.

Conclusions: Diffusion and activation of coagulation factors inside the clot can support propagation of thrombus edge in the absence of blood flow. The effect is limited by the availability of TF at the site of injury, the distance from the site of TF exposure, and FXI activation inside the clot. Presence of FIXa and FXIa in circulation removes these limitations and supports uncontrolled clot growth. These results suggest that control of FIXa activity and its generation by FXIa could limit growth of intravascular thrombi.

PB 4.59-4

Peritoneal mesothelium expresses functionally active thrombomodulin

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Background: The thrombin-thrombomodulin (TM) complex activates protein C (PC) and procarboxypeptidase B 2 (proCPB2 aka TAFI) that play anti-coagulant and anti-inflammatory roles within the vasculature. Extravascular expression of TM, however, has not been well studied. We hypothesized that TM is expressed on peritoneal mesothelial cells and leukocytes and thus facilitates PC and proCPB2 activation by thrombin within the peritoneal cavity

Methods: Mouse peritoneal macrophages were collected by lavage with PBS. Following PBS lavage, peritoneal mesothelial cells were detached from the peritoneum by intra-peritoneal trypsinization and lavage. Isolated MCs were maintained in culture for up to 4–5 passages. The phenotype of peritoneal macrophages and mesothelial cells was determined by immunofluorescence labeling in single cell suspension and in frozen abdominal tissue sections. PC and proCPB2 activation assays were performed in a 24-well culture plates on top of a single layer of mesothelial cells in culture. Levels of activated PC and CPB2 were measured using chromogenic substrates.

Results: Freshly isolated peritoneal mesothelial cells expressed high levels of TM while residential macrophages express TM weakly on their surface as detected by immunofluorescent staining and flow cytometry. Immunofluorescence studies of frozen sections of mouse abdominal wall, omentum, and intestine showed TM expression on the cells lining the peritoneum. Primary peritoneal mesothelial cells were adherent and displayed a typical polygonal, cobblestone-like morphology in culture. These cells expressed TM and endothelial protein C receptor (EPCR) on their surface, and the epithelial cell markers, calretinin, and cytokeratin intracellularly. Unlike endothelial cells, mesothelial cells were negative for the endothelial markers P-selectin (CD62p) and PECAM-1 (CD31). Thrombin activates PC and proCPB2 in the presence of cultured primary mouse peritoneal mesothelial cells. This activity is blocked by pre-treatment of mesothelial cells with a neutralizing anti-TM polyclonal antibody showing that thrombin activation of PC and proCPB2 on mesothelial cells is dependent on the presence of TM.

Conclusion: TM is expressed on peritoneal mesothelial cells and is functional, serving as thrombin's cofactor for protein C and proCPB2 activation. In the setting of peritoneal inflammation, thrombin generated locally will bind to mesothelial TM, leading to generation of activated PC and CPB2 and thus is actively involved in regulation of inflammation, fibrin clot formation and fibrinolysis within the peritoneal cavity.

PB 4.59-5

Role of platelets in the regulation of the spatial propagation of fibrin clot

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Background: Activated platelets affect blood coagulation by secreting contents of their granules and exposing phosphatidylserine on the outer leaflet of the cell membrane.
Aims: We investigated the influence of platelets on the process of spatial fibrin clot growth in the reaction-diffusion *in vitro* experimental model (Thrombodynamics assay).
Methods: Spatial clot growth was monitored by light scattering in recalcified platelet-rich plasma (PRP) or in platelet-free plasma (PFP) of healthy subjects following activation with immobilized tissue factor (TF) at either high or low density (80 or 8 pmole/m^2, respectively).
Results: When coagulation was initiated by high TF density, platelets only moderately increased spatial clot growth velocity (V) from 25.7 ± 1.9 to 38.7 ± 8.3 μm/min ($n = 3$, $P = 0.08$); a similar effect was achieved with artificial phosphatidylserine microvesicles (V = 38.1 ± 1.4 μm/min).
On the contrary, in experiments with low TF density activation, platelets greatly increased V (from 6.5 ± 2.3 μm/min in PFP to 42.6 ± 10.6 μm/min in PRP; $n = 4$, $P < 0.05$). In heparin-supplemented plasma (0.09 ME/mL) activated with high TF density V was 12.2 ± 2.3 μm/min in PFP and 27.3 ± 6.2 μm/min in PRP ($n = 9$, $P < 0.05$). Addition of prostaglandin E1 (platelet activation inhibitor) in heparinized PRP decreased V in 1.5 ± 0.4 times ($n = 3$, $P = 0.1$). Apyrase and wortmannin did not show any effect on the spatial clot growth.
Conclusion: Clot growth rate in a reaction-diffusion system is insignificantly affected by platelets in normal conditions (physiological TF density and inhibitors concentration). Nevertheless the physiological concentration of the platelets in the plasma decrease the influence of clotting factors such as TF density decrease or heparin presence making system less sensitive to clotting conditions.

PB 4.59-6

Coleus forskohlii extract attenuates the anti-coagulation activity of warfarin

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Background: *Coleus forskohlii* root extract (CFE) is a popular herbal ingredient in commercial dietary weight-loss supplements. The CFE used for such supplements has been standardized as 10% (w/w) active component forskolin. However, the safety of CFE remains unclear.
Aims: We studied the effects of CFE on cytochrome P450 (CYP) expression in the liver and its interaction with warfarin, which is a widely used anticoagulant.
Methods: ICR mice (male, 5 weeks old) were fed AIN93G-based diets containing various doses of CFE (dried roots of *C. forskohlii* obtained from Bangalore in southern India, extracted and standardized as 10% forskolin) or pure forskolin (Biomol, Plymouth Meeting, PA, USA). Plasma levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) were determined by enzymatic methods. Total cytochrome P450 content in liver microsomes was determined by optical density method, and activities of CYP subtypes were determined by high-performance liquid chromatography. CYP subtypes mRNA and protein levels in the liver were measured by real-time polymer chain reaction and Western blot analysis, respectively. Anti-coagulation activity was determined by prothrombin time, activated partial thromboplastin time, and thrombo test.
Results: When mice were fed various doses (0, 0.005, 0.05, 0.5, and 5%) of CFE for 3 weeks, the higher doses (0.5, 5%) of CFE decreased body weight and increased the relative liver weight, and significantly increased plasma levels of AST, ALT, and ALP. In addition, a high dose (0.5%) CFE significantly prolonged activated partial thromboplastin time. These data suggest that excess intake of CFE could induce hepatotoxicity and may inhibit coagulation. On the other hand, lower doses (0.005, 0.05%), which are comparable to doses taken in dietary weight-loss supplements in humans, did not change body weight, relative liver weight, or plasma levels of AST, ALT, or ALP. These data suggest that low-dose CFE does not induce hepatotoxicity. However, increased total content of hepatic CYP and activities of CYP2B, 2C, and 3A were observed with a lower dose (0.05%). Consistent with these findings, CFE increased CYP2B, 2C, and 3A mRNA expressions in the liver. CFE also increased CYP2C and 3A, but not 2B, proteins in the liver. Unlike the CFE, pure forskolin had little effect on CYP induction, indicating that an unknown factor is involved. When mice fed 0.05% CFE for 1 week were challenged with warfarin by gavage on the last 2 days of the treatment regimen, warfarin-induced anticoagulation was attenuated by CFE in parallel with induction of CYP, especially S-warfarin 7-hydroxylase.
Summary/Conclusions: These results indicate that intake of CFE, but not its active component forskolin, induces hepatic expression of CYPs and might affect efficacy of other drugs, including warfarin. To be safe, patients who take medicines should avoid using CFE, even though its effects might depend on the source of CFE or personal conditions.

PB4.60 – Cancer and Thrombosis – X

PB 4.60-1

Biomarker profiling of bladder cancer patients undergoing radical cystectomy. Relevance of thrombotic and inflammatory processes

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Introduction: Inflammation and hemostatic activation may contribute to postsurgical thrombosis in patients with bladder cancer. This study profiled antiphospholipid antibody (APA), procoagulant microparticle (MP) and antiglycosaminoglycan antibody (AGA) levels and the thrombin generation profile in bladder cancer patients.
Materials and Methods: 134 serum samples collected from patients undergoing radical cystectomy and samples collected from healthy male and female individuals ($n = 50$) were analyzed. APA were measured with by ELISA (American Diagnostica, Stamford CT). MP were measured using a functional procoagulant assay (Hyphen Biomedical, ParisFrance). AGA were measured with a commercially available assay from GTI (Madison, WI). Thrombin generation was measured using a fluormetric method (Technoclone, Vienna, Austria). The proteomic profile was analyzed by SELDI-TOF mass spectrometry.
Results: A higher prevalence of elevated biomarkers was seen in the cancer group compared to normals. The average MP value was 3.1 + 5.8 nM (range 0.1–34.6 nM) with 26 of 134 samples were above the normal values of 2.8 ± 1.1. The average APA value was 6.7 ± 11 (range 4–65 nM) with 32 above normal. The average AGA value was 0.22 ± 0.09 (range .01–.8O) with 10 above normal. SELDI analysis showed unique biomarkers in the area of 11–12 kDa, 15.1–15.4 kDa, and 15.8–16.2 kDa which were absent in the normal group. Higher thrombin generation was observed in the cancer group.
Conclusion: This data suggests that bladder cancer patients have subclinical activation of thrombotic and inflammatory processes. The MP may be generated by cellular damage whereas the APA and AGA suggest vascular endothelial damage and activation. The unique proteo-

mic biomarkers in these patients suggest endogenous protease activation. A clinical correlation in the subset with higher markers may provide insight into the biomarker role in prognosis, risk stratification and treatment of bladder cancer.

PB 4.60-2

Prevalence of unexpected pulmonary embolism at contrast-enhanced CT scan performed for cancer staging in patients with advanced lung cancer

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Background: Patients with advanced lung cancer have been reported to be at high risk for venous thromboembolism (VTE). In patients with cancer, a rate of unexpected pulmonary embolism (UPE) of about 1.5% has been reported.
Aims: The aim of the study was to determine the prevalence of UPE in patients with stage IIIB or IV NSCLC or extensive SCLC who underwent CT scans for cancer staging.
Methods: We reviewed the contrast-enhanced CT scans of the chest performed for routine cancer staging in consecutive patients with advanced lung cancer (stage IIIB or IV NSCLC or extensive SCLC) referred to the Division of Medical Oncology at thePerugia hospital between 2008 and 2012. All CT scans were reviewed by an ad hoc panel composed by three radiologists. PE was defined as unexpected when a filling defect in central, lobar, segmental or sub-segmental pulmonary arteries were observed in absence of clinical suspicion of PE.
Results: Overall, 223 patients were included in the analysis: 180 patients with IV-NSCLC, 24 patients with IIIB-NSCLC, and 19 patients with extensive SCLC. A total of 899 CT scans were reviewed. The prevalence of UPE was 19.7% (44/223): 34 (77.3%) in patients with IV-NSCLC, 7 (15.9%) in patients with IIIB-NSCLC, and 3 (6.8%) in patients with advanced SCLC. Patients with UPE were 26 males and 18 females and had a mean age of 58 years (range 24–78). UPE was monolateral in 30 patients and bilateral in 14 patients. UPE involved central pulmonary arteries in six patients, lobar arteries in 16 patients and segmental arteries in 19 patients. Three patients had an isolated sub-segmental UPE. The mean time between cancer diagnosis and UPE was 11.8 months. 27% of cancer patients with UPE had the positive CT scan at diagnosis and 50% within 3 months. A recurrence of UPE was observed in one patient.
Conclusions: Patients with IIIB or IV NSCLC or extensive SCLC have a high rate of UPE at CT scan performed for cancer staging. UPE was bilateral in about one third of patients. A minority of UPE involved isolated sub-segmental arteries.

PB 4.60-3

Impact of chronic kidney disease and treatment with LMWH on the risk of major bleeding in patients with cancer-associated venous thromboembolism

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Background: Information on the risks of recurrent venous thromboembolic events (VTE) and major bleeding during anti-coagulant treatment in patients with cancer-associated VTE and chronic kidney disease (CKD) is scarce.
Aim: We analyzed these risks in CKD patients with cancer-associated VTE and compared their risk with those of non-CKD patients.
Methods: 1684 patients diagnosed with cancer-associated VTE between 2001 and 2011 were followed for 180 days after VTE diagnosis. Patients were treated mainly with low molecular weight heparin (LMWH) or vitamin-K antagonists. Primary outcomes were recurrent VTE and major bleeding. Secondary outcome was fatal bleeding.
Results: Recurrent VTE occurred in 62/994 (6.2%), 29/548 (5.3%), and 3/142 (2.1%) patients without CKD (eGFR >60 mL/min), with moderate (eGFR 30–60 mL/min), or severe CKD (eGFR <30 mL/min), respectively. Hazard Ratios (HRs) for recurrent VTE in moderate and severe CKD compared with non-CKD patients were 1.1 (95% CI 0.7–1.8) and 0.5 (95% CI 0.2–1.7), respectively. Major bleeding occurred in 45/994 (4.5%), 32/548 (5.8%), and 17/142 (12.0%) patients without, with moderate or severe CKD, respectively. HR for major bleeding in patients with moderate and severe CKD compared with non-CKD patients were 1.6 (95% CI 1.0–2.6) and 3.7 (95% CI 2.0–6.7) and for fatal bleeding 3.6 (95% CI 1.2–11.4) and 12.5 (95% CI 3.7–45.1), respectively. These increased bleeding risks in CKD patients were mainly observed in patients treated with LMWH.
Conclusions: The risks of major and fatal bleeding were increased in CKD patients, with VTE and cancer, which was most prominent in those treated with LMWH. These results highlight that LMWH should be used with caution in this specific population.

PB 4.60-4

Von Willebrand factor, ADAMTS13 levels and prediction of venous thromboembolism in patients with cancer

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Background: Cancer patients are at high risk for venous thromboembolism (VTE). However, thromboprophylaxis in these patients is associated with an increased hemorrhagic risk. The Khorana score is a risk scoring model for prediction of VTE that includes clinical and laboratory parameters. It has been expanded by incorporating two biomarkers, soluble P-selectin (P-sel), and D-Dimer. Other laboratory markers like prothrombin fragment 1 + 2 (F1 + 2) have also been reported to predict VTE in cancer patients. von Willebrand factor (VWF) is a mutimeric protein that promotes platelet adhesion and aggregation in high shear stress conditions; it is also implicated in venous thrombosis. ADAMTS13 (a desintegrin-like and métalloprotéase thrombospondin type 1 motif) specifically cleaves VWF multimers and subsequently regulates its activity; previous studies displayed decreased levels of ADAMTS13 in cancer patients when compared to patients without cancer.
Aims: (i) to search for an association between VWF and ADAMTS13 levels and VTE in cancer patients; (ii) to compare Khorana score and expanded Khorana score before and after addition of VWF and ADAMTS13 levels.
Methods: multicenter case-control study. Subjects were ambulatory patients receiving chemotherapy for stage III or IV cancer, prospectively followed for 6 months for the development of VTE. Each case (patient who developed VTE) was matched with seven controls (patients who did not develop VTE) for age, sex, type of cancer and stage. The Khorana score and the expanded score were calculated. ADAMTS13 activity was measured using a fluorescence resonance energy transfer assay; VWF, ADAMTS13 antigen, P-sel, D-Dimer and F1 + 2 levels were measured using ELISA methods. Univariate

and multivariate analysis were performed to compare the levels between cases and controls. Logistic models were used to test whether VWF and ADAMTS13 levels would add significant prognostic information to Khorana and expanded Khorana score. Results are expressed as odds ratios (OR) and their 95% CIs.

Results: We studied 20 cases and 140 controls. VWF levels were significantly higher in cases when compared to controls (326 ± 185% vs. 242 ± 158%; $P = 0.02$), whereas ADAMTS13 levels were not significantly different (activity 87 ± 18.9% vs. 81.5 ± 18.9; antigen 579 ± 108 ng/mL vs. 564 ± 137 ng/mL). VWF and ADAMTS13 levels were not correlated ($r = -0.06$ Pearson). VWF levels significantly increased with increasing stage of cancer ($P = 0.03$) whereas ADAMTS13 activity levels only tended to decrease ($P = 0.06$). The addition of VWF levels significantly improved the Khorana score (OR = 3.5 [1.2–10.2]). ADAMTS13 activity levels significantly improved the Khorana and the expanded Khorana scores (OR = 5 [1.4–23.5]) and 5.8 [1.4–28.2], respectively).

Summary/Conclusions: Prospective studies are needed to confirm our results and determine if VWF and ADAMTS13 can be used as predictive markers of VTE in stage III/IV cancer patients. Our results also highlight a role of VWF and ADAMTS13 in the pathophysiology of VTE in cancer.

PB 4.60-5

Thrombotic events in children with malignancies in relation to treatment components of chemotherapy

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Background: Thrombotic events are an important factor for morbidity and mortality in pediatric patients with hematological malignancies and solid tumors. The pathogenesis of these complications is most often multifactorial and either related to the underlying malignancy, the use of central venous catheters (CVC), certain antineoplastic treatment elements and/or genetic risk factors.

Aims: To retrospectively analyze thrombotic events and underlying pathogenetic factors in a single center pediatric cohort treated for malignant disease.

Methods: Overall 498 patients with childhood cancer (age 2–17 year) diagnosed between 2002 and 2012 were studied with regard to occurrence of thrombotic events, their association with antineoplastic therapy and hereditary thrombophilia.

Results: We recorded a total of 30 thrombotic events in 22 children among our patient population comprising 10 deep vein thromboses (DVT), 2 pulmonary embolisms (PE), 3 cerebral sinus venous thromboses (CSVT), 2 cerebral infarction, 3 venoocclusive diseases (VOD) and 10 CVC occlusions. In exception of one patient, thrombotic events occurred closely associated with antineoplastic polychemotherapy regimes including asparaginase, anthracyclines, high dose mtx, actinomycin and glucosteroids. Screening for genetic risk factors was possible in 15/22 children. 7/15 revealed a hereditary procoagulative abnormality.

Conclusion: Thrombotic events can occur in children with various malignancies and chemotherapy regimen. Screening for genetic risk factors as well as careful management and supportive treatment is therefore essential for prevention of thrombosis in these patients supplemented by anticoagulative therapy.

PB 4.60-6

Impact of haemophilia on cancer detection and management: a retrospective study

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Background: Lymphoma or hepatocarcinoma secondary to blood-borne transmitted diseases are well known malignancies in persons with haemophilia (PWH). However with increasing life expectancy more PWH are facing other malignancies such as prostate or colorectal cancers, requiring a close collaboration between oncologist and the haemophilia specialist.

Aims: The aim of present study was to analyze the impact of haemophilia on cancer detection and its management in five European haemophilia treatment centers (Brussels, Geneva, Marseille, Montpellier and Paris-Bicêtre) from three countries.

Methods: We collected retrospectively in the last 10 year-period, clinical data on cases of cancer that occurred in PWH.

Results: A total of 46 malignancies occurred in 1267 PWH during the observation period. Eighteen tumors were fatal (39%). The most common types of malignancy were hepatocellular carcinoma (12/46) and urogenital tract tumors (9/46). A change in bleeding pattern revealed cancer in four patients only. No bleeding was associated with chemotherapy or radiotherapy. Few bleeding complications occurred with surgical procedures because of insufficient haemostatic coverage for some cases and in spite of adequate clotting factor substitution for others. After cancer diagnosis, five patients had to be switched from on demand to prophylaxis substitution. In the majority of cases the treatment protocol of cancer was not modified because of hemophilia.

Conclusion: Our study emphasizes that in adults PWH a change in bleeding pattern should raise the suspicion of malignancy and also that a PWH should benefit from the same oncologic treatment as other persons with cancer but no hemophilia.

PB4.61 – Cancer and Thrombosis – XI

PB 4.61-1

Recombinant thrombomodulin reduces the elevation of some biomarkers after allogeneic hematopoietic stem cell transplantation

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Background: Levels of cytokines, chemokines and soluble molecules fluctuate after allogeneic hematopoietic stem cell transplantation (HSCT). They can be influenced by various post-transplant complications, as well as by existing basal diseases, types of transplantation, and the conditioning regimen used.

Aims: In the present study, we investigated the effects of recombinant thrombomodulin (rTM) on levels of the cytokines, chemokines, and soluble factors registered in the 'SIGHT' research.

Methods: The subjects were 116 patients who underwent allogeneic HSCT (bone marrow = 69, peripheral blood stem cells = 20, cord blood = 37). Blood samples were collected before and after transplantation. Levels of cytokines (interleukin-6, tumor necrosis factor-α, high-mobility group box (HMGB) 1), chemokines (monocyte chemotactic protein (MCP)-1, RANTES), and soluble molecules (soluble vascular cell adhesion molecule (VCAM)-1, soluble E-selectin, plas-

minogen activator inhibitor-1, platelet-derived microparticles [PDMP]) were measured by enzyme-linked immunosorbent assay. The rTM was administered as a therapy for transplantation-associated coagulopathy (TAC). This protocol was completed in day 4–14 after HSCT and consisted of day doses of 380 unit/kg with every days. Control group was also used heparin or no anti-coagulation therapy.

Results : MCP-1, IL-6, and TNF-a exhibited more significant elevations on days 7–14 after HSCT. In contrast, the levels of HMGB1, sE-selectin, sVCAM-1, PAI-1 and PDMP exhibited significant changes on days 14-28. There were significant improvements in TNF-a, sE-selectin, sVCAM-1, HMGB1, PAI-1 and PDMP after rTM treatment, but not after heparin treatment.

Summary/Conclusion: We believe one of causes for TAC is pro-inflammatory cytokine including HMGB1. For this reason, it is thought that the direct anti-inflammatory effect of rTM's lectin domein plays an important role in therapeutic mechanism for TAC. The present findings suggest the possibility that rTM can play a therapeutic role for TAC after allogeneic HSCT.

PB 4.61-2

Peak factor Xa generation as a candidate biomarker for bevacizumab-induced thrombotic and bleeding events

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Background: Bevacizumab, an anti- vascular endothelial growth factor monoclonal antibody, is an important component of regimens against colorectal and other cancers but is associated with a high risk of both thrombotic and bleeding events. The mechanisms underlying this are incompletely understood.

Aims: We evaluated the impact of bevacizumab therapy on Factor (F) Xa, a critical enzyme in blood coagulation, in a prospective pilot cohort study.

Methods: We prospectively enrolled patients initiating systemic treatment with a bevacizumab-containing regimen for metastatic colorectal, renal, breast, or lung cancer or lymphoma. At baseline and after 1 month of therapy we measured individual concentrations of plasma factors II, V, VII, VIII, IX, X, antithrombin and tissue factor pathway inhibitor and estimated tissue factor-initiated peak FXa generation using a mathematical model. Simple *t*-tests were used to assess statistical differences between groups.

Results: The cohort comprised 40 patients including 45% women, with a mean age of 59 (12) years. We ascertained six venous thromboembolism (VTE) events (15%) and seven bleeding events (17%). Peak FXa generation at baseline was 2.0 nM ± 0.16 (SE). Over a 1 month period on bevacizumab, there was a −3% ±7 change in FXa generation, −6% ±8 for patients who did not develop VTE and 16% ± 18 among those with a VTE ($P = 0.29$). D-dimer also changed during therapy among those developing VTE (5% ± 8) compared to those without a VTE (−3% ± 3 respectively, $P > 0.05$). Among those with hemorrhage, there was a −22% ± 12 change in FXa compared to −2% ± 9 among those without hemorrhage ($P = 0.30$), with similar trends for D-dimer (−7% ± 8 vs. −2% ± 4, $P > 0.05$, respectively).

Conclusion: In this pilot study, total active FXa generation increased (albeit not statistically significantly) after 1 month of treatment with bevacizumab among those who developed a VTE; conversely, FXa generation decreased among those who developed minor or major bleeding. We propose that FXa generation is a candidate biomarker of bleeding and thrombosis risk during bevacizumab therapy and further studies to investigate this are warranted.

PB 4.61-3

Anticoagulant treatment of cancer patients with pulmonary embolism in the real world

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Background: Since 2004, international guidelines provide specific recommendations for patients with cancer and pulmonary embolism (PE), namely long-term treatment with low molecular weight heparin (LMWH). However, recent studies indicate that the use of LMWH mono-therapy might be far from optimal. Another important area of uncertainty is the duration of the anticoagulant treatment.

Aims: Therefore, we evaluated the real world clinical practice of type and duration of anticoagulant therapy in cancer patients with PE over a period of 10 years.

Methods: This was a retrospective cohort study based on data from the Dutch Pharmo database, which contains hospitalisation and pharmacy data of over 4 million patients. PE patients with or without cancer were identified according to the International Classification of Diseases Ninth Revision (ICD9 CM), based on at least one hospitalization for cancer within 2 years prior to and 1 year after the PE. Thereafter, every subject with PE and cancer was matched to two subjects with PE without cancer, for age, sex and year of diagnosis of PE. The primary objective was to assess the type and duration of outpatient anticoagulant treatment for PE. The secondary objective was to evaluate the incidence of hospitalizations for bleeding during follow-up, which were expressed as incidence rate per 1000 patient years.

Results: Between January 1998 and August 2008, 600 cancer patients with PE and 1200 patients with PE without cancer were identified. Overall, long-term LMWH was prescribed in 82 (13.7%) of the cancer patients and in 8 (0.7%) of the cancer free patients ($P < 0.001$). From 1998 to 2008, there was an increase in the use of long-term LMWH in cancer patients, but not in controls ($P < 0.001$ and $P = 0.76$, respectively). In patients with cancer and PE in 2007/2008, LMWH was prescribed in 42 (32%) cases, compared to 1 (1.7%) of the cancer patients with PE in 1998/1999. Median duration of treatment was 5.8 months (interquartile range (IQR) 3.1–8.8) in cancer patients, compared to 7.0 months (4.9–11) in patients without cancer ($P < 0.001$). Cancer patients on long-term LMWH were treated for a median duration of 5.1 months (IQR 3.4–9.7), compared to 5.9 months (IQR 3.0–8.7; $P = 0.36$) in the cancer patients on VKA. In the group of cancer patients, 22 hospitalizations for bleeding per 1000 patient-years of follow-up were counted, compared to 11 hospitalizations per 1000 patient-years in the non-cancer patients (OR 1.9; 95% CI 1.2–3.1).

Summary/Conclusions: Although the use of LMWH in patients with cancer and PE is increasing, in 2008, patients are still mostly treated with VKA, and not with LMWH as recommended by guidelines. Future studies should focus on reasons for this underuse and on the impact of long-term LMWH use on the quality of life of cancer patients. Additionally, most cancer patients with PE are not treated indefinitely, instead receive anticoagulants for 6 months or shorter.

PB 4.61-4

L-asparaginase induced laboratory and clinical hemostasis impairments and use of replacement therapy in children treated from acute lymphoblastic leukemia – single center experience

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L-asparaginase is an essential component of regimens used to treat patients with acute lymphoblastic leukemia (ALL). Reduced synthesis of proteins such as antithrombin, fibrinogen and other coagulation factors causes both thrombotic and hemorrhagic complications associated with L-asparaginase.

This was retrospective analysis of 42 children with acute lymphoblastic leukemia treated according the protocol ALLIC-BFM 2009 from the 1st of April 2010 up to 31st of December 2012 in our Institute. All 42 patients have received protocol IA, 13 patients have received protocol IB Augmented and 32 patients up to now have finished protocol IIB.

At the diagnosis the median value of prothrombin time was 80%, median value of activated partial thromboplastin time (aPTT) 26.5 s, median fibrinogen level was 3.4 g/L, and the median platelet count was 70×10^9/L. At that time there was no need for transfusions with fresh frozen plasma, cryoprecipitate or antithrombin concentrate. Several children received platelet transfusions, and there were neither thromboembolic nor major hemorrhagic complications.

During the first part of induction treatment (protocol IA), median values of PT and aPTT were almost the same as the values at the diagnosis (88.1% and 25.15 s), median fibrinogen value has dropped up to 1.1 g/L, antithrombin ranged from 23.2 up to 146% (median 66.3%), and D-dimers from 104 up to 8283 ng/mL (median 441 ng/mL). The median platelet count was 132×10^9/L. Fourteen children received different types of replacement therapy once, three, four, five or six times (10 single infusion of antithrombin, 17 single infusion of cryoprecipitate and four simultaneous infusion of antithrombin and cryoprecipitate, 31 infusions in total). One patient has developed partial sagittal sinus venous thrombosis, and sever hypoproteinemia with edema of the lower extremities was noticed in three patients.

During the second part of induction treatment (protocol IB Aug), median PT value was slight diminished (69.5%) and median aPTT was prolonged (32.15 s), median fibrinogen value has gradual increased up to 2 g/L. Antithrombin ranged from 28 up to 141.3% (median 44.3%), but the median value of D-dimers significantly dropped up to 156.5 ng/mL. Median value of platelet count was 98.75×10^9/L. Seven patients have received replacement therapy once, twice or three times (8 single infusion of antithrombin, 1 single infusion of cryoprecipitate and 1 simultaneous infusion of antithrombin and cryoprecipitate, 10 infusions in total).

During the first part of reinduction therapy (protocol IIA), median values of PT (110.2%), aPTT (24.8 s), antithrombin (88.6%), D-dimers (146 ng/mL) and platelet count (276×10^9/L) was in normal ranges. We only registered lowering of fibrinogen levels (median 0.9 g/L). Replacement therapy with cryoprecipitate received six patients during this part of treatment.

During augmentation and reinduction course there were no clinical manifestations of hemostasis impairment.

In conclusion, the most frequent and most sever impairments of hemostasis due to L-asparaginase were noticed during the first part of the induction period, so it is therefore clear that during this part of treatment there were the greatest need for replacement therapy. The rarest disorders of hemostasis (exclusively hypofibrinogenemia) were recorded during the reinduction phase

PB 4.61-5

Thrombotic events in acute promyelocytic leukemia – Single center experience

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Background: Acute promyelocytic leukemia (APL) is a distinct subset of acute myeloid leukemia characterized by severe coagulopathy at presentation. As a part of the clotting activation, thrombosis either at diagnosis or during the treatment is a less recognized and probably underestimated life-threatening manifestation of APL

Aim: To report trombotic events in 60 de novo APL patients and to identify factors predictive for thrombosis development.

Method: We retrospectively analyzed data on ED in 60 newly diagnosed, *t*(15;17)(q22;q12) or PML-RARA positive APL patients (median age 44 years, range 19–78; 31/29 female/male) managed at the Clinic of Hematology from 2004 to 2012 with all-trans retinoic acid combined with anthracyclines. The diagnosis of deep vein thrombosis (DVT) was established by Color Doppler ultrasonography. The diagnosis of cardiac ischemic condition was based on electrocardiographic and cardiac enzymatic modifications. Budd-Chiari syndrome was diagnosed by CT scan and central retinal vein occlusion by ophthalmoscopy and fundus angiography.

Results: Thrombotic events (TE) were registered in 10/60 (16.7%) APL patients: five with DVT, (catheter-related in two patients), two with cerebral infarction and one by one patient with acute myocardial infarction, Budd-Chiari syndrome and central retinal vein occlusion. The timing of TE was as follows: one patient exhibited TE before therapy initiation, seven during induction, one patient during II consolidation and one during maintenance therapy. Only 2/10 (20%) patients displayed coagulation impairment at the time of thrombosis. One patient with TE (cerebral infarction) died during induction.

The main clinical and biological characteristics of 10 APL patients with thrombosis were: median age 47.5 years (range 31–64); 3/9 female/male; median WBC count 5.6×10^9/L (range: 1.1–88); median platelet count 46×10^9/L (range: 18–93); Sanz' s risk stratification: high 3/11 (27.5%), intermediate 3/11 (27.5%), low 5/11 (45%); Disseminated intravascular coagulation (DIC) was confirmed in 8/11 (72.7%) patients, median ISTH DIC score (International Society of Thrombosis and Hemostasis Scoring System for disseminated intravascular coagulation) was 5.4 (range: 2–7); variant form of APL displayed in 1/11 (9.1%) patients; seven patients disclosed aberrant immunophenotypic features: expression of CD2 in five and CD15 in two patients; additional cytogenetic abnormalities 2/10 (20%) patients; FLT3-ITD was documented in 2/10 (20%) cases; differentiation syndrome in 2/10 (20%).

Patients with TE displayed significantly higher median WBC count (5.6×10^9/L vs. 3.6×10^9/L, $P = 0.04$), higer median platelet count (46×10^9/L vs. 29×10^9/L, $P = 0.006$) and more frequent CD2 expression (50% vs. 24%, $P = 0.002$). No correlation was found with age, French-American-British subtype, laboratory coagulation parameters (fibrinogen, prothrombin time, activated prothrombin time, D-dimer), ISTH DIC score, CD15 expression, additional cytogenetic abnormalities, FLT3-ITD and differentiation syndrome.

Conclusion: TE rate of 16.7% in our APL cohort showes that TE rate in APL is frequent, especially during induction treatment. This result emphasizes significance of D-dimmer monitoring after coagulopathy resolution. Besides, our study suggests the possible relationship between TE occurrence on one side and laboratory findings (WBC and platelet count; CD2 expression) on the other.

PB 4.61-6

Thrombotic complications in hematological malignancies

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Background: Patients with haematological malignancies have both a procoagulant state and an increased risk of thrombosis because of therapy. Population based surveys have identified haematological malignancies as a leading cause of thrombosis.

Aim: To evaluate the prevalence and outcome of patients with thrombotic complications in hematological malignancies.

Method: We identified patients with thrombosis from the case records of 636 indoor admissions with haematological malignancies from January 2010 to December 2011. We performed plasma homocysteine levels, factor V leiden, immunological markers including ACLA, ANA, and flow cytometric analysis for CD55 or CD 59 deficiency in all patients who had a thrombus. Proteins C, S and Antithrombin were analysed after patients were asymptomatic and off anticoagulation.

Results: Thirteen patients suffered a thrombosis. There were eight venous, six arterial thrombi including one patient who had both. Six patients had thrombus as presenting feature. Arterial thrombi involved brain in four and lung in two patients. Venous thrombi involved lower limbs in 4, sagittal sinus in two and renal vein in one patient who also had lower limb DVT. One patient each with waldenstrom's macroglobulinemeia (WM) and follicular lymphoma(FL) presented with leg DVT. However the patient with WM also had concurrent renal cell carcinoma and the patient with FL had liver cirrhosis. Among the remaining patients, seven had acute lymphoblastic leukemia(ALL) and four had acute myeloid leukemia(AML). Two patients with ALL had thrombus at presentation-one leg DVT and one anterior cerebral artery thrombus respectively. Remaining five patients with ALL suffered a thrombotic episode after starting chemotherapy. One of these patients had a pulmonary embolus and all the remaining four had intracerebral thrombi. The patient with ALL who had a pulmonary embolus was Philadelphia positive on Imatinib and had not received any L-asparaginase(L-ASP) prior to the thrombotic episode. All other ALL patients had received L-ASP. L-ASP was successfully continued in all patients under cover of heparin or warfarin except for one patient who suffered another episode of intracerebal thrombus despite anticoagulation. Two patients with AML had a thrombus at presentation of whom one presented with a parietal lobe infarct and the other, a young male of 28 years, had prior myocardial infarction and a documented factorV Leiden mutation. Of the other two AML patients who presented after chemotherapy, one patient was in induction phase and the other had started consolidation with high dose cytarabine at the time of thrombotic episode. Both these thrombotic episodes involved the upper limb (1 subclavian and 1 brachial) and were perhaps associated with the PICC line. No patient died because of thrombotic complications. Only one patient with ALL required a discontinuation of L-asparahinase. None of hese patients except for the one AML patient with Factor V Leiden mutation had a prior thrombotic episode.

Conclusions: An overt thrombotic episode was uncommon in our cohort of patients. There were predictive factors in none but one patient. L-ASP was most likely association in patients with ALL. L-ASP can be successfully continued under anticoagulant cover. A thrombotic episode had no bearing on outcome after chemotherapy.

PB4.62 – Antiphospholipid – V

PB 4.62-1

Variability in exposure of epitope G40-R43 of domain I in commercial anti-β2-glycoprotein I IgG ELISAs influences the diagnosis of the antiphospholipid syndrome

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Background: Diagnosis of the antiphospholipid syndrome (APS) depends on the detection of autoantibodies against β_2-glycoprotein I (β_2GPI), the most prominent antigen in APS. A main problem is the high variability between commercially available anti-β_2GPI assays. Anti-β_2GPI antibodies constitute a heterogeneous population, but predominantly antibodies reacting to a cryptic epitope Glycine40-Arginine43 (G40-R43) in domain I of β_2GPI are associated with thrombosis. b_2GPI is present in blood in a native conformation. After interaction with anionic surfaces it opens up, resulting in exposure of G40-R43. Therefore, in diagnostic assays b_2GPI should be presented in the open conformation enabling antibodies to react with G40-R43.

Aims: We hypothesize that the high variability between commercial anti-b_2GPI ELISA assays arises from variation in exposure of epitope G40-R43.

Methods: Two patient-derived monoclonal antibodies P2-6 and P1-117 were tested for their reactivity towards β_2GPI. Using both antibodies, we compared exposure of epitope G40-R43 on β_2GPI in commercial anti-β_2GPI IgG assays (A-E). Ten G40-R43-reactive and 10 negative patient samples were tested in assays A and B. Additionally, samples of 176 SLE patients, possibly suffering from APS, were tested for reactivity towards domain I of b_2GPI and for positivity in assays A and B.

Results: Using neutral vs. anionic ELISA plates, we have shown that antibody P1-117 specifically reacts with epitope G40-R43, exposed only in the open conformation, while antibody P2-6 recognizes β_2GPI irrespective of its conformation. In assay A, both antibodies showed equal reactivity towards β_2GPI, indicating that all the coated β_2GPI exposes epitope G40-R43. In other assays P1-117 displayed lower reactivity towards β_2GPI than P2-6, demonstrating a reduced exposure of G40-R43. To rule out that other assay features account for the observed differences in reactivity, we have re-examined the reactivity of both antibodies on coated plates of assay A and B using the protocol and reagents from each assay. In all combinations, reactivity of both antibodies on a plate was comparable to the results obtained with its own protocol and reagents. This suggests that the coating, rather than any other component of the assays, is responsible for the differences between the assays. Interestingly, all 10 G40-R43-reactive patients tested positive in assay A, vs. only three in assay B. 24 of the 176 SLE patient samples were found positive for reactivity against domain I of b_2GPI by ELISA. Of these 24, 23 tested positive in assay A, vs. only 19 in assay B. Decreasing the cut-off value of assay B did not ameliorate test results, suggesting that the differences in correct patient classification between the assays is not simply the result from different cut-off settings.

Conclusions: Exposure of epitope G40-R43 on β_2GPI is highly variable in commercial anti-β_2GPI assays. By testing patient samples we have indications that patients may be falsely assigned negative in assays characterized by a reduced exposure of G40-R43. Our results have major implications for the diagnosis of APS. Antibodies P2-6 and

P1-117 can serve as controls to ensure sufficient exposure of G40-R43. Alternatively, we encourage the development of alternative assays coating only domain I of β_2GPI.

PB 4.62-2

Elevated levels of endothelial cell microparticles in patients with antiphospholipid antibodies correlate with levels of anti-beta2-glycoprotein I antibodies

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Background: The antiphospholipid syndrome (APS) is characterized by venous or arterial thrombosis and/or recurrent fetal loss in the presence of circulating anti-phospholipid antibodies. It has been shown that these antibodies can cause activation of endothelial cells that leads to shedding of microparticles (MP). These MP have been ascribed procoagulant properties and increased MP levels have been observed in several disease states that share a phenotype of vascular dysfunction, inflammation and thrombosis such as cardiovascular disease, venous thrombosis and cancer.

Aims: The aim of this study was to characterize the levels of endothelial cell, monocyte, and platelet derived as well as tissue factor bearing MP in patients with APS. A secondary aim was to study whether MP levels correlate with titers of anticardiolipin antibodies and anti-β2 glycoprotein I antibodies.

Methods: Citrated whole blood from 47 patients with APS and 144 healthy controls were centrifuged at 1500 ***g*** for 15 min and the supernatant was centrifuged at 13,000 ***g*** for 2 min to obtain platelet free plasma (PFP). To detect MP and their origin, PFP was incubated with the following fluorochrome conjugated monoclonal antibodies and analyzed by flow cytometry (LSRII BD biosciences): Annexin V-APC, CD105-PE and CD144-PE (endothelial cells); CD41-PECy4 (platelets); CD14-PE (monocytes); and anti-Tissue Factor mAb-Alexa Fluor647. MP measurements were left skewed so a log 10 transformation was performed before comparing them with a *t*-test or running correlation analyses. $P < 0.05$ was considered significant for all analyses.

Results: Females comprised 52% of patients and 55% controls. The median age of patients and controls was 43 years and 29 years, respectively; however there was no association between age and MP numbers. Thirteen patients had APS along with SLE, 29 had a history of venous thrombosis, 12 had had strokes, and 5 of 21 female patients had a history of pregnancy loss.

Levels of Annexin-V, CD105 and CD144 (endothelial derived), CD41 (platelet derived) and TF positive MP were higher in patients ($47{,}000 \pm 7900$, $10{,}140 \pm 2164$, $46{,}690 \pm 8783$, $127{,}200 \pm 28{,}050$, and 7270 ± 2810 MP/mL respectively) vs. controls ($17{,}000 \pm 1800$, 3824 ± 374, $17{,}320 \pm 2636$, $54{,}910 \pm 7132$ and 2537 ± 747 MP/mL respectively). These differences were highly statistically significant (all $P < 0.02$). In contrast, there was no difference between CD14 positive MP levels (1035 ± 167 vs. 1409 ± 465, $P = 0.66$).

There was no significant association between age or clinical features (thrombosis, strokes, pregnancy loss) and MP levels. Interestingly, MP positive for Annexin V, CD105 and CD144 (endothelial markers) showed a positive correlation with IgG anti-beta 2- glycoprotein I (anti- β 2GPI) ($R = 0.60$, $P = 0.006$) and IgM anti- β 2GPI ($R = 0.58$, $P = 0.006$). These did not correlate with CD14 or CD41 positive MP levels and there was no association between MP levels and anti-cardiolipin antibodies.

Conclusions: Plasma of APS patients contains higher levels of endothelial cell and platelet derived microparticles and these correlate with anti- β 2GPI titers. This suggests that there is a chronic state of endothelial cell and platelet activation in these patients even in the absence of thrombosis and that anti-phospholipid antibodies likely play an important pathologic role in thrombosis.

PB 4.62-3

False positive results of Lupus Anticoagulant in plasmas of patients receiving LMWH or the new oral anticoagulants

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In the new guidelines for lupus anticoagulant (LA) diagnosis it is established not to study patients receiving unfractioned heparin, dicumarinic oral anticoagulants with INR >3.00 and to discontinue LMWH at least 12 h before searching a LA. In the last 2 years, oral direct thrombin and oral direct Xa inhibitors have been worldwide distributed for prevention and treatment of venous thromboembolism (VTE) and atrial Fibrillation (AF). So, it seems important to recognize the effect of these anticoagulants on results of LA tests. The aim of the present study was to describe the rate of false positive results of screening, mixing and confirmatory tests for LA in plasmas of patients that previously have normal coagulation tests results (LA-) and were receiving LMWH in therapeutic doses, Dabigatran etexilate (DAB) 220 mg/day because of an AF or Rivaroxaban (RIV) 10 mg/day for prophylaxis of VTE in elective hip replacement or 15 mg/day for AF. In patients with LMWH blood was taken at 4 h post dose, and for DAB and RIV between 1.5 and 4 h post dose. Tests evaluated were PT, APTT, ICA for APTT mixing study, DRVVT screen, ICA for DRVVT mixing study, normalized ratio (NR) for DRVVT screen/confirm, and NR for silica clotting time (SCT) screen/confirm. All tests were performed in a photo optical coagulometer (ACL TOP). The cut off points of tests and indexes were calculated according to guidelines as the 99th percentile of 50 plasmas from healthy donors. Plasmas from patients receiving LMWH ($n = 15$, anti Xa activity 0.5–1.6 U) presented: 26.6% PT activity <70%, 53.3% prolonged APTT and 62.5% of them did not correct with normal plasma (NP), 60% prolonged SCT screen, but only 10% positive NR SCT screen/confirm, 66.6% prolonged DRVVT screen, 50% of them without correction with NP, and 70% positive NR for confirmatory. Plasmas from patients taking DAB ($n = 15$) presented: 93.3% PT <70%, 100% prolonged APTT without correction with NP, 100% thrombin time very prolonged, 100% DRVVT very prolonged without correction with NP, and 80% of them with a positive NR DRVVT confirm, 100% of SCT screen prolonged but only 6.7% of positive NR SCT confirm. Among those receiving RIV at 15 mg/day ($n = 4$) all presented PT prolonged, DRVVT screen very prolonged without correction with NP and positive NR confirm, 75% presented slightly prolonged the APTT (<1.5 times), all SCT screen prolonged, but none positive NR for confirmatory test. Finally, plasmas from patients taking RIV 10 mg/day ($n = 20$), 75% presented PT <70%, only 10% prolonged APTT, 75% DRVVT screen prolonged, 80% of them did not correct with NP, and 73.3% with NR confirm positive, 20% of SCT screen prolonged but none of them with NR for SCT confirm positive. In conclusion, LMWH at therapeutic dose, DAB and RIV greatly affect recommended tests for LA testing not only in the screening and mixing studies, but also in confirmatory tests. It is mandatory to clearly access to the list of medications that patients are taken, and discontinue these anticoagulants before searching a LA to avoid false positive results.

PB 4.62-4

Anticoagulation for patients with antiphospholipid antibodies undergoing cardiopulmonary bypass – a novel strategy for optimisation of heparin anticoagulation

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Introduction: Cardiopulmonary Bypass (CPB), an essential component of many cardiothoracic surgical procedures, requires extremely high plasma heparin concentrations to prevent thrombosis within the extra-corporeal circuit, which may be life-threatening or result in significant morbidity. Consequently, heparin concentrations in the range of 3–4 IU/mL are required for CPB, which contrasts with the target of approximately 0.6 IU/mL used in the treatment of venous thrombosis. CPB surgery is also associated with a significant risk of major haemorrhage, particularly if excessive heparin is administered. Therefore, unfractionated heparin (UFH) and protamine doses are carefully monitored and titrated during CPB using near patient Activated Clotting Time (ACT) testing. It is recognised that Lupus Anticoagulants (LA) may influence this test. Thus, heparin monitoring in LA patients undergoing CPB surgery constitutes a common clinical problem.

Aims: No consensus exists on heparin monitoring in LA patients requiring CPB surgery. As LAs are heterogenous, their effect on the ACT is unpredictable. We wished to establish an appropriate ACT range for such patients by adopting an individualised approach and testing the response of the ACT to increasing heparin concentrations in such patients.

Methods: To elucidate the relationship between heparin concentration and ACT, we performed pre-operative ex-vivo spiking studies in seven patients with LA and prolonged APTTs who were planned to undergo CPB. In brief, whole blood was collected and spiked with increasing heparin concentrations (0–7 IU/mL). At each concentration, both ACT and anti-Xa levels were determined. In addition, peri-operative haemostasis was assessed in a prospective manner.

Results: Four patients has baseline prolonged ACTs. One patient demonstrated a non-linear response between increasing heparin concentrations and ACT, precluding this test's use as a means of determining intra-operative heparin concentrations and necessitating the use of anti-Xa monitoring. Four patients has flattening of their ACT curves, such that a standard ACT target of 400–480 s would have resulted in heparin concentrations significantly outside the desired 3–4 IU/mL range.

Conclusion: Cumulatively, these novel data demonstrate definitely that ex-vivo spiking studies are critical in order to optimise heparin dosing for LA patients undergoing CPB surgery. We propose that this methodology should be adopted as gold-standard practice.

PB 4.62-5

Anti-β2 Glycoprotein I autoantibodies and atherosclerosis in patients with ischemic stroke

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Background: β2-Glycoprotein I (β2GPI) is a major antigenic target of antiphospholipid antibodies, with natural anticoagulant properties. IgG and IgM anti β2GPI are biological markers of the antiphospholipid syndrome (APS) and are strongly associated with thrombosis. The possible relationship between anti-β2GPI (aβ2GPI) antibodies and atherosclerosis is interesting because β2GPI has been found in atherosclerotic lesions. Anti-β2GPI could be implicated in the onset and/or progression of vascular parietal lesions and consequently in the pathogenesis of stroke.

Some authors have described an association between IgA aβ2GPI antibodies and diseases related to atherosclerosis but studies are still few. The aim of the present study is to evaluate a possible association between aβ2GPI of IgG, IgM, IgA isotypes and atherosclerosis in a group of patients with ischemic stroke

Patients & Methods: A total of 41 patients (20 male, 21 females) aged 26–84 years (mean 56 years), with ischemic stroke proved by CT-scan were included in our study. All patients were investigated with Doppler carotid to detect significant atherosclerotic changes (vascular stenosis with luminal reduction ≥50%). The control group consisted of 80 healthy subjects. We evaluated aβ2GPI (IgG, IgM and IgA isotypes) in plasmas of patients and normal controls by an enzyme-linked immunosorbent assay (Orgentec).

Results: Positivity for aβ2GPI antibodies of was seen in 18 cases (44%) of patients and none of the plasmas from the healthy control group ($P < 0.001$). The most frequent isotype was IgA ($n = 14$, 34.1%), followed by IgM ($n = 10$, 24.4%) and IgG ($n = 7$, 17%). Among the 18 patients positive for aβ2GPI, 8 (45%) are positive for only one isotype: IgA in four cases (22%), IgG in three cases (17%), and IgM in one case (6%). All patients with IgM aβ2GPI were negative for rheumatoid factor. The combination of three isotypes was seen in three cases (17%) and two isotypes in seven cases (39%). There was a correlation between IgM and IgA aβ2GPI ($P < 0.001$) but not between IgG and IgA aβ2GPI or IgG and IgM aβ2GPI.

Atherosclerotic plaque was detected in carotid Doppler in 31 patients (75%). Positivity of IgA or IgM aβ2GPI are significantly associated with atherosclerosis ($P < 0.01$ and $P < 0.05$ respectively).

All patients without atherosclerosis are negative for IgA and/or IgM aβ2GPI. Positivity of IgG aβ2GPI is not associated with atherosclerosis.

Discussion: Our results corroborate those of other studies which demonstrate a significant prevalence of aβ2GPI in unselected patients with stroke. Our data showed a strong correlation between IgA and IgM aβ2GPI in this disease. This study suggests that elevated IgM and/or IgA aβ2GPI could be risk factors for atherosclerosis and might contribute to the occurrence of ischemic stroke. IgG aβ2GPI directed against domain I are implicated in the occurrence of thrombosis but not in atherosclerosis. Does IgA and IgM aβ2GPI recognize domain I or others domains of the molecule involved in the protective role against atherosclerosis ?

PB 4.62-6

Antiphospholipid and antinuclear antibodies in schizophrenic patients during antipsychotic treatment: findings from the ANTRE study

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Background: Psychiatric disorders themselves and treatment with first or second generation of antipsychotic medications have been associated with increased risk of venous thromboembolism (VTE). Significantly higher levels of antiphospholipid (APA) and antinuclear antibodies (ANA) were repeatedly found in schizophrenic patients. Antiphospholipid antibodies (anticardiolipin antibodies – ACLA, antibody to beta2-glycoprotein I – beta2 GPI and lupus anticoagulat – LA) are among the most important risk factors of venous and arterial thrombosis.

Methods: This case-control prospective ANTRE (ANtipsychotics ThRombosis Embolism) study evaluates a possible association between APA (ACLA, beta2 GPI, LA) and antinuclear antibodies (ANA) and increased risk of thrombosis in patients with psychosis. IgG, IgM beta2 GPI, ACLA antibodies, LA and total ANA were assessed in 46 antipsychotic-naive patient with acute psychosis

(27.7 ± 8.0 years, range 18–52) and compared to 46 healthy controls matched for age and gender. In the patient group, we measured all the above parameters again after 3 months, one and 2 years of antipsychotic treatment.

Results: Prior to the initiation of antipsychotic treatment, there was significant difference in the plasma levels of ANA, ACLA and beta2 GPI IgG between patients and healthy controls. Elevated ANA was significantly higher ($P < 0.05$) than in the controls at the baseline as well during the 2 years of follow up. The plasma levels of beta2 GPI (IgG and IgM) and ACLA IgG increased significantly ($P = 0.047$; $P = 0.013$ resp. $P = 0.007$) in the course of 2-years antipsychotic treatment. Values of ANA did not change during follow- up.

Conclusions: These results point towards the existence of an autoimmune mechanisms in the pathophysiology of schizophrenia. On the other hand the pathological mechanisms of VTE in schizophrenia patients could be possibly explained by the increased titres of APA especially IgG isotype. We suppose an influence of the antipsychotic treatment on the rise in the levels of the APA.

PB4.63 – Antiphospholipd – VI

PB 4.63-1

High specificity of Silica clotting time confirm/screen normalized ratio for LA diagnosis

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The new guidelines for the diagnosis of lupus anticoagulant (LA) stressed the importance of avoid false positive results for LA, trying to improve specificity. Reagents for silica clotting time (SCT) has been introduced in our country last year, a test describe as a combined Screen/confirm duet, Silica based, as was recommended to reach the diagnosis of LA. The aim of the present study was to evaluate the specificity of this test for the presence of LA. We included samples from patients referred to our laboratory for LA searching: 250 which tested positive for LA with our standard tests (DRVVT and APTT) and 84 samples which tested LA negative. As control groups we also tested: 25 samples from patients previously LA negative taken coumarin anticoagulants (ACO), 18 samples from cirrhotic patients, six from patients with DIC, 28 from patients with congenital deficiencies (2 Factor V, 2 Factor X, 7 haemophilia A, 5 haemophilia B, 5 Factor XI, 4 Factor XII and 3 patients with vWD), 10 samples from patients receiving LMWH at therapeutic dose, 6 samples from those receiving UFH, 15 Dabigatran etexilate 220 mg/day, and 7 Rivaroxaban 15 mg/day which presented SCT screen prolonged. SCT screen/confirm normalized ratio (NR) was calculated for each sample and results were evaluated using the cut off point (1.19) obtained according the guidelines as the 99th percentile of 60 plasmas from healthy donors. From the 250 LA positive samples SCT was positive in 72% of LA clearly positive ($n = 204$) and 22% of weak LA ($n = 46$). Among samples which tested LA negative ($n = 84$). 7 (8.3%) tested positive for SCT (five of them with NR close to the cut off point). From these six patients presented APTT and/or DRVVT slightly prolonged but not fulfilling mixing or confirmatory results to be considered as positive LA. Considering all control samples ($n = 115$) we observed only three positive SCT NR: one sample from a patient receiving LMWH, one receiving dabigatran and one with ACO. The specificity of the test was really high 97.4%, particularly taking into account we evaluated a wide spectre of samples which presented acquired or congenital abnormalities of blood coagulation.

Conclusion: We showed that SCT expressed as SCT screen/confirm NR with a cut off properly calculated is a very useful tool to improve specificity of LA testing, because it helps to differentiate the p

PB 4.63-2

Erythrocyte and platelet microparticles are associated with thrombotic complications of antiphospholipid syndrome

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Background: Microparticles (MP) derived from endothelial cells, leucocytes and platelets are increased in patients with antiphospholipid antibodies. It is currently unknown whether erythrocyte MP are increased in patients with antiphospholipid antibodies.

Aims: Thus the aim of our study was to quantitate circulating microparticules (MP) from endothelial cells, leucocytes, platelets and erythrocytes to establish their clinical correlation in patients with antiphospholipid antibodies with or without thombosis.

Methods: The patient population consisted of 33 patients with antiphospholipid antibodies, 23 of which had thrombotic antiphospholipid syndrome. Controls were 25 normal healthy subjects. All gave informed consent. In this study, we evaluated the number of MPs present in plasma from endothelial (EMP), platelet (PMP), leucocytes (LMP), and erythrocyte (ErMP) origin in healthy subjects in comparaison with two antiphospholipid patient group, thrombotic (APS patients) and non thrombotic (aPL patients). Data were acquired and analysed using a FACSCalibur flow cytometer with appropriate software (BD Biosciences, CA, USA).

Results: We compared the number EMP, PMP, LMP, and ErMP in two antiphospholipid patient groups, thrombotic (23 APS patients) and non thrombotic (10 aPL patients) both of which were all positive for aPL in comparaison with 25 healthy subjects. Levels of MP of various cellular origins in patients were compared with controls. EMP (pannel A) and LMP (pannel B) were increased in all patients with aPL (APS patients and patients without vascular thrombosis), $P = 0.04$ and $P = 0.0063$ respectively. On the contrary PMP (pannel C) and ErMP (pannel D) were similar to controls in thrombosis-free aPL patients ($P = 0.31$) while they were significantly increased in patients with APS and thrombosis, $P = 0.0063$ and $P = 0.042$ respectively.

Summary/Conclusion: Altogether our data show that circulating LMP and EMP levels are increased in patients with aPL without thrombosis but not PMP and ErMP. Circulating PMP and ErMP are only increased in APS patients. These results suggest that PMP and ErMP contribute to thrombosis in patients with antiphospholipid antibodies.

PB 4.63-3

Antibody titers and clinical outcomes in patients with single-double or triple positivity antiphospholipid antibodies

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Background: Antiphospholipid Syndrome (APS) is a clinico-pathologic diagnosis characterized by the presence of thrombosis; pregnancy complications; and persistent (>12 weeks apart) presence of antiphospholipid antibodies (aPLs): anticardiolipin antibodies (aCL), anti-beta 2 GP1 antibodies (anti-b2GPI), and a clot-based dilute Russell viper venom test (DRVVT). Prior studies suggest that having three positive tests (triple positivity) in previously asymptomatic patients leads to higher risk of clinical manifestations. The role of the DRVVT ratio in these groups is uncertain.

Aims: To compare the incidence of clinical manifestations and DRVVT ratios among patients with one or two positive tests and those with triple positivity.

Methods: Retrospective chart review from January 1, 2002 to December 31, 2012 of patients ages 18–95 who were tested for aPLs at New York

Presbyterian Hospital. Demographics, presence of autoimmune diseases and cardiovascular risk factors including coronary artery disease, hypertension, hyperlipidemia, smoking status, and diabetes mellitus was collected. We reviewed the clinical manifestations defined as thrombosis: myocardial infarction (MI), cerebrovascular accident (CVA), pulmonary embolism (PE), deep vein thrombosis (DVT) and pregnancy complications. Titers of aCL and anti-b2GPI IgG and IgM isotypes were considered to be positive if >20 GPL or MPL and >99th percentile respectively. We calculated average antibody titers for aCL and anti-b2GPI in the two groups. We also calculated DRVVT ratios for both groups, as (clot time without phospholipid excess)/(clot time with phospholipid excess). A ratio of 1.2 or greater was considered positive.

Results: We had 55 patients with single or double-positive aPLs, and 23 patients with triple positive aPLs. The two groups were similar in gender (65% vs. 61%), history of autoimmune diseases (16% vs. 13%) and cardiovascular risk factors. There was no statistically significant difference in the incidence of CVAs (14.5% vs. 17.4%), MIs (3.6% vs. 13%), PEs (12.7% vs. 21.7%), DVTs (31% vs. 39%), or other VTE events (3.6% vs. 13.04%). Similarly, we did not identify a difference between pregnancy losses in the first trimester (13.8% vs. 28.6%), second trimester (13.9% vs. 14.3%), or third trimester (0% in both groups). All aPL antibody titer levels were significantly elevated in the triple positive group compared to the single/double positive group. The titers were aCL IgG = 17.6 vs. 95.9, aCL IgM = 20.8 vs. 47.5, anti-b2GPI IgG = 10.7 vs. 73.5, anti-b2GPI IgM = 28.5 vs. 56.7, $P \leq 0.001$ for all four aPLs). The DRVVT ratios were higher in the triple positive group compared to the single and double positive group (1.654 vs. 1.383; $P = 0.01$).

Conclusions: While not statistically significant, there was a trend toward more MI, PE and first trimester pregnancy losses in the triple positivity group compared to the single and double positive aPL. All aPL titers were significantly higher in patients with triple positivity aPL including DRVVT ratios than in patients with single-double positivity. Larger prospective multicenter studies are needed to determine the set of antibodies and the role of DRVVT ratio in predicting clinical outcomes in patients with aPLs.

PB 4.63-4

Circulating endothelial cells are increased in patients with antiphospholipid syndrome

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Background: Circulating endothelial cells (CECs) are markers of endothelium damage and have been reported in antiphospholipid syndrome (APS). However it is unknown whether CECs are associated with thrombotic complications *in vivo*.

Aims: To determine whether CECs are increased in patients with antiphospholipid antibodies (aPL) in particular according to their thromboembolic history and underlying disease.

Methods: Clinical and laboratory variables were recorded from a prospective cohort. CECs were assessed by CellSearch®, Veridex; CECs phenotype: CD146+, CD105+, DAPI+, CD45- in patients with aPL and/or SLE. We determined clinical and laboratory variables significantly associated with a high rate of CECs (>20/mL). Informed consent was obtained from each patient.

Results: Forty-six patients were included (mean age 44 ± 15 years, 37 women). Twenty patients had SLE and aPL and 26 only aPL. Forty-two patients had a history of one or several thrombotic/obstetrical manifestations. The presence of a elevated CECs was associated with history of myocardial infarction (29.7 ± 28.1 vs. 10.6 ± 18; OR = 8.75[CI 95%; 1.2–63.9], $P = 0.04$), pulmonary embolism (21.3 ± 22.3 vs. 10.3 ± 18.7; OR = 8[CI 95%; 1.6–40.3], $P = 0.01$).

Summary/Conclusions: In patients with aPL, history of pulmonary embolism and myocardial infarction are significantly associated with endothelial perturbation evaluated by the number of CECs.

PB 4.63-5

Prevalence of antiphospholipid antibodies in psychiatric patients users and non-users of antipsychotics

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Background: Past reports have suggested that antiphospholipid (aPL) antibodies may emerge as a response to antipsychotics treatment, since a high prevalence of aPL antibodies in antipsychotics users has been observed. However, no control group of non-medicated psychiatric patients was included in these reports.

Aims: We aimed to determine the prevalence of aPL antibodies in psychiatric inpatients users and non-users of antipsychotics in order to assess a potential association between aPL antibodies and antipsychotics

Methods: In a cross sectional study we determined the prevalence of aPL antibodies in 333 psychiatric inpatients. We compared the proportions of positive aPL antibodies tests between users and non-users of antipsychotics with adjustments for potential confounders (age and psychiatric diagnosis). To detect the presence of lupus anticoagulant (LA) in the plasma, two coagulation tests were performed: dilute Russell viper venom time (STA-Staclot dRVV Screen/Confirm, Diagnostica Stago), and LA sensitive partial thromboplastin time (PTT-LA Diagnostica Stago). IgG and IgM antiβ2GP-1 were detected by ELISA with Asserachrom anti β2GP1, and anticardiolipin antibodies IgG and IgM were detected by ELISA with Asserachrom APA (all from Diagnostica Stago)

The protocol was approved by our hospital's Ethics Committee. After complete description of the study to the subjects, written informed consent was obtained from all patients, their relatives, or their guardian before inclusion.

Results: Patients' mean age was 57.3 ± 18.1 years (range 19–93 years), 153 (45.9%) were male. Of these 333 patients, 59 (17.7%) were diagnosed as having organic mental disorder (ICD-10 codes F0), 86 patients (25.8%) had schizophrenia (ICD-10 codes F2 and F8), 110 (33.0%) had mood disorder (ICD-10 codes F3), and 78 patients (23.4%) had another psychiatric diagnosis (ICD-10 codes F1 + F4 + F5 + F6 + F7). At admission, 185 patients (55.6%) were receiving at least one antipsychotic medication. The proportion of antipsychotics users carrying at least one aPL antibody was 27.0% (50/185) compared with 27.2% (40/148) in non-users ($P = 0.24$). The prevalence of each subgroup of aPL antibodies (LA, IgM and IgG anti-β2GP-I antibodies, IgM and IgG anti-cardiolipin antibodies) was not different between users and non users of antipsychotics (22.2% vs. 23.6%, $P = 0.62$, 3.8% vs. 2.0%, $P = 0.75$, 0.0% vs. 0.0%, P = NA, 1.1 vs. 1.4%, $P = 0.91$, 2.7% vs. 3.4%, $P = 0.71$ respectively with adjustments for age and psychiatric diagnosis). In each subgroup of psychiatric disorders, the proportion of patients with aPL antibodies was not different between users and non-users of antipsychotics except in patients with mood affective disorder. In this category, 14.9% of the 47 antipsychotics users and 33.3% of the 63 non-users had a positive test for at least one aPL antibody ($P = 0.04$). This difference was due to the presence of LA, which was detected in 10.6% of antipsychotics users and 30.2% of non-users ($P = 0.03$).

Conclusions: aPL antibodies were frequently found in patients with psychiatric diseases and no significant increase in the prevalence of aPL antibodies was observed in antipsychotics users. Long term prospective epidemiologic cohort studies are needed to establish the possible pathogenic implications of persistent aPL antibodies in psychiatric patients.

PB 4.63-6

Hypoprothrombinaemic Lupus Anticoagulant Syndrome masquerading as Acquired Haemophilia in a 5-year-old child

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Background: Hypoprothrombinaemic lupus anticoagulant syndrome (HLAS) is a rare phenomenon caused by acquired hypoprothrombinaemia in the presence of lupus antibodies (LA). They pose both bleeding and thrombotic risk. It can be associated with systemic lupus erythematosus or rarely in children due to infections, medications or other causes producing antibodies

Aim: We describe a previously healthy child who presented with bleeding symptoms, later diagnosed with HLAS and responded to appropriate therapy

Methods: We describe a previously healthy 5-year-old child who presented to her local hospital with the new onset of bruising and ecchymoses, 2 days after a viral illness. Initial investigations revealed a prolonged prothrombin time (PT) (INR 2.3) and activated partial thromboplastin time (APTT) (APTTr 3.0). Factor assays using a reagent with intermediate lupus sensitivity showed a Factor VIIIC of 6%, Factor IX of <1% and Factor XI of 4% with otherwise normal levels. LA screen was positive. She was given a tentative diagnosis of Acquired Haemophilia and transferred to our hospital for further investigation and management.

Results: Initial investigations showed normal full blood count including platelet count (280 × 10*9/L) but prolonged PT (17.3 s) and APTT (61 s). A 50/50 mix study gave an APTT of 37.2 s and PT 12.8 s. Fibrinogen and D-dimer were normal. LA screen was positive, however autoimmune screen was negative. Factor analysis revealed Factor II 8.3%; Factor V 140%; Factor VII 79%; Factor X 84%. APTT dependent factor levels using an agent with low lupus sensitivity, Actin FS, were normal or near-normal but with markedly non-parallel curves.

She was diagnosed with post-viral Hypoprothrombinaemic Lupus Anticoagulant Syndrome. Short-course corticosteroid therapy was initiated; there was no recurrence of the bleeding and she showed consistent improvement in Factor II -levels 1 week and 6 weeks post therapy: 38.8% and 91% respectively. LA screen remained positive.

Summary: Hypoprothrombinaemic Lupus Anticoagulant Syndrome is a rare but previously reported condition in childhood. In this case, the presentation was with large bruises and ecchymoses and apparently low FVIIIC and FIX levels giving the apparent clinical syndrome of acquired haemophilia. It was only with repeat testing using reagents less sensitive to lupus anticoagulants that the diagnosis became clear and even then assays were difficult to interpret due to marked inhibition.

PB4.64 – Arterial Vascular Disorders – V

PB 4.64-1

Increased fibrinogen rises thrombin generation and fibrin clot formation in obese Zucker rats

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Background: The metabolic syndrome (MetS), a cluster of risk factor including abdominal fat, hypertension, dyslipidemia and disturbed glucose and insulin metabolism is associated with modifications in the arterial wall such as endothelial dysfunction and atherothrombosis. Enhanced platelet reactivity and coagulation and decreased fibrinolysis have been proposed as potential mechanisms but the determinants linking MetS and hemostasis remains unknown in particular in age-dependent arterial wall stiffening.

Aims: The main objective was to determine the triggering mechanisms and the substratum of the prothrombotic state in MetS.

Methods: We have characterized the hemostasis phenotype of lean and obese Zucker rats, aged 25 (adult) and 80 weeks (very old). Platelet reactivity was assessed by aggregation studies with platelet-rich plasma (PRP) and washed platelets and by using ADP and collagen as agonists. Thrombin generation was monitored in PRP and platelet-poor plasma (PPP) using calibrated automated thrombography (CAT). The *in vitro* reactivity of the coagulation system was estimated by the endogenous thrombin potential (ETP). Fibrinolysis was measured by a classical clot lysis assay in which thrombomodulin was added.

Results: Prothrombin fragment F1 + 2 and thrombin/antithrombin (TAT) complexes were increased in obese rats at 25 weeks of age. Platelet count was increased in obese rats at both ages (789 ± 34 vs. 574 ± 37 10^9/L at 25 week-old and 834 ± 63 vs. 633 ± 29 10^9/L at 80 week-old rats) without change in platelet response to ADP or collagen. ETP in PPP was higher in 25 week-old obese rats than in lean rats of the same age (428 ± 29 vs. 328 ± 27 nM/min) and still higher at 80 weeks compared with age-matched group (422 ± 30 vs. 306 ± 11 nM/min). Similar differences were observed wit PRP. Plasma prothrombin and tissue factor levels were increased in obese rats whatever the age whereas antithrombin was unchanged. The most striking result in obese rats was the increase in ETP without increase in thrombin peak but with a delay in thrombin inhibition. Fibrinogen was increased in obese rats (4.0 ± 0.2 vs. 2.8 ± 0.1 g/L at 25 week-old and 4.9 ± 0.2 vs. 3.1 ± 0.1 g/L at 80 week-old rats). Addition of purified fibrinogen in lean rats resulted in an increase in ETP and delayed thrombin inhibition. Half-lysis time was prolonged in obese rats at 25 weeks (46.5 ± 1.2 vs. 41.5 ± 0.7 min) and increased with age (54.1 ± 1.1 vs. 49.3 ± 1.8 min in 80 week-old obese and lean rats respectively). A cytokine array showed an increase in pro-inflammatory cytokines in obsess rats whatever the age.

Summary/Conclusion: To conclude, we have shown that thrombin generation increased in obese adult and very old Zucker rats without change in platelet activation. Increased inflammation and related fibrinogen level and subsequent decreased fibrinolysis may account for this hypercoagulable state in obese rats.

PB 4.64-2

Impact of venous thromboembolism on future risk of atrial fibrillation – the Tromsø study

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Background: It has been widely accepted that pulmonary embolism may precipitate cardiac dysrhythmias although the degree of evidence is low. Pulmonary embolism may increase the right atrium pressure with subsequent atrial dilatation, eliciting stretch injuries that can in turn trigger atrial fibrillation (AF). Venous thromboembolism (VTE) is a common term for deep vein thrombosis and pulmonary embolism, since clinically silent pulmonary embolism often occur in the presence of a deep vein thrombosis. To our knowledge, the association between venous thromboembolism, including pulmonary embolism and deep vein thrombosis, and subsequent atrial fibrillation has not yet been explored in a prospective cohort study.

Aims: We wanted to investigate the impact of incident venous thromboembolism on future risk of atrial fibrillation in a prospective cohort study recruited from the general population.

Methods: The study included 29 774 subjects who attended at least one survey of the Tromsø study (1994–95, 2001–02, 2007–08). Baseline

information was obtained by questionnaires, blood samples and a physical examination. Incident events of VTE and AF were registered and validated from date of study inclusion to the end of follow-up, December 31, 2010. Cox proportional hazard regression using age as time-scale and VTE as a time-dependent variable was performed to estimate crude and multivariable hazard ratios (HR) with 95% confidence intervals (CI). Sex, body mass index, smoking, physical activity, total cholesterol, self-reported cardiovascular disease and diabetes were potential confounders included in the multivariable analyses. The study was approved by the regional committee for research ethics, and all participants gave their informed written consent to participate.

Results: Of the study participants, 540 (1.8%) had an incident VTE event, of which 50 (9.3%, age-adjusted incidence rate (IR) of atrial fibrillation per 1000 person-years: 17.76, 95% CI: 13.44–23.47) suffered from subsequent atrial fibrillation during a median follow-up of 15.6 years. There were 1619 subjects that had an atrial fibrillation without a prior VTE event during follow-up (5.8%, age-adjusted IR: 12.65, 95% CI: 12.05–13.29). Subjects with VTE had 64% higher risk of AF compared to subjects without VTE (multivariable adjusted HR: 1.64, 95% CI: 1.23–2.19). The risk estimates for atrial fibrillation were higher after pulmonary embolism (HR: 1.84, 95% CI: 1.17–2.90) than after deep vein thrombosis (HR: 1.50, 95% CI: 1.04–2.17). The incidence of atrial fibrillation was highest during the first 6 months after the VTE event (IR: 24.38, 95% CI: 12.31–48.26).

Conclusion: We found that incident venous thromboembolism was associated with future risk of atrial fibrillation. Our findings support the concept that overt and silent pulmonary embolism may lead to cardiac dysfunctions that, in turn, can trigger atrial fibrillation.

PB 4.64-3

Different role of hypercoagulability in myocardial infarction and ischemic stroke: a systematic review

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Background: Hypercoagulability increases the risk of arterial thrombosis. However, there is emerging evidence that this effect may differ between various manifestations of arterial disease.

Aims: To compare the effect of coagulation factors as measures of hypercoagulability on the risk of ischemic stroke (IS) and myocardial infarction (MI).

Methods: We performed a systematic review of the literature searching for papers reporting on prothrombotic measures as risk factors for IS and MI. The effect of a risk factor on IS (relative risk for IS, RR_{IS}) was compared with the effect on MI (relative risk MI, RR_{MI}) by calculating their ratio ($RRR = RR_{IS}/RR_{MI}$) with corresponding 95% confidence interval (CI). To increase the validity of our analysis, we only included studies that reported risks for both MI and IS. When a risk factor has a similar effect (either increasing, decreasing or no effect) on MI and IS, the RRR equals 1, whereas a RRR >1 indicates a greater effect on IS risk than on MI, and vice versa. Prespecified subgroups were based on age, sex, stroke aetiology, study type and population baseline risk.

Results: We selected 70 papers, describing results from 32 study cohorts, accounting for 351 markers of hypercoagulability. 205 of these markers involved pro-coagulant factors, 46 anti-coagulant factors, 63 markers of fibrinolysis and 37 markers of platelet function and other pathways. 135 out of 351 markers (38%) had an RRR between 0.9 and 1.1, thus likely affecting the risk of IS and MI equally. In contrast, 136 out of 351 (39%) had an RRR >1.1, indicating a larger effect on IS risk than MI risk. Of these, 45/136 (33%) had an RRR >1.5 and 20/136 (15%) >2. The RRR of 80/351 (23%) markers of hypercoagulability was <0.9. Pro-coagulant factors contributed for the greatest part of the difference between RRRs (RRR >1.1, RRR <0.9: pro-coagulant 84/205 [41%], 44/205 [21%]; anti-coagulant 11/46 [24%], 10/46 [22%]; fibrinolysis 21/63 [33%], 15/63 [24%]; others 20/37 [54%], 11/37 [30%]). Within pro-coagulant factors the largest RRRs were observed for F5 Leiden mutation (RRR 3.42, 95% CI 0.11–104), a variant of FVIII (F8 rs6655259, RRR 4.72, 95% CI 0.62–35.73), presence of lupus anticoagulant (RRR 8.13, 95% CI 0.61–108.76) and three variants of FXIII (F13A1 V34L, RRR 4.66, 95% CI 0.44–49.10; F13A1 T204P, RRR 11.1, 95% CI 5.64–21.82 and F13A1 rs3024462, RRR 3.71, 95% CI 0.62–22.35). A substantial part of the studies (15 studies, 216 factors) combined haemorrhagic and ischaemic stroke as a single outcome. When restricted to those that excluded haemorrhagic stroke, 71 out of 135 markers (53%) had a RRR >1.1 (RRR >1.5: 33/84 [39%] and RRR >2: 16/84 [19%]). Larger RRRs were also found in young study populations (RRR >1.1: 27/44 (61%); RRR >1.5: 21/29 (72%) and RRR >2: 14/29 (48%); five studies).

Conclusions: These results suggest that the effect of various markers of hypercoagulability on the risk of IS is larger than that on MI, particularly in the young.

PB 4.64-4

A novel and selective proteasome inhibitor modulate expression of molecules linked to coagulation and angiogenesis independent of NF-kB activation in tumor cells

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Amblyomin-X is a Kunitz-like recombinant protein which has shown inhibitory properties on factor Xa and tenase complex and also inhibits proteasome in human tumor cell lines but not in normal human fibroblasts. Proteasome inhibition generates aggresomes transported by dynein in which K48 and K63-linkage of ubiquitin signaling is involved. This signaling is also important to NF-κB pathway and studies have been reported that recruitment of dynein translocate p65/p50 heterodimer to act as an activator of a large number of genes like tissue factor (TF), TFPI and VEGF. The p50 subunit is generated by its precursor molecule p105 that is processed by the proteasome. TFPI not only inhibits thrombin generation by inhibiting FXa which then inactivates TF/FVIIa complex but also can act as adhesive ligand for cancer cells to extracellular matrices. Additionally, TF can enhance tumor metastasis and VEGF contributes to angiogenesis. The present study intends to evaluate the pathway that links coagulation and cancer in the antitumoral mechanism of action of Amblyomin-X. Cell culture: Normal human fibroblasts, Mia-PaCa-2 and SK-Mel-28 tumor cell lines. Real-Time PCR: SYBR® green based reaction. Aggresome formation: Commercial kit for flow cytometry and fluorescence microscopy. Ubiquitin signaling: Flow cytometry with specific K63 and K48 antibodies. Western blotting: Cell lysis and immunoblotting after SDS-PAGE with specific antibodies. NF-κB, dynein, Ubc13, VEGF and TF gene expression were upregulated in Mia-PaCa-2 after 24 h of Amblyomin-X treatment. Only NF-κB, Ubc13 and dynein genes were upregulated in SK-Mel-28. Fibroblasts have normalized the expression levels of the targets analyzed in 24 h. Protein expression ofsome targets has been modified in tumor cells while only p50 has been modulated in fibroblasts. Amblyomin-X induced aggresome formation only in tumor cells. The K63 linkage signal was increased in all cell lines and corroborates with Ubc13 upregulation while K48 linkage has decreased only in tumor cells. Amblyomin-X inhibits proteasome and induces aggresome formation only in tumor cells. The molecule seems to trigger NF-κB signaling as a cell response in fibroblasts but do not target proteasome and cell death. Our data indicates that NF-κB, although high expressed, can be inactive to transcript its targets like TFPI, TF and VEGF, since proteasome inhibition can prevent

degradation of IKB, a natural inhibitor of NFkB, which in turns, could remain sequestered and inactive. Moreover, protein expression of p50 subunit has also decreased in tumor cells and its precursor p105 has increased indicating thatproteasome inhibition had affected its proteolysis. Additionally, K63 signaling for aggresome formation and decreased K48 signaling as a cell response, contributes to inactivate NF-κB pathway. Therefore, increased mRNA levels of TF and VEGF in Mia-PaCa-2 indicates that these targets may have its gene expression activated by another pathway independent of NFκB activation, as a tumor response to Amblyomin-X treatment. Aggresome formation and increased TFPI expression may contribute respectively to cell apoptosis and metastasis reduction, effects already observed *in vivo* in previous data, in the mechanism of action of Amblyomin-X.

PB 4.64-5

Correlation between factor VII-activating protease and metabolic control in children and adolescents with Type 1 diabetes

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Background: Factor VII-activating protease (FSAP) is a circulating serine protease for which a potential role in the regulation of hemostasis and vascular remodeling processes is postulated. Higher levels and activity of FSAP have been reported in patients with deep vein thrombosis, ischemic stroke, or acute coronary syndrome.
Diabetic micro- and macroangiopathy, major complications of diabetes mellitus type I, are associated with endothelial cell alterations and hypercoagulability caused by formation of advanced glycosylation end products due to hyperglycemia. The central role of HbA1c levels for the prediction of micro- and macrovascular complications in patients with type 1 diabetes is generally accepted.
Aims: The aim of this study was to investigate a possible relation between plasma concentrations of FSAP and HbA1c levels in patients with type 1 diabetes mellitus (T1DM).
Methods: Microalbuminuria, levels of FSAP und HbA1c were investigated in 37 patients with type 1 diabetes. FSAP was also determined in healthy age matched controls.
Results: Median age of patients was 13.6 years (3.3–20.7); median duration of diabetes was 6.5 years (0.8–15.7), median microalbuminuria was 4.69 mcg/min (0.1–45.4), median HbA1c was 8.4% (5.6–11.5). FSAP levels were significantly higher in patients with T1DM, 18.3 mcg/mL (11.2–21.4), than in healthy controls, 15.8 mcg/mL (12.6–21.6), $P < 0.01$. In addition, we found a significant correlation between HbA1c and FSAP ($r = 0.42$, $P < 0.05$). This correlation stayed significant after adjustment for age and duration of diabetes. There was no correlation between microalbuminuria und levels of FSAP.
Summary: FSAP levels were significantly elevated even in children and adolescents with type 1 diabetes compared with healthy controls and correlated significantly with HbA1c levels. Our study strengthens the importance of metabolic control in the development of vascular alteration in patients with type 1 diabetes.

PB 4.64-6

Coronary microvascular dysfunction due to essential thrombocythemia and policythemia vera: the missing piece of the puzzle of their increased cardiovascular risk?

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Background and Aim: Essential thrombocythemia (ET) and Polycythemia vera (PV) increase the risk for cardiovascular events. We evaluated coronary flow reserve (CFR) by transthoracic Doppler echocardiography (TDE), as an index of coronary microvascular function, in ET and PV.
Patients and Methods: 49 ET and 17 PV patients (pts) (38 Fand5 F, aged 61 ± 16 and 58 ± 13 years respectively) without clinical evidence of heart disease, and 50 controls matched for age and gender were studied. Coronary flow velocity in the left anterior descending coronary artery was detected by TDE at rest and during adenosine infusion. CFR was the ratio of hyperaemic diastolic flow velocity (DFV) to resting DFV. A CFR ≤2.5 was considered abnormal. The median time from ET and PV diagnosis was 7 years (range 0–24) and 4 years (range 0–10) respectively. Patients with a reduced CFR underwent a computed tomographic coronary angiography.
Results: In ET and PV pts, CFR was lower than in controls (2.9 ± 1.0 and 2.3 ± 0.8 vs. 3.4 ± 0.7, $P < 0.005$ and $P < 0.0001$ respectively). CFR was ≤2.5 in 15 (31%) ET pts and 12 (71%) ET pts compared with controls (4%). At multivariable linear regression analysis adjusted for diagnosis (control, ET and PV respectively), age, gender and cardiovascular risk factors, diagnosis and age were the only determinants of CFR (diagnosis $\beta = -0.424$, $P < 0.0001$; age $\beta = -0.347$, $P = 0.004$ respectively). Patients with reduced CFR, undergone to coronary angiography failed to show any haemodynamic significant stenosis both of the left main coronary artery and of the left anterior descending coronary artery.
Conclusions: In ET and PV patients CFR is lower than in controls, this is due to a coronary microvascular dysfunction rather than to a coronary stenosis. Further studies on a wider population is needed to establish the links between coronary microvascular dysfunction and these myeloproliferative diseases.

PB4.65 – Hormones, pregnancy, women's issues – IV

PB 4.65-1

Impact on the initial pregnancy loss subtype on pregnancy outcomes in the conventionally treated purely obstetric antiphospholipid syndrome

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Background: In the purely obstetric form of antiphospholipid syndrome (APS) treated according to guidelines, the impact of the initial clinical heterogeneity on the subsequent pregnancy outcomes is uncertain.
Aims: We aimed to design a prospective study in women with a purely obstetric APS.
Methods: We performed a 10-year observational study of 1592 non-thrombotic women who had experienced three consecutive spontaneous abortions before the 10th week of gestation or one foetal death at or beyond the 10th week of gestation (*NOH-APS*, *Blood 2012;119 (11):2624–32*). A total of 517 women finally fulfilled the APS diagnosis criteria, leading to treat the subsequent pregnancy attempt using prophylactic low molecular weight heparin LMWH plus low dose aspirin LDA. We report the relevant risk factors for pregnancy out-

comes, with special emphasis on the initial clinical presentation (spontaneous abortions: $n = 206$ vs. foetal death: $n = 311$). The relevance of other pregnancy loss subtypes (primary vs. secondary), of the presence/absence of an associated systemic illness, of anthropometric characteristics (age >35 years, BMI >30 kg/m^2 or <18.5 kg/m^2, varicose veins), of metabolic characteristics (diabetes mellitus, hypercholesterolemia, hypertriglyceridemia), of life habits (smoking, oral contraceptives) and of haemostatic parameters (positive lupus anticoagulant LA, positive anticardiolipin IgG antibodies aCL-G and IgM antibodies aCL-M, positive anti-beta2-glycoprotein 1 IgG antibodies aB2Gp1-G and IgM antibodies aB2Gp1-M; positive *F5* 6025 or *F2* rs1799963 polymorphisms) was systematically evaluated by multiparametric logistic regressionanalysis.

Results: aB2Gp1-M was finally the single risk factor for spontaneous abortions ($P = 0.013$) whereas initial foetal loss was the single significant risk factor for subsequent foetal loss ($P = 0.014$).

Initial foetal loss ($P = 0.005$) and BMI >30 kg/m^2 ($P = 0.037$) were two independent risk factors for a subsequent pre-eclampsia (PE). None of the studied characteristics was a significant risk factor for PE before 34 weeks of gestation (WG).

Initial foetal loss was the single significant risk factor for a premature birth before 37 WG ($P = 0.0001$) and before 34 WG ($P = 0.0035$).

None of the clinical characteristics/biological markers was a significant risk factor for the birth of a small-for-gestational age neonate (SGA, customised percentile th WG to the 27th day after delivery).

The ischemic placenta diseases (IPD) being defined as a composite endpoint associating PE, placental abruption and SGA neonates, initial foetal loss ($P = 0.049$), aCL-M ($P = 0.025$) and varicose veins ($P = 0.028$) were independent risk factors for IPD.

An initial foetal loss was the single significant risk factor when the IPD definition was extended to stillbirth cases.

Summary/Conclusions: In women with the purely obstetric APS, the initial clinical presentation impacts on the risk of poor outcomes during the first pregnancy attempt conventionally treated.

Initial foetal loss indicates a higher risk of subsequent foetal loss, pre-eclampsia, premature birth, or ischemic placenta diseases than initial recurrent spontaneous abortions, despite treatment.

This fact may indicate insufficient treatment doses and principles in case of initial foetal loss, or the presence of unknown cofactors in APS patients with initial foetal loss. The analysis of the mechanisms of foetal loss is still needed, even in APS women.

PB 4.65-2

Severe preeclampsia: evaluation of D-dimer, PAI-1 and inflammatory cytokine

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Background: Preeclampsia (PE) is a multi-system disorder of pregnancy characterized by hypertension and proteinuria. A predisposition to endothelial dysfunction, which may trigger abnormal activation of the hemostatic and/or inflammatory systems, is thought to play a crucial part in pathogenesis of PE.

Aims: The aim of this study was to investigate the relationship between hemostatic and inflammatory parameters in women with severe PE.

Methods: D-Dimer, PAI-1 (American Diagnostica®), IL-8, IL-6, TNF-α (Human Inflammation kit Cytometric Beads Array (CBA) – BD Biosciences Pharmingen, USA) and IFN-γ (Human Th1/Th2 CBA – BD Biosciences Pharmingen, USA) levels were measured in severe PE ($N = 59$), normotensive pregnant ($N = 49$) and non-pregnant women ($N = 48$).

Results: D-Dimer and PAI-1 were significantly higher in severe PE vs. normotensive pregnant ($P < 0.001$ for both) and non-pregnant women ($P < 0.001$ for both). IFN-γ was higher in severe PE vs. normotensive pregnant ($P = 0.018$) and non-pregnant women ($P < 0.001$) and in normotensive pregnant vs. and non-pregnant women ($P = 0.024$). IL-6 was significantly higher in severe PE vs. non-pregnant women ($P < 0.001$) and in normotensive pregnant vs. non-pregnant women ($P < 0.001$). TNF-α did not differ among groups. D-Dimer and PAI-1 showed an elevated area under ROC curve (0.938 and 0.873). A weak correlation between D-Dimer vs. IL-8 ($r = 0.597$, $P < 0.001$) and PAI-1 vs. IFN-γ ($r = 0.397$, $P = 0.045$) were found in sPE.

Conclusion: D-Dimer and PAI-1 levels showed to be important tools for discriminating severe PE. However, no important correlation between these haemostatic markers and cytokines levels was found. More studies are necessary to improve the knowledge of hemostasis and inflammation in severe PE.

Financial support: CN.

PB 4.65-3

A safe and effective regimen for managing women at intermediate and high risk of pregnancy-related venous thrombosis

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Background: The optimal approach for venous thromboembolism (VTE) prophylaxis during pregnancy and postpartum in women at increased risk of VTE is still not established despite recent efforts to resolve this matter.

Aims: To evaluate the safety (incidence of postpartum hemorrhage [PPH]) and efficacy (incidence of pregnancy-related VTE) of a protocol recommending thromboprophylaxis with 5700 IU anti-Xa nadroparin once daily (or an equivalent dose dalteparin 5000 IU once daily) in pregnancies at intermediate and high risk of VTE.

Methods: This is a single center study performed at the Erasmus University Medical Center in women at intermediate or high risk of pregnancy-related VTE. They received low-molecular-weight heparin (LMWH) according to our protocol only postpartum (intermediate risk of VTE) or during pregnancy and 6 weeks postpartum (high risk of VTE). Pregnancy-related VTE was defined as VTE during pregnancy or within 3 months postpartum. Postpartum haemorrhage (PPH) was defined as blood loss >500 mL and severe PPH as blood loss >1000 mL. The study was not subject to the Medical Research Involving Human Subjects Act and was approved by the Medical Ethics Committee.

Results: One hundred and nineteen pregnancies in 93 women were considered at risk of trombosis of which 57 pregnancies had an intermediate risk and 62 pregnancies had a high risk of VTE. In our cohort, no VTE was observed in women using LMWH prophylaxis. No women were lost to follow-up. The occurrence of PPH was 21.7% (95% CI, 15.2–30.1%) and severe PPH 8.7% (95% CI, 4.8–15.3%). There was no significant difference in PPH between intermediate and high risk pregnancies. Estimated blood loss during delivery was comparable to previous Dutch studies.

Summary/Conclusion: VTE prophylaxis with the current dose of LMWH prophylaxis in pregnant women at an intermediate or high risk of pregnancy-related VTE proved to be safe, since we did not observe an increase in PPH. In our cohort of women the dose of LMWH was effective in preventing pregnancy-related VTE. A randomized trial comparing the current dose LMWH with lower-dose LMWH in pregnant women at intermediate and high risk of VTE is urgently needed.

PB 4.65-4

No increased fibrinolysis in women with menorrhagia

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Background: Menorrhagia is a common problem. At least 5–10% of women in reproductive age seek medical attention for menorrhagia. Bleeding disorders have been recognized as important etiologic and/or contributing factors in some women with menorrhagia. Increased fibrinolysis might also lead to menorrhagia.
Aim: To investigate fibrinolytic parameters, including clot lysis time (CLT) in women with menorrhagia.
Methods: The study was approved by the Institutional Review Board of the University Medical Center of Groningen. Informed consent was obtained from all patients and controls. We included 102 patients referred for menorrhagia (PBAC-score >100). Patients and controls (28 healthy volunteers without menorrhagia) had haemostatic testing in the 1st week after menstruation. For 79 patients and all controls fibrinolytic parameters (FXI, TAFI-act, PAI, t-PA, PI) and CLT were available. TAFI, PAI and PI act as inhibitors of fibrinolysis, CLT measures overall fibrinolytic potential.
Results: Median age was 46 years (range 23–59) in patients and 40 years (range 27–57) in controls ($P = 0.02$). In 33% ($n = 26$) of the patients a gynecological abnormality was found. Fibrinolytic parameters were comparable in patients and controls, except for TAFI (91% vs. 83.9% $P = 0.02$) and PI (104.8% vs. 96.9% $P < 0.01$), which were significantly higher in patients. CLT tended to be longer in patients than in controls (72.0 min vs. 67.1 min; $P = 0.21$). Patients had lower FXI levels than controls (mean: 100% vs. 124%; $P < 0.01$), although within the reference range. Patients with FXI levels <100% had significantly shorter CLT, lower TAFI, PAI and PI levels than patients with levels >100%. In patients, shorter CLT correlated with lower TAFI and PAI. In controls we observed only a correlation with PAI.
In women with menorrhagia without gynecological abnormalities we found significantly lower TAFI and PAI levels compared to women with gynecological abnormalities (TAFI: 88% vs. 96%, $P = 0.03$; PAI: 16.0 μg/L vs. 25.6 μg/L, $P = 0.03$).
Conclusion: Fibrinolytic capacity is not increased in women with menorrhagia. Overall, the fibrinolytic inhibitors TAFI and PI were even higher in patients than in controls. However, inhibitors of fibrinolysis are lower in subgroups of women with lower FXI and in women with unexplained menorrhagia. The clinical relevance is not clear yet.

PB 4.65-5

Is rotation thrombelastometry a useful method for monitoring of hemostasis in normal pregnancy and puerperium?

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Aims: Aim of the study was to analyse the changes of hemostasis in normal pregnancy and puerperium by rotation thrombelastometry (ROTEM) and compare these results with those in healthy non-pregnant women.
Methods: Prospective observational study was approved by the etics committee of Jessenius Faculty of Medicine. All study subjects gave their informed consent to participate in the study. Fifty normal pregnant women, mean age 30.6 [19–44] years, were enrolled into study and tested by ROTEM (INTEM and EXTEM) repeatedly during pregnancy and puerperium (T1: 11–12th week, T2: 17–18th week, T3: 25–26th week, T4: 35–36th week and T5: 6–7th week after delivery). Blood count and hemocoagulation tests were studied at the same time as well. In the control group there were 54 healthy women (blood donors), age 30.5 [18–45] years, examined by the same investigations. Statistical analysis was performed by SPSS 16.0 (Chicago, IL, USA).
Results: At almost all time during pregnancy there was the significant reduction of clotting time (CT), more pronouced in EXTEM compared to INTEM. In EXTEM test the significance of CT reduction was as followed: CTT2:CTT1 ($P < 0.005$), CTT3:CTT1 ($P < 0.00001$), CTT4:CTT1 ($P < 0.00001$), CTT5:CTT1 ($P < 0.0001$). In EXTEM test there was also the significant CT reduction of CTT3 ($P < 0.00001$), CTT4 ($P < 0.00001$) and CTT5 ($P < 0.0001$) compared to healthy non-pregnant controls. The maximum clot firmness (MCF) was very significantly increased in both EXTEM and INTEM tests compared to healthy non-pregnant controls (MCFT1-MCFT4 $P < 0.00001$; MCFT5 $P < 0.005$). In EXTEM test the MCF showed the significant increase between MCFT3:MCFT1 ($P < 0.05$) and very significant increase between MCFT4:MCFT1 ($P < 0.00005$). The differences between MCFT2:MCFT1 and MCFT5:MCFT1 were not significant.
Summary/Conclusions: Our ROTEM results testify for a tendency to hypercoagulation in normal pregnancy, especially in the 2nd and 3rd trimester of pregnancy (max. 36–37th week) as well as in puerperium. In our cohort of normal pregnant women the arrangement of hemostasis was not achieved completely even at 6–7th week after delivery (MCF5 not significantly increased). Acknowledgement: Study was supported by projects APVV 222-11, Vega 1/0016/12, UK/319/2012 and CEPV II (ITMS 26220120036) which was cofinanced from EC sources.

PB 4.65-6

Anticoagulation in pregnant women with history of unexplained miscarriage

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Background: Unexplained miscarriage affects 11–15% of women. Low molecular weight heparin (LMWH) is frequently given to prevent recurrent pregnancy loss. However, only limited data exist concerning LMWH treatment for prevention of miscarriage, especially in non thrombophilic women and in women with one miscarriage.
Aims: The aim of this study is to reveal the rate of live births in patients (with or without thrombophilic disorders) with unexplained miscarriage treated with LMWH during pregnancy.
Methods: 243 women with one ore more unexplained miscarriages were examined. Thirteen patients were lost for follow-up and therefore excluded from analysis, thus 230 women (average age at beginning of gestation: 33 years) with one ($n = 50$) or more ($n = 180$) unexplained miscarriages were examined.
Data were collected during first pregnancy under LMWH treatment. Women with an indication for anticoagulant treatment other than abortion and women with antiphospholipid antibodies were excluded. LMWH was given in prophylactic dosage.
The following thrombophilia parameters were determined: Factor V Leiden mutation, Prothrombin (G20210A) polymorphism, protein C, protein S, antithrombin, antiphospholipid antibodies.
Results: In 41% of pregnant women (94/230) thrombophilia was observed: 51 women with Factor V Leiden mutation, 26 with Prothrombin polymorphism, 3 with protein C deficiency, 13 with protein S deficiency and 1 with antithrombin deficiency.
The proportions of women who gave birth to a live infant were 73% in women with thrombophilia (69/94) and 77% in women without thrombophilia (105/136).

Women who had experienced one miscarriage had – after pregnancy under LMWH – a live birth rate of 88% (44/50), women with more than one miscarriage had a live birth rate of 73% (131/180).
Except vaginal bleeding, no bleeding complications occurred during LMWH treatment. Moreover, no haemorrhagic complications in association with delivery were reported. Furthermore there was no heparin- induced thrombocytopenia or thromboembolism.
Conclusions: Thrombophilia has been identified in about 41% of women with unexplained miscarriage. When LMWH was given during pregnancy, live birth rate in women with only one miscarriage was higher than in women with >2 miscarriages. Thus, thrombophilia seems to be one risk factor for occurrence of miscarriages. Heparin therapy to prevent miscarriage is safe, but it is still unclear whether LMWH improves the chance of live birth in women with unexplained miscarriage.

PB4.66 – Hormones, pregnancy, women's issues – V

PB 4.66-2
May-Hegglin anomaly in pregnancy: a systematic review

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Background: May-Hegglin anomaly is an autosomal dominant disorder, characterised by a variable degree of thrombocytopenia, large platelets and inclusion bodies in white blood cells. Bleeding manifestations are generally mild but severe bleeding episodes have been reported.
Aims: To perform a systematic review of the literature to assess the affect of May-Hegglin anomaly on pregnancy outcomes and bleeding manifestation
Method: A systematic review of literature for May-Hegglin anomaly during pregnancy was performed using electronic databases including PUBMED, MEDLINE, and EMBASE. The following keywords were used 'May-Hegglin anomaly', 'pregnancy', 'gestation' and 'inherited thrombocytopenia'. Initial literature review revealed 655 articles. Twenty-six articles were relevant to MHA in pregnancy, and were included in the review.
Results: The review revealed 26 articles (25 case reports and one case series) including 75 pregnancies (five twin pregnancies) in 40 women. In 11 women, first presentation was incidental thrombocytopenia during routine antenatal blood test. Of these, five women were misdiagnosed as ITP, including three who underwent splenectomy for resistant ITP. Postpartum haemorrhage (PPH) and bleeding after miscarriage were presenting symptoms in two women.
Antiplatelet antibody was found in three pregnancies. Only one of them required intervention with IVIG to prevent neonatal alloimmune thrombocytopenia. Postpartum haemorrhage (PPH) was reported in four pregnancies, three were primary PPH, one required blood transfusion, one required platelet and cryoprecipitate transfusion and the 3rd was managed conservatively. There was one secondary PPH that was treated conservatively. Neonatal outcome included 78 live neonates and 2 intrauterine fetal deaths. Thirty four neonates had thrombocytopenia and subsequently were diagnosed with May-Hegglin anomaly, 3 of them required prophylactic platelet transfusion as they developed a very low platelet count and one neonate with platelet count of 29×10^9/L received IVIG as the mother had a positive antiplatelet antibody during pregnancy. No obvious bleeding complications were reported among the neonates.
Conclusion: May-Hegglin anomaly can present challenges during pregnancy and be associated with adverse maternal and neonatal outcome due to bleeding complications. Joint management by obstetrians and haematologists is required to minimise these risks.

PB 4.66-3
Incidence of pregnancy outcomes in women with pregnancy loss subtypes bearing Leiden polymorphisms:comparison with the purely obstetric antiphospholipid syndrome

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Background: By comparison to what is observed in the purely obstetric form of antiphospholipid syndrome (APS) treated according to guidelines, the incidence of pregnancy outcomes in women with current constitutive thrombophilias is uncertain.
Aims: We aimed to design a prospective study in women selected on the same clinical criteria, according to the presence of APS, or of frequent constitutive thrombophilias.
Methods: We performed a 10-year observational study of 1592 non-thrombotic women who had experienced three consecutive spontaneous abortions before the 10th week of gestation or one foetal death at or beyond the 10th week of gestation (*NOH-APS*, *Blood 2012;119 (11):2624–32*).
We compared the frequencies of pregnancy outcomes among women positive for antiphospholipid antibodies (n = 517; treatment: prophylactic LMWH plus low-dose aspirin), women carrying the *F5* 6025 or *F2* rs1799963 polymorphism (n = 279; recurrent abortions: no treatment; foetal death: prophylactic LMWH), and women with negative thrombophilia screening results (n = 796; no treatment).
We report the results comparing APS women and women carrying the *F5* 6025 or *F2* rs1799963 polymorphism ('Leiden polymorphisms').
Results: In women with recurrent abortions, the relative risk of early abortion, foetal death, pre-eclampsia (PE; total PE, severe PE, PE before 34 weeks, HELLP syndrome, PE with a small for gestational age SGA neonate), placental abruption PA, any ischemic placental disease IPD, premature birth before 37 weeks or before 34 weeks and neonatal mortality, computed between non-treated 'Leiden polymorphisms' women (n = 93) and treated APS women (n = 206), were non-significant.
In women with foetal death, by comparison to treated 'Leiden polymorphisms' women (n = 186), treated APS women (n = 311) were at higher risk of premature birth before 37 weeks or before 34 weeks; of PE, severe PE, PE before 34 weeks, HELLP syndrome and PE with SGA neonate; of any IPD. A trend ($P < 0.15$) was observed for increased risks of foetal death, of eclampsia, of SGA neonates and of neonatal mortality. By comparison to non-treated women with negative thrombophilia screening results (n = 313), treated 'Leiden polymorphisms' women were a lower risk of foetal loss, of premature birth, of severe PE and of PE before 34 weeks, a trend being observed for lower risks of SGA neonates and of any IPD.
Summary/Conclusions: In women with recurrent abortions, patients bearing the *F5* 6025 or *F2* rs1799963 polymorphism attempting a new pregnancy without any treatment have similar pregnancy outcome rates than treated purely obstetric APS women. Specific therapeutic developments are unneeded in these Leiden positive women.
In women with foetal loss, treated patients bearing the *F5* 6025 or *F2* rs1799963 polymorphism attempting a new pregnancy are at strikingly lower risk of poor outcomes than treated purely obstetric APS women. They are also at lower risks than non-treated women with negative thrombophilia screening. Prophylactic LMWH given in our selected *F5* 6025 or *F2* rs1799963 positive women with unexplained foetal loss appear to exert some protective effect during pregnancy. This protective effect might depend of the molecular mechanism underlying the previous foetal loss, which identification is thus warranted.

PB 4.66-4

Mild and severe preeclampsia: platelet activation, platelet-leukocyte aggregates and monocytes-tissue factor expression in assessment

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Background: Preeclampsia (PE) is a serious complication of pregnancy associated with high maternal-fetal morbidity and mortality, whose etiology remains unclear. In pure form, it is characterized by hypertension and proteinuria after 20th weeks of pregnancy, in normotensive pregnant women. Studies suggest that platelet activation and aggregation contribute to the hypercoagulable state seen in this disease. Moreover, hemostatic and inflammatory changes associated with normal pregnancy are more exacerbated in PE.

Aims: To assess platelet activation markers and monocytes-TF expression in mild (mPE) and severe (sPE) preeclampsia.

Methods: A cross-sectional study (ETIC 0243.0.203.000-10). Mean fluorescence intensity (MFI) of CD41a, CD61, CD42a, CD62P and $CD62P^+$ (%) were evaluated by flow cytometry in 35 preeclamptic women (20 mPE and 15 sPE), 25 normotensive pregnant (NP) and 31 nonpregnant women (NW). Platelets-monocytes aggregates (APM), platelets-neutrophils aggregates (APN) and monocytes-TF^+ were evaluated in 20 PE (12 mPE and 8 sPE), 20 NP and 20 NW. Data were analyzed by ANOVA/LSD and Kruskal-Wallis/Mann-Whitney. Differences were significant at $P \leq 0.05$ and $P \leq 0.017$ (Bonferroni).

Results: CD62P, $CD62P^+$ (%), CD42a, APM (%), APN (%) and monocytes-TF^+ (%) did not differ among groups ($P = 0.69$; $P = 0.54$; $P = 0.57$; $P = 0.13$; $P = 0.02$; $P = 0.26$). CD61 and CD41a were lower in sPE vs. NW ($P = 0.00$). No difference were found for CD61 and CD41a in sPE vs. mPE ($P = 0.49$; $P = 0.48$), sPE vs. NP ($P = 0.79$; $P = 0.69$), mPE vs. NP ($P = 0.63$; $P = 0.73$) and for CD41a in mPE vs. NW ($P = 0.10$).

Summary/Conclusions: The results showed that PE is associated with reduced CD41a and CD61 expression on platelet surface. It can also be inferred that the circulating platelets are not activated and the activated ones would be trapped in the platelet aggregates, no longer being in circulation.

PB 4.66-5

Is bleeding disorders investigation mandatory in women with verified menorrhagia?

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Background: Menorrhagia is caused by bleeding disorders in substantial number of cases and it is very common clinical problem among women of reproductive age.

Objective: To estimate prevalence of coagulation disorders in females with menorrhagia as well as frequency of menorrhagia and its characteristics.

Method: 115 women (36.1 ± 9.6 years) with symptoms of menorrhagia have been studied. Menorrhagia has been verified using semiquantitive method – Pictorial Bleeding Assessment Chart (PBAC) with score >100 (equivalent >80 mL blood). Bleeding time (BT), coagulation screening (PT, aPTT), VWF:Ag and VWF:Ac, FII, FV, FVII, FVIII, FIX, FX and F XI activity, have been analyzed, as well as complete blood count including hemoglobin (Hb) and serum iron.

Results: Menorrhagia (PBAC >100) has been objectively verified in 64 women (55.7%). In comparison with those with normal menstruation, women with menorrhagia had higher SCOR of menstrual cycle (Md = 150.0 vs. Md = 50.0; $P < 0.001$) but not its duration (7.2 ± 2.1 days vs.7.3 ± 1.9 days; $P > 0.05$), lower FVII:Ac (78.0 ± 28.3% vs.100.5 ± 35.0%; $P < 0.001$) and lower FX:Ac (Md = 77.4%, 39.8–141.0% vs. Md = 87.8%, 42.0–143.0%; $P = 0.018$). Coagulation defects have been found in 12 women (10.4%) – decreased FIX:Ac in 4 (3.5%), decreased FVII:Ac in 1 (0.9%), decreased FX:Ac in 1 (0.9%), 1 woman (0.9%) was a hemophilia C carrier, while 5 women (4.3%) matched criteria for mild VWD type 1. Women with coagulation disorders had prolonged PT (Md = 13.1s, 12.2–14.8s vs. Md = 12.5s, 10.6–18, 3s; $P = 0.032$) (after adjustment for the presence of FVII and FX deficiency this finding was persistent for entire group). Anemia was diagnosed at 61 women (53.0%) and these patients had higher SCOR of menstrual cycle (Md = 109.0, 50–778 vs. Md = 100.0, 26–750; $P = 0.036$), they were older (38.6 ± 9.0 years vs. 33.5 ± 9.5 years; $P = 0.005$) and had higher values of FV:Ac (Md = 117.0%, 50.0–241.0% vs. Md = 80.7%, 50.0–199.0%; $P = 0.004$) and FVIII:Ac (Md = 135.0%, 22.0–596.0% vs. Md = 118.5%, 70.0–460.0%; $P = 0.066$) in comparison with those with normal blood tests.

Conclusion: About half of patients with positive history (55.7%) of heavy bleeding really had menorrhagia diagnosed with PBAC score. Ability of women for self-evaluation of blood loss during period was poor. Higher SCOR of menstrual cycle but not also its duration suggests that intensity, but not its duration contributes to abundant menstrual bleeding in women with menorrhagia. Coagulation abnormalities were present at 10.4% of the examined population, predominantly mild VWD. At more than half of the patients anemia was diagnosed and it was primarily associated with inflammation and higher age. We suggest that objectively verification of menstrual loss (PBAC) is mandatory and it seems that bleeding disorders investigation (screening for PT and VWD) predominantly in women with anemia may be useful in the diagnostic algorithm of menorrhagia.

PB 4.66-6

Thrombin generation, APC resistance, protein S and TFPI function in women with a history of a thromboembolism during oral contraceptive use

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Background: Venous thromboembolism (VTE) is a multifactorial disorder that arises as the result of genetic and environmental factors. Oral contraceptive (OC) use is a well-known acquired risk factor for VTE. Up to 40% women, who develop a VTE during OC use have a thrombophilia. Annual risk of a recurrent event among women with a previous hormone-related VTE is about 2%, which suggests persistence of the prothrombotic changes. Global coagulation tests are currently used to assess the over-all coagulation state and function of different coagulation pathways. Thrombin generation assays and activated protein C (APC) resistance tests are associated with a risk for primary and recurrent VTE.

Aims: To identify changes in APC resistance and thrombin generation measured at different assay conditions in a population of women, who had a VTE during OC use.

Methods: We compared thrombin generation, APC resistance as well as the functional activities of protein S and TFPI in plasma of healthy controls and women, who had a thrombotic event during OC use in their personal history. Thrombin generation was measured via calibrated automated thrombography at 1 pM and 10 pM of tissue factor. The function of protein S and TFPI was evaluated in thrombin generation-based assays and expressed as protein S and TFPI ratios. The study was approved by the Medical Ethical committee of the Kar-

olinska Institutet, Stockholm, Sweden. All participants gave written informed consent.

Results: 57 women with VTE during OC use in the anamnesis and 25 non-pregnant healthy control women who were not using hormonal contraceptives were recruited in the present study. 16 participants were excluded from the thrombosis group due to hormonal contraception (progestin-only pill or levonorgestrel-containing intrauterine device) or medication interfering with blood coagulation. Both groups were comparable with regards to age.

The thrombosis group had higher endogenous thrombin potential (ETP) in the presence of APC as well as APC resistance as compared to the control group ($P = 0.014$ and $P = 0.033$ respectively). Furthermore, women with a VTE in the anamnesis had an increased protein S ratio ($P = 0.032$), indicating an impaired function of protein S. Thrombin generation measured at 1 or 10 pM tissue factor and the TFPI ratio did not differ between the groups.

The Pearson Chi-Square test confirmed a significantly higher number of women with a thrombosis in the past among participants with values above the upper quartile (>75%) for the ETP determined in the presence of APC as well as for the protein S ratio ($P = 0.016$ and $P = 0.045$ respectively).

Summary: Women with a previous VTE had higher APC resistance as compared to the control group. This might be explained by a higher number of carriers of the factor V Leiden mutation in the thrombosis group. Furthermore, women in the thrombosis group had an impaired function of protein S that likely contributes to their prothrombotic phenotype.

PB4.67 – Inflammation: Basic – III

PB 4.67-1

Overexpression of activated protein C improves host defense during pneumococcal pneumonia

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Background: During pneumonia, inflammation and coagulation are activated as part of anti-bacterial host defense. Activated protein C (APC) has anticoagulant and anti-inflammatory properties and during pneumonia its levels are decreased. *Streptococcus pneumoniae* is the most common causative pathogen in community-acquired pneumonia.

Aims: We aimed to investigate the effect of endogenous overexpression of APC during experimental pneumococcal pneumonia.

Methods: Wildtype (WT) and APC-overexpressing (APC^{high}) mice were intranasally infected with *S. pneumoniae* (strain 6303). Mice were sacrificed after 6, 24 or 48 h and survival studies were performed. Lungs, liver, spleen and blood were harvested to measure bacterial loads, cytokines, histopathology and coagulation parameters. Additionally, bronchoalveolar lavages (BAL) were done and the BAL-fluid (BALF) and cells were analyzed.

Results: In comparison to WT mice, APC^{high} mice showed decreased bacterial dissemination to liver ($P < 0.05$) and spleen ($P < 0.01$), while no differences in local bacterial loads were measured. Additionally, although no differences in overall lung histopathology were seen, APC^{high} mice showed a significantly decreased total cell influx in BALF due to decreased numbers of neutrophils (both $P < 0.01$), which was accompanied by decreased levels of the pro-inflammatory cytokine TNF-α ($P < 0.05$). Finally, APC^{high} mice showed a strong trend toward a better survival when compared to WT mice ($P = 0.06$).

Conclusions: Overexpression of activated protein C improves host defense during experimental pneumococcal pneumonia. These data contribute to the knowledge of cell-protective effects of APC during severe infections.

PB 4.67-2

SMTP, a novel family of small molecule anti-inflammatory thrombolytic: structure-activity relationships with respect to plasminogen modulation and soluble epoxide hydrolase inhibition

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Background: The SMTP family small-molecules enhance plasminogen activation through conformational change (plasminogen modulation; PM). The SMTP molecule consists of a core unit (a tricyclic γ-lactam moiety with a geranylmethyl group) and an *N*-linked side-chain. SMTP-7, which is a potent thrombolytic with an excellent potential to treat ischemic stroke, is one of the SMTP compounds that comprises >50 congeners differing in the side-chain. The excellent SMTP-7 activity in treating stroke is attributable not only to PM activity (thrombolysis) but also to anti-inflammatory properties, which are implicated in soluble epoxide hydrolase (sEH) inhibition (sEHi). sEH catalyzes the conversion of the anti-inflammatory fatty acid epoxyeicosatrienoic acid (EET) to an inactive corresponding diol, and inhibition of sEH stabilizes endogenous EET, leading to anti-inflammation and neuroprotection.

Aim: The present study aimed at evaluating potencies of a variety of SMTP congeners with different *N*-linked side chains with respect to PM and sEHi activities to elucidate their structure-activity relationships for the design of ideal anti-inflammatory thrombolytic and for the discrimination of the structural requirement for sEHi from that for PM.

Methods: PM activity was determined by incubating plasminogen with urokinase-type plasminogen activator and a chromogenic substrate. sEH activity was determined by incubating mouse sEH with a fluorogenic substrate.

Results: Previous investigations have demonstrated that the *N*-linked side-chain of SMTP is crucial for PM activity. Therefore, we evaluated SMTP congeners with different side-chain for their potentials in PM. The results demonstrated that the presence of both an aromatic group and a negatively ionizable group in the side chain greatly potentiates PM activity. For example, SMTP-7, which has a dimer like structure, with butane-1-carboxylic acid-connected another core unit (which has an aromatic hydroxyl group) as a side-chain, was one of the most potent congeners with respect to PM activity. The *N*-linked side-chain structures of other potent congeners included 4-phenylcarboxylic acid (SMTP-19), 3-hydroxyphenyl-4-carboxylic acid (SMTP-22), 2-hydroxyphenyl-3-carboxylic acid (SMTP-25), and (*S*)-2-phenylacetic acid (SMTP-43). On the other hand, a congener with hydrogen as a side-chain (SMTP-0), consisting of the core unit alone, showed no PM activity but had potent sEHi activity. Thus the presence of the *N*-linked side-chain is not essential for sEHi, clearly discriminating structural requirement for sEHi from that for PM activity. The congeners with hydrophilic or nonpolar side-chains, however, were essentially inactive in sEHi, and the congeners with some isomers of hydroxyphenylcarboxylic acid (including SMTP-25) were more potent than SMTP-0, demonstrating that the side-chain played a role in the tuning of sEHi activity in SMTP congeners.

Conclusions: The *N*-linked side-chain of SMTP is the crucial determinant of its PM activity, while the minimum structural requirement for sEHi is the core unit of SMTP (ie. SMTP-0). Congeners with some isomers of hydroxyphenylcarboxylic acid as a side-chain are potent with respect to both PM and sEHi activities. Certain types of the side-chain affect sEHi both positively and negatively, contributing to the tuning of sEHi activity of SMTP congeners.

PB 4.67-3

Acute fluoxetine treatment increases leukocyte-endothelial interactions in murine peritonitis

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Background: Activated platelets release serotonin at sites of inflammation where it acts as inflammatory mediator. We found recently that serotonin enhances the recruitment of neutrophils to sites of inflammation. Chronic treatment with selective serotonin reuptake inhibitors (SSRI) depletes the serotonin storage pool in platelets in humans and mice, leading to reduced leukocyte recruitment in murine experiments.
Aims: Here, we examined the direct and acute effects of SSRI on leukocyte recruitment.
Methods: Four week-old C57Bl/6 mice underwent acute (40 mg/kg i.p. 2 h before surgery) treatment with the standard SSRI fluoxetine or vehicle. Serum and plasma serotonin concentrations were measured by ELISA. Leukocyte rolling and adhesion on endothelium was analyzed by intravital microscopy in mesentery venules at resting conditions and after inflammatory stimulation with lipopolysacharide (LPS, 20 mg/kg i.p.). Leukocyte extravasation in sterile peritonitis, triggered by 1 mL 4% thioglycollate i.p. for 4 h, was measured by flow cytometry of abdominal lavage fluid.
Results: Plasma serotonin levels were elevated with large variation among individual mice 2 h after fluoxetine treatment (96 ± 32 after vehicle vs. 336 ± 188 after fluoxetine application, $P = 0.2$, $n = 6$), while serum serotonin did not change. Without further stimulation, acute fluoxetine treatment increased the number of rolling leukocytes (63 ± 8 vs. $165 \pm 17/0.04$ mm^2/min) and decreased their velocity (61 ± 6 vs. 28 ± 1 μm/s, both $P < 0.0001$, $n = 10$). Acute fluoxetine treatment itself also induced leukocyte adhesion (0 vs. $3.5 \pm 0.8/0.04$ mm^2, $P = 0.004$). Stimulation with LPS decreased mean rolling velocity and multiplied leukocyte adhesion in both groups. Adhesion was further amplified by acute fluoxetine application (27 ± 3 vs. $36 \pm 2/0.04$ mm², $P = 0.008$, $n = 10$). Leukocyte migration in sterile peritonitis was not affected by acute fluoxetine treatment.
Conclusions: Surprisingly, acute fluoxetine treatment induced leukocyte adhesion, suggesting an unknown, direct effect on endothelium. This effect could also be mediated by a transient accumulation of serotonin in the plasma, when uptake into platelets is blocked. These observations warrant further investigation since they may have implications for the treatment of depression but also for anti-inflammatory strategies.

PB 4.67-4

Effect of C1-inhibitor glycans on the kinetics of target protease inhibition

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Background: The anti-inflammatory plasma protein C1-inhibitor (C1-inh) is the major inhibitor of the proteases of the contact phase and the classical and lectin pathways of the complement systems. C1-inhibitor is a heavily glycosylated protein with 6 *N*- and 7 *O*-linked glycans known. The sialic acids on the glycans are known to prevent the *in vivo* clearance of the protein but studies to date indicate that C1-inh glycans have no effect on its complex formation with target proteases. However, the effect of C1-inh glycosylation on the kinetics of complex formation with various proteases has not been studied in detail.
Aims: Study the kinetics of inhibition of target proteases by various glycoforms of C1-inh to ascertain a role for C1-inh glycosylation in protease inhibition.
Methods: Various C1-inh glycoforms are prepared either by recombinant protein expression in *Pichia pastoris* or by *in vitro* enzymatic treatment of plasma derived C1-inh (pdC1-inh). Kinetics of C1-inh inhibition of C1s, FXIIa and kallikrein is studied with chromogenic assays.
Results: Three glycoforms of recombinant human C1-inh are successfully produced in *Pichia pastoris*: wild-type (WT), truncated form without the N-terminal 98 amino acids, thus removing all *O*-linked and three *N*-linked glycans (NT98) and a mutant where the additional three *N*-glycans of the C-terminal serpin domain are also removed (NT98Δ). The wild-type and NT98 are similar to the pdC1-inh in their inhibition kinetics to C1s, FXIIa and kallikrein. However, NT98Δ shows significantly reduced association to the proteases. This mutant also exhibits lower heat stability than the WT and NT98. Together this indicates that the three *N*-glycans of the serpin domain play an important role in stability and function of C1-inh.
Studies are now underway to determine the inhibition kinetics of additional glycoforms of the pdC1-inh. Using enzymatic treatment either entire glycans or only the sialic acids are removed. Also a fucosylated form of pdC1-inh, to mimic the glycoform expected in acute phase inflammation, has been prepared.
Conclusions: The *N*-glycans present in the serpin domain of C1-inh are crucial for the stability and function of C1-inh.

PB 4.67-5

The modulation of astrocyte functions by activated protein C

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Background: Activated protein C (APC) is polyvalent cofactor-dependent serine proteinase. APC has no only exclusive anticoagulation activity, but also protective effects in systemic inflammation and neurodegeneration. Recently neuroprotective effects of APC on stressed neurons, hypoxic brain endothelium has been found and APC may be useful in therapy of stroke (Zlokovic et al., 2005, Gorbacheva et al., 2009, Wang et al., 2012). Astrocytes are the immune cells of human brain. Many pathological processes in brain are accompanied with structural and functional changes of astrocytes. It is known that reactive astrogliosis observed during various inflammatory diseases, encephalopathy, acute brain injuries and neurodegenerative pathologies. However, the participation of APC in regulation of astrocyte function is not yet clear. Thrombin (Th) promotes cell proliferation, which correlated with astrogliosis (Nicole et al., 2005). S100B protein express in high abundance and release by astrocytes (Sen, Belli, 2007), and abnormally elevated levels of S100B contribute to the prominent reactive gliosis.
Goal. In this study the influences of APC on the morphological and functional parameters of astrocytes at thrombin-induced activation were investigated.
Methods: Experiments were performed on cultural astrocytes. Primary cultures of astrocytes from cortex were obtained from brains of 1–3-day-old Wistar rats. Confocal microscopy, Western blot methods and MTT-assay of the cultured astrocytes at Th-induced toxicity and pretreatment with APC were used.
Results: Previously we and other researchers have shown that thrombin and APC can activate the same receptors and induce the different effects (Zlokovic et al., 2005, Gorbacheva et al., 2009, 2010, Wang et al., 2012). Here we demonstrate that the effect of APC on cultural astrocytes is opposite to the high concentration of thrombin. The high concentrations of thrombin were manifested during inflammation, injuries, etc.. Th may be account for the reactive astrogliosis which is

associated with proliferation and morphological changes. Our data point to dose-depended effects of thrombin on astrocytes. The concentration of thrombin higher then 10 nM induced the significantly activation of astrocyte proliferation (on 25% compare to control), the rise of S100 protein (in two times higher then in control), the formation of stress fibres and the amount of free fields in astrocyte cultures. These results indicate that thrombin alters cell migrational status and causes morphological changes in astrocytes. We detected EPCR on cultural rat astrocytes by the special anti-EPCR-antibodies for the first time. The finding of new receptor EPCR on astrocytes make up the possibility of APC mediates itself effects not only via PAR1 receptor, but via EPCR or two receptor PAR1 and EPCR. Pre-treatment and co-incubation of astrocytes with APC led to prevention of the effect of Th on cells. APC decreases to control values the level of S100B, the proliferation of astrocytes, the actin cytoskeletal rearrangement and the amount of free fields in astrocytes culture which were induced by Th.
Conclusion: Thus APC has not only neuroprotective effects but also can prevent the activation of astrocytes and astrogliosis during pathological condition. Our results demonstrate new aspects of APC as a protective agent for brain at trauma and neuropathology.

PB 4.67-6
Gene expression profiling of mouse platelet GPCRs under chronic inflammatory conditions

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Background: Some diseases associated to chronic inflammatory stimuli, such as diabetes, obesity and rheumatoid arthritis are associated to high cardiovascular risk. In this study a mouse model of chronic inflammatory stimulus *in vivo* was set up to evaluate modifications of the expression of platelet GPCRs under these conditions
Aims : Aim of this study was to evaluate whether changes of platelet transcritpome may occur *in vivo* as a consequence of inflammatory stimuli
Protocol of the study: Ten C57/Bl6 mice were treated with low dose lipopolysaccharide (LPS), i.e. 0.2 mg/kg s.c., or saline for five consecutive days. Whole blood from treated mice was obtained by vena cava blood draw. A highly purified platelet population was obtained by standard centrifugation and washing procedures followed by negative immunoselection for depletion of WBC, RBC and endothelial microparticles contaminating the platelet suspension. mRNA and cDNA were prepared using commercially available kits. GPCRs expression profiles of platelets from LPS- and from saline-treated mice were obtained by real time PCR Taqman technology. Changes of GPCRs expression on platelets were validated by functional studies on platelets, by measuring P-selectin and aIIb-b3 integrin exposures after agonist stimulation ex vivo, using conjugated MoAbs and flow cytometry.
Results: Treatment of mice with low dose LPS caused changes of the expression of some relevant GPCRs and of the platelet function. LPS treatment caused reduced expression of P2Y1 and of P2Y12 and of CysLT2, a GPCR found to be relevant for mouse platelet function. Accordingly, platelets from LPS-treated mice had reduced exposure of P-selectin and of aIIb-b3 integrin upon ADP and LTC4 stimulation. On the contrary, LPS treatment caused increased expression of PAR3 and PAR4 on mouse platelets. Accordingly, mouse platelets showed increased response to thrombin.
Conclusions: Low dose inflammatory stimuli cause changes of GPCRs expression in mouse platelets and changes of platelet responses to agonists. Similar changes might happen in human diseases with a chronic inflammatory stimulus associated to a high cardiovascular risk

PB4.68 – Inflammation: Clinical – III

PB 4.68-1
Hypercoagulability and hypofibrinolysis in patients with human immunodeficiency virus infection partially resolve after antiretroviral treatment

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Background: Patients infected with human immunodeficiency virus (HIV) are known to have an increased risk of arterial and venous thrombosis. This may involve changes in the hemostatic status, including hypercoagulability and hypofibrinolysis. Some data suggest that antiretroviral treatment (ART) reduces the increased risk.
Aim: We evaluated levels of coagulation factors, *in vitro* thrombin generation by automated calibrated thrombography and fibrinolytic potential assessed by clot lysis time (CLT) in patients with HIV infection before starting ART and during the first 6 months afterwards.
Methods: Blood was drawn after written informed consent was obtained just before ART was started, as well at 1, 3, and 6 months after starting ART.
Results: We included 40 males (87.5% of Caucasian origin). At baseline, FVIII:C and VWF:RCF were elevated (median levels: 205 and 196 IU/dL, respectively) and free protein S (FPS) was decreased (70 IU/dL). Compared to healthy controls, CLT, endogenous thrombin potential (ETP), peak thrombin, as well as the velocity index derived from thrombin generation curves were significantly increased in patients at $t = 0$ (median levels: 49.9 vs. 67.5 min; 440 vs. 604 nM*min; 121 vs. 176 nM and 61.3 vs. 85.2 nM/min, respectively). After 6 months of treatment, FVIII:C and VWF:RCF were significantly reduced to 159, 129 IU/dL and FPS significantly increased to 84 IU/dL. CLT, ETP, peak thrombin and velocity index all decreased but this did not reach statistical significance.
Summary/Conclusions: Patients with HIV infection were in a procoagulant as well as a hypofibrinolytic state, which partially improved after 6 months of ART.

PB 4.68-2
Thrombomodulin-modified thrombin generation in patients with diffuse peritonitis

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Background: The diffuse peritonitis (DP) is a serious and common infection. Its clinical course includes haemostatic abnormalities, ranging from insignificant laboratory changes to excess activation of haemostasis that may result in localized thrombotic complications or disseminated intravascular coagulation. Thrombin generation test (TGT) with the addition of thrombomodulin may be useful as a global method to assess the individual coagulation profile in DP patients.
Aims: The aim of our study was to investigate the process of thrombin generation in patients with DP.
Methods: Venous blood was obtained from 43 patients within 24 h of the diagnosis of DP. The control group comprised 65 healthy volunteers. TGT was performed in platelet poor plasma in a Fluoroscan Ascent® fluorometer at 5 pM tissue factor concentrations in the presence of human recombinant thrombomodulin using dedicated software. The following parameters were analyzed: lag time of thrombin generation (LT, min), endogenous thrombin potential (ETP, nM*min), maximum concentration of thrombin (Peak, nM), time to

reach the peak (TTP, min), and velocity index (VI = Peak/[TTP-LT], nM/min). Statistical analysis of the results was performed by non-parametric. Methods using the median (Me), 95% confidence interval (95% CI) and Mann-Whitney U test (Statistica 6.0).

Results: Except for LT and TTP, there was significant difference between patients and controls regarding ETP (Me (95% CI): 1496 (1133–1919) vs. 779.2 (594.5–919.5), $P = 0.001$), Peak (243 (203.6–312.5) vs. 153.64 (134.9–195.2), $P = 0.04$) and VI (135.5 (87–191) vs. 72 (57–90.5), $P = 0.005$). In addition, ETP, Peak and VI demonstrated a clear tendency to increasing in non-survivors than in survivors (Me: 1574.5 vs. 1493.1, 250.4 vs. 216.5 and 114.4 vs.101.1, accordingly, $P = 0.3$).

Summary: Patients with DP are characterized by considerable hypercoagulability. Thrombomodulin-modified TGT may be a potentially useful tool to identify patients with increased risk of DP mortality.

PB 4.68-3

Elevated platelet count, platelet activation and CRP in gulf war veterans' illnesses; evidence of a chronic inflammatory state?

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Background: Gulf war veterans' illnesses (GWVI) are chronic, multi-symptom disorders in veterans of war in Kuwait and Iraq in 1990–1991. GWVI are defined according to CDC criteria by symptoms in two or three categories – fatigue, mood-cognition, musculoskeletal pain – not attributable to other diseases. The cause is unknown. A previous study observed impaired platelet activation in a majority of subjects with GWVI who had evidence of blood coagulation activation (Hannan KL, et al. Blood Coagul Fibrinolysis 2000;11:673–8).

Aims: To further characterize platelet function in GWVI we studied 43 subjects (40 male) with GWVI (GWVI+) and 21 male subjects who served concurrently in the war zone but who did not meet diagnostic criteria for GWVI (GWVI-). All participants in the study were free of infections and other known inflammatory disorders.

Methods: The following studies were performed in all subjects: platelet count, immature platelet fraction (IPF), plasma thrombopoietin (TPO), plasma C-reactive protein (CRP), light transmission platelet aggregation and ATP secretion in response to 0.5 mM arachidonic acid, 0.5 μM U46619, 1.0 μM U46619, 0.5 μM ADP, 0.5 μM epinephrine, 1 μg/mL collagen, 10 μM TRAP 6, 50 μM TRAP 6, and spontaneous platelet aggregation.

Results: Significant differences between the group means of GWVI+ and GWVI- subjects were observed for the following: platelet count (236.1 ± 55.4 vs. $205.9 \pm 44.2 \times 10^3/mm^3$; $P = 0.036$); spontaneous platelet aggregation (7.8 ± 2.6 vs. $5.6 \pm 1.2\%$; $P = 0.011$); platelet ATP secretion in response to 50 μM TRAP 6 (17.9 ± 5.5 vs. 12.8 ± 3.3 nmoles ATP/10^9 platelets; $P = 0.013$; CRP (4.0 ± 4.6 vs. 1.5 ± 1.3 mg/L; $P = 0.020$. IPF, TPO, platelet aggregation and platelet secretion in response to other agonists did not differ between GWVI+ and GWVI- subjects. Subset analyses of platelet aggregation and secretion based on medication history revealed no significant differences between GWVI+ and GWVI- subjects. Among subjects who had elevated CRP (>2 mg/L) platelet aggregation in response to 0.5 μM U46619 was significantly elevated (62.0 ± 37.8 vs. $39.8 \pm 39.3\%$; $P = 0.029$).

Summary: Platelet aggregation and secretion were not impaired in GWVI+ subjects. Platelet count, spontaneous platelet aggregation, platelet secretion in response to thrombin receptor agonist, TRAP 6, and CRP were significantly elevated in GWVI+ subjects. In addition subjects with elevated CRP had significantly elevated platelet aggregation in response to thromboxane analog, U46619. The differences between GWVI+ and GWVI- subjects were independent of medication effects.

Conclusion: Platelet activation is not impaired in GWVI, but elevated plasma CRP, platelet count, and platelet U46619-induced aggregation are characteristics not previously described that suggest GWVI is a chronic inflammatory disorder.

PB 4.68-4

Gene expression analysis in patients with spontaneous deep venous thrombosis

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Background: Deep venous thrombosis (DVT) is a multifactorial disease and in about 30% of patients no risk factor can be identified. Association of inflammation and haemostasis is thought to play a significant role in the pathogenesis of DVT.

Aim: We hypothesized that distinctive patterns of gene expression in mononuclear cells from patients with DVT could be relevant to the pathogenesis of the disease.

Methods: cDNA microarray technology (CodeLink Bioarrays) was used to study the gene expression profile of five patients with spontaneous DVT, three of their siblings and one healthy individual. DVT patients were selected based on strict clinical characteristics, including no hereditary predisposition or known risk factors. Functional analysis of differentially expressed genes was used to identify biological processes possibly differentially regulated in DVT.

Results: Preliminary analysis showed that 2% of approximately 55,000 transcripts contained in the array had significant differences in expression when spontaneous DVT patients were compared to their asymptomatic siblings and healthy individuals (2-fold change, $P < 0.05$). When all these genes were taken into account we were able to identify the deregulation of genes associated with both haemostasis and coagulation (F2RL2, LPA, FOXA2, FBLN5, PROZ, GNA12 and PAPSS2). Aiming to better describe our data, we divided genes in two groups: up (607 genes) and down-regulated (499 genes) in DVT patients as compared to siblings and healthy individual. Up-regulated genes are involved in 50 biological processes, including, chemotaxis (e.g., CCL1, CCR3, FER, IL8, NUP85, PLA2G1B) cell motility/migration (e.g., APOB, IL8, TGFBR1) and protein transport (eg., STX3, AKAP12, SMURF1). On the other hand, down-regulated genes are involved in 25 biological processes, such as, cell adhesion (e.g., CD93, CD97, ACTN1, ITGA11) and blood circulation (e.g., EPB41, LPA, NFE2). Interestingly, some genes found by Lewis et al. (2011) were similar in our study (FOXP1, ITPR2, MZF1, UBE2D2, LTB).

Conclusions: Evaluation of the significance of these distinctive gene expression profiles between patients with spontaneous DVT compared to their asymptomatic siblings and healthy individuals could reveal novel insights into the pathophysiology of the first DVT episode and of DVT recurrence, as well as new biomarkers or therapeutic targets. Prospective studies are needed to validate these results.

PB 4.68-5

Up-regulation of tissue factor, adhesion molecules, nitric oxide and adiponectin in end stage renal disease. A paradoxical interplay

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Introduction: End stage renal disease (ESRD) represents the fifth (final) stage of chronic kidney disease characterized by an established kidney failure (GFR <15 mL/min/1.73 m^2). Severe cardiovascular and cere-

brovascular manifestations progressively occur in ESRD, resulting in acute coronary syndrome and stroke. The ESRD patients also exhibit a hypercoagulable state despite the use of heparin during maintenance hemodialysis suggesting the activation of other components of the hemostatic network. To further understand the pathophysiology of ESRD, this study was designed to measure the circulating levels of tissue factor (TF), adhesion molecules, such as p-selectin (P-Sel), soluble ICAM (s-ICAM), nitric oxide and adiponectin (AD).

Methods: This study included 119 ESRD patients undergoing maintenance hemodialysis in conjunction with an ongoing IRB approved protocol on the profiling of inflammatory markers in this syndrome. Citrated blood plasma samples were collected from these patients prior to the routine dialysis session. Blood samples were centrifuged at 3000 g and platelet poor plasma was frozen at −80 °C for further analysis. Blood samples from normal healthy male and female volunteers ($n = 53$) served as controls. Nitric oxide levels (NO) were measured using a commercial kit from R&D systems (Minneapolis, Minnesota) and ELISA based methods for TF, P-Sel, s-ICAM and adiponectin were also purchased from R&D systems. A chromogenic substrate method was used to measure the anti-Xa activity in both the controls and ESRD patients. Tissue factor mediated thrombin generation (TGA) was measured using a fluorogenic substrate method (Technoclone Vienna, Austria).

Results: Tissue factor levels were found to be increased in the ESRD group (20.4 ± 6.1 pg/mL) vs. the control (11.9 ± 2.8 pg/mL). The nitric oxide level was markedly higher in the ESRD group (32 ± 17 μM) vs. the controls (7 ± 3 μM). Wide variations in the range of nitric oxide were noted (3–60 μM), whereas in the normals the range was much narrower (5–12 μM). The p-selectin levels were also elevated in the ESRD group (46 ± 20 ng/mL) vs. the control (31 ± 3 ng/mL). The soluble ICAM levels were higher in the ESRD group (250 ± 112 ng/mL) vs. the control (180 ± 19 ng/mL). The ESRD group also showed a wide variation in the ICAM level, ranging from 20 to 530 ng/mL. Interestingly, the adiponectin levels were also increased in the ESRD group (19.2 ± 9.3 μg/mL) vs. the control (11.2 ± 4.1 μg/mL). The pre-dialysis samples of the ESRD patients exhibited detectable levels of heparin like activity as evident by the inhibition of Xa (13 ± 14% inhibition), which is approximated to >0.1 U/mL heparin.

Summary/Conclusions: These studies suggest that TF, NO, p-selectin and s-ICAM levels are increased in the ESRD patient. It is of interest to note that despite that a significant number of ESRD patients were diabetic; the AD levels were increased in this group. This may be due to the repeated administration of heparin during dialysis sessions and the use of anti-diabetic and anti-hypertensive medications. Thus, these results suggest that while ESRD represents a pro-inflammatory/hypercoagulable state, the repeated administration of heparin and other drugs may contribute to the regulation of the hemostatic process and inflammatory balance. Since adiponectin has anti-atherogenic and anti-inflammatory roles, it may contribute to the regulatory processes to control inflammatory and thrombotic mediators in ESRD.

PB 4.68-6

Increased expression of caspase 4 gene in mononuclear cells from patients with deep venous thrombosis

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Background: Deep venous thrombosis (DVT) is a common and potentially fatal condition affecting 1% to 2% of the population worldwide, with an annual incidence of 1 in 500. While hereditary or acquired risk factors are identified in most patients with DVT, about 40% of patients present yet unknown risk factors.

Aims: In the present study, we evaluated the gene expression profile from mononuclear cells of patients with spontaneous DVT (SPONT), risk factor-associated DVT and patients with antiphospholipid syndrome (APS).

Methods and Results: In this study, we performed a comparative analysis of gene expression from mRNA extracted from mononuclear cells of 15 patients with previous DVT of the lower limbs. Patients were divided in subgroups: (i) SPONT without any known hereditary thrombophilia ($n = 5$); (ii) risk factor-associated DVT (either acquired or hereditary thrombophilias) ($n = 5$); and (iii) patients with APS-associated DVT ($n = 5$); all compared to the healthy volunteers. Using bioarray technology, 60 upregulated and 56 downregulated genes were identified in DVT patients when compared to healthy volunteers. Azurocidin 1 (AZU1), Caspase (iv) (CASP4), Cartilage-associated protein precursor (CRATP), Vascular endothelial growth factor (FLTI), Immunoglobulin Heavy Chain Constant region mu (IGHM), Glutathion S-transferase, microsomal 2 (MGST2) and RNA polymerase II subunit (POLR2J) genes were selected for validation with quantitative real-time PCR (qRT-PCR).

Conclusion: The increased expression of CASP4 was confirmed in the same sample of DVT patients without any known risk factors ($P = 0.050$; $n = 4$). CASP 4 has been described induce NF-kB activity and acts in the activation of Caspase 1 which participates in the conversion of proIL1b and proIL-18 to IL1-b and IL-18. In addition, IL-18 may induce the adhesion of T cells, increase the immune response of natural killer cells, enhance production of interferon-g and induce chemotaxis and activation of monocytes. Increased CASP4 expression could thus be involved in the pathogenesis of DVT in these patients. Further studies are warranted in larger cohorts to confirm these findings.

PB4.69 – Non-Inherited Risk Factors VT – V

PB 4.69-1

Prevalence of clonal populations of hematopoietic cells with paroxysmal nocturnal hemoglobinuria phenotype in patients with splanchnic vein thrombosis

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Background: Venous thromboembolism is a serious complication in patients with paroxysmal nocturnal hemoglobinuria (PNH) and frequently occurs in unusual sites, such as the splanchnic and cerebral veins. In particular, PNH is a well recognized cause of Budd Chiari syndrome. It has been hypothesized that the presence of mutant PNH clones can be associated with an increased risk of splanchnic vein thrombosis (SVT) even in the absence of overt disease, but the prevalence of PNH clones in non-selected SVT patients remains unknown.

Aims: In a multicentre cross-sectional study, we aimed to determine the prevalence of PNH clones in a group of patients with SVT and without overt PNH.

Methods: Patients with objective diagnosis of SVT within the previous 2 years were eligible for the study. Patients with known or clinically suspected PNH and patients receiving treatments potentially interfering with laboratory assessment of PNH clone were excluded. Information was collected on demographic characteristics, on the site of SVT and the presence of risk factors. All patients underwent blood sampling and the presence of PNH clone was centrally assessed using flow cytometric analysis of granulocytes and monocytes, counting the number of FLAER -ve events Because of the high-sensitivity tech-

nique used, those samples with FLAER -ve events higher than the 95th percentile underwent a second evaluation to avoid false-positive results.
Results: A total of 199 SVT patients were enrolled in the study, 116 (58.3%) were males, mean age was 54 years (range 17–94). Site of thrombosis was portal in 100 patients, mesenteric in 67, splenic in 37, and supra-hepatic in 10. Thrombophilia was diagnosed in 70 (35.2%) patients, whereas JAK2 mutation was present in 28 of 125 (22.4%) screened patients. Other risk factors for SVT included cirrhosis in 28 patients (14.1%), recent surgery in 22 patients (11.1%), and haematological disease in 21 patients (10.6%); in 71 (35.9%) patients SVT was defined idiopathic. Mean time elapsed between SVT diagnosis and study testing was 21 months. Cells with the PHN phenotype (defined as clustering of FLAR -ve events, >5 cell per 50,000 events, confirmed in two independent samples) were detected in 2 (1.01%, 95% CI 0.18–3.99) patients. SVT occurred in the superior mesenteric vein in one patient and in the portal vein in the second patient. SVT was secondary to IBD in one patient and was idiopathic in the second patient. Time between SVT diagnosis was 2 months and 24 months, respectively.
Conclusions: PNH clones can be detected in patients with a history of SVT and no clinical manifestations of disease. Future studies are needed to explore the potential role of this finding in the pathogenesis of SVT and to better define in larger cohorts the cost-effectiveness of screening for PNH clones in this population.

PB 4.69-2
Systemic hypoxia and risk of venous thromboembolism – the Tromsø study

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Background: Previous autopsy and venography studies indicate that venous thrombosis is initiated in the valvular sinus of veins in an environment characterized by local hypoxia and intact endothelium. Experimental studies have shown thal local hypoxia promotes endothelial expression of adhesion molecules and activation of monocytes with subsequent formation of microparticles with expression of ligands for adhesion receptors at the endothelium and expression of tissue factor (TF) which may initiate targeted thrombus in the valvular sinuses. To the best of our knowledge, however, no previous study has investigated the association between systemic hypoxia and future risk of venous thromboembolism (VTE).
Aims: We wanted to investigate the association between systemic hypoxia, defined by oxygen saturation, and future risk of VTE in a population-based prospective cohort study.
Methods: Pulse oximetry was measured in 8684 men and women who participated in the fifth or sixth Tromsø study, conducted in 2001–2 and in 2007–8. Events of VTE were registered from data of study enrolment until the end of follow-up, December 31, 2010. The best of three pulse oximetry measurements were used in analyses, and values below 70% were considered as invalid. Cox proportional hazard regression models were used to assess age-adjusted and multivariable hazard ratios (HR) with 95% confidence intervals (CI). Age, sex, body mass index and smoking status were included in the multivariable analyses. The study was approved by the regional committee for research ethics, and all participants gave their written consent to participate.
Results: In total, 188 VTE events were registered during a median of 6.62 person-years (Incidence rate: 3.57, 95% CI: 3.10–4.12). The risk of VTE increased by 18% per 3% decrease in oxygen saturation (multivariable HR: 1.18, 95% CI: 0.91–1.55). Subjects with oxygen saturation <97% (21% of the population) had a higher risk of VTE compared to subjects with oxygen saturation of 97% or above (multivariable HR: 1.54, 95% CI: 1.13–2.09). Low oxygen saturation was especially associated with provoked VTE (multivariable HR: 1.65, 95% CI 1.12–2.43) whereas the risk estimates for unprovoked VTE were lower (HR: 1.38, 95% CI: 0.83–2.30).
Conclusions: Our findings may imply an inverse association between systemic oxygen saturation and future risk of VTE. It is not known, however, whether the risk of VTE is a direct consequence of low systemic oxygen saturation or is due to unrecognized confounders.

PB 4.69-3
RDW-CV and MPV as a risk indicators for deep venous thrombosis

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Background: Inflammation has been related to venous thromboembolism, but it is unknown whether MPV and RDW are associated with venous thrombosis risk.
Aims: To study the association of MPV and RDW as indicators of inflammation with venous thromboembolism
Methods: We included 1915 healthy new donors and 150 new VTE patients who visited the blood bank or anticoagulation centre in Nijmegen or Arnhem, the Netherlands, between september 2011 and february 2012. Blood samples were taken at the first visit, and then after 1 month, 3 months and 6 months if still under treatment for patients, whereas donors were sampled at each visit, varying over time. Donors were participants in the ZINC study. All blood samples were analysed with an automated cell counter at the quality control lab of the blood bank in Nijmegen. Patient and donor characteristics were collected from the files at entry.
Results: More patients than donors were male, and patients were 30 years older. At the first visit, MPV was somewhat lower in patients than in controls, and increased over time. Red cell diameter width was statistically significantly higher in patients than in controls, and remained higher. Further results will be presented at the ISTH.
Summary/Conclusion: MPV was not associated with venous thromboembolism in our study, whereas RDW was. One explanation may be that the MPV had changed prior to inclusion in our study due to the thromboembolic even tor its treatment. RDW remained high in patients with thromboembolism. The fact that we included patients on average 6 days after their thromboembolic event and first treatment may have resulted in these findings.

PB 4.69-4
Clinical presentation of isolated distal deep vein thrombosis differs significantly from proximal disease states

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Background: There is ongoing lack of consensus on the management of isolated distal deep vein thrombosis (IDDVT). While some clinicians treat as they would for proximal disease, other experts manage the condition as a distinct entity. Limited comparative literature exists on risk profile and presenting clinical features in direct support of this assertion.
Aims: We sought to determine the clinical and aetiological presentation of IDDVT and compare it to that of proximal disease.
Methods: An observational, ambulatory prospective cohort study. Consecutive patients attending an established outpatient thrombosis service during the year 2011 were included. Historical, aetiological and clinical data were collected routinely by attending physicians on a

standardized proforma during the initial clinical encounter. This data was anonymised and stratified by outcome at vascular ultrasound. Descriptive and regression analyses were performed to identify and quantify predictors of distal vs. proximal thrombotic disease.

Results: 1888 patients attended the service with suspected deep vein thrombosis, with a retrospective pre-test probability of 8.3% for acute thrombotic disease. Distribution of acute disease was shared evenly between distal (78 cases [49.7%]) and proximal (79 cases [50.3%]) thrombi.

Distal cases were significantly more likely to be provoked ($P = 0.025$), right sided ($P = 0.039$) and to present with localised pain ($P = 0.003$). Distal cases were less likely to present with entire leg swelling or calf swelling >3 cm (both $P < 0.01$). Laboratory markers of clot burden and inflammation were all significantly reduced in the IDDVT group compared to proximal cases.

Conclusions: Patients with IDDVT are significantly different to those with proximal disease, regarding clinical presentation and risk profile. Our results concur with previous evidence supporting the idea of IDDVT as a distinct disease entity. Diagnostic algorithms which place clinical importance on accurate diagnosis of IDDVT could potentially use these findings to guide investigative strategy and influence therapeutic management.

PB 4.69-5

Pulsed methylprednisolone therapy markedly increases thrombin generation potential in a rabbit experiment

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Background: We use pulsed methylprednisolone (m-PSL) therapy for various diseases, including intravenous immunoglobulin therapy-resistant Kawasaki disease, encephalopathy and immune thrombocytopenic purpura. Heparin is often administered along with pulsed m-PSL therapy for thrombosis prevention. However, the effects of steroids on blood coagulability and the underlying mechanisms remain to be clarified.

Aims: To clarify the effects of steroids on blood coagulability, we measured thrombogenicity using the thrombin generation test (TGT) before and after pulsed m-PSL injections in rabbits.

Methods: First, we injected m-PSL (30 mg/kg/day, 3 days) into the ear veins of rabbits (Japanese white species, male). The dose of m-PSL was determined according to the treatment protocol for humans. As the control, we injected rabbits with PSS (physiologic saline solution). We examined the parameters of TGT, prothrombin time (PT), activated partial thromboplastin time (APTT), fibrinogen, antithrombin, soluble fibrin (SF), D-dimer, factor VII activity (FVII:C), factor VIII activity (FVIII:C), factor IX activity (FIX:C), and factor XI activity (FXI:C), before and after the injection. Next, we investigated the effects of adding m-PSL or recombinant activated factor VII preparation (rFVIIa) on the thrombin generation potential of rabbit plasma *in vitro*.

Results: Among the TGT parameters, the lag time was shortened (0.8 ± 0.11 times) and the endogenous thrombin potential (ETP) (1.31 ± 0.31 times) and peak (1.91 ± 0.69 times) were increased in the pulsed m-PSL group ($N = 29$) as compared to the control group ($N = 12$), indicating a marked increase in thrombin generation potential. Plasma FVII:C was increased (1.68 ± 0.47 times) in the pulsed m-PSL group. The increased thrombin generation potential could be reproduced *in vitro* by the addition of rFVIIa preparation, but not by adding m-PSL. Neither SF nor D-dimer levels were increased after pulsed m-PSL administration.

Conclusion: In conclusion, our results suggest that administering m-PSL pulsed therapy to rabbits increases FVII:C and markedly increases thrombin generation potential. However, neither SF nor D-dimer levels were increased, indicating that thrombosis was not induced by pulsed steroid therapy alone. Therefore, we propose that administration of an anticoagulant during steroid pulse therapy may be clinically appropriate when thrombotic risk factors are associated with the underlying disease.

PB 4.69-6

Incidence of venous thromboembolism (VTE) after major surgeries and proposal of evidence-based Korean guidelines for the prevention of VTE

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Background: VTE is a well-recognized, public health issue in the world. There are well-known international guidelines for thromboprophylaxis. But those are mostly based on the epidemiologic data of Western population and those are not currently applicable for Korean medical environment.

Aims: This study was performed to evaluate the incidence rate of symptomatic VTE after major operations such as orthopedic and non-orthopedic surgeries for the frequent cancers and benign diseases in Korea.

Methods: We used the database of the Korean Health Insurance Review and Assessment Service which is a unified insurance system covers almost Korean. The post-surgical claims data for recent 5 years from 2007 to 2011 were extracted, searched for major surgeries using both the operation and major diagnosis codes. Total 1,009,694 cases were selected. Finally VTE cases within 5 weeks after operation were sorted out using both diagnosis codes of VTE and prescription codes of heparin or low-molecular-weight heparin. VTE means all VTE including deep vein thrombosis (DVT) and/or pulmonary embolism (PE). VTE other than DVT and PE such as upper extremities or splanchnic vein thrombosis were excluded. Overall incidences of VTE, DVT and PE were 0.71%, 0.44% and 0.45%, respectively. Each surgery was grouped as moderate, low and very low risk of VTE according to VTE incidence. Total knee or hip replacement, hip fracture surgery, operations for colorectal, pancreas, ovary or esophageal cancer belonged to high risk and recommended for the use of LMWH, UH or mechanical prophylaxis. Surgeries for stomach, hepatobiliary, cervix, lung or brain tumor were at low risk and needed mechanical prophylaxis. Surgeries for breast, kidney, bladder, prostate cancer, and benign diseases had a very low risk of VTE and need early ambulation only.

Conclusion: Based on these results, we propose the second Korean guidelines for the prevention of VTE after surgery.

PB4.70 – Non-Inherited Risk Factors VT – VI

PB 4.70-1

The risk of venous thrombosis after a symptomatic arterial event in an older population

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Background: Age increases the risk of venous thrombosis (VT) and arterial thrombosis; several studies have shown that a prior arterial thrombosis increases the risk of subsequent VT. Whether this is the case in an older population is not known.

Aims: To determine whether an association is present between a history of a symptomatic cardiovascular event and the risk of VT in an older population.

Methods: These analyses were performed in the AT-AGE study (Age and Thrombosis Acquired and Genetic risk factors in the Elderly). The AT AGE study is a population based two-center case-control study that included 406 patients with a first deep venous thrombosis of the leg or a pulmonary embolism in Leiden, The Netherlands and Vermont, US. The study was approved by the Medical Ethical Committees of the Leiden University Medical Center and of the University of Vermont, USA, respectively. All participants signed the informed written consent. Control subjects aged 70 years and older were recruited from general practitioners (n = 433). Individuals with active malignant disease were excluded. Information on a history of arterial disease was obtained from an interview at home. Arterial events were defined as cardiovascular events, i.e., a history of angina, myocardial infarction, heart bypass surgery, and cerebrovascular events, i.e., a history of stroke or transient ischemic attack. We evaluated the risk of thrombosis associated with a history of an arterial event. For cases, time between the most recent arterial event and the occurrence of venous thrombosis was calculated, and for control subjects the time between the most recent arterial event and the index date. The risk of VT after a symptomatic arterial event was calculated for different time intervals (time since arterial event: <1 year, 1–5 years, and >5 years). Odds ratios (OR) and 95% confidence intervals (95% CI) were assessed using logistic regression analysis, after adjustment for age, sex, body mass index (BMI continuous) and study site (Leiden vs. Vermont).

Results: Among 404 cases (age 78.7 ± 5.6 years, range 70.0–100.9) and 431 control subjects (age 77.5 ± 5.4 years, range 70.3–96.3), a positive history was reported by 138/404 (34.2%) cases and 133/431 (30.9%) control subjects (OR 1.1, 95% CI 0.8–1.5). The year of occurrence of the arterial event was available in 125 cases and in 123 control subjects. Median time since last arterial disease was 6 years (range 0–58 years) for the cases and 8 years (range 0–36 years) for the control subjects. Of the cases 18 (6.3%) and 13 (4.2%) control subjects had an arterial event in the year prior to the index date (OR 1.3, 95% CI 0.6–2.8). No increased risk of thrombosis was found either for the time intervals of 1–5 years (OR 0.9, 95% CI 0.6–1.5) and >5 years (OR 1.2, 95% CI 0.8–1.8) prior to the VT.

Conclusion: Overall, we observed no association between a history of arterial disease and risk of venous thrombosis in this elderly population.

PB 4.70-2

Risk of atherothrombotic events in patients after deep vein thrombosis

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Background & Aims: Several studies have shown elevated incidences of atherothrombotic events (ATE) in patients with unprovoked venous thromboembolic events (VTE). This association remains understudied in patients with isolated deep vein thrombosis (DVT). We evaluated the incidence of ATE in patients with DVT and compared it to patients with provoked DVT and controls without DVT.

Methods: Patients with compression ultrasonography (CUS) proven unprovoked DVT, provoked DVT, and symptomatic patients, in whom DVT was excluded by CUS, were followed and scored for the occurrence of ATE.

Results: 170 patients with provoked, 74 patients with unprovoked DVT and 991 patients without DVT were included. During follow-up 128 ATE occurred (incidence 6.5/100 patient-years). Adjusted hazard ratio (HR) was not different between patients with DVT and without DVT (1.4; 95% CI 0.76–2.4). In contrast, patients with unprovoked DVT suffered ATE more frequent than provoked DVT patients (3.16; 95% CI 1.1–9.1) and control patients (HR 2.8; 95% CI 1.3–5.7). Notably, when fully adjusted for known ATE risk factors the risk differences between controls, provoked and unprovoked DVT patients diminished: HR 1.1 (95% CI 0.47–2.5) and 1.7 (95% CI 0.80–3.6) respectively.

Conclusion: Our study showed that risk of ATE in patients with unprovoked DVT was higher than in patients with provoked DVT or control patients. Interestingly, after full adjustment for multiple known risk factors, the significant difference between unprovoked DVT patients and provoked DVT patients or control patients diminished. This implicates that the correlation between ATE and DVT is non-causal and the measured cardiovascular risk factors are confounders in this correlation.

PB 4.70-3

An age-related prospective cohort study in patients with retinal vein occlusion: risk factors, antithrombotic treatment and outcome

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Background: Cardiovascular (CV) risk factors and thrombophilic states are involved in the onset of retinal vein occlusion (RVO), but their role in relation to patient's age is debated. Treatment with antithrombotic drugs is controversial, although RVO has been surmised as a predictor of a subsequent vascular event.

Aims: To evaluate risk factors, the effects of antithrombotic therapy and the occurrence of vascular events after a first episode of RVO, according to patient's age at RVO onset.

Methods: Patients with central or branch RVO confirmed by fluorescein angiography were enrolled in this prospective cohort study after obtaining informed consent according to Helsinki Declaration. Common CV risk factors (hypertension, diabetes, hyperlipemia, obesity, smoking), congenital and acquired thrombophilia were evaluated. Patients received anticoagulants (LMWH and warfarin) or aspirin for at least 3 months and they were followed every 6–12 months. Vascular events after RVO were recorded.

Results: One-hundred patients with central RVO and 32 with branch RVO were enrolled. Five (8.3%) patients younger than 50 years and 4 (5.5%) over 50 years had a hereditary thrombophilic defect, and the difference was not significant. Antiphospholipid antibodies, hype-

rhomocysteinemia, or PAI-1 increase were present in 29% of patients with no age-related differences. One or more CV risk factors were found in 35 (58%) patients of the younger group, and in 66 (91%) of the older group ($P < 0.001$). Antithrombotic treatment lead to both a satisfactory recanalization of occluded veins and visual acuity improvement especially in younger patients. Vascular events occurred in 19 (14%) cases after 4 ± 3.3 years from RVO; they were more frequent in older than in younger patients (22% vs. 5%, P 0.005).
Conclusion: Distribution of CV, but not of other prothrombotic risk factors, seems to be influenced by age in RVO patients. Patients with a first episode of RVO, especially those older than 50 years, are likely at risk of a subsequent vascular event.

PB 4.70-4
Association of perioperative inflammation and coagulation status with delayed VTE after major surgery

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Background: Patients presenting for major abdominal surgery are at increased risk of subsequent venous thromboembolism (VTE). Inflammation and hypercoagulable state are both implicated in the pathogenesis of VTE. It is unclear if perioperative inflammatory biomarkers and viscoelastic coagulation testing are predictive of future VTE.
Aims: We conducted a posthoc analysis of a study on major abdominal surgery patients to assess if the severity of perioperative inflammation and hypercoagulable state on viscoelastic coagulation testing was correlated with VTE at 6 months after surgery.
Methods: This study was conducted with full IRB approval. Patients were randomized to 1 of 2 heparin regimens for postoperative VTE prophylaxis while in the hospital (subcutaneous heparin or low-dose heparin infusion). Blood samples were drawn in the OR prior to incision, and then daily for 5 days. Patients were followed for 6 months for symptomatic VTE (any pulmonary embolism + symptomatic DVT).
Results: 110 patients were randomized. Follow-up was completed in 100% of patients. 18 patients had symptomatic VTE at 6 months. In bivariate analysis of the intention-to-treat population, age, gender, ICU length of stay, smoking status, in-hospital VTE prophylaxis regimen, and BMI had no association with 6 month VTE. Peak IL-6, IL-8, IL-10, and TNF-a levels also showed no correlation with 6 month VTE. The Sonoclot clot rate on day 5 showed a trend towards elevation in patients subsequently diagnosed with subsequent 6 month VTE (40.4 ± 15.1 U/min in patients with VTE vs. 31.9 ± 16.4 U/min in patients without VTE, $P = 0.07$), although this difference did not reach significance. Within the group randomized to low-dose heparin infusion, this difference became significant (48.4 ± 11.8 U/min in patients with VTE vs. 29.9 ± 17.4 U/min in patients without VTE, $P = 0.01$).
Conclusions: In this small study, clinical variables and inflammatory cytokines levels showed no association with delayed postoperative VTE after major abdominal surgery. However, the Sonoclot clot rate, a measure of activated fibrin polymerization, showed a trend towards elevation on the 5th day after surgery in patients who eventually developed symptomatic VTE within 6 months. Further prospective study of the Sonoclot and other viscoelastic coagulation tests for the identification of surgical patients at high risk of VTE after hospital discharge is necessary.

PB 4.70-5
Root cause analysis of hospital-acquired venous thromboembolism: a quality improvement initiative

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Background: Hospital-acquired venous thromboembolism (HA-VTE) is a serious but largely preventable complication of hospitalization that can result in significant morbidity, mortality and expenditure of resources. A hospital-wide initiative involving guidelines, order sets, education, and audits and feedback has been in place for over 5 years to improve the use of appropriate thromboprophylaxis at a university hospital. Despite these efforts, monthly audits demonstrate that an average of 13% of inpatients do not receive appropriate thromboprophylaxis. In an effort to further improve quality of care, an additional initiative was implemented to identify cases of HA-VTE, conduct a root cause analysis (RCA) for each to identify causative and contributing factors, and provide feedback to the healthcare team involved in the specific patient's care. HA-VTE was defined as symptomatic and occurring in hospital or within 2 months of discharge.
Aims: To improve the quality of patient care with respect to prevention of VTE through the identification of cases of HA-VTE, conduction of RCA and feedback to the healthcare team.
Methods: Cases of symptomatic, objectively-confirmed deep vein thrombosis (DVT) and/or pulmonary embolism (PE) that occurred more than two calendar days after admission and up to 2 months after hospital discharge were identified through the ultrasound imaging department. All cases with HA-VTE were reviewed and classified as potentially prevention HA-VTE (PPHA-VTE) or unpreventable HA-VTE based on hospital thromboprophylaxis policy. For patients with PPHA-VTE, written feedback was provided to the healthcare team and data was entered into an ongoing database to determine both temporal and patient group trends.
Results: In 2012, 73 cases of HA-VTE (average of 1.4 per month) were identified. There were 32 cases (44%) of DVT and 41 cases (56%) of PE. 51 (70%) of the cases were diagnosed during admission and 22 (30%) following discharge. No fatal PEs were identified. RCA of the cases revealed that thromboprophylaxis had been prescribed according to hospital policy in 71%. Approximately half of the cases in which 'appropriate' prophylaxis had been provided were seen in general surgery, gynecologic oncology and trauma patients. 7(22%) of the cases of DVT and 14 (34%) of the cases of PE were determined to be potentially preventable. Approximately half of these cases were seen in general or orthopedic surgery patients. The reasons for which cases were deemed potentially preventable included: no prophylaxis given (19%), inappropriate delay in initiation (43%), incorrect dose of thromboprophylaxis (29%), and inadequate duration (10%). At this stage of data collection and review, there is insufficient evidence of trends to suggest a change in thromboprophylaxis policy.
Summary/Conclusion: This feasible methodology provides insight into the burden of HA-VTE not seen with other strategies. Over the year, among the 73 clinically-important HA-VTE events, 29% were associated with suboptimal thromboprophylaxis. Feedback to the healthcare teams responsible for the care of these patients has led to discussions and strategies for further quality improvement. Ongoing data collection will help guide modifications to our hospital-wide thromboprophylaxis policy and suggest more targeted quality improvement strategies to ensure optimal patient care.

PB 4.70-6

Splanchnic vein thrombosis in Siriraj hospital: etiology and outcome

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Background: Splanchnic vein is composed of portal, splenic, hepatic, and mesenteric veins. Splanchnic vein thrombosis (SVT), an uncommon condition, has multiple etiologies such as malignancy, some hematologic condition, intraabdominal infection with variable outcome.
Aims: To evaluate etiology of splanchnic vein thrombosis and mortality rate at 1 year.
Methods: Patients with a diagnosis of SVT based on ICD-10 code since January 2008 to December 2009 were included. We performed retrospective chart review for etiologies, clinical manifestation, laboratory results, treatment and outcome of SVT.
Results: The study included 147 patients (91 men) with a median age of 56 years (range 18–90). The sites of SVT included portal vein (89.8%), mesenteric vein (18.4%), splenic vein (12.9%), and hepatic vein (4.1%) whereas 27.9% had multiple vessel thrombosis. Most common etiology was malignancy (68.0%, such as hepatocellular carcinoma [54.4%] and cholangiocarcinoma [8.2%]) followed by cirrhosis (52.4%), local factor (7.5%, such as hepatobiliary infection [5.4%]) and myeloproliferative disorder (2.7%). The most common clinical manifestation was abdominal pain (55.7%) followed by ascites (23.8%), hepatomegaly (23.8%) and weight loss (21.7%). All-cause 1-year mortality rate was 56.5%. Multivariate analysis found that malignancy was significantly associated with 1-year mortality rate (81.2% vs. 10.2%; adjusted HR 13.5, 95% CI 5.2–35.0, $P < 0.001$). Preferred management in malignancy associated SVT was conservative treatment (70.7%). Some patients (19.7%) were treated by anticoagulant mostly in non-malignant cause and they had bleeding complication rate of 13.8%. Among 54 patients who had repeated imaging within 1 year, 37% showed improvement of thrombosis.
Conclusion: Most common etiology of splanchnic vein thrombosis was hepatocellular carcinoma. One-year mortality rate of splanchnic vein thrombosis was 56.5%. Malignancy related SVT had a higher mortality rate than non-malignancy related SVT significantly.

PB4.71 – Paediatric Thrombosis – V

PB 4.71-1

Thromboembolic events emerging during the treatment of childhood acute lymphoblastic leukemia

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Background: Thromboembolic events are serious complications of childhood acute lymphoblastic leukemia (ALL) therapy, that may result in significant morbidity and occasionally mortality. The rate of thrombosis in 1752 children with ALL from 17 prospective studies was 5.2%. In these studies, the relationship between thromboembolic events and treatment with steroids and L-asparaginase were reported.
Aim: The aim of this study is the evaluation of the prothrombotic risk factors in children with ALL under modified BFM protocol.
Patients and Methods: Three hundred and thirty children with newly diagnosed ALL were recruited in observational cohort study conducted between January 2004 and May 2012. Patients with ALL were searched for any accompanying prothrombotic risk factors. All symptomatic thromboembolic events diagnosed were recorded.
Results: Nine patients of the overall 330 (2.7%) had thrombosis, of them 66.7% had venous thrombosis, and 33.3% had arterial thrombosis. Deep venous thrombosis of the lower extremity was present in one case. Other eight patients had cerebral thrombosis (three arterial and five venous). All the cerebral thrombosis had developed at the induction phase of the therapy. Prothrombotic risk factors were detected in 6 (66.7%) patients (2 had protein S, 1 had protein C, 2 had homozygous, 1 had heterozygous Factor V Leiden deficiency. One patient had prothrombin 20210A mutation). One of the patients with arterial cerebral thrombosis had died. The rate of death due to thrombosis in children with ALL was found as 0.3%.
Conclusions: Thrombosis in children with ALL is an important complication with high morbidity and mortality. As the patients that we follow, when the rate of central venous catheter use has declined, the rate of thrombosis also decrease and then cerebral thromboembolic events become significant relatively. Patients with the prothrombotic risk factors should be followed- up carefully for the possible emergence of cerebral thrombosis and strategies of antithrombotic prophylaxis should be investigated in this setting.

PB 4.71-2

Does management of warfarin by patients with home INR testing improve health related quality of life in children/families

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Background: Health Related Quality of Life (HRQOL) refers to how an individual's wellbeing may be affected by health and therapeutic management strategies. HRQOL is essential to develop appropriate therapeutic choices, generate research strategies, and adjust policies for improvement of healthcare adherence and outcomes. HRQOL is the 'gold-standard' measurement for patient relevant outcomes. Management of warfarin by patients (MWP) with home INR monitors (i.e. families make their own dose adjustments based on guidelines) has demonstrated that families make suitable dosing adjustments, and have improved control as illustrated by time in therapeutic range. The child and family may also have greater investment in their care with increased responsibility to adhere to therapeutic guidelines. The effect of MWP on HRQOL as a contributing factor to adherence must be determined.
Aims: To determine whether HRQOL improves with the long term implementation of MWP.
Methods: The warfarin KIDCLOT PAC QL© was administered to families on self-management over the period of years; baseline, 1, 3 and 5 years after the initiation of MWP. Each patient and parent was compared to themselves over the years. Focus groups were held during group anticoagulation clinics to ensure patients had opportunity to express their values, perceptions, concerns, and preferences. Three separate focus groups were held: MWP, adolescent MWP, and patient whose warfarin was dose adjusted by healthcare providers. Two staff members/group. Responses were coded to identify themes by two independent researchers.
Results: The mean impact on HRQOL was 29% pre, 33% at 1 year and 28% at 2 years on MWP for parents. The mean impact on HRQOL was 30% pre, 25.7% at 1 year, and 23.5% for children/teens. Focus groups revealed although caregivers felt anxiety about the increased responsibility ('makes me nervous because I feel more responsible') in managing their child's warfarin, a strong preference for MWP existed, as reflected in comments 'makes you more knowledgeable', 'increased flexibility', and a 'greater degree of independence'. Teens indicated that they had 'better awareness' and were 'more independent'. One family decided to leave MWP due to anxiety but then returned to MWP practice after a few months. Five year data is pending as group anticoagulation clinics are held in spring due to access to healthcare in a northern environment.
Conclusions: MWP provides the child/family more investment in managing their health condition as indicated by focus group themes. The KIDCLOT PAC QL © parent proxy inventory measured an increased impact of warfarin on HRQOL in the first year of MWP then a return

to baseline. Focus groups revealed that the patents felt the responsibility of MWP suggesting caregivers have improved awareness and knowledge of the impact of warfarin on their lives. There was a strong preference for MWP secondary to increased understanding, independence, and confidence documented in the focus groups. Finally, MWP nurtures awareness and autonomy in teens. Increased duration of MWP may result in decreased stress, anxiety and increased HRQOL through continued patient/family knowledge acquisition with the support of the health team.

PB 4.71-3

Postthrombotic syndrome in children with limb venous thromboembolic events

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Background: Postthrombotic syndrome (PTS) is a chronic complication of deep vein thrombosis (DVT), increasingly recognized in children during the last decade. PTS may affect patient's quality of life for many years, due to its devastating sequelae.
Aim: To evaluate the incidence, clinical characteristics, management, and potential predictive factors for PTS in children with a history of DVT.
Patients and Methods: Data of children with DVT in upper and lower extremities, referred to our Unit from January 2000 to January 2012, were retrospectively analyzed. Children suffered malignancies and followed at oncology departments of our hospital were excluded from the analysis, because of incomplete data. For evaluation of severity of PTS the modified Vilalta scoring system was applied.
Results: Thirty children (60% males) were diagnosed with DVT at a median age of 5.5 years. The median follow-up period was 6 years (2–13 years). DVT was present in upper extremities in only three patients, while the rest (90%) suffered a lower extremity DVT with extension to inferior vena cava in 9 (33.3%) and concomitant pulmonary embolism in 3 (11%) patients. Positive family history of DVT or thrombophilia was reported in six cases. PTS was developed in nine patients (30%) −5/9 were boys. Moreover, one additional patient endured amputation of both his limbs due to extensive necrosis. PTS, occurred after a median period of 3 months (1–12 months) following DVT, was initially graded mild in six children and moderate in 3. In comparison to upper extremity DVT, lower extremity thrombotic event was associated with development of PTS ($P < 0.005$). Highly elevated D-dimers were present in all nine PTS cases at DVT diagnosis. Extensive occlusion ($n = 9$), presence of thrombophilic factors -especially a natural inhibitor deficiency ($n = 4$) – and recurrence of DVT ($n = 4$) were associated with development of PTS. All PTS patients had been treated upon DVT diagnosis with anticoagulants for a median period of 24 months (3–60 months), while graduated elastic compression stockings were applied in 8/9 for 1–4 years, soon after PTS development. Appropriate diet was initiated in one obese female adolescent for achieving the optimal body weight. During a median observational period of 5 years (1.5–10 years), the outcome of PTS was: complete recovery in one boy with moderate PTS, improvement from moderate to mild in one boy with initially diagnosed moderate PTS, while PTS grade was defined as unchanged in all six patients with mild and in one with moderate PTS. The better than expected outcomes in all cases may be attributed to early therapeutic interventions.
Conclusion: Given the chronic debilitation following PTS of lower limb in children, it is crucial that future larger prospective multi-centre studies be organized, with the aim to provide better understanding of pathophysiology, risk factors and possible preventive tools for paediatric PTS, in order to improve the long- term quality of life of young patients.

PB 4.71-4

Epidemiology of thrombosis in children with cancer

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Background: A large amount of research has focused on the epidemiology of thrombosis in adult cancer populations however little has been done in paediatric populations. Many studies examining children with cancer, particularly ALL have identified factors that increase the risk of thromboembolism (TE) including chemotherapy, central venous lines (CVL's), prothrombotic defects, age and surgery. However, there are no clear guidelines for identification of high-risk groups, prophylaxis or management of TE complications in paediatric cancer patients.
Aims: To review the literature regarding the epidemiology, mechanisms, risk factors, prophylaxis and outcomes of thrombosis in children with cancer and to identify areas where more research is needed.
Methods: The PubMed database was searched from 1.01.1990 to 21.12. 2012 using the search terms: cancer, children, epidemiology, paediatric, thrombosis, and thromboembolism.
Results: The incidence of symptomatic VTE in children with cancer is 2.1–7.9%, 30% of which are associated with CVL's. The incidence of asymptomatic VTE is ~40%. The most common location of VTE is CNS representing ~50% of TE events. DVT, particularly upper extremity DVT is common and has been strongly associated with CVL's. Lower extremity DVT, also commonly reported, is associated with prolonged duration of immobilisation in hospitalised patients. The incidence of PE accounts for 20% of VTE events and arterial thrombosis is quite rare at ~3.6%.
Key characteristics that increase the risk of thrombosis in paediatric cancer patients include the cancer type (ALL, AML, lymphoma and sarcoma), age (older patients and neonates), the presence of a CVL, presence of pulmonary/intra thoracic disease, chemotherapy (concomitant treatment of L-asparaginase and corticosteroids particularly during induction therapy, as well as growth factor agents) and prothrombotic defects (FV Leiden mutation, prothrombin G20210A and MTHR variant, particularly children with haematological malignancies).
Outcomes for paediatric cancer patients who have suffered from TE vary but include post-thrombotic syndrome, PE, recurrent TE, destruction of upper venous system and death.
Recent studies suggest that higher D-dimer levels, platelet count and activity allow better prediction of a child's hypercoagulable state and therefore their risk of TE.
Conclusion: The factors involved in aetiology of thrombosis in children with cancer are many and complex. Focused studies aimed at enabling accurate risk stratification are required to allow recommendations for thromboprophylaxis to be developed.

PB 4.71-5

Quality of life of children participating to a formalized INR self-monitoring vitamin K antagonist educational program

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Background: Children under vitamin K antagonist (VKA) should benefit from a formalized educational program using self-monitoring of their International Normalized Ratio (INR). In France, since the 2008 Public Health Law, children and/or their family who wish to participate to INR self-monitoring must integrate a formalized authorized educational program to anticoagulation therapy led by a paediatric cardiologist.

Aim: Our national reference centre for congenital heart diseases, while building this program, aimed to regularly evaluate the quality of life (QoL) of children.
Methods: All children and parents participating to our INR self-monitoring VKA educational program were invited to complete a QoL questionnaire during each group session. Generic pediatric QoL questionnaires were used (QUALIN for infants <2 years old, PedsQL for children aged 2–18). Both parents independently participated. PedsQL Child self-report QoL questionnaires were used for children above 5 years, under trained nurse supervising. Each family participated to 1–3 educational sessions. This study received the approval of the Ethics Committee. Relations between QoL and patients' characteristics were studied. Patient's clinical instability during the program and duration of life with chronic illness were analyzed as biases which both classically influence QoL assessment.
Results: 111 children (54 girls, 57 boys) participated to our INR self-monitoring program between 2010 and 2012. Indications for VKA were classical within paediatric population: valve replacement (n = 47), total cavo-pulmonary connexion (n = 33), dilated cardiomyopathy (n = 13), Kawasaki disease (n = 8), others (n = 10). No family refused to be enrolled in this study. 476 QoL questionnaires (27 QUALIN, 449 PedsQL) were completed by 265 different persons (80 children, 107 mothers, 78 fathers), depending on the number of group sessions for each family. There were no significant relationships between QoL and patient's sex, type of AVK (warfarin or fluindione), number of group sessions, chronic illness duration or moment of diagnosis (prenatal or postnatal). Qol scores were significantly lower among children with congenital heart disease (n = 80). Fathers and mothers' QoL scoring are rather well correlated but are significantly lower than their child's self- assessment. There are almost no differences of QoL between children under transient VKA treatment (n = 37) and those treated for life (n = 74).
Conclusion: Routine QoL assessment well applies to educational programs with strong joining of families and children. As all patients included in this study are in self-monitoring, we could not evaluate the impact of self-monitoring on patients' QoL. However our results show that classical morbidity factors, which are mostly relevant for doctors, are not always correlated to QoL, which is mostly relevant for patients. We are now finalizing to collect data from a matched population to compare QoL of children under VKA to controls. Our centre leads five official education programs (pulmonary hypertension, anticoagulation, transition to adulthood, pacemaker-Defibrillator and chronic cardiac failure) and aims in further studies to compare QoL of children participating to such programs to those who don't.

PB 4.71-6
Portal vein thrombosis in children and adolescents

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Background: Portal vein thrombosis (PVT) is an important cause of portal hypertension (PH) in children. Complications include upper gastrointestinal bleeding from the rupture of esophageal varices, hypersplenism secondary to splenomegaly, growth retardation, and portal biliopathy. Management may include portosystemic shunting. Unlike neonatal portal vein thrombosis which has low reported rates of portal hypertension of <5%, while gastrointestinal bleeding occurs in up to 80% of children with PVT.
Aims: The aim was to describe the presentation, treatment, and outcomes of an unselected cohort of children presenting with portal vein thrombosis, to determine if the outcome differed with age of presentation.
Methods: The study was approved by the hospital research ethics board and written consent waived. A retrospective chart review of infants and children with PVT presenting to the Hospital for Sick Children from January 2008 to January 2012, identified from the clinical thrombosis database was conducted. Neonates were excluded, and age was limited to 31 days to 18 years at time of presentation. Clinical and radiologic data were collected. Descriptive statistics and Fisher exact testing were completed to compare the two groups; infants (age ≤1 year) and children (age. >1 year).
Results: 36 children with PVT were identified. At the time of presentation, 14/36 patients (38%) had underlying liver disease (biliary atresia (n = 6), sclerosing cholangitis (n = 3), metabolic disease (n = 1), chemotherapy induced liver disease (n = 2), hepatic infiltration (n = 2). The majority of PVTs, 19/36 (52%), were identified incidentally, on abdominal ultrasound completed during work up for other medical illness; (fever and sepsis (n = 7), pre- or post- liver transplant (n = 5), elevated liver enzymes (n = 2), pre Kasai (n = 1), abdominal pain (n = 10). Risk factors (intra-abdominal infection, sepsis or abdominal surgery) were present in 25/36 (70%). Infants (n = 20) had a mean age (±SD) of 5 months (±3), children (n = 16) had a mean age of 10 years (±5). Mean follow-up in infants was 24 months (±24) and in children was 36 months (±37). There was a history of a prior umbilical venous catheter in 10/20 (50%) infants and 1/16 (6%) children (P = 0.0091). Cavernous transformation was found on initial imaging in 2/20 (10%) infants and 7/16 (44%) children (P = 0.049). Gastrointestinal bleeding at time of initial presentation was present in 0/20 infants and in 5/16 (31%) children (P = 0.012). There was no difference between infants and children in hypersplenism (19%) gastrointestinal bleeding in follow-up (11%), PH at time of presentation (20%), PH in follow-up (31%), thrombus resolution (58%), need for portosystemic shunting (8%), growth failure (20%) or thrombophilia [low protein C, protein S, antithrombin] (44%). There was no difference in radiologic resolution with anticoagulation (67%) or without anticoagulation (57%).
Conclusions: In this single institution cohort, limited by the small number of patients, rates of portal hypertension and gastrointestinal bleeding were similar in infants and children, but higher than reported rates in neonates. PVT in infants had a greater association with previous umbilical catheter, and presented less often with cavernous transformation or gastrointestinal bleeding than in children.

PB4.72 – Thrombophilia – V

PB 4.72-1
Clinical characteristics of double heterozygous for factor V Leiden and prothrombin mutation. Findings from the RIETE Registry

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Background: Patients with the double heterozygousy (DH) for factor V Leiden (FVL) and prothombin G20210A (PT 20210A) are rare. Consequently, there is scarce information on their clinical characteristics when presenting with a first episode of acute venous thromboembolism (VTE).
Patients and Methods: RIETE is an ongoing registry of consecutive patients with symptomatic, acute VTE. We compared the clinical presentation of double heterozygous patients for FVL and PT 20210A with single heterozygous and with patients with no thrombophilia.
Results: In all, 6057 patients were tested for FVL and PT 20210A. Of these, 724 were heterozygous for FVL, 552 heterozygous for PTG20210A, 88 double heterozygous, and 4693 tested negative. Their median age was 49 ± 18, 51 ± 18, 45 ± 18, and 56 ± 19 years, respectively. About half of the patients (51%, 45%, 50% and 48%, respectively) had idiopathic VTE, with no difference between subgroups. Among women, 36%, 35%, 39% and 23% respectively were

using estrogens; 6.5%, 6.8%, 10% and 3.3% were pregnant; and 3.0%, 4.8%, 7.7%, and 2.1% postpartum. As for the VTE presentation, 35%, 52%, 34% and 51% respectively presented with acute pulmonary embolism (PE). PE patients with FVL or DH less likely presented with tachycardia (17% and 14% vs. 34% and 33%, respectively) or hypoxemia (17% and 14% vs. 34% and 33%, respectively) than those with PT 20210A or no thrombophilia.
Conclusions: Double heterozygous for FVL and PT 20210A have the first VTE earlier than non thrombophilic and single heterozygous patients. Estrogen use is the most frequent associated risk factor, with the same frequency for double or single heterozygous patients. Double heterozygous patients and heterozygous for FVL have a lower frequency of pulmonary emboli and less hypoxemia.

PB 4.72-2
Challenges in the diagnosis of type II antithrombin deficiency with heparin-binding site defects

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Background: Antithrombin (AT) is a potent inactivator of thrombin and the major inhibitor of blood coagulation. Hereditary AT deficiency is a rare autosomal dominant disorder with a predisposition to recurrent venous thrombosis. Two types are distinguished, quantitative (type I) and qualitative (type II) deficiencies. The latter can be subclassified in mutations that affect binding to heparin (Heparin-Binding Site, HBS), the reactive site (Reactive Site, RS) or both (Pleiotropic Effect, PE). When screening for AT deficiency, measurement of AT activity is the test of choice. Current methods quantify AT activity by chromogenic tests measuring the inhibition of thrombin or FXa by AT in the presence of heparin. Patients with HBS defects can show varying activity levels depending on the assay conditions used and may therefore not be detected. Although HBS deficiencies are associated with a milder thrombotic phenotype, if present in homozygous state or associated with other more prevalent thrombophilic risk factors, such as factor V Leiden or G20210A prothrombin mutation, thrombotic risk is markedly increased.
Aims: To study the suitability of several commercial AT activity assays for the identification of HBS variants and to ameliorate detection of these HBS deficiencies by adjusting assay conditions.
Methods: Our study included 29 patient samples with different HBS variants, all confirmed by molecular analysis. We evaluated three anti-Xa based activity tests (HemosIL® Liquid AT, IL; Coamatic® AT, Chromogenix; Innovance® AT, Siemens) on the ACL Top® 500 instrument and one anti-IIa activity test (Biophen® Antithrombin, Hyphen Biomed) on the ACL Elite® Pro analyzer. We also tried to optimize assay conditions for the Coamatic® test by varying sample dilution, incubation time and analyzer.
Results: The sensitivity of the anti-Xa assays for HBS deficiencies was 59%, 76% and 100% for HemosIL® Liquid AT, Coamatic® AT and Innovance® AT, respectively. Two specific mutations are responsible for this high variability between assays, namely the p.Pro41Leu and p.Arg47His mutations. The anti-IIa assay showed a sensitivity of 47% and was only able to detect some of the p.Leu99Pro and p.Arg47Cys variants. The anti-IIa assay yielded higher activity levels when compared to an anti-Xa assay (Coamatic® on ACL Elite® Pro) with an absolute average increase of 25%. Adapting the sample dilution (1/80 instead of 1/100) in the Coamatic® test did not show any improvement in sensitivity. Lower activity values were obtained with this assay by setting a short fixed incubation time (110 s instead of 100–140 s) with a resulting increase in sensitivity of 76% to 86%. The sensitivity further increased to 96% when another analyzer (ACL Elite® Pro instead of ACL Top® 500) was used. This is probably due to differences in reagent constituents' final concentration in the reaction cuvet.
Conclusion: Several commercial assays fail to detect AT deficiency HBS variants, emphasizing the importance of the assay conditions. Some deficiencies, especially the p.Pro41Leu and p.Arg47His mutations, can be missed. Adaptation of assay conditions, namely the incubation time, showed a significant improvement in sensitivity. However, one should be cautious with these adjustments, as they might evoke a decrease in specificity of the assay.

PB 4.72-3
Molecular markers of blood hipercoagulability and values of overall coagulation potential in double heterozygotes for the FV Leiden and FII G20210A mutation

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Background: Double heterozygosity for the FV Leiden and FII G20210A mutation is relatively rare thrombophilic condition with prevalence in Caucasian population of about 0.1%. This condition is generally considered as strong thrombophilia, and some authors recommend indefinite anticoagulant treatment after first thrombosis in carriers of this mutations. However, published data regarding risk of thrombosis in double heterozygotes are scarce and reported relative risk for first venous thrombosis ranges from 1.4 (Meinardi JR, 2001) up to 20 (Emmerich J, 2001).
Aim: Aim of this study was to investigate concentration of molecular markers of blood hypercoagulability, and values of overall coagulation potential in individuals who are carriers of both FV Leiden and FII G20210A mutation.
Methods: Among 1850 selected patients with thrombosis or pregnancy loss tested in one center between 2003 and 2010 for both FV Leiden and FII 20210A mutations, we identified 28 double heterozygotes, giving prevalence of 1.6%. Concentration of TAT, prothrombin fragment F1 + 2 and D-dimer were measured in plasma of 16 double heterozygotes by ELISA methods. Overall coagulation potential test, which reflects global haemostatic balance, was measured according methods described by He S et al. (Thromb Res 1999;96:145–56). Results of these tests in 16 double heterozygotes were compared with results of the same tests obtained in 133 heterozygous and 12 homozygous carriers of FV Leiden, 38 heterozygous carriers of FIIG20210A, 53 primary antiphospholipid syndrome (APS) patients and 77 healthy control persons. In all symptomatic individuals blood for analysis was drawn at least 3 months after cessation of anticoagulant therapy or termination of last pregnancy.
Results: Mean concentrations of all three investigated markers of blood hypercoagulability: F1 + 2, TAT complex and D-dimer were higher in double heterozygous group than in other investigated groups. Mean levels of investigated markers were as follows: in double heterozygotes- F1 + 2 1.89 ± 0.89; TAT 7.46 ± 4.68; D-dimer 139 ± 169; in FV Leiden heterozygotes- F1 + 2 1.30 ± 0.56; TAT 5.14 ± 2.15; D-dimer 95 ± 126; in FV Leiden homozygotes- F1 + 2 1.23 ± 0.54; TAT 4.83 ± 1.24; D-dimer 77 ± 40; in FII G20210A heterozygotes- F1 + 2 1.5 ± 0.73; TAT 5.65 ± 2.81; D-dimer 90 ± 114; in primary APS- F1 + 2 1.16 ± 0.51; TAT 6.17 ± 3.23; D-dimer 81 ± 116; and in control individuals- F1 + 2 0.94 ± 0.2; TAT 4.57 ± 1.68; D-dimer38 ± 29). Mean overall coagulation potential in investigated groups was as follows: in double heterozygotes-19.4 ± 6.4; in FV Leiden heterozygotes-17.9 ± 3.8; in FV Leiden homozygotes-16.1 ± 2.6 in FII G20210A heterozygotes-17.1 ± 3.5; in primary APS-17.8 ± 4.7; and in control individuals – 14.5 ± 2.7.
Conclusion: Results of our study indicate an increased level of basal activation of coagulation system in symptomatic double heterozygotes for the FV Leiden and FII G2012A mutation which is higher or comparable to patients with FV Leiden, FII G20210A mutation or patients with primary antiphospholipid syndrome. High values of

overall coagulation potential indicate a shift of haemostatic balance toward blood hypercoagulability in double heterozygotes even in basal state, suggesting presence of strong prothrombotic condition.

PB 4.72-4

Role of promoter polymorphisms of Glutathione Peroxidase (GPX3) gene in the development of Deep Vein Thrombosis in Asian Indian population

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Background: Amongst the various known acquired and inherited factors that alter the haemostatic balance, oxidative stress (OS) plays a pivotal role in the development of thrombosis. OS mediates the pathogenesis of venous thrombosis by allowing the reactive oxygen species (ROS) to enhance platelet hyperactivity and depleting nitric oxide (NO). NO is a well known vasorelaxant that has antithrombotic effects which upon depletion may lead to deep vein thrombosis (DVT). An efficient ROS scavenging mechanism comprising of various enzymatic and non enzymatic antioxidants is determining in the prevention of oxidative assault. Plasma Glutathione peroxidase (GPx3) is a major antioxidant enzyme in plasma, scavenging ROS arising during normal metabolism or after oxidative insult. A deficiency of this enzyme increases extracellular oxidant stress, promotes platelet activation, and may promote oxidative posttranslational modification of fibrinogen that leads to DVT.
Aims: To find out the role of promoter *GPX3* polymorphisms in the development of DVT in Asian Indian population.
Methods: Patients were recruited from outpatient clinics and wards of the Department of Haematology at the All India Institute of Medical Sciences, New Delhi, India. We studied 100 consecutive DVT patients (M:F = 67:33, age range = 18–65 years) confirmed by Doppler ultrasonography, computerized tomography (CT) scan (for cerebral venous thrombosis). Patients with pulmonary embolism, malignancy, pregnancy and diabetes were excluded from the study. Non-related sex and age (+5 years) matched healthy individuals (n = 100) of north Indian origin were also included in the study in order to determine risk posed by the polymorphisms. Most cases (73) were of lower limb thrombosis, and 15 cases were of upper limb thrombosis. Thrombosis at unusual sites was present in 12 cases, these included cerebral (5), abdominal (4), subclavian (1), renal (1) and retinal vein (1). All variants (−942 A/C, −927 T/C, −861 A/T, −568 T/C, −518 T/C) were genotyped in the whole study population (100 patients and 100 controls) by restriction fragment length polymorphism (RFLP) and allele specific PCR. This study was approved by the local ethics committee of the All India Institute of Medical Sciences and was in accordance with the Declaration of Helsinki.
Results: All the polymorphisms were in Hardy-Weinberg equilibrium. Amongst the five SNPs only two −568T/C and −861 A/T SNP show association with the DVT. −861A/T shows both genotypic (P = 0.0151, chi-square = 5.23;O.R. = 2.45) as well as allelic association (P = 0.002, chi-square = 11.32;O.R. = 3.12) while −568T/C shows only allelic (P = 0.03 chi-square = 4.04;O.R. = 4.03) association with DVT.
Conclusion: Susceptibility to DVT in North Indian Asian patients may be associated with some variants (−568T/C and −861A/T) of *GPX3*gene. Relatively small sample size and borderline P-values might bias the results obtained and that the association might be confounded by difference in the two groups or due to varied environmental exposures, which are known to modify the influence of *GPX3* variants on disease risk. However this needs to be replicated and confirmed in a larger multicentric population which will account for the genetic heterogeneity of the Indian population.

PB 4.72-5

Thromboembolic disease in overweight and obese patients

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Background: Overweight and obesity are increasing in the general population; both represent additional risk for thrombotic disease.
Aims: Present information on frequency and associated risk factors for first and recurrent arterial, venous or combined thromboembolic events in overweight and obese patients referred to our center for hypercoagulability assessment.
Methods: Retrospective review of clinical and laboratory data from 249 consecutive patients with hypercoagulability diagnosis. Subjects are classified as overweight, obese or extreme obese based on body mass index by NIH guidelines and stratified by the type of thrombosis and recurrences. Results are presented as frequencies and percentages.
Results: We found 231 subjects with thrombotic disease, 170 (73.6%) were overweight (59), obese(82) or extreme obese (29) with one or more clotting events, 40% females, age of first episode between 17 and 86 years old (mean 47.5+/− 15.1), recurrences in 91.7% of cases, 55 subjects with more than two episodes. Venous thromboembolism was found in 61.2%, while only arterial in 41 patients. Thrombophilic genetic markers were found in 54 subjects; in 64.7% six or more thrombotic risk factors were simultaneously present.
Conclusions: Three of four patients with thromboembolic disease are either overweight or obese with extremely high frequency of recurrence. Genetic markers of hypercoagulability are present in one third and they add to the high presence of other risk factors.

PB 4.72-6

Association of JAK2 V617F mutation and tromboembolic events among patients with essential thrombocytemia and idiopathic myelofibrosis in a Brazilian center: evidence of a retrospective study

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Background: Thromboembolic events are the leading cause of morbidity and mortality in patients with bcr-abl negative myeloproliferative diseases. Recent studies indicate a possible association of JAK2 V617F mutation with the risk of vascular phenomena in this group of patients, especially in individuals with essential thrombocythemia (ET), and less consistent in those with idiopathic myelofibrosis (MF). In this retrospective study we evaluate patients with ET and MF followed up at a large tertiary hospital of the State of Sao Paulo, the prevalence of VTE in this population and its association with JAK V617F mutation.
Results: 105 patients were followed up at our institution with ET, being 71% females. Of this total, 53 (50.4%) had heterozygous JAK2 V617F mutation, and 28 (52.83%) of those patients had thrombosis (18 arterial thrombosis – AT and 10 venous thrombosis-VT). In 52 subjects with TE without the mutation (50.9%), 13 (25%) had previous thrombosis (6 AT and 7 VT). The presence of JAK2 mutation was a risk factor for thrombosis in patients with ET (P = 0.0049). In the group of patients with MF (20 women and 19 men), 19 (48.71%) were positive for the mutation. Only eight patients in this group had thrombosis (7 AT and 1 in VT). In subjects with MF without JAK2 mutation, 4 (20%) developed thrombosis (3 AT and 1 VT). In patients with MF the presence of JAK2 V617F mutation was not an important factor for the development of thrombosis (P = 0.1760).
Conclusion: The myeloproliferative diseases are characterized in most cases by long survival, and its treatment, which includes prolonged use of cytoreductive agents, may be associated with progression to acute leukemia and teratogenicity. These variables lead us to carefully con-

sider the assessment of the thrombotic risk, in order to individualize the treatment in this population so heterogeneous. The presence of the mutation in patients with TE appears to be associated with increased incidence of thromboembolic events, similar to published data. However prospective studies are needed to validate these results and optimize the therapeutic strategy.

PB4.73 – Thrombophilia – VI

PB 4.73-1

Identification of a new thrombophilic disorder that affecting a correct N-glycosylation causes antithrombin deficiency

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Since the description of antithrombin deficiency as the first thrombophilic factor in 1965, an intense search for new defects has been done, with quite frustrating results. This search has identified rare mutations and three common polymorphisms on few haemostatic elements. Genome wide association studies suggest that it is unlikely that new common polymorphisms could play a relevant role in thrombosis. Therefore, rare mutations on genes that might influence key haemostatic proteins are good candidates. To identify new thrombophilic defects we focused our interest on antithrombin since even minor deficiency of this key endogenous anticoagulant significantly increases the risk of thrombosis. Thus, we recruited 29 patients with venous thrombosis and antithrombin deficiency, 4 severe (50%), and 25 moderate (70–85%) that had no mutations in the gene encoding antithrombin (*SERPINC1*). Analysis of plasma by western blot revealed an antithrombin isoform with lower molecular weight in four patients (14%). Glycomic analysis confirmed an impaired glycosylation (reduced sialic acid and galactose content) in all cases. This defect was not specific of antithrombin, as we detected abnormal glycoforms on other proteins (α1-antitrypsin, transferrin and prothrombin). These data encouraged to compare these samples with the commonest congenital disorder of glycosylation (PMM2-CDG), a rare autosomic recessive disorder with a large clinical spectrum including mental and psychomotor retardation, that causes defects in the assembly, transfer, and processing of *N*-linked oligosaccharides. In all four cases, electrophoretic, HPLC and Q-TOF patterns were fully compatible with a PMM2-CDG. Molecular analysis of *PMM2*, the gene affected in this disorder, revealed one case with compound heterozygosis (p.Arg141His and p.Cys241Ser), providing an accurate diagnosis of PMM2-CDG. This patient, with a mild mental retardation, had severe antithrombin deficiency (50%) and developed four thrombotic events, the last two on stable oral anticoagulant therapy. This report, together with previous thrombotic events described in children with PMM2-CDG, sustains that this disorder should be considered as a rare thrombophilic disorder. Interestingly, the remaining three cases only had heterozygous mutations in *PMM2* (p.Arg141His, IVS4 + 21 G>C and IVS2 −13 G>A). These three cases are adult males who developed early thrombosis, but without additional clinical features of PMM2-CDG. Moreover, they all shared moderate hepatic dysfunction due to alcohol abuse. These results strongly suggest a new disorder we called CDG-like, result of the combination of a congenital defect in heterozygosis affecting the *N*-glycosylation pathway (*PMM2*) with an acquired factor also disturbing this pathway (alcohol also interferes with the correct glycosylation) that lead to a CDG phenotype with a high risk of thrombosis but without neurological consequences. Finally, the CDG-like disorder might be underestimated, as the 5 years follow-up of the p.Arg141His CDG-like patient revealed a normal transferrin pattern and no antithrombin deficiency in some samples.

Conclusions: The analysis of antithrombin in thrombophilic patients with antithrombin deficiency has allowed the identification of a new thrombophilic disorder that, affecting the *N*-glycosylation pathway indirectly disturbs this key anticoagulant serpin. Accordingly, our study suggests that a diagnosis of PMM2-CDG or CDG-like might be suspected in patients with antithrombin deficiency and no mutations in the *SERPINC1* gene.

PB 4.73-2

Long-term follow-up in four homozygous protein C deficiencies with late clinical onset

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Background: Homozygous protein C (PC) deficiency is a very rare and severe hereditary thrombophilia. It is associated with purpura fulminans, massive thrombosis and disseminated intra-vascular coagulation (DIC) in the newborn. However, in a few number of cases, late diagnosis of the deficiency has been made when skin necrosis was observed at the start of oral vitamin K antagonists (VKA) treatment. Long-term anticoagulation is generally recommended in these patients.

Aim: Aim of the study was to assess the antithrombotic prophylaxis and long-term follow-up in severe PC deficiencies with late clinical onset.

Results: Results in one man and three women with genetically confirmed homozygous type I PC deficiencies with late clinical onset are reported. Patients are 35–79 years old. The first episode of venous thromboembolism (VTE) was a proximal deep vein thrombosis (DVT) observed between 17 and 45 years and all patients experienced skin necrosis when VKA (fluindione, acenocoumarol, warfarin) were introduced. One woman had three pregnancies without thromboprophylaxis and without VTE. Deficiency was detected at time of VTE in three cases, and in the 4th one, it was detected because of family history of VTE but homozygous status was detected later when she developed skin necrosis. PC levels without oral anticoagulant treatment are ranging from 10 to 23%. Homozygous amino acid replacements have been found in positions 168 (Pro-Leu), 267 (Ala-Thr), 301 (Gly-Ser) and 178 (Arg-Gln).

Patients have had recurrent episodes and have been followed for 25, 25, 22 and 3 years. Only one patient is on long-term phenprocoumon (long-acting VKA) for 25 years and had no recurrent VTE episode, although ulcers eventually occurred. In another patient, re-introduction of VKA failed on two occasions because of recurrent skin necrosis and she is still on prophylactic dose of LMWH after 23 years. In one patient, VKA were not re-started because of severity of skin necrosis and she is on LMWH for 3 years. The last patient has had over the past 22 years intermittent LMWH treatment at prophylactic doses for episodes of superficial vein thrombosis, after a knee surgery and for long haul flights.

Conclusion: None of these four homozygous patients had purpura fulminans or massive thrombosis as a first episode at birth. All of them had skin necrosis at start of VKA administered for VTE when they were adults. They are genetically confirmed homozygous but PC levels are measurable and patients had a late clinical onset: this latter finding was not known before the first patient we described in 1984. Skin necrosis at the start of VKA treatment is very rare and should be an alert to measure PC plasma level, and PS level as well. These four patients have had different long-term preventive approach. New target-specific oral anticoagulants might be an alternative to VKA treatment.

PB 4.73-3

Inherited thrombophilia as a risk factor for gestational vascular complications

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Background: The gestational vascular complications (GVC) are associated to a variety of factors, including the pregnancy physiologic hypercoagulability, acquired prothrombotic risk factors (ARF) and, to an extent that is still under investigation, the presence of thrombophilia. The use of anticoagulant prophylactic therapy (AcPT) is still controversial and the case-by-case approach is yet the preferred attitude.
Aims: In a group of women with GVC of unknown aetiology we aimed to evaluate the role of inherited thrombophilia, to identify ARF and to observe the impact of the AcPT in the success of the following pregnancies.
Methods: We studied the polymorphisms Factor V Leiden, PRT G20210A, PAI-1 4G/5G, ACE I/D, F13A1 Val34Leu and the haplotype M2 within the ANXA5 gene in two groups: the control group of 69 healthy women with none GVC and the patients group of 106 women with a history of at least one GVC. Women with anticoagulant factors congenital deficiency, hyperhomocysteinemia, antiphospholipid syndrome, autoimmune diseases, anatomical defects of the reproductive system, foetal chromossomopathies or foetal losses associated to infections were excluded from the study. Eighteen patients had recurrent miscarriage, 11 foetal death *in utero*, 37 preeclampsia or HELLP syndrome (PE/HELLP), 9 intra-uterine growth restriction (IUGR) and 31 had more than one GVC (>1GVC).
Results: We found a statistically significant association between the genotype PAI-1 4G/4G and the occurrence of GVC ($P = 0.019$; OR = 2.613; IC95% [1.149, 5.492]). When analysing the different subgroups we found the same polymorphism associated to the occurrence of PE/HELLP ($P = 0.011$; OR = 3.478; IC95% [1.294, 9.351]) or >1GVC ($P = 0.023$; OR = 3.175; IC95% [1.135, 8.879]). Regarding the ARF in the patients group, we found that 2 (1.9%) had diabetes, 29 (27.4%) had arterial hypertension and 40 (37.7%) obesity. 58.5% of the patients had at least one ARF, 8 (7.5%) had only ARF and in 8 (7.5%) we did not find neither ARF nor any of the studied polymorphisms. In the group of 30 women presenting at least one of the polymorphisms that had subsequent gestations, 13 were submitted to AcPT. Among the 17 without AcPT, 7 (41.2%) presented GVC (4 PE, 2 PE + IUGR and 1 early miscarriage); only 2 woman (15.4%) with AcPT had IUGR.
Conclusion: In our study only the genotype PAI-1 4G/4G had a statistically significant correlation with GVC, in particular with PE/HELLP. The fact that no prothrombotic risk factors were found in 7.5% of the patients indicates that there are still unidentifiable causes to these pregnancy complications. Although the anticoagulant prophylactic therapy seems to be associated with fewer and less severe GVC, the group is too small to allow any conclusions. Further studies are ongoing.

PB 4.73-4

Effect of genetic variants in the TAFI gene on TAFI levels, the efficiency and safety of anticoagulant therapy in patients with venous thromboembolism in Russian population

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Background: Several studies have reported that thrombin activatable fibrinolysis inhibitor (TAFI) levels may be partly determined genetically. Results from these studies are conflicting. The influence of TAFI genetic variants on the efficiency and safety of anticoagulant therapy is not yet clear.
Aims: To determine the frequency of the TAFI gene polymorphisms and theirs effect on TAFI levels, the efficiency and safety of anticoagulant therapy in pts with venous thromboembolism in Russian population.
Methods: One hundred and eleven pts (76 men) in the age 18–76 (mean 54 ± 14) years with deep vein thrombosis (DVT) and/or pulmonary embolism (PE) were included in the study. Pts received unfractionated or low molecular weight heparin for at least 5 days followed by long-term warfarin therapy (international normalized ratio 2.0–3.0). TAFI levels were measured at baseline by a chromogenic assay with reagent kits «STA STACHROM TAFI» (Diagnostica Stago). Three TAFI genetic polymorphisms (–438G/A, 505G/A and 1040C/T) were investigated. The follow-up period was 18 months. Endpoints were DVT/PE recurrences and hemorrhagic complications. Results of TAFI levels are presented as a median (interquartile range).
Results: In all pts, median of TAFI levels was 106 (90; 133)%. The frequency of –438GG, –438GA, –438AA genotypes was 67.6%, 29.7% and 2.7%; 505GG, 505GA, 505AA genotypes – 47.8%, 49.5% and 2.7%; 1040CC, 1040CT, 1040TT genotypes – 50.5%, 41.4% and 8.1% respectively. Univariate regression analysis showed that all three polymorphisms were associated with TAFI levels. For the –438G/A and 1040C/T variants, the carriers of rare alleles (–438A and 1040T) were associated with lower TAFI levels than common alleles (–438G and 1040C) – 98 (82; 118) vs. 106 (89; 137)%, $P = 0.004$ and 99 (87; 119) vs. 106 (89; 135)%, $P = 0.002$ respectively. While for the 505G/A, the carriers of the rare allele (505A) was associated with higher TAFI levels – 113 (94; 143) vs. 106 (89; 132)%, $P = 0.067$. During 18 months of anticoagulant therapy, the frequency of DVT recurrences was 17%, and the frequency of hemorrhagic complications was 30% (major bleedings – 2%, minor bleedings – 28%). There were no PE recurrences. We found no associations among all TAFI genetic variants and the risk of DVT recurrences and hemorrhagic complications during 18 months of anticoagulant therapy.
Conclusion: In pts with venous thromboembolism in Russian population, the –438G/A, 505G/A and 1040C/T polymorphisms of the TAFI gene associate with TAFI levels but do not influence on the risk of DVT recurrences and hemorrhagic complications during 18 months of anticoagulant therapy.

PB 4.73-5

The prevalence of thrombophilia in patients with isolated superficial vein thrombosis

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Background: Superficial vein thrombosis (SVT) shares some common risk factors with deep vein thrombosis (DVT). However, SVT has been considered banal and therefore has drawn little scientific interest until recently. Little is known about the prevalence of thrombophilia in SVT patients.
Aims: The aim of our study was to evaluate the prevalence of thrombophilic disorders in a group of patients with isolated SVT of the legs.
Methods: We examined 74 outpatients (age 58.4 ± 14.8 years, 70.3% women) referred to vascular clinic with SVT on the legs. We obtained family and personal history and performed clinical and ultrasonographical examination, as well as laboratory thrombophilia workup. For statistical evaluation we used Student t-test and χ^2 test.
Results: In most cases (90.5%) SVT occurred on varicose veins, on both legs almost equally (47.3% right side; 50% left side; 2.7% bilaterally). Duration of symptoms was in quite a wide range (9.6 ± 9.1 days). In 71.6% of patients SVT had not been preceded by any trigger, the rest of cases had been provoked by immobilization, injury, erysipelas, tumour, oestrogen therapy or pregnancy. In eight

cases (10.8%) SVT approached saphenofemoral junction at the distance ≤3 centimetres. Positive family history of SVT was reported by 33.8%; that of DVT by 35.1% patients while positive personal history of SVT had 47.3% and that of DVT 28.4%. Factor V Leiden (FVL) was found in 13 persons (17.6%); in three cases even in a homozygous trait. Other thrombophilias were less frequent – mutation in prothrombin gene G20210A was revealed in 6.8%; antithrombin deficiency in 7.1%; protein S deficiency in 4.1%, protein C deficiency in 1.4% and antiphospholipid antibodies in 2.7%. The prevalence of thrombophilic disorders was higher in men and younger individuals with SVT but the only factor significantly associated with thrombophilia was positive family history of DVT.

Summary/Conclusions: In the group of 74 outpatients with SVT we found relatively high prevalence of thrombophilic disorders (39.2%), the most prevalent one was FVL. The presence of thrombophilia in these patients was significantly associated with positive family history of DVT. Our results suggest that patients with SVT and positive family history of DVT be potentially considered for thrombophilia screening. These findings deserve further evaluation in a larger study and may become a small contribution to our changing approach to SVT management.

PB 4.73-6

Evaluation of the INNOVANCE Free PS Ag assay: an easy and reliable assay for the automated determination of free protein S

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A new latex particle-enhanced immunoassay for the quantitative determination of free protein S, INNOVANCE® Free PS Ag, has been developed. Validated assay applications are available on Siemens BCS® and BCS XP systems as well as on Sysmex® CA-1500, CA-7000 and CS-2000i/2100i systems. INNOVANCE Free PS Ag reagent components are liquid and ready to use. They can be stored on board the systems up to 48 h (BCS/BCS XP, Sysmex CA-1500/CA-7000 systems) or 72 h (Sysmex CS-2000i/2100i systems), and may be used up to 8 weeks after first opening of the reagent vials.

INNOVANCE Free PS Ag assay applications are linear over the measuring range of 10–150% of the norm free protein S with a limit of quantification of 5% of the norm. No high-dose hook is seen up to a theoretical concentration of 550% of the norm free protein S.

The INNOVANCE Free PS Ag assay measures free protein S molecules without showing cross reactivity to protein S/C4BP complexes. No interference by rheumatoid factors up to 3640 IU/mL or by heterophilic antibodies up to 3641 ng/mL was detected.

No interference by endogenous and exogenous interference substances was observed up to concentrations of: 1000 mg/dL hemoglobin, 60 mg/dL bilirubin, 2570–3066 mg/dL (depending on the system used) triglycerides, 625 mg/dL cholesterol, 7.5 IU/mL unfractionated heparins, 10 IU/mL low molecular weight heparins, 11 g/L fibrinogen, and 24.8 × 10E-7 per mL platelets.

Different INNOVANCE Free PS Ag lots show highly comparable results over the whole measuring range.

Thus, the INNOVANCE Free PS Ag assay offers an easy and reliable automated method for the determination of free protein S in human plasma.

Disclosure of Interest: Authors are coworkers of Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany.

INNOVANCE Free PS Ag assay is not availbale for sale in the U.S.

PB4.74 – Fibrinolysis and FXIII

PB 4.74-1

Fibrinolysis wave as a possible cause of rethrombosis

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Background: Plasminogen activators are used for the treatment of thrombotic events in patients with thromboembolism and stroke. However, there are possible complications of this therapy like secondary thromboembolism or rethrombosis, the cause of which is not clear.

Aims: In this work we evaluated the underlying mechanisms of fibrinolysis and coagulation system interaction, which might be responsible for the secondary thromboembolism, using a newly developed model of spatial fibrin clot growth and lysis.

Methods: A thin layer of unstirred human blood plasma contacting with the surface with immobilized tissue factor was monitored to register the fibrin clot growth and its lysis. Plasma was supplemented with streptokinase, urokinase or tissue plasminogen activator at different concentrations close to the concentrations used in thrombolytic therapy to test their ability to cause clot lysis. Time when the clot started to grow and when it started to dissolve, and rates of these processes where used to evaluate the influence of plasminogen activators on the systems of plasma coagulation and fibrin clot lysis.

Results: Fibrinolysis started in area of activation of coagulation and propagated towards the direction of clot growth. The spatial rate of clot lysis (66 ± 9 μm/min) did not depend on the concentration or the type of thrombolytic agent (measurements were performed in the concentration range of TPA 0.75–20[μg/mL], urokinase 180[UI/mL], streptokinase 200[UI/mL]). In contrast to the lysis rate, the time when fibrinolysis started increased with the decrease of TPA concentration (1.7 ± 0.3 min for 20 μg/mL TPA; 29 ± 14 min for 0.75 μg/mL TPA).

Conclusion: The subject of this study corresponds to situation if any clotting occurs during the plasminogen activator therapy. A possible mechanism of secondary thrombosis may be related with the wave of lysis, which started from the place of clot growth, detaching the clot from the vessel wall and thus forming an embolus for rethrombosis. Plasminogen activator concentration defines only the time when the lysis will start, but not the overall efficacy of the drug, i.e. when the lysis started, the time of clot dissolution will be the same for all activator concentrations.

PB 4.74-2

Polymorphism thr325ile in thrombin-activable fibrinolysis inhibitor gene in dyslipidemic subjects from Brazil

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Background: Thrombin-activable fibrinolysis inhibitor (TAFI) is a plasma zymogen synthesized in the liver, which plays an important role in hemostasis, acting as a potent inhibitor of fibrinolysis in its activated form. Studies have shown that increased plasma concentrations of TAFI could be a risk factor for thromboembolic and coronary artery disease (CAD). These diseases are a major cause of morbidity and mortality worldwide, constituting a public health problem. The role of hyperlipidemia is well known in the evolution of CAD, since its presence increases the formation of atherosclerotic plaque and subsequent thrombus formation. Many individuals with alterations in lipid profile are undiagnosed or undertreated, persisting

with an altered lipid profile and thereby increasing the risk of coronary events.
Aims: In this study the frequency of the Thr325Ile polymorphism (+1040C/T) in the TAFI gene in dyslipidemic and normolipemic individuals was evaluated and correlated with TAFI plasma levels.
Methods: 107 dyslipidemic patients from 30 to 60 years were evaluated and compared with a control group of 101 normolipemic subjects of similar age. All subjects gave informed consent and the study was approved by ethics committee from the Federal University of Minas Gerais, Brazil. The molecular analyses were performed by PCR-RFLP. Citrated plasma TAFI levels were determined using an ELISA test. Statistical analyses were performed using SPSS 13.0 software, by the chi-square test, Mann-Whitney and Spearman rank correlation.
Results: The T allele frequency in the case group was 0.327 compared with 0.248 in the control group, but the difference was not significant ($P = 0.213$). Also for the genotype frequencies there was no significant difference between the two groups ($P = 0.210$). However, a significant difference was found between medians of TAFI plasma levels between the dyslipidemic group (8.5 mg/mL) and the normolipemic group (7.21 mg/mL), with P value <0.01. There was a negative correlation between TAFI antigen levels and the presence of the TT genotype ($r = -0.209$, $P = 0.002$). A negative correlation was also observed between the levels of TAFI and the presence of the allele T ($r = -0.159$, $P = 0.021$).
Conclusions: These results suggest that dyslipidemia may be associated with higher levels of TAFI while the +1040T allele seems to be associated with lower levels of TAFI.

PB 4.74-3
Residual perfusion defects in patients with pulmonary embolism and fibrinolytic system

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Background: The relative balance between clot formation and lysis is considered to reflect the thrombotic potential but few methods are available to study this issue. Little is known about the pathophysiology of persistent vascular obstruction abnormalities after pulmonary embolism (PE). In previous studies we have observed a slight increase in CLT of pateints with previous PE vs. controls.
Aims: To assess the behaviour of fibrinolytic system components in patients with complete or incomplete restoration of pulmonary perfusion after an episode of acute PE.
Patients and Methods: We studied 80 patients after stopping oral anticoagulant therapy for an acute episode of PE. Ventilation/perfusion lung scan and echocardiography were performed to detected a percentage of residual vascular obstruction (RVO). Persistent perfusion defects were defined as a los of pulmonary vascular obstruction ≥10%, according a previously validated method of analysis of scintigrafic data. The Analyses of Clot Formation, Morphology, and Lyses were performed using the Clotting and Lysis assay according to Carter (2007) and clot lysis time CLT according to Lisman (2005) TAFI (ag), PAI (ag) and t-PA (ag) plasma levels were measured with commercially available ELISA whereas α2-Antiplasmin (α2-AP), plasminogen and factor XIII were measured using Berichrom reagents with the BCS system. Fibrinogen was evaluated with Clauss assay and plasmin mediated lysis of fibrin β-chain according to Morris (2006). ECLT was measured according to Buckell (1958).
Results: Ventilation/perfusion lung scan was available for 71 patients. Patients with RVO ≥10% were 18/71(25.4%) and without RVO were 53/71(74.6%). Median plasma levels of t-PAag, TAFIag, α2-AP, fibrinogen, lysis of fibrin β-chain and fXIII were similar between patients with and wthout RVO. CLT was not different between the two groups. Finally, by analysisng plasma turbidimetric parameters through EuroCLOT assay, statistically significant differences ($P < 0.05$) were observed between patients with RVO and without. A similar behaviour was observed in PAIag plasma levels (0.005) and ECLT($P = 0.049$).
Conclusion: An alteration of endogenous fibrinolysis, regarding to behaviour of Lysis assay and PAI-1 plasma levels, seems to be a special feature of PE patients with incomplete restoration of pulmonary perfusion.

PB 4.74-4
Molecular basis of 21 severe FXIIIA deficiency cases: 11 novel mutations detected

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Background: Congenital FXIII deficiency can be due to defects in either *F13A* gene (known as type 2 defect) or *F13B* gene (type 1 defect). Bleeding disorders as a result of mutations in the *F13B* gene occurs rarely (<5% of reported factor XIII deficiency cases). The incidence of severe FXIII deficiency is one in 3–5 million people and is inherited in an autosomal recessive pattern. This rare bleeding disorder affects people of all races and there is often a history of consanguinity within most of the families of FXIII deficient patients. FXIII deficiency is a serious lifelong bleeding diathesis, common symptoms ranging from neonatal umbilical stump bleed to impaired wound healing, prolonged bleeding from injury site, intracranial haemorrhage, menorrhagia and recurrent miscarriages in women.
Aims: To study the molecular basis of FXIII deficiency cases and to provide genetic diagnosis in affected families.
Methods: We have analysed 21 severe FXIII deficient patients, diagnosed on the basis of their clinical history, normal screening coagulation and clot solubility assay. Genomic DNA was extracted by the phenol chloroform method, and mutations were detected by direct DNA sequencing of the *F13A* gene. 15 of 21 patients had history of primary consanguinity.
Results: 17 mutations were detected in 21 FXIII deficient patients, of which 8 were missense (6 novel, 2 recurrent), 6 nonsense (3 novel, 3 recurrent), and 2 patients showed a novel single base pair deletion; two patients showed a known splice site mutation in exon 14. A large deletion in exon 3 is suspected in 2 unrelated patients because of repetitive failure of PCR amplification of this exon. Six polymorphisms were detected in these patients, of which one is novel.
Conclusion: We have identified *F13A* gene mutations in 21 FXIII deficient patients, and observed a high heterogeneity in the mutation profile. The data obtained would assist in establishing a National Mutation Database, and enable an accurate carrier and antenatal diagnosis in affected families.

PB 4.74-5
Mutation analysis in patients with decreased fibrinogen level

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Background: Fibrinogen is a 340-kDa glycoprotein synthesized in hepatocytes that circulates in plasma at a concentration of 1.5–4.5 g/L. The three genes encoding fibrinogen Bβ (*FGB*), Aα (*FGA*), and γ (*FGG*) are clustered in region of approximately 50 kb on human chromosome 4.

Aims: The objective of this study is the molecular characterization as well as genotype-phenotype analysis of patients with decreased fibrinogen level.
Methods: We examined 17 patients (9 [53%] females, 8 [43%], males). Each patient was asked about the type of clinical manifestations (bleeding, thrombosis). The *FGA, FGB, FGG* genes were analysed by automated direct sequencing of all coding regions and exon/intron splicing sites on ABI 3130 sequencer. In all patients, the functional concentration of fibrinogen (Clauss method; Multifibren*U, Siemens) and thrombin clotting time (BC-Thrombin Reagent, Siemens), were determined. Additionally, in group of eight patients rotational thromboelastometry (ROTEM®-delta; FIBTEM, Pentapharm), was performed. Von Willebrand factor (VWF) and factors VIIII, IX, XI activities, were analyzed in study subjects with bleeding symptoms. In patients with thrombosis we looked for common thrombophilic defects: *F5* p.R506Q and *F2* c.G20210A genotypes, antithrombin and protein C activities, free protein S antigen level.
Results: In our patient population 5/17 (29%) were asymptomatic, 4 (24%) – stated a tendency for bleeding, 6 (35%) – had thrombotic complications and 2 (12%) females – reported miscarriages. Direct sequencing approach revealed six novel mutations (not registered in GEHT www.geht.org as well as HGMD www.hgmd.cf.ac data base) in six patients (*FGG*: c.78 + 5G>A, p.D178H, p.R223I, p.G240R, p.D314Y, p.W395S), and three previously reported mutations (*FGA*: p.R35H, *FGB*: p.G444S, *FGG*: p.A108G) in eight patients. All patients with identified mutation were heterozygous. In three cases, no changes in analysed sequence had been found. Fibrinogen concentration was 0.4–1.4 g/L (normal range: 2–5 g/L). Thrombin time was prolonged in all patients (22–45.5 s; normal: <21s). Activities of VWF and factors VIII, IX, XI in four patients with bleeding tendency, were in normal ranges. In six study subjects with thrombosis, no thrombophilic defect has been revealed. Thromboelastometry (ROTEM®) showed reduced maximum clot firmness in five patients (MCF-FIBTEM 4–8 mm; norm 9–25 mm). In this group 2/5 patients were asymptomatic (causative mutation c.78 + 5G>A; no mutation in second one), 2/5 had miscarriages (p.A108G, p.R223I), 1/5 has thrombotic event (p.W395S). In three patients with the same mutation p.R35H, MCF-FIBTEM was normal: 11–16 mm; one was asymptomatic and two experienced thrombosis.
Summary: Studies on fibrinogen gene mutations might enable better understanding of pathophysiology and clinical course of inherited hypo-/dysfibrinogenemia as well as assist in optimal tailoring of haemostatic/antithrombotic treatment.

PB 4.74-6
Role of genotyping in FXIII deficiency

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Background: FXIII deficiency is a rare autosomal recessive disease, caused by genetic defects in the A subunit located on chromosome 6 (p24–25) or the B subunit located on chromosome 1(q32-32.1). More than 95% of the patients with FXIII deficiency are affected by a mutation in the A subunit. Currently more than 70 A subunit mutations have been identified. Mutations in the B subunit are very rare, only seven are known. Two different tests are available to detect FXIII deficiency. The clot solubility test is a qualitative test only able to detect severe deficiency (<1%). The quantitative test analyses FXIII activity levels by indirectly measuring the amount of measuring ammonia released by FXIII(a) in the first step of transglutaminase reaction. Patients with severe FXIII deficiency have plasma levels between 1 and 5%. Recently we were confronted with a young immigrant patient suspected with a tumor in his upper leg but showed to have a large hematoma. Screening assays showed slightly elevated PT and APTT levels, increased D-dimer (2400 ng/mL) levels and a low Hemoglobin concentration. Further analysis showed that the patient was suffering from FXIII deficiency. The qualitative assay was negative corresponding to <1% FXIII. The quantitative assay however showed levels of 4–7% FXIII, suggesting a less severe phenotype.
Aim: To clarify the discrepancy between the quantitative and qualitative assay results.
Methods and Results: We sequenced the genes of both subunits of factor XIII. Our patient had no mutations in the B-domain but carried a homozygous missense mutation in exon 3: c233G>A (p.Arg78His) of the A-domain. This mutation is located in the beta-sandwich, which is highly conserved. International registries have reported that other mutations in the same region have shown to be pathogenic. The patient is currently treated with FXIII-A_2B_2 concentrate (Fibrogammin P) every 6 weeks.
Conclusion: Our case illustrates that in patients with a discrepancy between qualitative test result (i.e. <1%) and quantitative analysis of FXIII activity, genotyping is recommended. As the clot solubility test can only detect severe deficiency, test results can be normal in patients with mild to moderate FXIII deficiency. Additionally, the quantitative assay has a cut off point around 5% making it less sensitive to detect severe FXIII deficiency. In patients with unknown FXIII deficiency genotype, diagnosis can be missed if only a one analysis is performed. We recommend to sequence FXIII gene subunits when a discrepancy between qualitative and quantitative tests is found or if one only FXIII activity assay is performed.

PUBLICATION ONLY

PO 001

Correlation of hemostatic parametres with age in patients with myocardial infarction

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Background: Age is a well-known risk factor for thrombosis though the mechanisam of more frequent thrombosis occurence in older patients has received little attention in clinical research so far.
Aim: Identifying specific hemostatic parametre changes related to the age of myocardial infarction patients.
Methods: The study involved a group of 173 patient with myocardial infarction older than 45. We investigated blood count parametres and hemostatic markers (antithrombin, protein C, FVII, FVIII, vWF, FX, FXII). The statistical analysis was done using primarily Spearman's correlation test.
Results: The average patient age was 61.28 ± 10.38 years. There was detected a negative age correlation with haemoglobin levels ($P = 0.002$), white blood counts ($P < 0.001$) and antithrombin ($P < 0.001$). A positive age correlation with vWF ($P = 0.023$) was determined. The levels of FVIII showed only a tendency towards higher levels ($P = 0.056$). Protein C, FVII, FX, FXII levels did not demonstrate any correlation with age.
Conclusion: Detecting phatogenic parametres responsible for acute myocardial infarction occurence may contribute to the establishment of adequate preventive measures and therapy optimalisation. It is necessary to conduct more comprehensive research to gain more reliable results in this field.

PO 002

Diagnosis of acquired hemophilia due to pregnancy

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Background: The acquired hemophilia (AH) is potentially life-threatening bleeding disorder caused by the spontaneous development of autoantibodies against plasma coagulation factors, most frequently to FVIII (as in our case). Diagnosis can be difficult because is a very rare condition (approximately one person per million each year), the patient does not have the usual personal or family history but have distinctly different clinical symptoms who are differ from those of hereditary hemophilia.
Aims: Importance of performance of laboratory tests for a rapid and adequate diagnosis of AH.
Methods: Presented is a case of AH in a 24-year-old woman (PF) diagnosed after delivery. Analyzed were: screening haemostasis, correction of aPTT with normal plasma, biological activity of FVIII and LAC and evaluation of inhibitors of FVIII by Bethesda coagulation method.
Results: A few days after the delivery, 24-year-old woman was admitted at the Outpatient Department in the Institute of Transfusion Medicine -Skopje. She had prolonged postpartum bleeding and bruises. Screening haemostasis showed: aPTT = 110″ (normal rang: 27″–37″) and PT = 12″ (normal rang: 10″–15″); the aPTT does not correct with normal plasma (88″). We suspected AH or LAC. LAC was negative. The diagnosis was confirmed by the finding of a decreased level of FVIII = 0, 19% (normal 50–120%), and presence of inhibitors to FVIII = 166 BU (normal < 0, 60 BU). Woman was hospitalized at the Clinic of Hematology. After 8 days treatment aPTT was 74″ (71″), and FVIII = 0, 21%; 25th day of treatment aPTT = 55″ (41″), FVIII = 2, 09% and inhibitors to FVIII = 9 BU. After 1 month treatment the laboratory findings were normal: aPTT = 33″, FVIII = 55% and inhibitors to FVIII = 0, 45 BU without relapse.
Summary/Conclusions: Rapidly and accurately – a timely diagnosis will enable clinicians to apply treatment strategies – control of bleeding and eradication of the inhibitor. Collaboration with an experienced Hemophilia centre is recommended.

PO 003

Haemostatic changes in cirrhotic patients

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Introduction: Liver cirrhosis leads to the impairment of coagulation protein synthesis. The aim of this study was to assess the alterations of haemostatic parameters in the evolution of liver cirrhosis scored according to Child's classification.
Methods: A case control study including 51 cirrhotic patients and 51 healthy subjects matched by age and sex was conducted. Disease severity was estimated according to Child Pugh classification. Prothrombin time (PT), activated partial thromboplastin time (APTT). Procoagulant factor activity (factor I, VII, II, V, VIII, XII) and inhibitor factor activity (Protein C, protein S) were detected with clotting assay. Antithrombin activity was detected with chromogenic assay. Search for Anticardiolipin and antiB2cardiolipin antibodies wasinvestigated. Statistical analysis was performed using SPSS software version 19.0.
Results: There were 25 males and 26 females. Mean age at inclusion was 56.8 years old [range16–86]. Patients were categorized as class A in 13 patients (25.5%), class B in 23 patients (45.1%), and class C in 15 patients (29.4%). Mean level of PT and APTT were significantly prolonged in cirrhotic patients than in controls. Procoagulant factors and anti-coagulant factors were significantly lower in cirrhotic patients: factor VII (54.87 vs. 96.7; $P < 0.0001$), factor II (42.7 vs. 99.6%; $P = 0.002$), factor V (50.3 vs. 97.8%; $P = 0.008$), factor XII (59.4 vs. 93.6%; $P < 0.0001$), antithrombin (50.2 vs. 100.6%; $P < 0.0001$), protein S (49.6 vs. 81.6%; $P < 0.0001$) and proteinC (45.7 vs120.6%; $P < 0.0001$). However, mean level of factor VIII was significantly higher in cirrhotic patients (115.87 vs. 95.8%; $P = 0.03$). On the other hand, anticardiolipin antibodies were more frequent in cirrhotic patients than in controls (47.9 vs. 12.5%; $P = 0.005$) but not B2 glycoprotein antibodies (2.1 vs. 0%). When comparing patients according to disease severity, factor (VII, II, V, XII), protein S and protein C decreased significantly from class A to C (respectively, $P < 0.0001$, $P = 0.012$, $P = 0.009$, $P = 0.002$, $P = 0.001$, $P = 0.001$). However, factor VIII and protein S increased without reaching a significant level.
Conclusion: Patients with liver cirrhosis are characterized by decreased levels of most of procoagulant factors. In fact, prolonged PT has been considered until recently as an index of bleeding. However, decreased levels of anti-coagulant factor in association with the elevated level of factor VIII suggest that patients may have a tendency to thrombosis. Accordingly, these hemostatic modifications lead to a fragile balance which could bend to either bleeding or thrombosis.

PO 004

Factor VIII inhibitor and pulmonary embolism developing in a patient after meningioma resection: clinical and laboratory aspects

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Background: Acquired haemophilia A is caused by the presence of a factor VIII inhibitor in a non-haemophiliac individual. It is a rare but potentially life-threatening event, with an estimated incidence of 1 per 1.48 million persons per year. These inhibitors can occur in association with auto-immune disorders, malignancy, post-partum or arise in individuals without known pre-disposing factors.
Aims: This paper describes the clinical and laboratory features of a case of FVIII inhibitor and pulmonary embolism developing in a patient following meningioma resection.
Methods: Basic laboratory investigations were coagulation screens, mixing tests and Bethesda assay for factor VIII inhibitors. Lupus anticoagulant testing was by diluted Russell's Viper Venom time (DRVVT) mixing tests according to published guidelines. Coagulation factors were assessed by one-stage assays.
Results: A previously well 65-year old female presented to the emergency department with a 2-month history of headaches. MRI revealed a right frontal lobe extra-axial mass measuring approximately 5.5 × 6.0 × 6.0 cm with a 2.2 cm midline shift and subfalcine herniation. On resection, a meningioma was diagnosed. On postoperative day 5, the patient was anti-coagulated with low dose molecular weight heparin (LMWH) for DVT prophylaxis. By day 70, at the same time she was developing excessive haematomas of 3–4 cm around the LMWH injection sites, she also developed a pulmonary embolism. This was treated with therapeutic dose LMWH, followed by warfarinisation. After 2 weeks of therapeutic LMWH/warfarin, a progressive increase in the APTT was noted, peaking at 141 (normal range 25–37). The APTT had been normal prior to exposure to LMWH. A haematology consultation was requested. Mixing tests revealed a slow acting inhibitor with APTT on immediate mix of 44 s rising to 66 s after incubation. One-stage factor assays showed a FVIII:C level of 1 U/dL and FIX:C of 48 U/dL. A FVIII inhibitor of 9.1 BU/mL was detected by day 100, peaking at 22 BU/mL by day 110. The presence of a lupus anticoagulant was excluded by a normal dRVVT ratio of 0.95 (< 1.20). In view of these changes anticoagulation was immediately reversed and an IVC filter was inserted. The patient subsequently suffered repeated major bleeding episodes requiring emergency treatment with recombinant FVIIa and packed cells. Immunosuppressive therapy with cyclophosphamide, prednisone and rituximab was commenced. By post operative day 140, the FVIII inhibitor had reduced to less than 0.5 BU/mL indicating successful clearance of the inhibitor. The patient remains inhibitor-free with normal FVIII:C and is off all immunosuppression. The IVC filter has been removed.
Summary/Conclusions: The development of a FVIII inhibitor causing acquired haemophilia A is an uncommon but serious cause of bleeding. This patient had two known predisposing factors for the development of an inhibitor: malignancy and surgery. Early recognition of the inhibitor was essential to allow cessation of the patient's anticoagulation therapy. Eradication of the inhibitor was achieved with a combined approach of steroids, cyclophosphamide and rituximab.

PO 005

Unusual thrombotic complications in patients with acute promyelocytic leukemia

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Background: The risk of thrombotic events persists in patients with acute leukemias during remission induction chemotherapy and even after achieving complete remission. The treatment of acute promyelocytic leukemia (APL) with all-trans retinoic acid (ATRA) results in rapid resolution of the coagulopathy and bleeding, but paradoxically induces thrombosis in a small number of patients.
Material and Methods: Case 1. We present a 45-year old man, with low risk APL in whom thrombosis of v. centralis retinae occurred in complete cytologic and molecular remission while on maintenance therapy with ATRA. While in complete remission inJanuary 2012 he noticed vision disturbances which gradually progressed to complete loss of vision on left eye. Ophtalomologic examination detected occlusion of left v.centralis retinae with oedema of maculae. Screening tests for thrombophilia have been done: Fibrinogen 5.28 g/L, PV 89%/75–120%), partial thromboplastin time 26 s (25–35 s), KCT 94.1 s (70–90 s), LA_1, 41.3 s (< 45s), Antithrombin III 130% (80–120%), PCG 0.46 (> 0.8), PC 142% (70–140%), APCR 1.7 (2.1–3.5), PS 104% (63–135%). With PCR-RFLP method it was found that the patient was heterozygous carrier for factor 5 Leiden mutation (G1691A) and heterozygote for MTHFR C677T with hyperhomocysteinaemia. The risk for thrombotic events is especially evident in APL where various thrombogenic factors have been identified, but also co-morbid conditions such as arterial hypertension, diabetes and hyperlypidemia may be contributing factors for thromboses as well as inherited thrombophilia.
Case 2. A-49-year-old female presented with a history of headache, weakness and fatigue and CT cerebral scan revealed a subdural haematoma above right cerebral hemisphere. Physical finding was normal. Laboratory examinations revealed: Haemoglobin (Hb) 82 g/lm white blood cells (WBC) 5.7 × 10^9/L, platelets 47 × 10^9/L. D-dimer 15 μg/L, PT 66&. Bone marrow aspirate contained 73% blast cells with Auer rods in some cells, POX+. Immunophenotype of mononuclear bone marrow aspirate showed C34, CD38, cMPO, cCD68, CD117, CD13, CD33, CD11a, CD11b, CD4, CD64, CD56, CD2+. Cytogenetic analysis revealed *t*(15;17) in three mitoses and molecular examination presence of PML/RAR alpha+ transcript. The patient was treated according to PETHEMA protocol with idarubicin and ATRA. During the period of aplasia she developed thrombosis of v.hepatic sin (Budd Chiari syndrome) typ II which was with LMWH treated. The patient achieved complete remission of APL. She was further treated with LMWH until complete resolution and recanalisation of hepatic vein.
Conclusion: APL, a subtype of AML is characterized by life-threating bleeding and occasionally with thromboembolic events occuring typically during induction remission therapy with ATRA and idarubicin. In younger patients with APL accompanied with an unusual thrombotic event haemostatic and molecular tests for inherited and acquired thrombophilia should be performed.

PO 006

Transiently prolonged prothrombin times with reduced factor VII levels in acutely ill children

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Background: Over the course of 3 years, eight children with prolonged prothrombin times (PT) were identified, with further testing demonstrating a consistent reduction in factor VII and a variable reduction in a range of vitamin K dependent clotting factors (V, IX & X). Correction of the PT was demonstrated when the patients returned to normal health. A literature search revealed limited case reports of vitamin K depletion in adults, suggesting linkage to poor dietary intake of Vitamin K rich foods, but of unclear clinical consequence, specifically for haemorrhage.

Aims: To determine the clinical circumstances and possible causation of prolonged PT's in children, the relationship to dietary intake of Vitamin K rich foods and the potential consequences for haemorrhage.

Methods: Patients were identified by abnormal laboratory PT (with activated partial thromboplastin time) screening tests on admission to hospital. The children ranged in age from four to seventeen years. Six were boys and two were girls. Dietary intake was formally assessed in three patients and by retrospective examination of clinical correspondence for the remaining five.

Results: Eight acutely unwell children had prolonged PT's (range 15.6s–22.2s [reference range 10.0–15.0s]). Four cases were associated with bleeding, of which two were post tonsillectomy, one with tonsillitis and one following abdominal trauma with bleeding identified on laparoscopic investigation. Of the remaining four children, none of whom had bled, two had meningitis, one with pneumonia and one with a perforated appendix who underwent surgery. All eight children had lowered factor VIIc levels (range 21–54 IU/dL [referance range 65–150 IU/dL]). Three children considered at risk for haemorrhage received vitamin K1, one post laparotomy, one with meningitis and with one pneumonia, with no subsequent bleeding episodes. In two of these children the PT rapidly responded to intravenous Vitamin K1, while one child with more persistently lowered factor VII levels received 4 weeks of oral Vitamin K1 with correction of the PT and factor VII. Of the other five children the PT and factor VII levels improved when they returned to normal health. In three of the children, formally assessed for dietary intake, their parents reported chronically poor dietary intake of Vitamin K rich foods prior to the acute illness. Of the remaining five children, it was concluded that there was probable dietary disturbance in the days prior to hospital admission.

Conclusions: Transient prolonged PT's and low FVII's may be identified in acutely unwell children, in association with both chronic and probably acute poor dietary intake for Vitamin K. This may be of significance if a child faces a haemostatic challenge, as the depletion of Vitamin K dependant factors may increase the likelihood of bleeding. Assessment of a child's dietary and haemorrhagic history is advisable for acutely ill children. Further study needs to be undertaken in children on the dietary consequences of acute illness, the effects on vitamin K intake and coagulation parameters, to determine whether this may contribute to haemorrhagic risk.

PO 007

Assessing the range of dietary vitamin K intake in children undergoing tonsillectomy

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Background: Vitamin K is essential for production of the coagulation factors II, VII, IX and X, such that deficiency or antagonism can compromise haemostasis. Known causes of vitamin K deficiency include liver or malabsorptive disease, and iatrogenic causes. Dietary deficiency is not currently considered a risk factor. This Department has recently observed eight acutely ill surgical and medical paediatric patients with transient prolonged prothrombin times (PT) and factor-VIIdeficiency, unexplained by illness or drug treatment, in whom it was concluded that dietary deficiency of vitamin K was the likely causation.

Aim: To assess vitamin K intake in paediatric patients undergoing tonsillectomy and the possible consequences for haemostasis.

Methods: A pro-forma survey was undertaken by the parents of children undergoing tonsillectomy, at the time of a pre-admission clinical assessment. As there is no well-established measure in common clinical use for assessing vitamin K intake, a score was developed based on the subjective assessment by the parents for the frequency for intake of a range of foods, common to the local diet, in the preceding week. The vitamin K intake for each food was used, divided according to defined content of vitamin K per common measure (USDA National Nutrient Database) and ascribed as high, moderate or low, each category gaining 3, 2, or 1 points whilst a standard portion was assumed. Results were expressed as a score whereby the vitamin K content points were multiplied by the frequency of food consumption over the preceding week. The scores were histographically profiled and analysed for probability distribution and skewness. In addition parents were asked to comment on their child's diet when they were acutely unwell with tonsillitis. Subsequently the clinical and laboratory record at the time of surgery was assessed for complications and interventions including coagulation screening.

There were eighteen subjects, of whom 15 had progressed to tonsillectomy. The profile for the range of scored results for vitamin K intake was negatively skewed (skewness: −0.131) showing the majority of subjects had a high score, suggesting an adequate vitamin intake. Three children had four minor complications of surgery including: epistaxis (n = 1), vomiting (n = 2) and a vasovagal event (n = 1). No subject required coagulation screening at the time of surgery so PT prolongation and coagulation factor deficiency were not analysable. Fifteen children had had recurrent tonsillitis, in 61% of whom their parents reported probable reduction of vitamin K intake when they were ill.

Conclusion: The variability of vitamin K content in the diet of children prior to tonsillectomy diet has been highlighted. It is possible that during an acute episode of tonsillitis or prior to surgery, paediatric patients may undergo a dietary change which does not favour an adequate vitamin K intake and may result in transient changes in coagulation parameters and be a risk for haemorrhage. Further dietary studies of vitamin K would necessitate a larger sample size with an improved validated clinical measure for assessing intake.

PO 008

Acquired hemophilia A with severe anemia

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Background: Acquired hemophilia A (AHA) is a rare bleeding disorder caused by an autoantibody to coagulation factor (F) VIII. It is charac-

terized by soft tissue bleeding in patients without a personal or family history of bleeding.

Aim: To increase awareness of this disorder among clinicians, because bleeds in AHA are often spontaneous, and the severity does not correlate with the factor VIII level or strength of the inhibitor.

Methods: Diagnosis and treatment methods are based on clinical experience and involve coagulation studies and the use of coagulation factor concentrates.

Results: Diagnosis and treatment: A 88-years-old woman presented to our hospital, after a fall, with hematoma and functional impotence of the left lower limb. Here hemostasis was previously normal. She did not have any family history of bleeding disorders. Laboratory tests revealed a white blood cell count of 8350 cells/μL with an initial hemoglobin level of 5.6 g/dL and platelet count of 313 × 103 cells/μL. Coagulation studies revealed a normal prothrombin time, and prolonged aPTT of 70 s. The presence of an inhibitor of coagulation was diagnosed via prolonged aPTT and a mixing study that did not correct with the addition of normal plasma PTT when an immediate mixing test was performed; with a ratio of patient's plasma to normal plasma of 1:1. Quantitative assays factor VIII activity 4% and the presence of factor activity inhibitor measured at 1.5 Bethesda units in the serum. Acquired hemophilia A (AHA) was diagnosed.

Four units of red blood cells were transfused, and 75 UI/kg/day of human factor VIII during 3 days, oral prednisone therapy 1 mg/kg/day, plus human erythropoietin 'Darbepoetin alfa' 40 μ once a week, every Friday during 5 weeks. Here aPTT, and factor VIII gradually improved.

Summary/Conclusion: Clinicians should suspect a diagnosis of acquired hemophilia in patients with unexplained persistent and profound bleeding from soft tissue and mucosa and in any patient who presents with bleeding and a prolonged aPTT without other cause, and without any history of bleeding. Our patient was discharged with prednisone as immunosuppressive therapy with treatment plan for a slow tapering of steroids as well as careful monitoring of here coagulation parameters disorders.

Further diagnostic investigations in terms of finding an underlying cause for the acquired hemophilia were done, we did not find any etiological factors associated with AHA [autoimmune disease, drugs, solid tumor, vaccination, hematologic malignancies, skin disorders, respiratory disorders, infections, others (diabetes, surgery), idiopathic]. Treatment should be focused on controlling the immediate bleeding episode and suppressing the immune reaction against the coagulant factor. Immunosuppressive therapy with steroids or cyclophosphamide for inhibitor eradication should begin immediately after diagnosis is made.

PO 009

Multiple myeloma presenting with acquired factor VIII inhibitor – a case report

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Introduction: Acquired factor VIII inhibitor (AFI) is a very rare entity with an estimated incidence of 0.2–1 per million and a mortality rate ranging from 8% to 22%. This condition occurs in association with drugs, viral and parasitic infections, in autoimmune conditions such as graft vs. host disease, solid tumors and myeloproliferative and lymphoproliferative disorders. Only five cases of AFI have been associated with multiple myeloma (MM). Acquired factor VIII inhibitor usually presents with bleeding (cutaneous and mucosal) while hemarthrosis, which is commonly seen in patients with congenital hemophilia, is very infrequent in AFI.The diagnosis of the acquired inhibitor is usually made with the laboratory finding of an elevated activated partial thromboplastin time (aPTT) that cannot be corrected by plasma mixing study (incubating for 2 h at 37 °C equal volumes of patient plasma and normal plasma), and further confirmed by low factor VIII activity/antigen levels along with elevated factor VIII inhibitor levels using the Bethesda assay. Treatment is usually based on steroids and cyclophosphamide.

Case report: A 39-year-old man was referred to our hematology unit to evaluate a diagnosis of myeloma. Two months prior to this presentation he started with hematomas in his body and pain in your right thigh. He visited many medical services looking for a diagnosis.

The patient had no history of bleeding disorders. The physical exam was normal.

Amagnetic resonance was performed and showed lytic lesion in acetabulum and some images suggesting blood collections in psoas and gluteo muscles.

His initial labs showed a normal hemogram and the coagulation profile demonstrated a normal protrombin time and an elevated activated partial thromboplastin time of 58s (control, 31s).

Serum protein electrophoresis showed a biclonal band between beta gamma region. An IgA paraprotein was identified by Imunofixation. Bone marrow aspiration showed normal celularity with 28% of plasma cell infiltration.

In our Unit he was submited to further evaluation. X-ray films of the bones revealed no lytic areas. The serum Beta2 microglobulin level was normal. The liver function was normal, also serum calcium and serum dehydrogenase. Activated partial thromboplastin time was repeated and could not be corrected by mixing with normal plasma. Additionally, other coagulation tests including fibrinogen and factor assays (IX, XIII, X) were in normal rangeslFactor VIII activity level was found to be 4% while the Bethesda assay confirmed the presence of an AFI.

A diagnosis of IgA multiple myeloma (Durie Salmon stage IA and International Scoring System stage I), associated with acquired FVIII inhibitor was made and treatment with cyclophosfamide, dexametasone and thalidomine was started.

He is receiving the second cycle and activated partial thromboplastin time measured in the last visit was 49s (control, 34s) and he hasn't been no bleeding or new hematomas.

Conclusion: This case demonstrates that AFI can be seen in plasma cell disorders such as MM, and can be the first manifestation of these diseases. Therefore this association should be remembered in patients with hematological malignancies and excessive bleeding not related with low platelet counts and with abnormal coagulation tests.

PO 010

Factor X inhibitor: an unexpected bleeding disorder with a therapeutic challenge

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Background: Isolated factor X (FX) deficiency is a rare coagulopathy. It may be hereditary or even more rare an acquired condition. Acquired FX deficiency states are usually secondary to systemic amyloïdosis in which FX binds irreversibly to amyloid fibrils or in association with some malignancies. Sporadically, transient selective FX deficiency has also been reported in the absence of amyloidor cancers.

Patient and Results: A 86-year-old patient was referred to our department for persistantepistaxis and gum bleeding. His medical history consisted of high blood pressure, appendectomy, total hip replacement and ileus requiring surgery. None of these surgical procedures had been complicated with abnormal bleeding. Clinical examination showed several hematomas: left forearm, right testis and right buttock. Initial laboratory testing revealed a platelets count of 196 × 10^9/L, normal fibrinogen concentration (4.6 g/L) but a low haemoglobin level

of 7.3 g/dLrequiring blood transfusion. There was no sign of vitamin K deficiency. Furthermore, serum protein electrophoresis and immunoelectrophoresis were normal and there was no immunoglobulin free-light chain in serum, indicating neither amyloïdosis nor monoclonal gammopathy.
Laboratory abnormalities includedsignificantly prolonged prothrombin time (PT), activated partial thromboplastin time (aPTT) and an aPTT mixture assay was slightly above the reference interval. Specific factor assays revealed a profoundly decreased FX activity (0.01 IU/mL), whereas FX antigen was 0.3 IU/mL. Although a Bethesda inhibitor assay for a FX inhibitor was negative, patient plasma fully inhibited FX activity in Russell's viper venom and tissue factor/factor VIIa-based FX chromogenic assays. Moreover, an immunosorbent assay showed the presence of anti-FX IgG antibodies, which did not react with murine FX or other human vitamin K-dependent coagulation factors. Typing studies revealed that patients' immune response towards FX involves preferentially IgG1 and IgG4. Pre-incubation of FX with patients' IgG did not alter FX clearance following injection in mice.
Therapy with an infusion of Prothrombin Complex Concentrates was initiated but immediately stoppedbecause of an anaphylactic shock, and treated successfully by epinephrine and corticosteroids. The treatment further resulted in normalization of FX levels.
Conclusions: Prothrombin complex concentrates are given as replacements in cases of acquired or inherited deficiency. However, our study showed that allergy or anaphylaxis should be considered especially when the patient has an idiopathic, isolated acquired FX deficiency with no history of bleeding, and no primary pathology that could cause FX deficiency. Corticosteroids alone (1 mg/Kg/d) permitted to obtain an increase of FX activity and a clinical improvement with no further hemorrhagic event.
These data also demonstrate that acquired FX inhibitors appearing in transient selective FX deficiency in the absence of amyloid or malignancies can display specificity against restricted functional domains of FX.

PO 011

Recombinant activated factor VII in treatment of bleeding complications in thrombocytopenic patients

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Background: Hemorrhage in patients (pts) with thrombocytopenia usually is treated with platelet transfusions. In cases of an inefficiency or impossibility of platelet transfusion recombinant activated factor VII (rFVIIa) may be useful.
Aim: Aim of the study was to evaluate efficacy of rFVIIa to control of bleeding in thrombocytopenic pts and relationship between clinical response and coagulation tests.
Methods: We observed 6 pts with thrombocytopenia and hemorrhagic syndrome who didn't answer platelet transfusions due to alloimmunization or who couldn't receive platelets because of religious beliefs (Jehovah's Witness). All pts were treated with single bolus dose (40–90 mcg/kg) of rFVIIa (Koagil VII, Masterclone, Russia). APTT, INR, FVII:C level,
endogenous thrombin potential (ETP) and Thromboelastography (TEG) were evaluated before rFVIIa administration, in 15 min, in 60 min and in 120 min after rFVIIa administration.
Results: In all pts clinical response was achieved. In 3 pts gastrointestinal bleeding was stopped. In patient with subarachnoid hemorrhage after injection of rFVIIa good clinical response was achieved despite low platelet level (6 10^9/L). Administration of rFVIIa allowed to execute invasive procedures (lumbar puncture, catheterization of central vein). In patient with aplastic anemia (Jehovah's Witness) and very low blood platelet count (1 × 109/L) two repeated injection of rFVIIa reduced hemorrhagic syndrome (epistaxis, petechia) and, allowed to perform catheterization of central vein. The plasma concentration-time profile of FVII:C and ETP demonstrated a dose dependent increase. The peak of increase of FVII:C (from 59% to 545%) and ETP (from 1000 to 1200 nM min) was noted in 15 min after rFVIIa administration. However maximal clinical response and normalization of TEG parameters (R, MA) were observed only in 60 min, when the level of FVII:C started decreasing (to 430%). In 120 min level of FVII:C remained higher than norm (370:), but parameters of TEG came back to initial levels.
Conclusion: rFVIIa treatment showed an improvement in the control of bleeding in pts with thrombocytopenia who for various reasons couldn't receive therapy by platelet concentrates. Clinical response and improvement of parameters of TEG didn't coincide with increase of a level of FVII:C in plasma.

PO 012

Long-term follow up in Acquired Hemophilia A: clinical courses and outcomes observed in a single Hemophilia Centre

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Background: The prospective study from the European Acquired Hemophilia Registry (EACH2) reported the clinical outcomes of a large number of patients after < 1 year follow up.
Aim of the study: To report the clinical courses and outcomes in patients with Acquired Hemophilia A (AHA) enrolled during 5 years in Hemophilia Centre of Pavia.
Methods: Since June 2007 to July 2012 AHA has been diagnosed in 18 pts (8 women and 10 men) aged between 27 and 93 years. Twelve pts were over 70 years. AHA was idiopathic in 9 pts (50%), attributed to Autoimmune diseases in 4 pts (22%), to malignancies in 3 pts (17%), to pregnancy (PPAHA) in 2 pts (11%). The pts have been treated on demand with by passing agents (aPCC or rFVII) during acute bleeding and with immunosuppressive drugs [PDN ± CTX and in 3 cases of failure with rituximab), to eradicate the inhibitor.
Results: One man (62 year) with malignancy and one woman (89 years) with idiopathic AHA died after 2 months from AHA diagnosis for causes not related to hemorrhagic complications. Stable Complete Remission (CR) of AHA was obtained in 12 pts (66%) after a period of time variable from 1 month to 2 years. Three pts achieved remission only during PDN treatment (clinical follow up 26 months, 10 months and 6 months) Among the 8 pts (44%) in CR followed for more than 3 years, 2 pts relapsed: in the first pt with previous PPAHA after 35 months of CR, not in relation to another pregnancy, in the second pt with idiopathic AHA after 25 months of CR. Two pts died respectively after 4 years and 1 year from achieving CR for causes not related to AHA.
Conclusion: The causes of death in patients with history of AHA is frequently not related to this disease. CR is obtained in a large percentage of cases, nevertheless relapses can occur even after a long period from CR, so that a clinical follow up is lifelong recommended.

PO 013

Haemostatic parameters including Factor VIII levels in different types of tuberculosis in Northern India

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Background: Vascular occlusions- cerebral and peripheral- are encountered in our patients with tuberculosis with upto 10% being peripheral venous thrombosis and cerebral arterial in 6- 41%. Haemostatic abnormalities in these clinical subjects is inadequately studied especially from developing countries which report 95% of the 5.8 million new cases annually (WHO 2009).

Aim: To study the coagulation parameters including coagulation Factor VIII level in different types of tuberculosis at baseline and after intensive phase antitubercular treatment.
Methodology: One hundred and twenty eight adult patients over 12 years of either sex with newly diagnosed tuberculosis of different sites- categorized as pulmonary, CNS, disseminated and others- were included after written informed consent. Along with detailed clinical assessment, the haemostatic parameters assessed included CBC, Prothrombin Time (PT), Activated Partial Prothrombin Time (APTT), Factor VIII level, Fibrinogen and D-dimers. These parameters were repeated after 2 months of intensive standard antitubercular therapy (ATT).
Results: Mean age of the 128 subjects was 31.55 ± 15.03 years, range 12–75 years. Pulmonary, CNS, disseminated and other types of tuberculosis comprised 30.5%, 28.9%, 17.2%, and 23.4% respectively.
Abnormal PT and APPT at baseline was seen in 64 (50%) and 23 (18%) cases respectively whereas the mean coagulation Factor VIII level was 125.08 ± 72.83%. The Factor VIII was deranged in 45 (35.15%) cases, being high in 28.12% and low in 7.03%. Baseline fibrinogen levels were altered in 73 (57%), with 62 being elevated beyond 400 mg%. D-Dimers were high in 57.8% patients. Platelets were disturbed in 66 (51.5%), with thrombocytopaenia in 47 and thrombocytosis (exceeding 400×10^9/L) in 19 cases.
After intensive phase ATT for 2 months, the PT reverted to normal in 32 (50%) of 64 abnormal at baseline across all tuberculosis ($P < 0.01$). APTT did not show a similar trend. Coagulation Factor VIII levels did not change significantly after ATT (125.08 ± 72.83 vs. 126.21 ± 37.65 IU/mL) whereas the fibrinogen levels decreased significantly from 391.43 ± 167.34 to 272.33 ± 92.66 mg% ($P < 0.01$). Cases with elevated D-dimers came down to 12.5%. All the abnormalities in platelet counts- thrombocytopaenia as well as thrombocytosis-reverted to normal within the 2 months of ATT.
Subgroup analysis on the four different types of tuberculosis mirrored these results except for the PT in disseminated tuberculosis where the *P* value was 0.08.
Conclusions: Platelet disturbances were seen in half of the patients with tuberculosis, thrombocytopaenia being thrice as common as thrombocytosis. Elevated PT/INR was present in half of all cases whereas APTT was deranged in 18%.
Coagulation Factor VIII levels were high in 28% cases, high fibrinogen levels in 48% cases and high D-dimers in 58%. These elevated fibrinogen, D-dimers, and coagulation Factor VIII levels observed in patients with different types of tuberculosis may favour a risk to hypercoagulable state and which improved with anti-tubercular therapy.

PO 014
Snake venom induced coagulopathy

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Background: Snake venom induced mortality and morbidity is a neglected health hazard in tropical and sub-tropical regions particularly in rural areas of the world. Snake venom secreted from highly specialized venom glands, has evolved to capture prey. Primarily, the venom is evolved to immobilize, kill and digest the prey and is a potent defensive weapon. It is a complex mixture of enzymatic and non-enzymatic components affecting various organs and haemostatic system.
Aim: The present study aims at evaluating snake venom induced coagulopathy, with differential effect of 'big four' venomous snakes of India (*Naja naja*, *Daboia russelli*, *Echis carinatus* and *Bungarus caeruleus*).
Method: The effect of 'big four' snake venoms on haemostasis was evaluated using pooled human citrated plasma and citrated plasma obtained from venom injected rats. Routine clotting assays (PT, APTT), Fibrinogen content, specific factor assays were used to determine the effects on haemostasis and visco-elasticity tests for clot formation were used to evaluate pro-coagulant/anticoagulant nature of snake venoms.
Results: The findings revealed differential action of venom from big four snakes towards coagulation cascade. The results showed that of *D. russelli* and *E. carinatus* venoms exhibited pro-coagulant activity, whereas *N. naja* and *B. caeruleus* venoms are anticoagulant in nature. *E. carinatus* venom showed strong pro-coagulant and *D. russelii* venom showed defibrinating effect. *N. naja* venom showed stronger anticoagulant than *B. caeruleus* venom. Further, the mechanism by which *N. naja* venom exhibits anticoagulant effect was evaluated. Clotting times in PT and APTT assays were prolonged with the increasing concentration of the venom, with minimal effects on fibrinogen level, FX and FVII activities. Interestingly, FVIII is significantly decreased with the increasing concentration venom and this decrease was time-dependent. FVIII was completely inactivated after 12 min at 1 mg/mL of venom. Similar results were obtained in plasma from venom injected rats (60 min after injecting respective lethal dose of *N. naja*, *E. carinatus*, *D. russelli* and *B. caeruleus* through intraperitoneal route), with slight variations. In contrast, no effect was seen 30 and 45 min after venom injection. Thus, *N. naja* venom exhibited anticoagulant effect under both *in vitro* and *in vivo* conditions. Similarly, *D. russelii* venom exhibited defibrinogenating activity in both conditions. In contrast, *E. carinatus* venom exhibited pronounced pro-coagulant effect *in vitro* and anti-coagulant effect *in vivo*. *B. caeruleus*venom exhibited negligible effect towards rat plasma.
Conclusion: The marked differential effect on coagulation cascade will be useful in treatment of snakebite victims, based on the characteristic haemostatic effect. The administration of specific anti snake venom based on the observed effects will be an important strategy in treatment and management of snakebite victims.

PO 015
Bleeding into tongue – case history of acquired haemophilia A

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Background: Acquired haemophilia A is a rare form of acquired coagulopathy caused by autoantibodies against coagulation factor VIII. It is characterized by major bleeding complications often life-threatening.
Aims: The authors report case history of acquired haemophilia in 85 years old woman with medical history of hypertension, ischemic heart disease, ischemic stroke without neurological consequences and no past bleeding history during operation (appendectomy and hysterectomy for myomas) and no family history of coagulopathy. The first sudden onset of bleeding was observed in the oral cavity – mostly into the tongue and under tongue oral mucosa when she was admitted to the department of internal medicine for generalized worsening.
Methods: Laboratory investigations showed a prolonged APTT-R (activated partial tromboplastin time ratio) 4.1 and normal PT (prothrombin time) and TT (thrombin time). Fibrinogen and antithrombin were normal. Failure of correction on mixing study prompted further evaluation of individual clotting factors activity: FVIII (0.8%), FIX (47.7%), FXI (52.7%), FXII (38.8%). The low FVIII:C (FVIII activity) was confirmed by testing with Actin FS, Siemens (insensitive to lupus antikoagulant) – 1.8%. The FVIII inhibitor titer was quantified 13.6 BU (Bethesda units). However, lupus anticoagulant was also confirmed by platelet neutralization procedure (PNP).
Result: Bleeding complications were well controlled by bypassing agents rVIIa at a dose 100 μg/kg every 3 h during the first day and by aPCC (activated prothrombin complex) at a dose 90 IU/kg twice daily when hematuria or soft tissue bleeding and diffuse bruising manifested. Immunosuppressive therapy was initiated with methylprednisolone 500 mg infusion first 3 days and continues with combination prednisolon 1 mg/kg and cyclophosphamide 1 mg/kg. After 6 weeks

from therapy initiation antibody titer did not drop significantly. Inhibitor eradication was successful after the use of second line agent rituximab 375 mg/m² weekly for 4 weeks. The inhibitor completely disappeared after 128 days from diagnosis and patient is still in remission (9 months of dispenzarization). Underlying associated disease was not found except only laboratory manifested lupus antikoagulant.
Summary/Conclusion: Rituximab used as a second line therapy in elderly patient was well tolerated, only infection complications were observed after this treatment – bronchopneumonia and urine infection. Patient is still in remission in this time.

PO 016

Acquired hemophilia A: literature review and report of two cases

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Background: Acquired hemophilia A (AHA) is a hemorrhagic disorder caused by autoantibodies, generally IgGk4, directed against circulating factor VIII (FVIII) of coagulation which neutralize its procoagulant function.
Is a rare bleeding disorder with an estimated anual incidence between 1 and 1.5 per million per year, with a biphasic behavior, with a first peak occurring among young adults due to cases in women in the postpartum period, and a second major peak in elderly patients.
In approximately 50% of cases, FVIII autoantibodies occur in patients lacking any relevant concomitant disease and remain idiophatic, while the rest cases may be associated with hematologic malignancies, solid cancers, autoimmune disorders, infections, drugs or other illnesses.
The diagnosis is based on the initial detection of an isolated prolongation of activated partial thromboplatin time (APTT) which does not normalize by mixing study and a reduced FVIII levels, with evidence of FVIII inhibitor activity.
The treatment of AHA is based on two aspects: treatment of acute bleeding and eradication of the acquired FVIII-inhibitor.
Patients: Two clinical cases are presented, one acquires and one secondary. An 87 years old man with no past medical history and a 60 years old man with rheumatoid artritis, AHAI an refractory anemia. Both consulted for mucocutaneous bleeding, objectifying lengthening of TTPA isolated, not corrected with mixing test, decreased en FVIII level and detection inhibitor against FVIII. The 87 years old patient also presented TVP in the right leg with positive lupus anticoagulant. Both were treated with hemostatic treatment with rFVIIa. Treatment was prescribed corticosteroids vs. inhibitor (1 mg/kg/weight) y rituximab (375 mg/m^2/per week X 4 doses). In a case was added previously cyclophosphamide. Both patients responded with increased in FVIII level above 40% and cessation of bleeding manifestations.
Discussion: AHA is a rare autoinmune bleeding disorder. Bleeding into the skin, muscles and soft tissues are the principal clinical manifestations.
The diagnosis should be considered in patients with unexplained bleeding and prolonged activated partial thromboplastin with no previous personal or family history of bleeding. In laboratory the mixing test (mixture of patient plasma and normal plasma) is important, in AHA APTT does not normalize after this test with reduced FVIII levels and an inhibitor against FVIII.
AHA treatment is based on three aspects: 1) treatment of bleeding with bypassing agents as a first-line treatment (aPCC and rFVIIa), 2) eradication of the inhibitor with immunosuppressive agents (prednisone alone or with cyclophosphamide as a first-line treatment) and 3) treatment of the underlying disorder. Rituximab may be useful as first-line therapy in patients for whom immunosuppressive agents fails or are contraindicated.
After treatment AHA must be monitored with regular APTT, FVIII activity and FVIII inhibitor titer measurement with accurate clinical evaluation.
AHA is a rare coagulation disorder that usually presents to clinicians without previous experience. Prompt recognition is very important to initiate an early management.
Noted for its rarity deep vein thrombosis with hemorrhagic disease in one of our cases.

PO 017

Our experience in the use of prothrombin complex concentrate in patients with acute haemorrhage

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Background: Prothrombin complex concentrate (PCC) is recommended for rapid reversal of vitamin K anticoagulants which is essential when acute bleeding or emergency surgery occurs. Prothrombin complex concentrate produce more rapid effect with a better clinical outcome, and do not cause volume overload compared with fresh-frozen plasma (FFP).
Aim: Our goal is to report our institutional experience with Prothrombin complex concentrates for the treatment of acute bleeding in the surgical settings.
Methods: This is a retrospective analysis in a University Clinical Center Maribor with 1288 beds. We report a case series of 64 patients with acute haemorrhage who were treated with PCC in the last 2 years.
Results: During 2011–2012 sixty-four patients received PCC, 75% (fourty-eight of sixty-four) men and 25% (sixteen of sixty-four) female. The most common causes of bleeding among patients were cardiac or vascular surgery (17), abdominal surgery (12), multi-trauma (12), obstetrical complications (1), neurosurgery (14), urology (4), orthopedic surgery (1) and patients in internal intenive care unit (3). Forty-five percent (twenty-nine of sixty-four) patients were on anticoagulant therapy with warfarin or acenocoumarol. Ten of them were neurosurgery patiens, three cardiovascular, six multi-trauma, six abdominal surgery, three urology and one hematologic patient. Six of twenty-nine patients who were receiving anticoagulants were overdosed while twenty-three patients were in therapeutic range. The median dose of PCC administred was 1800 units per patients (25–50 IU/kg body weight). Most patients with INR in therapeutic range achieved an INR of 1.5 or less after one prothrombin complex concentrate infusion while patients with INR above therapeutuc range did not.
Conclusions: Rapid reversal of excessive anticoagulation with PCC should be undertaken in patients with serious bleeding at any degree of anticoagulation. These patients have much better outcome than patients treated only with FFP and vitamin K.

PO 018

Acquired haemophilia: why the delay in diagnosis?

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Background: Acquired haemophilia is a rare but potentially life-threatening bleeding disorder resulting from the autoimmune depletion of a coagulation factor, most commonly factor VIII. Patients without any previous bleeding history presents with sudden onset of haemorrhage. Urgent laboratory diagnosis and quantification of the inhibitor is necessary in order for appropriate treatment to be instituted.
Case study: A 32 year old patient presented to a tertiary care facility at 37 weeks gestation with spontaneous labour and a breech presentation. The patient underwent an uneventful Caesarean section. Post surgery the patient's abdomen was distended with ongoing haemorrhage from the surgical scar. The haemoglobin remained low (< 6 g/

dL) despite multiple blood transfusions resulting in a re-look laparotomy and bilateral uterine artery embolisation. The first PI/PTT was performed 4 days after admission with a twice normal aPTT but no correction study was done. The prolonged aPTT was confirmed on repeat testing with the first correction study performed 12 days post admission. The aPTT failed to correct and factor VIII and IX levels were performed 14 days post admission followed by quantification of the factor VIII inhibitor. At the time of diagnosis the patient had received more than 50 units of packed cells, multiple platelet and FFP units and developed a femoral artery aneurysm at a vascular access site. After the correct diagnosis the patient received appropriate haemostatic agents and therapy to eradicate the inhibitor.

Discussion: Delayed diagnosis of acquired haemophilia can have serious consequences. This delay has also been documented in other centres with a range of 3 days to 9 months from the onset of bleeding to correct diagnosis. Omission of baseline haemostatic parameter testing and lack of thorough analysis of abnormal laboratory results contribute to the delayed diagnosis.

Keywords: Acquired haemophilia; life-threatening; laboratory diagnosis.

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PO 019

Bleeding tendency associated with a coagulation inhibitor in two patients with monoclonal gammopathy of undetermined significance

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Background and Aims: Acquired haemostatic defects affecting coagulation, fibrinolysis or platelet function may occur in patients who have abnormal levels of monoclonal protein (M-protein) in the blood. We present the cases of two patients with a monoclonal gammopathy of undetermined significance (MGUS) – [WHO ICD-O Code (9765/1)], who presented with a history of moderate bleeding and an abnormal APTT.

Methods and Results: Patient 1 was a 70 year old female who presented for elective insertion of a permanent pacemaker. She had developed a bleeding tendency later on in life, mainly related to invasive procedures and surgery. She was known to have a MGUS with an IgA Lambda M-protein of 7 g/L and considered to have a lupus anticoagulant (LA) on testing performed elsewhere. The APTT (Actin FS, a lupus insensitive reagent) was prolonged at 57s (Reference Interval 25–35s) and there was no correction in mixing studies, demonstrating the presence of an inhibitor. The prothrombin time and fibrinogen level were normal but the thrombin time (TT) was slightly prolonged at 13 s (RI 10–12s. The intrinsic factors were markedly decreased (1–4%), rising only slightly higher when tested with higher dilutions of the patient plasma, suggesting the presence of a LA like inhibitor. The inhibitor also interfered with factor specific inhibitor assays (Bethesda). However, LA testing (RVV and APTT with Actin FSL and Actin FS) performed in our laboratory, was negative. Chromogenic Factor VIII was 114%. The patient was managed with infusion of fresh frozen plasma (FFP) prior to the procedure, which went ahead without any bleeding complications whilst in hospital or following discharge.

Patient 2 was a 53 year old male with a several year history of mild to moderate bleeding after surgical procedures which were managed with infusions of FFP. The APTT was always prolonged with an inhibitor type profile similar to Patient 1 in that intrinsic factors were moderately reduced in the presence of a LA-like inhibitor but LA testing was negative on different occasions except for one. Some years later, he was found to have an IgG Lambda M-protein of 9 g/L with a diagnosis of MGUS.

Conclusions: It was concluded in these 2 cases of patients with MGUS and a history of bleeding, that the abnormal APTT was due to the presence of the M-protein (one an IgG and the other an IgA), the mechanism of action remaining unclear. These laboratory studies illustrate the difficulties in distinguishing a lupus like inhibitor from other inhibitors. With both patients, intrinsic factor assays were affected by the presence of the inhibitor to various degrees with increasing factor levels measured in higher dilutions of the patient plasma. This interference with factor assays was also evident in Bethesda inhibitor assays but no specific factor inhibitor could be demonstrated. Nor was there any consistent laboratory evidence of the presence of a lupus anticoagulant. Both cases were managed successfully with FFP infusions, although the rationale for the efficacy of FFP also remains unclear.

PO 020

Acquired Von Willebrand syndrome: about one observation

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Introduction: Von Willebrand syndrome is an acquired rare bleeding disorder developing in an elderly patient with no history hemorrhagic staff or family. Mechanisms are multiple, the most common autoimmunity, adsorption on the surface of tumor cells or accelerated proteolysis.

Objective: Difficulty therapy in a patient with Von Willebrand-acquired. Observation: patient aged 69 with a history of mono arthritis in 1972 operated for cholelithiasis in 1976 tonsillectomy in 1995 extraction of a molar in 1995, 2007 giant hematoma of the right buttock after intramuscular injection. In 2010, the etiological severe haemorrhagic syndrome following the bite of a language concluded acquired von Willebrand disease. Laboratory tests showed normal bleeding time 7 min (ivy-incision), an aPTT of 51 s (M/T = 1.64) while the TP is normal rate factor VIIIC collapsed to 12.60% and Von Willbrand factor VWF Ag: 7%, vWF-RCo 3%, the ratio RCo/Ag < 0.43, the systematic search for autoantibodies discovered an anti-VWF 8 IU (neutralization test) associated with anti VIII-7UB by Bethesda Nijmegun method. In France, in April 2012 angina secondary to thrombosis of the circumflex artery. In the pre-opéraoire is diagnosed with von Willebrand disease. The patient was operated under emergency Willebrand factor without incident.

Discussion: (1) The acquired Willebrand disease: age, lack of personal history and/or family history of hemorrhagic disease. Our patient has a mono arthritis, decreased VWF-RCo and FvW-RCO/Ag ratio < 0.7. VWF antibody that is found in 16% (Ref 1) cases is found in our patient is most associated with anti-VIII. (2) In our patient, the mechanism character is selected according to the criteria defined by the International Register of von is probably related to mixed proteolysis accelerated by the existence of a thrombus in the circumflex artery (21% of cases in the literature) and an autoimmune mechanism in the presence anti-VIII and anti-VWF (2–6% of cases in the literature). The existence of a mono arthritis is a chronic course of disease in favor of a system that would settle low noise. (3) The susceptibility of our patient bleeding risk and thrombotic risk may be increased by age, and history of hypertension and dyslipidemia. (4) feature in our patient, it presents a antiFVW and anti VII acquired therapeutic problem.

Conclusion: Von Willebrand syndrome is a disease acquired exceptional. But we must think in the elderly and therefore to search systematically.

Annuler les modifications

PO 021

Inhibitor characterization in acquired haemophilia and its influence on bleeding profiles and treatment response: CIHA-01 project

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Background: Acquired haemophilia (AH) is a rare autoimmune bleeding disorder induced by autoantibodies against clotting factors. The most frequent target is factor VIII (FVIII). Clinical picture is an acute or recent onset of bleedings in subjects without personal or family history of bleeding disorders, associated with a long activated partial thromboplastin time (aPTT). Bleeding pattern is different from congenital haemophilia, typically with more mucocutaneous and soft tissue events. There is no correlation between residual FVIII, inhibitor titer and severity of bleeding, we propose that FVIII target epitopes could influence inhibitor characteristics, bleeding profile and treatment results.
Aims: To recognize FVIII epitopes target in AH.
To analyse any correlation between these epitopes, inhibitor kinetic and titre, bleeding severity, haemostatic and eradication treatment response.
To identify changes in inhibitor characteristics throughout AH treatment.
Methods: This is a prospective, descriptive, non-interventional observational study. Ethics Committee evaluation and informed consent are requested from all patients. Expected number of patients: 30 (expected diagnosis rate of 40 cases-year, and an inclusion rate of 35%). Sampling schedule: At diagnosis, 14 days post-diagnosis, 56 days after remission and free from eradication treatment. If eradication fails and an alternative therapy is initiated, sampling schedule will be repeated. Demographic data to include: age, sex, underlying disorders, bleeding profile, haemostatic and immunosuppressive treatment, transfusions and treatment adverse events. Haemostatic parameters: FVIII activity (one stage coagulation method), mixing test (one stage based on Kasper method), FVIII inhibitor titer (Bethesda method) and epitope mapping by immunoblot analysis.
Results: Patients recruitment started in June 2011 and it is still open. By December 2012, 26 centers have applied for inclusion. Nineteen patients have been included. Four of them died before second sample extraction. Six subjects have completed the study. Nine patients are on sampling period. In the six evaluable patients, median age is 63 years-old (range 19–82). AH was idiopathic in all cases but one (drug associated). The median pick inhibitor titre is 21 BU (Bethesda Units) (range, 17–400). Kinetic was type 2 only in one case. Three patients show reactivity against light chain and A1 and A2 domains, two patients against light chain and other one against A2 domain. First line treatment was prednisone or prednisone and cyclophosphamide in all cases. Two patients required second-line treatment. One patient did not respond to eradication therapy even after 3° lines treatment. He was a 65 years-old, idiopathic HA, kinetic type 1 and 30BU inhibitor of antibody against the light chain. Only one patient changed antibody specificity during immunosuppressive therapy. He is a drug-associated AH, 400BU titer, type 1 kinetic, specificity against light chain and A1 and A2 domain. He failed 1° line treatment and in sample just before 2° line treatment, specificity moved to light chain.
Conclusions: By the moment is not possible to get any conclusion. We hope to collaborate with knowledge to predict clinical profile and treatment response in AH.

PO 022

Accuracy of thromboelastometry analysis in detecting patients treated with vitamin k antagonists

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Background: The accuracy of thromboelastometry analysis to rule-out patients treated with oral anticoagulants (OAC) such as vitamin k antagonists (VKA) is unknown. Existing studies suggest limited sensitivity, but no accuracy analysis has been done. A low sensitivity may limit the clinical value of thromboelastometry in trauma patients because VKA are common, are associated with an increased risk for bleeding, and represent an important risk factor for mortality in this setting.
Aims: We investigated the extent of accuracy as determined by sensitivity, specificity and the negative likelihood ratio of thromboelastometry analysis in detecting patients treated with VKA and we calculated the probability that a patient is treated with VKA given a negative test result.
Methods: To preclude confounding variables such as dilutional coagulopathy, thrombocytopenia or acute traumatic coagulopathy, the investigation was conducted in stable patients without other risk factors for bleeding. The study was approved by the local ethical review board and all participants provided written informed consent. We enrolled patients consecutively that were evaluated for risk of recurrent venous thromboembolism between February 2011 and October 2012 at our institution. Participants were stratified according to (a) oral anticoagulation with VKA and (b) no anticoagulation. Thromboelastometry analysis was conducted using two ROTEM® delta analysers. Detection of VKA was defined as EXTEM measurements above reference ranges established by others (clotting time > 79 s; maximum clot firmness < 50 mm). Patients were considered as OAC patients if international normalised ratio (INR) was 1.7 or above at the time of thromboelastometry analysis.
Results: For this preliminary analysis, data from 63 patients treated with VKA (17.6%) and 294 patients without anticoagulation were available. Two hundred and seventeen of the 357 patients were female (60.8%), median age was 43.5 years (IQR 30.4–59.2 years). OAC was done almost exclusively with phenprocoumon. Thromboelastrometry analysis was abnormal in 42 patients with OAC and in three patients without, and it was normal in 21 patients with OAC. Sensitivity was 66.7% (95% CI: 53.7–78.1%), specificity was 99.0 (95% CI: 97.1–99.8%), and the negative likelihood ratio was 0.51 (95% CI: 0.37–0.68). When using a pre-test probability of 17.6%, the probability that a patient is treated with VKA given a negative thromboelastometry result is 9.8% (95% CI: 7.8–12.7%).
Summary/Conclusions: The results of our investigation indicate that thromboelastometry analysis is of limited value in ruling-out that a patient is treated with VKA. Therefore we suggest, that thromboelastometry analysis does not substitute INR determination in trauma patients.

PO 023

Haemodilution with Nacl solutions below 600 mOsm/L Induce hypercoagulability while concentrations above 1200 mOsm/L progressively impair coagulation as evaluated with thrombelastography *in vitro*

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Background: Solutions of Nacl at various concentrations are the mainstay of trauma resuscitation. Though acute trauma associated coagulopathy has been attributed to haemodilution, the role of fluid osmolality remains large unexplored.
Methods: Citrated whole blood was collected from 10 healthy volunteers and haemodiluted (20%v/v) with (mOsm/L) 300,600, 1200, 1500,

and 1800 of Nacl solutions *in vitro*, undiluted sample acting as control. Thrombelastography[TRADEMARK] (Haemoscope, Niles) after recalcification was used to assess coagulation. Kruskal-Wallis was used for statistical analysis.

Results and Discussion: Nacl solutions concentrations of 300 and 600 mOsm/L shortened r and k values while alpha angle and maximum amplitude were increased, but not significant. Solutions at 1200, 1500 and 1800 mOsm/L lengthened r and k values with reduction of alpha and maximum amplitude. It is evident that concentrations below 600 mOsm/L are either hypercoagulable or do not impair coagulation. In contrast, solutions above 1200 mOsm/L progressively induce hypocoagulability. Significance level was < 0.01.

Conclusion: At 20% v/v haemodilution, Nacl solutions below 600 mOsm/L maintain, while concentrations above 1200 mOsm/L impair whole blood coagulation. Impairment is more on clot strength than clot formation time suggesting effect of on either fibrin polymerization or platelet function. Further tests are warranted to clarify the observation herein.

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PO 024

Monitoring anticoagulation during Extra-Corporeal Membrane Oxygenation (ECMO) in patients with acute respiratory failure

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Background: Extra-corporeal membrane oxygenation (ECMO) is a viable alternative to mechanical invasive ventilation in selected patients with acute respiratory failure. To avoid blood clotting in the circuit, patients need to be anticoagulated with intravenous heparin in continuous infusion to maintain an activated partial thromboplastin time (aPTT) ratio between 1.5 and 2.5. A standard dose of antithrombin concentrate (1000 U/24 h) is often supplemented when plasma levels drops below 50%. In such patients aPTT monitoring can be hampered by several confounding factors, mainly due to the acute inflammatory condition. Hence, alternative laboratory tests should be looked for.

Aim: To asses the reliability of aPTT in monitoring anticoagulation with heparin during ECMO, compared to an anti-factor Xa assay.

Methods: Observational study of 12 consecutive patients during veno-venous ECMO with daily blood sampling.

Results: Mean ECMO duration was 9 ± 4 days. Overall, a moderate correlation between aPTT and anti-Xa assay was observed ($r = 0.52$). Anti-Xa levels were positively correlated with antithrombin levels ($r = 0.61$).

Conclusions: To monitor heparin anticoagulation in patients on ECMO, aPTT and anti-factor Xa measurements can be considered equivalent.

PO 025

Multiple inhibitory effects associated with IgG kappa multiple myeloma

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Background: Haemostatic alterations are often detected in patients with monoclonal gammopathies such as multiple myeloma (MM). They frequently show abnormal coagulation tests (thrombin times, fibrin degradation products (FDP), platelet aggregation, and bleeding time), due to the interference of the paraprotein present in their plasma.

Aims: The aim of this report is to present the case of a patient with multiple myeloma whose prothrombin time (PT), activated partial thromboplastin time (APTT) and thrombin time (TT) were significantly prolonged and did not correct by mixing with normal plasma.

Case Report: A 47-year-old man with IgG kappa multiple myeloma was referred to our institute, because he had had episodes of haematuria and epistaxis without previous history of bleeding in major surgeries.

Results: Coagulation screening of patient's plasma showed prolonged APTT (ratio 4.6; RV: 0.87–1.13), PT (ratio 1.5; RV: 0.82–1.13) and TT (ratio 1.6; RV: 0.75–1.25), and slightly high fibrinogen clotting activity (430 mg/dL). Mixing tests did not correct neither the APTT (ICA 72; control < 10), the PT (1.31; control 1.19) or TT (1.38; control 1.1) suggesting an inhibitory effect on different coagulation pathways. FDP were negative.

Screening (APTT and dRVVT) and confirmatory tests were performed showing results compatible with positive lupus anticoagulant like effect (LA-like). No time dependent inhibitory effect (characteristic of the anti FVIII inhibitors) was detected.

Moreover, FVIII and IX coagulant activities were very low (2 UI/dL and 1.2 UI/dL respectively) probably due to the interference of LA-like effect and/or the paraprotein effect; an apparent activity increase was observed on progressive dilutions of patient's sample for both factors.

Coagulation activity of the extrinsic pathway factors was normal except for FV (52 UI/dL). Mixing tests did not correct FV activity and no apparent activity increase was observed on progressive dilutions of patient's sample. These behaviour was compatible with a specific inhibitory effect on FV activity.

In addition, the inhibitory effect on TT was normalized by progressive dilutions of patient's plasma, suggesting an interference of the paraprotein on the polymerisation of fibrin.

Conclusion: Interferences on coagulation tests due to the paraprotein effect have been previously described mainly on thrombin time; LA-like activity was also reported in multiple myeloma patients. Anti-FV autoantibodies have been identified rarely in patients with monoclonal gammopathies The results of the case presented here suggest multiple inhibitory effects. In addition to the interference on fibrin polymerisation and LA-like activity, an inhibitory effect against FV was also detected; further studies are necessary in order to characterize this effect. None of them can be clearly associated with the clinical symptoms of the patient.

PO 026

A sensitive mixing test to screen for clotting factor inhibitors

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Background: Mixing studies are helpful for clotting factor inhibitor screening in cases with APTT prolongation. However, the standard consensus procedure is lacking.

Aim: The objective is to develop a sensitive inhibitor screening assay in bleeding patients. The test is preferably insensitive to lupus anticoagulants (LA) in this setting.

Methods: Plasma samples investigated for APTT prolongation ($N = 90$) were tested for factor VIII or IX inhibitors and LA. Positive mixing was defined as an ability of one part of patient plasma to prolong APTT of one part of pooled normal plasma (PNP) after 2-h incubation. Two controls, which were also incubated for 2 h, were PNP mixed with test plasma immediately before the APTT test and PNP mixed with buffer. The cutoff APTT ratio difference of 0.05 was the

laboratory percent coefficient of variation (%CV). Three lots of APTT reagents were investigated.
Results: There were 18 (20%) clotting factor inhibitor cases. The mean titer was 1.7 ranging from 0.67 to 4.0 Bethesda units. Fifteen samples of LA were regarded as negative. The mixing study using any lots of APTT reagents showed 100% sensitivity for clotting factor inhibitors. The sensitivity for LA was lower. The majority of factor inhibitors were time-dependent, while the LA inhibition was mostly immediate. One lot of the reagent displayed 93.1% specificity, but the other 2 lots showed higher false positive rates.
Conclusion: We described a sensitive screening test for clotting factor inhibitors. However, optimal lots of APTT reagents need to be determined to obtain excellent specificity.

PO 027

Two cases of acquired haemophilia A associated with chronic myelomonocytic leukaemia

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Background: Acquired haemophilia A (AHA) is an uncommon, but potentially fatal bleeding diathesis caused by autoantibody against circulating coagulation factor VIII (FVIII). The underlying causes of AHA can be identified in approximately half of patients, of which malignancies account for 10–20%. Heretofore, there has been only one case report of AHA concomitant with chronic myelomonocytic leukaemia (CMMoL).
Aims: We reported two more cases of AHA with CMMoL in our hospital and reviewed possible causal relations between these two rare blood disorders.
Methods: We retrospectively reviewed patients diagnosed as AHA in King Chulalongkorn Memorial Hospital from 2003 to 2012. Of 14 documented AHA, eight patients had no apparent underlying conditions, while three had systemic lupus erythematosus, two CMMoL and one indolent B-cell non-Hodgkin lymphoma. Surprisingly, two cases of CMMoL were found as an underlying aetiology connected to AHA.
Results: Case 1: A 41-year-old male presented with left gluteal abscess and excessive bleeding after drainage. Screening coagulogram revealed isolated prolonged aPTT uncorrected by mixing study. Autoantibody against FVIII was detected at a level of 107.5 Bethesda units (BU). Complete blood count demonstrated leukocytosis with marked monocytosis. Bone marrow study was compatible with CMMoL. He received cyclophosphamide combined with prednisolone. Unfortunately, he did not show any response and subsequently died due to suspected intracranial hemorrhage. Case 2: A 54-year-old male presented with multiple large spontaneous ecchymoses, hematoma at the entire left arm after venipuncture, and gross hematuria. Initial laboratory investigation revealed leukocytosis with marked monocytosis as well as isolated prolonged aPTT. The quantitative assay for FVIII inhibitor revealed a level of 21 BU. The bone marrow examination was consistent with CMMoL. He received prednisolone monotherapy for inhibitor elimination and rapidly achieved complete response 3 weeks after the initiation of immunosuppressive therapy. The pathogenesis of CMMoL-associated AHA remains unknown. The tumour cells may express neoantigens, which their structures mimic FVIII, and subsequently trigger the development of FVIII autoantibodies. In addition, abnormal T-cell responses to antigen presentation as well as dysregulation of B- and T-cell interactions, especially when neoepitopes from malignant clones are over-exposed, may result in the development of FVIII inhibitors. An alternative explanation is immune dysregulation resulting in autoimmune complications. Autoimmune phenomena have been sporadically reported in patients with CMMoL such as vasculitis, polyarthritis, Coombs negative haemolytic anaemia and immune thrombocytopaenia. These autoimmune disorders were able to be successfully treated with immunosuppressive therapy. In addition, autoantibodies against circulating coagulation factors such as FVIII and factor XI in patients with CMMoL were previously reported.
Conclusion: We reported two case of CMMoL-associated AHA. The pathological basis involved in the development of FVIII autoantibodies in CMMoL remains poorly understood. The optimal managements for this rare association require further studies.

PO 028

Haemostatic function in hypothyroid state

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The link between haemostatic disorders and thyroid function is uncertain. In some cases hypothyroidism is associated with acquired von Willebrand syndrome and may have several haemostatic abnormalities such as modification of the coagulation proteins and bleeding tendency.
The aim to investigate haemostatic function in patients with overt hypothyroid state.
Methods and Objectives: Fifty-one patients were included – 31 with hypothyroid state 18–71 years old (53.5 ± 10.6), eight male, 23 female and 20 control persons with light thyroid gland structure changes, but without thyroid dysfunction 29–76 years old (53.9 ± 9.7), eight male 14 female. Quik test, APTT, thrombin time, fibrinogen (Clauss), antithrombin, plasminogen, protein C, protein S, factor VIII activity, von Willebrand factor antigen, D-dimers were measured (STA Compact, Stago reagents). The results are presented as median, 25 and 75 percentiles (Me [25%; 75%]); statistic Mann-Witney U-criteria was used.
Results: There were no any significant changes of Quick test, thrombin time, antithrombin, plasminogen, protein C, protein S and D-dimers in hypothyroid patients. But APTT as screening test and some additional tests showed hypocoagulation tendency. APTT was prolonged in hypothyroid group up to 30.1 [29.0;33.0] s compared with controls – 28.0 [26.0;30.0] s, $U = 183.5$, $Z = -2455$, $P = 0.014$. Factor VIII activity was significantly reduced up to 69 [58;80]% (97[75;119]% in controls, $P = 0.016$); von Willebrand factor antigen chanced in the same manner – from 102 [64;120]% up to 65 [56;79]%, $P = 0.006$. From the other hand fibrinogen was higher in thyroid hypofunction but inside the reference range: 3.9 [3.3;4.5] g/L vs. 3.3 [3.1;3.6] g/L, $P = 0.028$.
Conclusion: There are clear tendency to hypocoagulation in patients with decreased level of thyroid function, which may be suspected according to APTT results and confirm by factor VIII activity and von Willebrand factor antigen. It seems to be important in any cases of surgery or haemorrhagic syndrome in this patients.

PO 029

Acquired factor VIII inhibitor associated to prostate cancer in elderly

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Background: Acquired factor VIII inhibitor is a rare bleeding disorder and accounts for 1.3–1.5 cases per million per year, with the mortality rate around 22%. It occurs most frequently in elderly people, and the incidence increases with age. Clinical features are characterized by a large spectrum of bleeding, from subcutaneous bruising to life threatening bleeding, such as muscle, intracranial or gastrointestinal hemorrhages. Search for an underlying cause is negative in up to 50% of the cases. Some relevant conditions associated are: rheumatoid arthritis, systemic lupus erythematosis, malignancy, pregnancy and pemphigoid. Treatment is based on the control of bleeding and eradication of the

inhibitor. We describe a case of acquired FVIII inhibitor and lupus anticoagulant in a patient with prostate cancer, succesfully treated with corticosteroids plus azathioprine.

Case report: A 77-year-old man with controlled reumatoid arthritis and prostate cancer was referred with a history of bleeding after exeresis of a node in the left forearm and spontaneous multiple hematomas (upper and lower limb, and chest). Coagulation tests revealed a prolongation of activated partial thromboplastin time (APTT), and failure to correct after 1:1 mixing with normal plasma FVIII activity was severely decreased (< 1%), with increased FVIII inhibitor level (512 BU). Other coagulation factors were normal. Lupus anticoagulant was negative at the time of the diagnosis. Treatment with activated prothrombin-complex concentrate (FEIBA), steroids and azathioprine led to improvement of his clinical symptoms. After 5 months of immunossupressive therapy it was observed normalization of coagulation parameters (FVIII > 50%, inhibitor negative). Surgery for the cancer was not indicated for urologic issues related to the age. Before 3 months of the complete withdrawal of immunosuppressive therapy, he presented another decrease of FVIII activity with no correction of APTT after mixing study, and a positive test for inhibitor. In order to investigate the cause of the lower level of FVIII, it was performed lupus anticoagulant test and substrate chromogenic FVIII assay (SCA) to quantify the inhibitor was performed. The presence of LA was confirmed, FVIII (SCA) was normal and FVIII inhibitor was negative using this methodology. After 2 months without immunossupressors, the inhibitor was still negative (< 0.6BU).

Summary: Treatment of acquired FVIII is usually performed with corticosteroids plus cyclophosphamide. The decision to use azathioprine was based on the mortality rate of 22% due to infectious complications, possibly related to myelotoxicity of cyclophosphamide, and the frequent occurrence of factor VIII acquired inhibitors in elderly people. Lupus anticoagulant is common in cancer, and it can interfere with the determination of coagulation factors. It is important to use sensitive assays to avoid inadequate treatment. As the cancer was not eradicated, it is possible that the inhibitor relapses. This is an interesting case of acquired FVIII inhibitor associated to cancer in an elderly patient, which treatment with prednisone and azathioprine resulted in eradication of the inhibitor.

PO 030

Parameters of hemostasis in patients with deep frostbites and malnutrition

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Aim: The aim of the study is to provide indicators of hemostasis in patients with deep frostbites and malnutrition.

Research Methods: Conducted a prospective nonrandomized controlled study in 59 patients of both sexes with the local cold injury, that were hospitalized to City Clinical Hospital -1 of Chita during the winter period of 2009–2011. The composition of therapy consisted of anticoagulants of direct action under the protocol. The object of the study was the blood of patients. Blood sampling was not carried out up to reactive period of local cold injury. Inclusion criteria: deep frostbites, hospitalization up to reactive period of the local cold injury, hospitalization in the early and late reactive periods of the local cold injury. Exclusion criteria: age younger than 16 years; age older than 60 years; sepsis; acute renal failure. The control group included 23 healthy volunteers of similar age and sex.

Patients were divided into two groups of nutritional status. Nutrition assessment was conducted on amount of balls of the table Luft V.M. and Kostyuchenko A.L. 'Indicators of the severity of malnutrition' at the beginning of hospitalization. The first group included 22 patients with eutrophia, that have secured a total of 28–30 balls. The second group consisted of 37 patients with malnutrition, the result of which does not exceed 27 balls. Twenty patients of the first group and 32 patients of the second group were hospitalized in the first 2 days after the injury. Assessment of platelets was performed twice in these patients: in the early reactive period of the local cold injury (ERP) and in the late reaction period (LRP) of the local cold injury again.

Research methods were assessment and quantitative comparison of platelets, expression of tissue factor (TF) and tissue factor pathway inhibitor (TFPI), antithrombin-III (AT-III). These data are presented as median and interquartile range. Comparison of the groups was used the Mann-Whitney test.

Results of the study: Comparison of platelets were revealed higher ($P < 0.001$) in both groups, then control group 301 (255;356) in 1 mkl in the ERP and LRP. Comparison of platelets was revealed lower ($P = 0.042$) in patients with malnutrition, than in patients with eutrophia: 187 (117;291) vs. 214 (196;325) in 1 mkl. Were no differences of comparison of platelets ($P = 0.832$) between groups 1 and 2: 471 (287;648) and 445 (278;600) in 1 mkl. TF expression were revealed higher ($P < 0.001$) in both groups, then control group 33.1 (28.9,37.8)%. Were no differences of this parameter between groups 1 and 2: 54.8 (45.4;61.8) and 61.6 (51.4;70.5)%. TFPI was revealed higher ($P = 0.021$) in patients with eutrophia, then control group 3.2 (2.7;3.6) and ($P = 0.022$) then the group with malnutrition: 2.9 (1.7;3.8) vs. 3.8 (3.6;4.1) ng/ml. AT-III was revealed lower in patients with malnutrition: 72.0 (55.0;97.1)% vs. 101.7 (87.9;111.7)% of control group ($P = 0.005$) and vs. 101.7 (87.9;111.7)% of the group with eutrophia ($P = 0.047$).

In conclusion, patients with deep frostbites and malnutrition have changes parameters of hemostasis.

PO 031

Some hemostatic parameters in patients with severe influenza A/H1N1, requiring artificial ventilation

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Background: Change in blood coagulation system is an essential consequence of the cytokine cascade, resulting from activation of immunoreactive system in response to the introduction infected. Strains of some viruses, including influenza virus, have not only epithelial, but endothelial activity. Endothelial cells are involved in the implementation of a wide range of biological effects, including an active effect on all parts of hemostasis.

Objective: To investigate the change in blood coagulation system in patients with severe pneumonia against influenza A/H1N1, on mechanical ventilation.

Materials and Methods: A study in 16 patients aged 25–50 years with influenza A/H1N1 pneumonia complicated by severe. The control group consisted of 20 healthy volunteers. All patients were treated in the intensive care unit of City Clinical Hospital 1 in Chita in November 2009 study was conducted on 1–2, and 7–8 per day of hospitalization. Evaluated fibrinogen, APTT, INR, SFMC. Statistical analysis was performed using the software package Microsoft Excel.

Results: Found that patients with severe pneumonia against the background of influenza A/H1N1 on the 1–2 day of onset fibrinogen levels decreased by 17% relative to the control, APTT decreased by 26%, INR increased by 1.2 fold, the number SFMC increased by 87%. While the 7–8 day fibrinogen increased by 63%, APTT decreased by 15%., INR increased by 1.1 fold, the number of SFMC has increased by 7.2 fold compared to the control group. The development of pneumonia in patients during influenza A H1N1, certainly accompanied by the presence of systemic inflammatory response syndrome (SIRS), implying an increase in plasma immunoreactive system mediators (leukotrienes, prostaglandins, etc.). This can lead to multiple organ failure. Lungs contain the most, compared to other organs, the number of endothelial cells. As a result, the direct damage to the lung tissue by virus, and the presence of multiple organ failure, affecting the endothelium, which leads to a deviation of the functioning of blood coagulation and the development of hypercoagulability.

PO 032

A retrospective study of treatment and outcome of consecutive patients with Acquired Haemophilia A

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Background: Acquired Haemophilia A (AHA) is a rare autoimmune bleeding disorder with an incidence of 1.34–1.48 per million per year. Bleeding results from the development of inhibitory autoantibodies to FVIII and is fatal in 8–22%. Prompt diagnosis is crucial so that treatment can begin immediately. This comprises control of acute bleeding, inhibitor eradication and treatment of the underlying disorder. There is limited data in Asian patients with the largest report comprising only 46 patients.

Aims: This is a retrospective review of our cohort of 15 patients with AHA in our institution.

Methods: All consecutive AHA patients treated at NUH between Jan 2006-Dec 2012 were included. Clinical and laboratory data were extracted from case records. AHA was diagnosed using UKHCDO 2006 criteria. Major bleeding was defined using ISTH 2005 criteria. Complete remission (CR) was defined as normalization of FVIII level, undetectable inhibitor and no immunosuppression.

Results: Fifteen patients were included, six females and nine males. Their median age was 68 (range, 38–92 years). All presented with spontaneous bleeding, 10 with major bleeding. The 3 commonest sites were skin ($n = 12$), muscle ($n = 8$) and genitourinary ($n = 3$). An underlying cause was found in three patients – post-partum, acute myeloid leukaemia and connective tissue disease. Median FVIII level was 4% (range, < 1–9%), median inhibitor titre was 80 BU/ml (range, 9–576 BU/mL). Twelve patients received bypassing agent FEIBA and 1 received FVIII concentrate. two patients did not receive any bypassing agents as they had minimal bleeding. All major bleeding episodes responded to treatment. Median number of days of FEIBA treatment was 6.5 (range, 0–20 days), and median dose of FEIBA per kg weight was 69 U/kg/day (range, 39–167) for the first 3 days. Thirteen patients received immunosuppression with prednisolone and cyclophosphamide and 2 with prednisolone alone. All patients normalized their FVIII level with 9 achieving CR (60%) at a median of 190 days (range, 17–231 days). One patient relapsed after CR. Four patients had immunosuppression-related toxicity including 3 Grade 3 sepsis and 1 fatal sepsis. One patient who achieved CR died on AML treatment.

Conclusion: Despite the older age and presentation with major bleeding in 66.7%, the outcome of our case series is excellent. All bleeding episodes responded to treatment and all patients normalized their FVIII levels with immunosuppression. Although a pan-European registry (EACH2) recently reported on 501 patients, there are only Three published case series in Asia with 70 cases in total. Similar to other Asian patients, our patients were less likely to have a secondary cause (20% of cases) and more likely to have durable CR (60%). In contrast, the EACH2 study a secondary cause was found in 48% and relapse rate was 33%. However, the median time to FVIII > 70 IU/dl and CR were longer for our cohort at 82.5 and 190 days respectively compared with 40 and 74 days respectively for EACH2 patients. This may reflect the lack of an underlying cause and/or different immunosuppression tailing regimens.

PO 033

Activated prothrombin complex concentrate in acquired haemophilia A: an Italian registry-the F.A.I.R. study

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Background: Acquired Haemophilia A is a disease characterised by bleeding manifestations and alterations in coagulation tests (lengthening of the activated Partial Thromboplastin Time-aPTT) in patients having no previous personal or family history of coagulation disorder. It is an autoimmune disorder, due to the presence of auto-antibodies specifically directed against factor-VIII (FVIII-inhibitor). It is a rare condition, with a reported incidence of around 1–4 cases per million/year. The risk of AHA increases significantly with age (range 55–78 years), with an approximately equivalent sex ratio. This disease is associated with high rate of mortality, usually due to delay in diagnosis and inadequate management of hemorrhagic episodes. Recent data have shown that excellent or good haemostasis was obtained with Activated Prothrombin Complex Concentrate (aPCC, FEIBA®, Baxter) administered at doses of 50–100 U/kg every 8–12 h in 76–100% of treated bleeding though no standardised treatment protocols are available.

Aim: The purpose of this study is to evaluate the dosage, efficacy and safety of aPCC, used for bleeding episodes, in an Italian population of 50 AHA patients.

Patients and Methods: The FAIR study (Feiba in Acquired haemophilia A, an Italian Registry) is an observational, non interventional, retrospective/prospective study on safety, efficacy and dosage of Feiba in AHA. Fifty patients with confirmed diagnosis of Acquired Haemophilia A according to standard laboratory methods and treated with aPCC are expected to be enrolled in this study. It is planned to analyse 25 patients retrospectively and 25 prospectively. Ten sites are expected to be involved in the study, after the approval of local ethics committees. General information (i.e. patients' medical history, FVIII levels and Inhibitor titre and information about bleeding episode management, adverse events) is collected into an electronic case report form (eCRF). Subjects have to be older than 18 years and they shall have given a written informed consent.

Conclusion: Data from the Italian Haemophilia Registry database will be used to better assess the dosage, efficacy and safety of aPCC in patients with acquired haemophilia A in order to make standardised protocols available for treatment of bleeding episodes.

PO 034

Evaluation of an automated method for the measurement of dabigatran in plasma

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Background: Dabigatran (Pradaxa®) is a novel oral anticoagulant currently licensed for use inEurope for the primary prevention of venous thromboembolic events in adults who have had an elective total hip or knee replacement surgery. It is also used for the prevention of stroke or systemic embolism in adults with non-valvular atrial fibrillation with one or more risk factors. Its anticoagulant effect is achieved via direct inhibition of thrombin. Dabigatran is cleared primarily by renal excretion with a half life of 13 h with normal renal function. It may be necessary to monitor levels of Dabigatran in instances where clearance is compromised, in life threatening bleeding or in situations where thrombosis persists despite anticoagulation.

Aims: A reliable reproducible assay is required to measure levels of Direct Thrombin Inhibitors (DTI) in plasma. A commercial CE

marked kit (Hemoclot Thrombin Inhibitors CK002L, Hyphen BioMed) for the quantitative measurement of direct thrombin inhibitors in citrated human plasma was verified for use with the ACL TOP automated coagulation system (Instrumentation Laboratory).
Method: The method is based on the ability of Dabigatran to inhibit a constant and defined concentration of thrombin. Citrated plasma is diluted with saline and mixed with Normal Pooled Plasma (NPP) at a ratio of two parts NPP to one part diluted plasma and a thrombin time is performed on this mixture. The level of Dabigatranis calculated by reading from a standard curve using calibrators with known levels of the drug. The assay was controlled at the lower and upper range of measurement for the calibration curve. Evaluation included calibration precision and stability, sensitivity and assay precision.
Results: Linear calibration curves ($R^2 > 0.985$) were achieved for the assay with an inter-assay calibration precision (CV%) at < 5%. Peak plasma concentrations are in the range of 0.10–0.40 μg/ml and trough concentrations are in the range of 0.02–0.15 μg/ml. Two calibration curves were constructed with an achieved sensitivity ranging from 0.05 to 0.50 μg/ml. Assay precision was acceptable with a CV% of < 10%. Overall performance characteristics were satisfactory for this assay.
Conclusion: In specific instances where measurement of Dabigatran is necessary, the Hemoclot Thrombin Inhibitor assay is deemed suitable for use in our laboratory.

PO 035

Level ranges for a new latex agglutination immunoassay for free protein S antigen in a pregnant women cohort

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Background: In normal pregnancy the haemostasis balance modifies towards hypercoagulability, to decrease bleeding complications on delivery. There is an increase in concentration of most clotting factors, a decrease in some of the natural anticoagulants, namely protein S (PS), and reduced fibrinolytic activity. PS deficiency is a well known risk for thrombosis. Free PS (fPS) assay measures unbound PS fraction and can be used as a surrogate marker for PS activity.
Aims: We evaluated a pregnant women cohort to establish range values of fPS during normal pregnancy for a new immunoassay, INNOVANCE® Free PS Ag kit (Siemens, Marburg, Germany).
Patients and Methods: A study cohort of 56 pregnant women without personal or familial thrombophilia, mean age 31 years (15–41), was divided in four groups according to number of weeks of pregnancy: three patients in first group (until 22 weeks), 9 in second group (23–28 weeks), 24 in third group (29–35 weeks), and20 in fourth group (36–41 weeks) were evaluated. Blood samples were collected by venipuncture into 3.8% sodium citrate tubes. Plasma was separated into aliquots within 4 h of collection and stored at −70 °C until analysis. Free Protein S was measured in duplicate on a BCS® XP System by latex agglutination test, using INNOVANCE® Free PS Ag kit (Siemens, Marburg, Germany).
Results: A histogram showed a normal distribution of fPS levels in the study cohort. Subgroup analysis was performed: in first group fPS was 56.0% (44.8–67.3), in second group 53.4% (44.0–62.7), in third group 50.4% (47–53.7) and 49.6% (44.0–55.2) in fourth group. Despite a decreasing tendency of fPS until term, no statistically difference was found between the four groups. Due to the weak contribution of the results of the first group, we used the results of the other groups (23–41 weeks) to determine the normal range of fPS in pregnancy. Comparing this cohort to a previously studied control group of blood donor women without hormonal contraceptive methods and fitting age range, a statistically difference ($P < 0.001$) was found, with a mean fPS of 50.9% (28.4–70.6%, CI 90%) in the cohort analyzed vs. a mean fPS of 86.5% (57.8–126.7%, CI 90%) in control group.
Conclusion: Our results of fPS in pregnant women are similar to previously reported. The use of distinct normal ranges for specific populations allows risk stratification of thrombosis, namely in pregnant women.

PO 036

At what time derived fibrinogen must replace the clauss assay

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Background: Dabigatran is an oral, reversible thrombin inhibitor that has shown promising results so far, with the advantage that laboratory monitoring is not needed/deemed. However, clinicians and pathologists must be aware how coagulation assays are affected when handling emergency interventions, bleeding complications or overdoses.
Aims: To show how a derived fibrinogen assay that usually has a bad behavior in measuring high and low levels of fibrinogen can be useful, when some of thrombin reagents used for clauss fail to detect fibrinogen levels in patient's samples with dabigatran.
Methods: Three common coagulations assays (TP; APTT and Fibrinogen) were done in two elderly (male: 88 and female: 83 years old) when admitted in our hospital. Both presented a personal history of atrial fibrillation, hypertension, metabolic disease and abnormal renal function and both were medicated with dabigatran (150 mg bid). For both patients, TP and APTT were prolonged but only in one patient clotting time for APTT was not achieved; the levels of fibrinogen by clauss method were less than 0.30 g/L for both patients. In face of these results pathologists wanted to know the levels of dabigatran regarding that only one of the patients had bleeding complications. The levels of dabigatran found were above 700 ng/mL, higher for the hemorrhagic patient which was consistent with APTT but as Ddimer was normal and there wasn't any explanation for the low level of fibrinogen a derived fibrinogen was performed, and normal results for fibrinogen were found, between 3.0 and 5.0 g/L. It was performed derived fibrinogen vs. clauss in other patients medicated with dabigatran for concentrations between 70 and 540 ng/mL, and verified that only above 400 ng/mL of dabigatran the results of fibrinogen were very different between the two methods. It was also performed for those samples the clauss with another reagent and the results were similar to the derived fibrinogen, although lower for the clauss method.
Conclusions: Current knowledge is that none of the global tests are elective to assess the anticoagulant effect of dabigatran: APTT shows poor dose-response linearity and intermediate responsiveness to increase dose beside be depend of the reagent used; TP presents good linearity but poor responsiveness; TT has excellent linearity but excessive responsiveness and when fibrinogen is performed the results with clauss method for overdoses are not reliable, they are extremely affected by the reagent used and can be extremely dangerous when offered to the clinician, in view of our results we think the derived fibrinogen could be a good alternative to assess the anticoagulant stage in overdoses.

PO 037

Effect of primary tube underfilling and hemolysis on PT, APTT, antithrombin and D-Dimer assays; implementing the pre-analytical sample integrity checks of the Sysmex CS-2100i System*

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Background: Pre-analytical visual inspection of primary blood collection tubes is a subjective and inconsistent procedure at best. A new

generation of hemostasis analyzers offers pre-analytical sample-integrity checks (PSI) to detect incorrect primary-tube fill volumes as well as interfering levels of hemolysis, icterus, and lipemia. We looked at the effect of incorrect tube fill and hemolysis on several hemostasis assays with the optical based system above as well as a mechanical detection based analyzer, and questioned whether PSI checks can replace visual inspection in routine lab workflow.

Methods: Normal and heparin-spiked blood samples were modified by various levels of hemolysis or by controlled underfilling of collection tubes. Samples were then tested for PT (Thromborel® S reagent), APTT (Dade® Actin® FSL reagent) and antithrombin (INNOVANCE® Antithrombin assay) on both the Sysmex® CS-2100*i* (CS-2) and STA Compact® (STA) analyzers.

In a separate part of the study, normal samples were spiked with 0.5 μg/mL D-Dimer and then submitted to the same levels of underfilling and hemolysis (INNOVANCE® D-Dimer assay on CS-2, Liatest® D-Dimer assay on STA).

Finally, the pre-analytical integrity of 1548 blood samples from several outpatient clinics were inspected visually by lab technologists and by the CS-2 PSI checks prior to testing.

Results: Underfilling and hemolysis resulted in longer PT on both analyzers. APTT was prolonged with tube underfilling and shortened with increasing hemolysis on both analyzers. Moreover, APTT of heparinized samples normalized when free hemoglobin levels exceeded 400 mg/dL. Tube underfilling caused lower AT results with both systems. Hemolysis did not affect AT results on the CS-2, but caused decreasing AT results on the STA before becoming unreadable. D-Dimer measurements on both systems were hardly affected by underfilling. In contrast, hemolysis caused dramatically increasing D-dimer results on STA, but no noticeable effect on D-Dimer results on the CS-2.

All sample tubes with improper fill volumes (> 110% or < 90%) or hemolysis (≥ 0.5%) were detected by the PSI checks of the CS-2.

During inspection of the 1548 samples, 267 were found to have low fill volume by visual checks and 226 by PSI checks on the CS-2. Comparison of negative samples (no underfilling) with positive samples (underfilling detected by both visual inspection and PSI) demonstrated significant differences ($P < 0.05$) for the above assays. Samples detected visually but not with PSI-checks did not differ in result from the negative samples.

Conclusions: PT, APTT, AT and D-Dimer assays are sensitive to improper tube filling and/or hemolysis, irrespective of the type of analyzer. Pre-analytical factors can interfere significantly with the assay result, even to the extent that a different diagnosis is suggested.

These results suggest that assessment of underfilling and hemolysis by the PSI checks on the CS-2 analyzer may provide an objective, reliable and efficient alternative to visual inspections.

*Sysmex CS-2100*i* System is not available for sale in the U.S.

PO 038

A modified coagulation time (activated partial thromboplastin time) assay for the measurement of very low levels of factor VIII activity

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Background: It is essential to accurately determine the level of blood coagulation factor VIII (FVIII) activity to evaluate the severity of haemophilia A. In general, FVIII activity is assessed by activated partial thromboplastin time (APTT) coagulation time with the use of FVIII deficient plasma. However, the precision of the assays are usually not standardized and hence guaranteed at the levels less than 1% of FVIII. The reasons for this are thought to be due to the differences in FVIII sensitivity of the APTT reagents and varying characteristics of FVIII-deficient plasma, e.g., their residual FVIII activities.

Aims: The accurate measurement of FVIII activity, even at low ranges less than 1%, is presumed to be possible by the modified APTT assay when we select suitable combination of APTT reagent and FVIII-deficient plasma. For this purpose, we sought to identify the APTT reagent and FVIII-deficient plasma, enabling high sensitivity in the detection of FVIII activity and the resultant measurement of the FVIII activity in its very low range.

Methods: The assay was performed with APTT reagents as follows: HemosIL SynthASil reagent APTT (Instrumentation Laboratory), Coagpia APTT-N (Sekisui Medical), Thrombocheck APTT-SLA, and Thrombocheck APTT (Sysmex). Besides normal human pooled plasma obtained from healthy volunteers, HemosIL Calibration Plasma and Coagtrol N (Sysmex) was used as normal plasma. The assessment of FVIII activity was performed with FVIII-deficient plasma products as follows: HemosIL Factor VIII Deficient Plasma and two kinds of factor VIII-deficient plasma (Sysmex and George King Biomedical). FVIII activity of normal human pooled plasma was defined as 100% arbitrarily, and the FVIII activity of each normal plasma was determined. Plasma of 50% FVIII activity was prepared by separate combinations of each normal plasma and each FVIII-deficient plasma. Thereafter, serial dilutions of mixed plasma were assayed by various APTT reagents on Coapresta-2000 (Sekisui Medical). Factor VIII activity was evaluated by APTT coagulation method using three FVIII deficient plasmas, and by chromogenic method using COATEST SP (Chromogenix).

Results: Prolongation of coagulation time based on APTT was widely varied among the samples of serial dilution of the mixed plasmas, and significant difference was observed at the levels less than 5%. We assumed that larger extension rate of APTT prolongation results in higher sensitivity and more accurate measurement of FVIII activity. It was found that the combination of the George King's deficient plasma and HemosIL SynthASil was favorable. As for the detection of factor VIII activity, the combination of reagents and plasmas were similar to the result from APTT study. There was remarkable difference among FVIII deficient plasmas, and measurement level of FVIII activity was lower using the George King's deficient plasma and COATEST SP. However, there are no reference materials for measurement of FVIII activity. Thus, further studies are necessary to assess this preference in order to quantitate the true value of FVIII activity.

Summary/Conclusion: Appropriate combination of reagents and plasmas based on their characteristics may improve the sensitivity of FVIII activity measurement.

PO 039

Interference in von Willebrand factor latex immunoturbidometric assays, lupus anticoagulant assays and one-stage factor assays in a patient with splenic marginal zone lymphoma

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Background: Latex immunoturbidometric assays (liatests) are commonly used assays for the measurement of von Willebrand factor (VWF) antigen and some types of VWF activity assays. Because these assays are based on agglutination of antibody-coated latex particles by target antigen at typically use low sample dilution, there is potential for interference by non-specific agglutinating agents in the test plasma. The most common of these is rheumatoid factor, but others include human anti-mouse antibodies.

Aims: To describe a case of grossly discordant results observed between a VWF antigen liatest and a VWF activity liatest in a patient with splenic marginal zone lymphoma.

Methods: VWF antigen (VWF:Ag) assays were performed on the STA-R analyser by liatest. VWF activity (VWF:Ac) assays were performed on the CA-1500 analyser by a method employing two steps: firstly, binding of plasma VWF to a recombinant glycoprotein 1b (rGp1b) containing two gain-of-function mutations; secondly aggluti-

nation of the VWF-rGP1b complex by latex particles coated with anti-Gp1b (Siemens Innovance). Collagen binding activity was measured by enzyme-linked immuno-sorbent assay. Ristocetin cofactor activity (VWF:RC) was assessed by aggregometry with formalin fixed platelets and ristocetin. Lupus anticoagulant (LA) tests were by APTT and DRVVT-based assays.

Results: The INR was 1.8 and APTT 63 s (RI: 25–37 s).The VWF:Ag could not be measured at standard sample dilution (1/2) due to analyser flags. At 1/15 dilution the VWF:Ag was 1134% (RI: 55–200%) reducing to 386% at 1/50 dilution. Free protein S antigen was also not measurable due to a marked 'dose-hook' effect seen at several dilutions. At standard dilution (1/3) the VWF:Ac level was 11% (RI: 50–185%). The VWF:Ac rose progressively with dilution and peaked at 17,152% at 1/384 sample dilution, falling to 5171% at 1/1536 sample dilution. VWF:RC was 345% (RI: 50–200%) and VWF:CB was 218% (RI: 45–180%). Both LA sensitive and LA insensitive APTTs were 15–20 s prolonged with non-correction in 1:1 mixing tests and final LA ratio of 1.00 (negative for LA). The DRVVT screen clotting time was normal on neat plasma. Assays for coagulation factors VIII, IX and IX showed non-parallelism. Coagulation factors II, V, VII and X showed normal levels. The patient had a greatly raised IgM anticardiolipin antidody (ACA) titre of 4640 MPL (RI: < 20 MPL). Total IgM was mildly elevated at 3.49 g/L (RI: 0.5–3.0 g/L).

Summary/Conclusions: This is an unusual case of interference in several types of coagulation assays, the exact cause of which is not clear. Of particular interest was the marked effect of the inhibitor on the rGp1b-based VWF:Ac assay. The patient most likely has elevated VWF levels of approximately 300% based on VWF:RC levels. The interference may possibly be due to the markedly elevated IgM anticardiolipin antibody. Scientists should be aware of potential interference in immunoturbidometric assays caused by unusual inhibitors, possibly IgM ACA, which may only be suspected from discordant results seen in assays based on differing principles.

PO 040

Hypercoagulability markers in renal transplanted patients: association to inflammatory response

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Background: Renal transplant is able to trigger an intense inflammatory response and alterations in the hemostatic system. However the pathophysiological mechanisms leading to these changes are not completely understood.

Aims: The aim of this study was to investigate hypercoagulability and inflammatory biomarkers in patients undergoing renal transplant without clinical signs of rejection according to renal function.

Methods: We evaluated the plasma levels of regulatory (IL-4, IL-5 e IL-10) and proinflammatory cytokines (IL-8, IL-6, IL-1b, TNF-α, IL-12p70, IFN-γ) by flow cytometry BD® Biosciences Pharmingen, USA and hemostatic parameters (thrombomodulin/TM, von Willebrand factor/FvW, ADAMTS13 and D dimer/D-Di) by ELISA American Diagnostica® Inc. This study was approved by the Ethics Committee at Federal University of Minas Gerais, Brazil and informed consent was obtained from all participants. The research protocol did not interfere with any medical recommendations or prescriptions.

Results: Results showed an increase of D-Di and tendency to increase of IL-6 levels in the subgroup with creatinine plasma level > 2.0 mg/dL compared to subgroup with creatinine < 1.4 mg/dL. The regulation of inflammation in the short term post-transplant was mediated by IL-5 and the pro-inflammatory state in the long was mediated by IL-12. Multinomial regression analysis revealed that ADAMTS13 was associated with creatinine plasma levels. A multivariate logistic regression analysis showed that the Di-D plasma levels were associated with glomerular filtration rate.

Conclusion: These results suggest that the D-Di was a promising marker to estimate renal function. Besides, ADAMTS13 has shown promise for estimating renal function.

PO 041

Correlation between hemostatic molecular markers under warfarin anticoagulation

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It is well known that novel oral anticoagulants (NOAC) can be monitored with exact precision by neither conventional prothrombin time nor activated partial thromboplastin time. To investigate the possibility of new surrogate markers for NOAC efficacy, we evaluated the utility of two hemostatic molecular markers indicating thrombin or fibrin generation among patients with nonvalvular atrial fibrillation (NVAF) undergoing chronic warfarin anticoagulation.

We recruited patients with NVAF who were treated with warfarin for at least 6 months continuously. Two hemostatic molecular markers, prothrombin fragment 1 + 2 (F1 + 2) and soluble fibrin monomer complex (SFMC), and PT-INR were measured simultaneously.

Blood specimens from 69 patients with NVAF (mean age = 73.4 ± 9.3, 22 females) were taken into investigation. Among INR ranges between 1.11 and 3.76 (mean INR = 1.94), there existed a significant negative correlation between INR and F1 + 2 ($r = 0.4668$, $P < 0.0001$), whereas most samples (67/69) showed an SFMC measurement under the sensitivity limit (< 0.3 μg/mL) and a lack of correlation with INR and F1 + 2 of SFMC.

This study revealed the utility of F1 + 2 as a new surrogate marker for chronic anticoagulation including that with NOAC, whereas SFMC is not tenable as such due to poor sensitivity.

PO 042

Use of the thrombin generation test in patients with ischemic cerebral pathologies

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Background: The patients referred to the neurology department of the hospital of Legnano with suspected ischemic undergo imaging studies and laboratory investigations; of these is also included screening for thrombophilia, which aims to highlight anomalies in hemostatic system. This screening includes assays (with functional methods) of the major physiological inhibitors of coagulation (antithrombin, protein C and protein S) and the main mutations associated with increased risk of thrombosis (FV Leiden and FII20120A). In addition to these parameters we have included, in the thrombophilic screening, the dosage of prothrombin fragment 1 + 2 (F1 + 2) and thrombin-antithrombin complexes (TAT) for the evaluation of the activation of coagulation and fibrinolysis.

Aims: Recently we have included in the protocol of thrombophilic screening also the test of thrombin generation in order to assess the ability to detect the presence of a hypercoagulable state.

Methods: Fifty-eight consecutive patients were investigated pertaining to the neurology department with suspected ischemic pathology. The surveys were conducted during the period January to December 2012. On the day of sampling the following tests were performed: PT, aPTT, fibrinogen, protein C, protein S, and D-dimer; the other tests were performed subsequently on aliquots of plasma stored frozen at −80 °C until the moment of the analysis. The thrombin generation test was performed on CAT (Calibrated Automated Thrombogram) System of Thrombinoscope, Stago group. As activator of coagulation was used tissue factor 5 pM.

Results: We evaluated the following parameters: Lag time, ETP, Peak, tt Peak, Velocity Index e Start Tail, The group of patients had mean

values, SD and ranges for the above parameters respectively equal to 2.93, 0.88, 2.0–7.3, 1600, 338, 905–2582, 280, 86, 79–583, 5.93, 1.37, 4.3–11.7, 102.8, 47.9, 18.2–291, 23.4, 3.6, 16.7–39.7.
The control group represented by 61 blood donors instead showed mean values, SD and ranges for the parameters above, respectively equal to 2.85, 0.32, 2.33–3.33, 1448, 193, 1176 -1676, 253, 43, 186–320, 5.9, 0.78, 5.0–7.3, 88.1, 3.1, 45–187, 22,5, 4.9, 19–25.
Using a statistical sofware (MedCalc) were compared the values thus obtained with the Mann-Whitney test and the values were equal to 0.451, 0.201, 1.143, 0.548, 0.265 AND 0.868 respectively for Lagtime, ETP, Peak, Velocity Index and Start Tail.
Conclusion: Was not reached statistical significance for any of the parameters considered, but the values achieved for Peak and ETP in some patients with ischemic stroke were significantly higher than the maximum limit found in the control group. Further studies should be conducted on a larger number of patients, but the results obtained in our study are already suggestive of useful employment of the test in patients with acute cerebral ischemic disease.

PO 043

Is it necessary to monitor dabigatran efficacy?

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Background: Dabigatran etexilate acts as a reversible direct thrombin inhibitor without activity of antithrombin as a cofactor. At present the recommendation for prevention assume dabigatran use as an alternative to vitamin K antagonist therapy. For patients with a lower risk of bleeding is appropriate and effective dose of 150 mg twice daily, at a higher risk of bleeding is secure a lower dose of 110 mg twice daily. In patients with only one serious risk of thromboembolism we can use dabigatran in a single daily dose of 110 mg. Dabigatran is rapidly absorbed from the gastrointestinal tract with concentration peak 2 h after administration. In the Czech Republic the recent approval of dabigatran for the prevention of ischemic stroke in patients with chronic atrial fibrillation is accompanied by rather widely expanded application of this drug. Simple dosage without monitoring is one of the advantages of dabigatran. On the other hand in the case of life-threatening bleeding or urgent surgery haematologists should have rapid coagulation test at hand to solve the question how to evaluate haemostasis in these patients.
Aims: To have own experience with dTT in patients with bleeding complication.
Methods: Calibrated quantitative diluted thrombin time (dTT; Hemoclot Thrombin Inhibitors; Hyphen BioMed, France) was used for the quantitative determination of dabigatran etexilate. Measurements were performed on coagulation analyzer STA-R Evolution. We examined 51 patients in the age range 45–90 years treated with dabigatran 150 mg twice daily in the period from March to September 2012. Blood samplings were analysed after a minimal 3-day dosing and 12 h after last administration.
Results: Prolonged tests APTT and TT compared with the level of dabigatran measured by dTT were found quite different in all our patients. dTT remains the only specific test determining the actual haemocoagulative status.
Conclusion: Our small analysis verified, that the simple coagulation method based on inhibition of constant defined thrombin concentration should be always available in the coagulation laboratory because it helps to clinicians to determine the impact of dabigatran on the immediate patient's bleeding complications. Expansion of elderly complicated patients receiving dabigatran is expected and so this simple dTT test can bring another experience in the short future.
Therefore, this method is successfully used in our routine laboratory praxis.

PO 044

Evaluation of assay performance of the rivaroxaban screening assay TECHNOCLOT® RIVAROXABAN SCREEN

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Background: Rivaroxaban is a new oral direct Factor Xa (FXa) inhibitor. The increasing use of the new oral anticoagulants administered for prevention of VTE in patients after orthopedic surgery creates the need of their measurement in the clinical routine. Anti-Xa assays calibrated with special Rivaroxaban calibrators are used for precise determination of rivaroxaban concentrations in patient samples.
It has been shown that rivaroxaban interferes with routine clotting assays, like PT, APTT or factor assays. For correct interpretation of the results it is necessary to have information on rivaroxaban in the sample.
A simple sreening assay was developed to screen for the presence of rivaroxaban in patient samples.
Aim: The aim of this study was to evaluate the performance of the new clotting assay for screening rivaroxaban in patient plasma samples.
Method: Technoclot® Rivaroxaban Screen is a clotting assay based on the activation of FXa in the patient sample. Rivaroxaban beeing a direct Xa inhibitor will prolong the clotting time in patient sample in comparison with normal plasma sample. The normalized ratio will give the information on rivaroxaban presence in the sample.
Patient samples and normal plasma spiked with different concentrations of rivaroxaban were used for assay evaluation.
Results: In normal samples the normalized Ratio was found to be between 0.8 and 1.2.
Samples with rivaroxaban gave ratios of > 1.3, the ratios being correlated to the rivaroxaban concentration. Patient sample with 52.2 µg/L had a ratio of 1.39.
Conclusions: Our data demonstrate that Technoclot® Rivaroxaban Screen is a simple method suitable for screening samples with rivaroxaban even in laboratories were only routine clotting tests are performed.

PO 045

New immunodepleted plasmas for determination of Factor VIII and IX activity

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Background: STA®-ImmunoDef VIII and IX (Diagnositca Stago) are new immunodepleted human plasmas to be used with STA®-C.K. Prest® for the determination of factor VIII and factor IX activity in plasma.
Aims: Performances of these reagents were evaluated on patient samples (including Haemophiliac patients) across a wide range of factor VIII and factor IX activities. Two laboratories were involved: Leicester, UK for factor VIII and Dresden, Germany for factor IX.
Methods: For each factor, calibrations were performed using STA®-Unicalibrator (one unique calibration curve) and Internal Quality Controls (IQC) were tested using STA®-System Control N + P on STA-R®. For factor VIII study, 144 patient's plasmas were tested in 15 runs: 63 Deficient VIII samples (including 16 Severe Haemophilia A patients), 53 normal samples and 28 other samples (including 10 interferences).
For factor IX study, 144 patient's plasmas were tested in 13 runs: 57 Deficient IX samples (including 16 Severe Haemophilia B patients), 66 normal samples and 21 other samples (including 11 interferences).
Samples were tested with the new products along the working range, STA®-ImmunoDef VIII (0.7–400%) and STA®-ImmunoDef IX

(0.7–300%), and results were compared to the ones obtained with a reference method STA®-Deficient VIII or IX with STA®-C.K. Prest® on STA-R® analyser.
Correlations were analyzed through linear regressions and differences were plotted in Bland & Altman graphs. During the analysis, the stability of calibration was evaluated by comparing results obtained with a systematic calibration and results obtained with the calibration of the first day.
Results: Analysis of IQC results shows good day to day reproducibility: CV < 5% based on factor levels. IQC results also suggest that systematic calibration is not required.
The correlations between new and current reagents show equivalence of results.

- Factor VIII: $y = 0.973x + 1716$ ($r = 0.994$)
- Factor IX: $y = 0.984x + 2.635$ ($r = 0.989$)

Results for clinical haemophilia segregation were also in good agreement, especially for Severe Haemophilia.
Conclusion: Results show good consistency between STA®-Immuno-Def and STA®-Deficient used with STA®-CK Prest® for all the assay working range. Besides, these studies strongly suggest there is no need for a systematic calibration. Finally, results confirmed that Haemophiliac patients are well segregated with these new products.

PO 046

Coagulation profile in polycythemia vera and essential thrombocythemia patients in Medan Indonesia

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Thrombosis, the main complication of Polycythemia Vera (PV) and Essential Thrombocythemia (ET) patients, can significantly affect the prognosis and quality of life. In this cohort study we study the coagulation profile of 35 PV and ET patients. We measured the TAT-Complex, Beta thromboglobulin (βTG), von willebrand factor (vWF), fibrinogen and D-dimer. Results TAT-Complex was increased in 22 cases (63%, $P = 0.011$), βTG was increased in 35 cases (100%, $P = 0.443$), vWF 7 cases (20%, $P = 0.030$), fibrinogen 10 cases (29%, $P = 0.000$). Thrombosis were excluded with D-dimer negative, and found out there's 10 cases of thrombosis. Most of the cases has the history of thrombosis (32 cases, 91%) with arterial thrombosis 13 cases (39%), venous thrombosis 10 cases (28.6%) and microvascular disturbances 21 cases (60%). History of thrombosis show statistically significant ($P = 0.011$) to develop thrombosis.
Conclusion: Most of the PV and ET patients showed significant hypercoagulable state towards thrombosis.

PO 047

Adverse efects of PEG-Asparaginase treatment in paediatric population and relation with APOE polymorphisms and hereditary thrombophilia

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Background: Asparaginase, including its peguilated formulation (PEGAsp) is effective in induction/consolidation schemes for Acute Lymphoblastic Leukaemia (ALL). It is associated with adverse effects such as allergy, thrombosis, pancreatitis and deranged lipid metabolism, which can be severe and delay treatment.
The identification of subgroups at risk would allow us to define preventive and early intervention strategies to address PEGAsp treatment associated complications.
Aims: Analise the incidence of adverse effects related to PEGAsp administration for ALL in paediatrics and its relation with hereditary thrombophilia risk factors and *APOE* polymorphims; to identify subgroups at risk.
Methods: Analysis of children (< 18 years) with ALL (B and T) treated in our Centre since May/2007 with regimen DFCI05–01, which includes PEGAsp; ALL risk stratification: standard (SR), high (HR) and very high risk (VHR).
Severe adverse effects (sAE)- those causing PEGAsp administration delay or suspension: allergy, extreme hypertriglyceridaemia (HTG, TG > 11 mM, fasting), pancreatitis and thrombotic events.
Genetic thrombophilia risk factors assessed: PRT A20210G, FV Leiden and MTHFR C677T, by PCR-Mutiplex. *APOE* genotype (polymorphisms e2, e3, e4) was determined by direct sequencing.
Results: Forty-eight children were studied (SR-23, HR-19, VHR-6). A 54.2% ($n = 26$) suffered sAE, of which 2 were allergic reactions, with a higher rate of sAE in highest ALL risk groups: HR-84.2%, VHR-66.7%, as compared with SR-26%. In the 24 children with non-allergic sAE, 14 HTG (SR-2, HR-10, VHR-2) and 7 pancreatitis (SR-3, HR-4) were documented. Twenty-three percent of patients suffered a thrombotic event, 91% of which were documented in the highest risk ALL groups (HR-8, VHR-2, SR-1).
Heterozygous (htz) PRT A20210G was found in one child (SR) and htz FV Leiden in another (HR); eight children were homozygous (hmz) MTHFR C677T (SR-3, HR-5).
Genotype *APOE* was determined in 46 children: 74% e3/e3 ($n = 34/46$), 15.2% e3/e4 ($n = 7/46$), 8.7% e2/e3 ($n = 4/46$) and only one e4/e4 (2.1%).
We did not find any correlation between *APOE* genotype and HTG or pancreatitis.
Of those 10 high ALL risk patients with thrombosis, 1 was htz FV Leiden, four patients carried allele e4 (e3/e4) (one with htz FV Leiden); 6 were e3/e3 (3 hmz MTHFR C677T and 1 with strong anticoagulant lupic). Among the HR/VHR groups, only 2 children, who carried both the allele e4 and hmz MTHFR C677T, did not suffer any thrombotic event during the observation period, one of which was under prophylactic enoxaparin due to known risk factors (MTHFR C677T homozygosy and oral anticonceptives therapy).
Conclusion: In this series of 48 children with ALL treated with PEGAsp, we documented a 54.2% frequency of sAE, with a higher incidence in high ALL risk. As previously described, the overall incidence of sAE was associated with high ALL risk score and an older age at diagnosis. Thrombotic events occurred at a high frequency in our patients (23%), 91% in children with a high ALL risk score (HR/VHR).
A larger series is warranted to confirm a possible emerging hypothetical association between *APOE* and/or *MTHFR* polymorphisms and thrombotic events among our high risk ALL risk paediatric population.

PO 048

Long-term use of Low-Molecular-Weight Heparins (LMWH) for cancer-associated venous Thromboembolism: Clinical Practice and Patients' Perception in the TROPIQUE study

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Background: Long-term treatment with LMWH is the recommended standard for treatment of venous thromboembolism (VTE) in cancer patients. In a previous study, the prescription of LMWH in France was shown to comply poorly with established recommendations [1]. Few data are available on the long-term prescription in clinical practice and treatment follow-up in cancer patients. Study objectives are to document the prescription and use of curative doses of LMWH, to

assess whether treatment follow-up is consistent with established recommendations and to assess patients' perception of the therapy. Clinical outcomes such as VTE recurrence and bleeding are also documented.

Methods: This is an ongoing prospective, observational, multicenter study in cancer patients with symptomatic VTE and an indication for treatment with LMWH for at least 6 months. Adult patients, aged 18 years or more, willing to participate, with diagnosed malignancy and recent symptomatic VTE in whom a treatment with LMWH has been initiated within less than 7 days, are consecutively included in the study. Patients already treated with LMWH for more than 7 days or with a contra-indication to LMWH are not eligible to participate. Main study outcome measures include the proportion of patients treated with LMWH according to the French established recommendations as well as patients' expectation and satisfaction based on the validated Perception AntiCoagulant Treatment Questionnaire (PACT-Q) [2].

Assuming a normal patient distribution and the unfavorable hypothesis that only 50% of patients are treated according to the national recommendations, 384 patients are needed to obtain conclusive results with a precision of 5% and a risk error of 5%. Incidences of 7% of VTE recurrence and 6% of major bleeding are expected. A total of 400 patients are therefore planned to be included in the study. PACT-Q scores will be calculated per item at baseline (PACT-Q1) and globally at study end or at treatment discontinuation (PACT-Q2). Scores will be expressed in means and medians.

As of end of January 2013, 50 sites were activated and 70 patients were enrolled. Completion of enrollment is expected by June 2013.

Conclusion: The results obtained from this study will add significantly to the knowledge regarding the long-term treatment with LMWH to prevent recurrent cancer-associated VTE in real clinical practice and patients' treatment perception as PACT-Q are applied for the first time to this patient population. Important prospective data on the long-term management of cancer patients with symptomatic VTE will be generated, and analyses of clinical parameters will add important information that may help to further tailor therapy and improve compliance with established recommendations.

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PO 049

Evidence of the 'off-label' usefulness of FEIBA® to manage the critical gastro-intestinal bleeding in patients with haematological malignancies

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During the clinical course of haematological malignancies in patients with acute leukemias at onset of disease as well as in or after the intensive chemotherapies, the massive gastro-intestinal (GI) haemorrhages constitute life-threatening complications. In this scenario, several coagulation abnormalities and severe thrombocytopenia are considered causative factors of GI bleeding. The complex treatments include administration of platelet concentrates (PC), red blood cell (RBC) units, antifibrinolytic drugs, plasma infusions, somatostatin acetate and H_2-antagonist intravenously, even recombinant activated FVII (rFVII) boluses with unsatisfactory results. In the past decade we experienced that FEIBA®, at 90 IU/KG infusions were capable to stop dramatic GI bleeding in patients with haematological malignancies not respondent to various therapeutic attempts.12 consecutive patients (seven females and five males), age ranging 19–79 years, with acute myeloid leukaemia (n = 4), non-Hodgkin lymphoma (n = 3), at onset of disease (n = 4) and during intensive combined chemotherapy (n = 8) were considered during 14 episodes of massive GI bleedings. In all patients the haemostatic profile showed as expected several coagulation changes as PT and aPTT slight prolongation, D-dimer elevation and fibrinogen in normal range, sometimes Protein C and Protein S together with Antithrombin III variable reduction. No sepsis findings were documented. Severe thrombocytopenia (< 20.000/μL) and marked anaemia (Hb < 7 g/dL, Hct < 21%) were also present. By FEIBA® administration (90 IU/Kg) at first infusion (n = 3), at second infusion (n = 5) after 6 h, at third infusion (n = 2) after 12–18 h, at fourth infusion (n = 4) after 24 h, we achieved GI haemorrhages complete control with safe and efficacious outcomes, even without PC in six patients. Interestingly, in five patients we carried out during their critical haemorrhagic occurrences an endoscopy with videocapsula to explore the site of GI tract lesion. We noted small bowel origin of bleeding (n = 3) and in caecum/ascendent colon (n = 4).Obviously, the surgeon excluded the operation as well as the digital subtraction angiography (DSA) to perform trnscatheter embolization even in emergency owing to massive bleeds and/or critical conditions of the patients. It is noteworthy that after FEIBA® in 4 of these same patients we demonstrated by endoscopic videocapsula non GI haemorrhages, thus providing strong evidence that bleeds has been resolved. Despite the mechanisms by which FEIBA® determines bleedings' stopping in life-threatening haemorrhages of patients with haematological malignancies are to be defined, from our observation and for critical practice we propose FEIBA® employment safely in GI dramatic and massive bleeds, even if this indication is off-label, but efficacious based on clinical endpoints and suggest use of this drug in patient who needs a rapid control of GI bleeds.

PO 050

Association of Factor V Leiden mutation with thrombosis in newly diagnosed cases of acute leukemia in Pakistan

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Background: Acute Leukemia is commonly occurring of blood cancer with very high morbidity and mortality rate through out the globe. Newly diagnosed acute leukemia patients may have significantly high risk of thrombosis.

Aims: The purpose of study was to estimate the risk of thrombosis with frequency and association of Factor V Leiden in newly diagnosed cases of acute leukemia and as well as in healthy individual served as control.

Patients and Method: Case control study was conducted in haematology and research departments of National institute of blood disease and bone marrow transplantation from March 2011 to December 2012 after approval of Institutional review board. Total 90 cases including newly diagnosed cases of 27 with AML, 38 with ALL and 25 healthy blood donors served as control were selected with signed consent that were already screened from other thrombophilia markers including Protein C, Free Protein S, AT III and activated protein C system. Activated protein C system was tested using ProC-Global assay (Dade Behring, Marburg, Germany). A decreased response, normalized ratio (NR) below 0.80 is suggestive of presence of homozygous Factor V Leiden mutation. Genomic DNA was isolated by standard Qiagen column. Factor V Leiden mutation confirmed by restriction fragment length polymorphism (RFLP) for a 220 base pair (exon 10-intron10) fragment of factor V. The Fragment was amplified by polymerase chain reaction using the specific primers. Amplified product was digested by restriction enzyme Mnl1 and separated by electrophoresis on 6% acrylamide gel and stained with silver nitrate. Simple descriptive and chi square test was applied for the statistical analysis of data by using SPSS version 17.0.

Results: Only 2 cases of acute myeloblastic leukemia were identified as homozygous Factor V Leiden mutation with thrombo-embolic events out of 65 patients of acute leukemia with mean age of 25.38 ± 13.85 years. The frequency of FVL G1691A polymorphism was 7.4% in acute myeloblastic leukemia patient compared to none in control and acute lymphoblastic leukemia patients ($P < 0.05$).
Conclusion: Our study suggests that chances of finding FVL mutation leading to thrombosis are higher in acute myeloblastic leukemia than acute lymphoblastic leukemia in newly diagnosed patients. Prospective studies with larger sample size are required to validate our findings.

PO 051

Thrombotic complications associated with chemotherapy: an analysis of 356 patients

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Background: Thrombosis is a well recognized complication of cancer and is associated with severe organ damage and a high mortality. It is well known, that chemotherapy potentiates the risk of thrombosis. The type and the stage of cancer, as well as patients' age are also among the risk factors.
However, many studies were conducted in this field; still the mechanisms for chemotherapy and cancer-associated thrombosis are not well known and there are a lot of gaps to be filled.
Aims: The main aims of this study were to investigate the association between chemotherapy and thrombosis, to determine the frequency of those thrombotic complications and to identify patients with cancer at a high risk for developing these thrombotic episodes.
Methods: During 4 years of this study (from 2008 to 2012) 356 cancer patients were treated and observed at the Clinic of Chemotherapy of 'Muratsan' Hospital Complex of Yerevan State Medical University, from which 48 patients were children until 18-year-old. Retrospective data was collected concerning thrombotic complications associated with chemotherapy and the analysis was done. All the patients with prior thrombotic events before chemotherapy treatment were excluded from our study group.
Results: In children group there was no incidence of thrombosis during and after chemotherapy. Among 308 adult patients 9 cases (2.9%) of thrombosis were evaluated. Three patients (25%) from 12 patients with testicular cancer, 3 (9.1%) from 33 lung cancer patients, 2 (11%) from 17 patients with B-cell lymphoma and 1 (1.5%) in 66 breast cancer patients had thrombotic complications. One of those three patients with lung cancer resembled pulmonary thromboembolism.
Summary/Conclusions: However, the incidence of thrombosis in whole group of study is not so high, but it is significant in some subgroups, especially in testicular cancer, B-cell lymphoma and lung cancer. Therefore, early prophylaxis of thrombosis in mentioned groups of patients is quite important and can reduce thrombotic complications and thrombosis-associated morbidity and mortality.

PO 052

Diagnosis of functional and structural platelet disorders – implication for oncology

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In the past years, the prevalence of malignant neoplasms of the oral cavity and nasopharynx has grown up to 28.8% per 100 thousand of population and lethality during a year since the moment of cancer diagnosis in this location reached 40.3%. In the majority of patients with advanced oral and nasopharyngeal tumors, radiation therapy (RT) remains a single method of treatment. One of the main causes of the contemporary RT failure is a presence of chronically hypoxic radioresistant cell fraction within a tumor. The low-level laser radiation is used for radiomodification of these cells. However, till now, there are none of approaches worked out allowing objective assessment of therapeutic results and prediction of the disease outcome. Studying the mechanisms of thrombosis and metastasis in oncologic practice convincingly showed an important role of thrombocytes in these processes. Chemo- and radiotherapy also influence hemostasis condition. The objective of the study is elaboration of the method to estimate the efficiency of treatment of oncologic patients with different stages of the disease receiving radiotherapy combined with low-level laser radiation as a radiosensitizer.
A total of 45 patients with malignant oral tumors (MOT) were examined in dynamics: 20 patients were treated with only RT, 25 – with RT in combination with intravenous blood laser radiation (radiation power 25 mw, wave length 632.8 nm). The control group included 30 practically healthy people. All patients underwent distant gamma-therapy (DGT) using uncommon regimen of dose fractioning in summarized focal doses isoefficient 72–73 Gy. Analysis of the morphofunctional condition of live unfixed and unstained thrombocytes was carried out in the real time using express-method of vital computed phasemetry on the bass of device-program complex for cellular diagnostics using cytomorphometry and microelectrophoresis. Morphological structure size parameters and adhesive activity of circulating population was assessed. ADP-induced aggregation was used as a reference method.
In patients examined, the number of immovable cells, slightly or highly activated, and degenerative cells was, on the average, 27.6, 44.4, 25.5 and 2.5 vs. 63.1, 20.9, 12.3 and 3.7% of the norm. Increase in the mean diameter values of circulating thrombocytes by 23.4, 26.8 and 30.3% was noted as well as of perimeter by 17.6, 21.3 and 24.6%, and square by 30.2, 32.5 and 38.1% in patients with II, III and IV MOP stages, accordingly. The cellular height and volume reflecting granules condition decreased by 10–15%. With growth of DGT dose, the number of active thrombocytes increased and the size parameters also changed. Inclusion of intravenous blood laser radiation into the course of radiotherapy enabled normalization of thrombocyte structural-and-voluminous characteristics (growth of the height and volume, diminishing of diameter, perimeter and square) and reliable reduction of their activating potential.
Thus, application of the express-method of vital computed phasemetry of thrombocytes in oncologic practice allows objective determining the level of intravascular activation of thrombocytes. Morphometric and functional thrombocyte parameters can serve criteria of the efficiency of the radio- and laser therapy carried out.

PO 053

LMWH generics as first line drugs in prophylaxis of thrombotic complications in cancer patients

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Background: The most perspective methods of prophylaxis of thrombophilia and thrombosis in cancer patients is administration of low-molecular weight heparin (LMWH). That is connected with a number of its features: 30% of LMWH activity are performed through AT III and 70% through TFPI; LMWH activates fibrinolysis by exemption t-PA from endothelium; LMWH is inhibit leukocytes procoagulant activity; LMWH less often cause the heparin-induced thrombocytopenia. LMWH is effective in case of warfarin resistance. LMWH administration does not required constant laboratory control. The purpose: To specify an optimum schedule of administration LMWH for

prophylaxis of thrombotic complications in cancer patients in perioperative period.

Materials and Methods: Two hundred and twenty-six cancer patients at random was divided in three groups in accordance to scheme of heparin administration: I – LMWH (Hemapaxan) 24 h prior to surgery treatment, further once a day on 0.4 mL (4000 IU) within 10 days in the postoperative period – 75 patients II – LMWH (Hemapaxan) once day on 0.4 mL (4000 IU) within 10 days in the postoperative period – 77 patients III – Unfractionated heparin on 5000 IU 3 times a day within 10 days in the postoperative period – 74 patients Lab Methods a quantitative estimation of platelets aggregation in the presence of Adrenaline, Collagen and ADP in various concentration (10–3, 10–5, 10–7) to determine degree of their activation. Determination of a platelets marker of activation – platelets antiheparin factor 4 (PF4). DIC-syndrome markers: D-dimer, TAT, prothrombin fragments F1 + 2.

Results: Before surgery treatment the rate of subcompensated DIC-syndrome was 18.5–50% depending of localization and degree of tumor expansion. After surgery treatment the rate of subcompensated DIC-syndrome considerably increased, and was found signs of decompensated DIC-syndrom. In total the rate of patients with DIC-syndrome was 52–75%. In the first group normalization of levels of thrombophilia markers (TAT, PF4, F1 + 2) was observed in 3–5 days after surgery treatment. In the II group thrombophilia markers tended to normalization in 5–7 days after operative intervention. In III group normalization of thrombophilia markers was found out only after 7 days. Some patients remained increased level of D-dimer up to 10 days. Besides that, in 28 patients (13.7%) was found extensive painful hematomas in a place of heparin injection.

Conclusions: Perioperative period is the extremely dangerous for thrombotic complications risk and demands careful control of a hemostasis system functions. At present till now still there is no uniform tactics of thrombotic complications prophylaxis in perioperative period. The scheme offered by us: LMWH (we have studied the effectiveness of biosimilar grug – Hemapaxan) 24 h prior to operative intervention, further once a day on 4000 IU within 10 days in the postoperative period can be recommended for all cancer patients as the program-minimum, however for 10th day of application of such scheme it is necessary to control of a condition of hemostasis system in order to determine necessity of continuation of prophylaxis application of LMWH. Besides that, we consider necessary to provide the analysis of genetic forms of thrombophilia and APS for all cancer patients with clinically expressed thrombotic complications.

PO 054

Diffuse intracranial haemorrhage in Factor V deficiency: two case reports

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Background: Factor V (FV) deficiency is a rare autosomal recessive bleeding disorder and its prevalence has been estimated to be 1 out of 1,000,000 populations. Factor V deficiency can present from mild to severe bleeding symptom such as bruising, nose and mouth bleeds. In severe forms of factor V deficiency, joint bleeding and risks of central nervous system bleeding can be seen in newborns.

Aim: We report two cases of severe FV deficiency, presenting with intracranial hemorrhage (ICH).

Methods: Case 1 is a 6- month male infant who was admitted to emergency Hospital because of lethargy and pallor. He had normal condition after a Normal Spontaneous Vaginal Delivery. The patient's parents were not relatives. Coagulation screening showed a prolonged Prothrombin time (PT) of 25s and upper limit of normal Partial Thromboplastin time (PTT) of 40s and His hemoglobin was 5 g/dL. Computed tomography (CT)-Scan was undertaken and revealed a large intraparenchymal in the left frontoparietal lob, with significant edema and mid line shifted to the right side (Figure 1). Coagulation factor assays revealed a FV plasma activity < 0.5%. FFP treatment was stopped for 24 h because of poor venous access after 1 month of ICH. Unfortunately patient passed away 1 day later.

Case 2 is a 4- month old boy who was admitted in Hospital with skin bleeding, convulsion and poor feeding. There was no history of a hemorrhage diathesis in their family, and parents were second degree relative. He had ICH and spontaneous bleeding from gums. A non-stop bleeding had occurred after circumcision at 20 days after birth and he had been treated with FFP without diagnosis. Coagulation tests showed prolonged PT and PTT and plasma FV activity was below one percent. FFP (15 cc/Kg) was transfused to patient once a day and he became clinically stable. After 1 month, FFP infusion was reduced to once a week after 1 month of therapy.

Conclusion: An intraventricular hemorrhage associated with FV deficiency was first reported by Whitelaw et al. in 1984. Deficiency of FV leads to in severe bleeding symptoms such as ICH. So, FV activity should be considered in newborn or infants with ICH as well as FX, FVII and FXIII assays that clearly are common causes of ICH in newborns. FFP is the only method of treatment in patents with FV deficiency, and prophylaxis with FFP is recommended in patients with severe bleeding symptoms or in cases of ICH.

PO 055

Congenital combined deficiency VII and V associated with thrombocytopenia

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Although they are very rare, the combined deficits V and factor VII congenital deficiencies and combined deficiency VII and other factors the factor IX, X, XI, lack of protein C are known (1). We report the case of an association of congenital combined deficiency VII and V associated with mild thrombocytopenia. Patient and methodsIt is a patient aged 42 years, nonconsanguineous parents, ménarchie at the age of 12 years, menorrhagia scarce, married, G4P1, scalable current pregnancy 33 weeks, with a history of death in two- utero at 7 months and the other 2 months during the first two pregnancies, a girl aged 5 years in good health born by cesarean section requiring two blood units and 2 units of fresh frozen plasma. Has a herniated disc without any treatment. Not taking medication. The only problem serious bleeding was observed at the age of 7 years after injury of the hand. If not the patient had multiple dental extractions without special precautions, no bleeding postpartum at different deliveries, no epistaxis, no gingivorragie. C is in search of the etiology of deaths in- utero we found a moderate thrombocytopenia 98,000/mm^3, TP 58%, APTT:32 s. (Witness 34 s), Fibrinogen 4.60 g/L. The assay showed a differential deficit at 36% V, VII deficiency in 36%, 104% II, the X-92%, VIIIc 286%, 116% plasminogen. The dosage of 78% ATIII, Protein C at 105%, Protein S A71% 261% RPCA. The search for lupus anticoagulant negative. Given the large size of platelets, a research Dystrophy Jean Bernard Soulier negative by flow cytometry. The study of the family could not be made for the grandparents and parents of the patient. Consists of siblings three sisters and five brothers: one older sister 44 years this D deficiency in 52% V, Y brother aged 33 has a V deficiency in 54%, A brother aged 40 has a combined deficit in V (34%) and VII (56%), explorations have not been made in other siblings.

Discussion: VII deficiency has been described associated with unexplained mental retardation, a epicanthal folds, the ductus arteriosus and other anomalies that remain unexplained. The rarity of a disease can teach us many things in physiology and pathophysiology study that deficits and/or frequent illness. In addition, these deficits must be combined very few in mind before a certain replacement therapy apparently isolated deficit remaining inadequate or ineffective.

PO 056

Registry of inhibitors in mild and moderate haemophilia A patients in Spain

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Background: Inhibitor development occurs less frequently in mild and moderate haemophilia A patients than in severe ones. The development of alloantibodies against factor VIII (FVIII) in these patients is often associated with important clinical problems such bleeding phenotype changing into a more severe form. We have tried to determine the characteristics of the patients who develop inhibitors in this pathology in our country.

Patients and Methods: All patients with mild and moderate haemophilia A and inhibitors registered at several centers of hemophilia in Spain in the last 20 years were included. Demographic and hemophiliac data (date of birth, ethnicity, FVIII level, family history of haemophilia and inhibitor development, F8 gene mutation) were obtained. We also obtained data related to inhibitors: initial and maximum historical titres, kinetic reaction type, inhibitor against exogenous o both endogenous and exogenous FVIII, exposure days (ED) and intensive replacement therapy before inhibitors. We collected data of treatment to bleeding episodes and immune tolerance induction if performed.

Results: Fifteen patients (four with moderate and eleven with mild haemophilia A) developed inhibitors in this period. All our patients were Caucasian. In twelve of them information about F8 gene mutation is available. Six cases have a mutation considered as high risk for inhibitor development (four R2150H and two R593C mutations). Average age at which inhibitors developed was in the age range of 28 (3–64) and 38 (6–47) for moderate and mild haemophilia patients. Eleven patients (73%) had an inhibitor considered as high titre. In the majority of the patients (8/13) inhibitor development occurs after more than 50 ED. Only 6 out of the 15 patients underwent eradicator treatment, 5 of which (83%) responded positively.

Conclusions: Several patients present mutations associated to high risk for inhibitor development. As well as in severe haemophilia, the majority of inhibitors show high titres. Apart from this disease, inhibitors may develop at an advanced age showing many ED. Many patients do not undergo proper treatment and the inhibitor may disappear. The mild and moderate haemophilia patients with inhibitors showed quite a significant difference as compared to severe haemophilia A patients.

PO 057

Fluctuation in factor VIII levels in a patient with hemophilia B

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Background: In here, we want to share an interesting patient with Hemophila B in whom Factor VIII levels are fluctuating. 31 years old male patient was following with hemophilia B and Hemophila A since 2 years old. He had been diagnosed as combined factor deficiency because of low Factor VIII (7%) and low Factor IX (5%) levels. He was followed by adult hematology department after 22 years old. When we checked his Factor VIII levels were such as: 7%, 138%, 208%, 117%, 52%, 63%, 15%, 64%, 29%, 80%, 80%, 19%, 19%, 101% during last 29 years. We checked whether Fresh Frozen Plasma (FFP) replacements were done just before these measurements, the situation was safe. There was not any replacement. Factor inhibitors for VIII and IX were always negative. His Factor IX levels were consistent such as 5%,%7 during last 29 years.

The level of vWF Ag was 97%, in the same way there was not FFP replacement just before this measurement. His blood group is O Rh +. Patient's mother, father and sister's blood groups are also O Rh +.

According to patient's history his mother and sister have got same situation as low Factor VIII and low Factor IX. However, we could not reach their old charts.

Summary: Finally, if our patient would carry hemophilia A gene, his factor VIII levels never can be as high as 138% or 208%. Because of his blood group there is a big suspicion for the reason of these fluctuations; it can be a von Willebrand disease in O blood group patient. After this evaluation we accepted patient as only as Hemophilia B not together with Hemophilia A despite low Factor VIII levels such as 7%, 15%, 19% and 29%. We planned to study of ristocetin cofactor activity. We are still thinking that whether there is a big waving in his vWF Ag level or vWF activity results in very low (7%) and high Factor VIII levels (208%).

PO 058

Structural comparison of a new recombinant rFVIII molecule, turoctocog alfa, and commercially available FVIII products

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Background: Turoctocog alfa is a new recombinant FVIII molecule (rFVIII) with a truncated B-domain consisting of 21 amino acids from the naturally occurring B-domain.[1] Commercially available rFVIII products are either produced from the full-length gene resulting in a heterogeneous mixture of heavy chains with different lengths of the B-domain attached (Advate® or Kogenate FS®) or from a construct with a 14 amino acid B-domain attached (ReFacto AF®). The essential role of the B-domain is to facilitate the secretion from the FVIII synthesizing cells. As the B-domain is not required for procoagulant activity, the biological activity of turoctocog alfa and Advate® *in vitro*[2] and *in vivo*[3] is comparable.

Aim: To compare the structure and homogeneity of turoctocog alfa to commercially available rFVIII products.

Methods: The FVIII-related components present in Advate®, Kogenate FS®, ReFacto AF® and turoctocog alfa have been analysed using SDS-PAGE with and without thrombin digestion followed by *N*-terminal amino acid sequencing of excised bands, size exclusion (SE)-HPLC and reverse phase (RP)-HPLC.

Results: SDS-PAGE of turoctocog alfa showed the expected major bands corresponding to the heavy chain (HC) and light chain (LC) in addition to a minor band corresponding to the single chain (scFVIII). The same band pattern for HC, LC and scFVIII was observed for ReFacto AF®, while several high molecular weights bands corresponding to HC with different length of the B-domain attached were observed for Advate® and Kogenate FS®. Following thrombin digestion, all rFVIII products showed the expected bands corresponding to the A1 and A2 domains and the activated LC. In addition to intact A2, ReFacto AF® contained two components with lower mass likely due to *C*-terminal cleavage within A2.[4] For turoctocog alfa the major components in the RP-HPLC profile consisted of HC and LC and a small amount (approximately 3%) of scFVIII. Heterogeneity of HC was observed for all rFVIII compounds. Turoctocog alfa had two components, one with *C*-terminal at amino acid 740, i.e. at the end of the A2 domain, and one containing the 21 amino acid B-domain. Advate® and Kogenate FS® both contained several HC degradation products corresponding to HC's with different lengths of the B-domain attached. For Advate®, a specific LC component containing part of the B-domain was observed. The SE-HPLC of turoctocog alfa and

ReFacto AF® have similar profiles, while variation due to the heterogeneously processed B-domains was observed for Advate® and Kogenate FS®.

Conclusion: The major difference of the commercially available rFVIII products is the variation in length of the B-domain: Advate® and Kogenate FS®, encoded by the full-length gene, showed highly heterogeneous profiles in SE- and RP-HPLC, while ReFacto AF® and turoctocog alfa both exhibit more homogeneous profiles. For Advate®, a specific LC variant containing part of the B-domain was observed. After thrombin cleavage all rFVIII compounds contain the expected pattern of A1, A2 and activated LC, however, ReFacto AF® also contains smaller size A2 domains. In summary, turoctocog alfa is a highly homogeneous rFVIII product.

[1]Thim, Haemophilia 2010;16:349–59.
[2]Christensen, Haemophilia 2010;16:878–87.
[3]Martinowitz, Haemophilia 2011;17:854–9.
[4]Sandberg, Thromb Haemost 2001;85:93–100.

PO 059

The activity of glycoPEGylated recombinant FVIII (N8-GP) can be measured in both two-stage chromogenic and one-stage clotting assays

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Background: N8-GP is a 40KDa glycoPEGylated recombinant human FVIII. The PEG group is selectively attached to a unique O-linked glycan in the B-domain of turoctocog alfa, which upon activation is released and generates activated FVIII (FVIIIa) similar to native FVIIIa.[1] N8-GP and FVIII have similar potency in animal bleeding models, however N8-GP has longer duration of effect. In humans N8-GP has a 1.6-fold prolonged half-life compared to rFVIII. N8-GP is in late stage clinical development for bleeding prophylaxis in haemophilia A.

Aim: To evaluate N8-GP relative to non-PEGylated rFVIII in two-stage chromogenic and one-stage clot assays.

Methods: Different FVIII compounds (turoctocog alfa, N8-GP, Advate®, and Haemate®) were analysed in two-stage chromogenic assays and in one-stage clotting assay with different APTT reagents. Reference materials (plasma, rFVIII, N8-GP) were calibrated against WHO FVIII standard.

Results: The activity of N8-GP and turoctocog alfa was comparable when measured in two-stage chromogenic assays. Thus, chromogenic assays do not appear to be affected by the PEG group of N8-GP. In the one-stage clot assay the activity measured for turoctocog alfa, Advate® and Haemate® was within 100 ± 25% of labelled value irrespectively of APTT reagent used. APTT reagents containing ellagic acid in general gave activities for N8-GP within 100 ± 25% of labelled value, e.g. the use of Actin FS® and STA Cephascreen® resulted in recoveries of N8-GP close to 100%. Other reagents, in particular the silica-based reagents, resulted in activities below 75%. The latter suggested some interaction of silica-based APTT reagents with the PEG group of N8-GP. Full recovery was achieved for all APTT reagents when using the N8-GP standard. Addition of free PEG to the assay buffer did not influence the performance of rFVIII in the one-stage clot assay, i.e. the interference is only observed when the PEG moiety is attached to rFVIII.

Summary/Conclusion: As for rFVIII (e.g. turoctocog alfa), the activity of glycoPEGylated recombinant FVIII (N8-GP) can be reliably measured against the WHO FVIII standard in two-stage chromogenic as well as in one-stage clotting assays with ellagic acid-based APTT reagents. Other APTT reagents provided reduced recoveries, probably due to interference of the PEG group of N8-GP with APTT reagent. The reagent specific variations can be normalised by introduction of an assay specific conversion factor or by using a N8-GP standard. The data illustrate the need for evaluating performance of new FVIII products in various activity assays using different reagents.

1. Stennicke et al. Blood 2013, doi:10.1182/blood-2012-01-407494

PO 060

Selective measurement of PEGylated human recombinant factor VIII (BAX 855) in laboratory animal plasma

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Background: Quality-determining attributes of protein therapeutics such as half-life, immunogenicity or resistance against proteolytic degradation, can be improved by the covalent attachment of polyethylene glycol (PEG), i.e. PEGylation. Monitoring the resulting PEGylated protein in preclinical settings requires a composite assay type that not only measures the parent, unmodified protein, but also its PEG moiety introduced by PEGylation.

Aim: We therefore developed a ligand-binding assay (LBA) to selectively measure PEGylated human recombinant factor VIII (rFVIII; BAX 855), and validated it for use in pharmacokinetic studies in macaques, rats and FVIII-deficient mice.

Method: The LBA uses a combination of an anti-PEG antibody and a polyclonal anti-human FVIII antibody-peroxidase conjugate as a reporter antibody. PEGylated rFVIII BAX 855 is selectively captured via its PEG moiety by the plate-bound anti-PEG antibody and then detected by binding of the reporter antibody. Concentration-dependent responses were obtained with a calibration curve ranging from 0.18 to 2 ng FVIII-bound PEG/mL. We validated the LBA in the matrix of macaque, rat and FVIII-deficient mouse plasma, according to the EMA guideline on bio-analytical assay validation.

Results: Using the combination of anti-PEG capturing antibody and anti-FVIII reporter antibody allowed us to specifically measure human PEGylated rFVIII in all three plasma matrices. This was posible even in macaque plasma where usually a high degree of cross-reactivity interferes with the specific immunological measurement of human FVIII. The assay's selectivity allowed measurement of BAX 855 without interference by endogenous FVIII in wild-type animal models. Results of the assay validations proved the LBA suitable for its intended use in all three species: Mean accuracy, expressed as the recovery of BAX 855 spiked at five relevant concentrations to the plasma of macaques, rats and FVIII-deficient mice, was 91.9%, 96.4% and 91.7%, respectively, with all individual values in a ± 20% range, even at the lower limit of quantification. Intra- and inter-run precision also complied with accepted recommendations for assays of this type. The three animal plasma matrices showed no influence on the linearity of the dilution series obtained for BAX 855-containing plasma samples. The LBA's mean total errors, giving the sum of absolute accuracy and precision, were 11.0%, 16.4% and 15.2% for rat, E17 mouse and macaque plasma spiked with BAX 855 at FVIII-bound PEG concentrations ranging from 10 to 400 ng/mL, and were < 35% at the assay's lower limit of quantification.

Summary/Conclusions: A ligand-binding assay was developed and successfully validated for measuring the PEGylated rFVIII preparation BAX 855 in the plasma of laboratory animals. The concept of using paired antibodies, and in particular, the application of an anti-PEG antibody to capture the PEGylated protein, can be extended to measurement of other PEGylated biologicals using an appropriate reporter antibody.

PO 061

Modification-dependent activity assay: A new assay type for selective activity measurement of PEGylated human factor VIII (BAX 855)

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Background: Quality-determining attributes of protein therapeutics such as half-life, immunogenicity or resistance against proteolytic degradation can be improved by the covalent attachment of polyethylene glycol (PEG), i.e. PEGylation. Optimally, PEGylation does not or only minimally reduces the biological activity of the modified molecule. Measurement of the activity of the resulting PEGylated protein can be achieved with a new type of activity assay, which only measures the protein's activity as long as the PEG moiety is present. This assay type is therefore termed modification-dependent activity assay (MDAA).

Aim: We developed and validated a modification-dependent activity assay to selectively measure the FVIII activity of the PEGylated human recombinant factor VIII (rFVIII) preparation BAX 855 in normal and in FVIII-deficient human plasma without interference by non-PEGylated FVIII.

Method: The MDAA combines the use of an anti-PEG antibody with an FXa-based chromogenic FVIII activity assay. PEGylated rFVIII BAX 855 is selectively captured by the plate-bound anti-PEG antibody, and non-PEGylated compounds including endogenous FVIII when present are removed by washing. The activity of the bound PEGylated FVIII is then determined by chromogenic FVIII activity assay. The assay, which, due to its underlying principle, shows absolute specificity for PEGylated FVIII, was validated for measurement of BAX 855 in human plasma samples according to the EMA guideline on bio-analytical assay validation.

Results: The six-point calibration curve of the MDAA ranged from 2.2 to 69 mU FVIII/mL; thus, it was as sensitive as conventional chromogenic assays. Using this MDAA for measurement of samples from a preclinical pharmacokinetic study in a FVIII-deficient mouse model (E17 mice) provided almost overlapping time vs. activity curves compared with those obtained with the conventional chromogenic FVIII assay. Results of the validation proved the MDAA suitable for its intended use: Mean accuracy, expressed as the recovery of PEGylated rFVIII BAX 855 spiked at five concentrations ranging from 0.07 to 2 U/mL to normal human plasma and FVIII-deficient plasma was 97.1% and 96.1%, respectively, with all individual means in a $\pm$ 12% range even at the lower limit of quantification (LLOQ). Intra- and inter-run precision clearly complied with accepted recommendations with relative standard deviations of less than 12%, even at the LLOQ. The selectivity of the capturing step was confirmed by competition with PEG and an anti-PEG antibody: In both cases, a dose-dependent signal reduction was obtained.

Summary/Conclusions: A highly selective MDAA was developed and successfully validated for measurement of the PEGylated rFVIII preparation BAX 855 in normal human plasma and FVIII-deficient plasma. This particular concept of specifically capturing PEGylated rFVIII before measuring its activity can be extended to other PEGylated proteins. The results obtained with this newly developed assay type will allow to determine whether or not the PEGylated protein is still intact.

PO 062

Differentiation of the natural, full length recombinant FVIII molecule from B-domain deleted recombinant FVIII with respect to its hemostatic potency

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Background: A variety of recombinant FVIII (rFVIII) preparations that provide efficacious replacement therapy in hemophilia A patients are available. These preparations include full-length rFVIII (FL rFVIII), which is equivalent to the naturally occurring FVIII molecule, and genetically engineered B-domain deleted rFVIII (BDD rFVIII). Determination of an accurate FVIII activity is crucial for manufacturing, and especially for product dosing in clinical settings and testing of patient plasma. Several studies have demonstrated that assessing reliable FVIII activity for BDD rFVIII is a significant challenge due to the major discrepancy in results obtained with FVIII chromogenic and 1-stage clotting assay. Moreover, FVIII 1-stage clotting activity depends on the type of aPTT reagent used.

Aims: The objective of the study was to evaluate differences in potency determination between FL rFVIII and BDD rFVIII with respect to their dependency on the type of aPTT reagent used in the 1-stage clotting assay.

Methods: Potency of FVIII was measured by a FVIII chromogenic activity assay and 1-stage clotting assay, using different commercial available aPTT reagents. Both the 8th international standard for blood coagulation factor VIII concentrate (NIBSC #07/350) and human normal plasma served as assay reference standards. The performance of the two different types of rFVIII products was compared by calculating the ratios between FVIII 1-stage clotting and chromogenic activities.

Results: Depending on aPTT reagent type, FVIII potencies varied within a relatively broad range, independent of which type of assay reference standard was used. The resulting ratios between FVIII 1-stage clotting and chromogenic activity showed a broader variance for the BDD rFVIII products than for FL rFVIII products.

Summary/Conclusions: Although further investigation is required to substantiate our observation, dependence on aPTT reagent type appeared more pronounced for BDD rFVIII than for FL rFVIII. As clinical laboratories use a number of commercial aPTT reagents with different compositions, determination of the correct potency and concentration in plasma post infusion of BDD rFVIII might be more challenging than for the natural, full-length rFVIII molecule.

PO 063

Factor XI deficiency: a family report

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Background: Factor XI (FXI) deficiency is an autosomal recessive inherited coagulation disorder associated with a mild bleeding tendency, absence of spontaneous bleeding and this manifestations are weakly correlated with plasmatic FXI level.

Methods: We describe an asymptomatic family withe FXI deficiency. The patient 65 year old man; diagnosed after a systematic blood test, his wife, her two girls and son were tested. Factor XI level were determined by one-stage plasma clotting assay. Von Willebrand Factor (VWF) activity were determined by visual method.

Results: The patient and her two girls have a severe deficiency (FIX level 2%). The wife patient and her son have a minor deficiency (respectively FXI levels 56% and 46%). Previous studies suggested that VWF activity may influence bleeding tendency. The absence of bleeding history in this familly and the result of VWF activity level (mean 116%, range 81% $-$135%) that suggest a role of VWF.

Conclusion: This result suggests the necessity to perform assay of VWF activity in investigation of factor XI deficiency, to evaluate a bleeding tendency.

PO 064

Factor XI deficit: cases report

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Background: Factor XI deficit is a uncommon condition with a estimated incidence of one case per million. Bleeding as clinical manifestation are polymorphic, and also the relationship between the level of factor XI and bleeding events.
Aims: To describe clinical presentations, diagnosis, level of factor XI and treatment the nine patients in our center.
Methods: The factor levels were done by one stage clotting method (at least two dilutions). Fibrinogen levels were made with a Clauss method, and the Factor VW levels with antigenic and immuneturbimetric methods. All test were made with an optic automated coagulometer.
Results: The mean age was 48 years old (range 9–73), 7 was female. The diagnosis as screening preoperative test when prolonged APTT was found in six patients, post surgical bleeding 2, as metrorrhagia in 1 of cases. None presented as spontaneous or severe bleeding before the diagnosis was made. The factor XI measured were between 9% and 50%, two patients presented severe Factor XI deficit with a level less than 15% (one with metrorrhagia and the other without bleeding manifestations) and seven patients the deficit was mild with a factor XI level between 30 and 50%. No molecular test and platelet Factor XI were available in our country. In six patients we used fresh frozen plasma, in two anti fibrinolytics and in one DDAVP, previously to the surgical procedure since there is no factor XI concentrate in *Argentina*. None of them had post surgical bleeding.
Conclusion: (1) Factor XI is an uncommon condition, but must be ruled out in a patient with a prolongued APTT with correction in mix assays; (2) In pre surgical intervention are effective fresh frozen plasma, antifibrinolytics and DDAVP as an alternative to Factor XI concentrate.

PO 065

Prevalence of Venous Thrombo-Embolism & Related Morbidity and Mortality among hospitalized patients in Saudi Arabia (SAVTE Registry)

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Background: Venous Thrombo Embolism (VTE) is a serious and potentially fatal condition. Its prevalence and incidence has not been clearly defined on a large scale bases in Saudi Arabia. Appropriate prophylaxis can lead to favorable and cost-effective results.
Aims: The primary objective is to determine the percentage of VTE patients who received appropriate anti-thrombotic prophylaxis according to ACCP guidelines. The secondary objective is to determine the mortality due to VTE events, type of VTE events, and percentage of patients prescribed the anti-coagulant therapy and adherence to it after discharge.
Methods: Seven major tertiary hospitals in the Kingdom of Saudi Arabia have participated in this study. During the period from 1 July 2009 till 30 June 2010, all cases of VTE recorded in the hospitals, were collected using patients' medical records and computerized databases of the hospitals. Only patients with confirmed diagnosis of VTE were included in the analysis.
Results: One thousand two hundred and forty-one of confirmed VTE were included in the study analysis. A 58.3% of them were DVT only, 21.7% were PE and 20% had both DVT and PE. A 21.4% and 78.6% of confirmed VTE occur in surgical and medical patients respectively. A 40.9% of total VTE cases received prophylaxis.
Only 63.2% of surgical patients and 34.8% of medical patients had received appropriate prophylaxis ($P < 0.001$). Mortality rate was 14.3% of all patients. A 89.4% of survived patients were on anti-coagulation therapy at discharge, and 71.7% were adherent to it on follow-up.
Conclusions: VTE is a significant cause of morbidity and mortality in hospitalized patients. All efforts should be made to reduce the gap between guidelines and practices in implementing appropriate VTE prophylaxis.

PO 066

Acute Deep Venous Thrombosis secondary to May-Thurner syndrome: a case report

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Background: We report a case of a 31 year-old female with pain and swelling of the left lower extremity as the initial presentation of acute deep venous thrombosis (DVT) secondary to May-Thurner Syndrome, a rare case with unknown overall prevalence or incidence, that results in iliofemoral DVT due to an anatomical anomaly where the left common iliac vein is compressed between the right common iliac artery and the lumbar vertebrae. She had a two-day history of pain and swelling of the left lower extremity, difficulty ambulating, warmth, and purplish discoloration of the skin. On physical examination, the left lower extremity had purplish discoloration, varicosities, increased capillary refill time, grade three pitting edema, warmth, tenderness from the medial part of the thigh to the calf and limitation of movement. Its circumference was greater than the right lower extremity by 10 cm. Pulses were full and equal, with intact reflexes, sensory, and no masses or inguinal lymphadenopathy.
Aims: May-Thurner Syndrome is often unrecognized due to the prevalence of other more easily recognized risk factors for DVT. A more invasive study, such as venography, is necessary to correctly identify and manage this anatomical anomaly to prevent debilitating sequelae.
Methods: Duplex scan of lower extremities showed acute DVT partially occluding the left common iliac and distal external iliac veins, proximal to distal femoral vein, and popliteal vein. CBC, PT, and PTT were unremarkable. She had elevated Factor VIII levels at 201%, and low protein S level at 19%, prompting investigation for thrombophilia which yielded negative findings. On further work-up, venography revealed stenotic, anomalous vein originating from the left iliac vein, obstruction from the left common iliac vein to mid-proximal femoral vein and collaterals in the common femoral vein to the left common iliac vein. Workup for anti-phospholipid antibody syndrome, systemic lupus erythematosus, malignancy, lymphedema, and infection were negative.
Results: Management was aimed at clearing the thrombus present and correcting the underlying compression of the left iliac vein. Methods of clearing the thrombus include anticoagulation and thromboreductive strategies. Initial anticoagulation with Enoxaparin was overlapped with Warfarin for long-term anticoagulation. She also underwent catheter-directed thrombolysis with Alteplase, and ilio-femoral, popliteal, and posterior tibial thrombectomy after IVC filter insertion; however, treatment failed as evidenced by persistence of pain and swelling on the left lower extremity, and occurrence of pulmonary embolism. Subsequently, femoro-femoral vein bypass using cross-over great saphenous vein and AV fistula creation to the femoral venous bypass graft were successfully performed, followed by chronic anticoagulation with Rivaroxaban. Clinical improvement was noted with completely resolved pain and swelling on the left lower extremity after 6 months.

Follow-up duplex scan of lower extremities also showed marked improvement.

Summary/Conclusions: A comprehensive diagnostic approach is essential to accurately identify this anatomical anomaly as a cause of iliofemoral DVT, especially in a young female with unilateral pain and swelling of lower extremity. Long-term anticoagulation and thromboreductive strategies, while indicated, are not adequate to prevent long-term sequelae in May-Thurner Syndrome, and a more invasive therapeutic approach, surgery, is indicated.

PO 067

The role of clinical scores in the diagnosis of pulmonary thromboemboli

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Background: Pulmonary thromboembolism (PTE) is a common disease, that requires prompt diagnosis and treatment due to the high morbidity and mortality. Clinical scoring methods are used for the diagnosis of PTE in addition to the algorithms that includes clinical, radiological and laboratory parameters. Wells and the modified Geneva clinical scores are two of these methods.

Aim: The aim of this study was to compare the roles of these two methods in the diagnosis of PTE.

Methods: The demographic and clinical characteristics, risk factors, Wells and modified Geneva scores, arterial blood gas values, radiographic and computed tomography angiography findings of patients hospitalized in our clinic with the pre-diagnosis of PTE between January 2010 – December 2012 were retrospectively analyzed. The role of Wells and modified Geneva clinical scores in the diagnosis of PTE was evaluated and compared with each other by Mann–Whitney-U test using the SPSS 17.0 program, $P < 0.05$ was considered statistically significant.

Results: Two hundred and twelve patients, 104 (49.1%) female and 108 (50.9%) male, hospitalized with the diagnosis of PTE were included in the study. The mean age of patients was 60.6 ± 17.7 yeras, 124 of these patients (58.5%) were treated with the diagnosis of PTE and 88 (41.5%) of them consisted of patients who were diagnosed besides PTE. Twenty-three (18.5%) of cases were in low, 99 (79.8%) were in moderate and 2 (1.6%) of them were in high clinical probability with the Wells scoring method. On the other hand, 98 (79%) of patients were in moderate and 26 (21%) of them were in high clinical probability with the modified Geneva scoring. The mean Wells and modified Geneva scores were 2.74 ± 1.08 and 2.83 ± 6.51. It is determined that Wells score and the modified Geneva score were statistically significant in the diagnosis of PTE ($P = 0.03$, $P < 0.0001$).

Conclusion: As a result, a variety of clinical scoring methods are important in achieving the diagnosis of PTE. In our study, Wells scoring method is found more valuable than the modified Geneva scoring.

PO 068

Retrospective evaluation of pulmonary thromboembolism cases

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Background: Pulmonary thromboembolism (PTE) is a common disease, that requires prompt diagnosis and treatment due to the high morbidity and mortality.

Aim: The aim of this study is to evaluate retrospectively the clinical, laboratory and radiological findings of patients that diagnosed and treated as PTE.

Methods: The demographic and clinical characteristics, risk factors, arterial blood gas values, radiographic and computed tomography angiography findings of patients hospitalized in our clinic with the diagnosis of PTE between January 2010 – December 2012 were retrospectively analyzed.

Results: The most common risk factors were older age (> 65) (48.4%), deep venous thrombosis (44.4%), immobilisation (27.4%) and surgical operation in last 4 weeks (14.5%). The most common symptoms observed in our patients were dyspnea (75.0%), cough (29.8%), chest pain (27.4%), hemoptysis (21.0%) and unilateral lower extremity pain (14.5%), respectively. Chest X-ray reveals parenchymal lesions in 82 (66.1%), pleural effusion in 57 (46.0%) and linear atelectasis in 28 (22.6%) of the cases. Serum d-dimer level was high in 121 (97.6%) cases. Fifty-one (41.1%) of patients had hypoxemia and 69(55.6%) had hypocapnia arterial blood gasses. Thrombosis was detected in 44.4% of cases at lower extremity Doppler ultrasonography. A 62.9% of the patients were diagnosed with V/Q scintigraphy while 37.1% of them were diagnosed with computed tomography angiography of the thorax.

Conclusion: We consider that clinical evaluation is also important besides the radiological and laboratory examinations in PTE suspected cases. The morbidity and mortality can be reduced if PTE is kept in mind especially in patients with risk factors.

PO 069

D-dimer, P-selectin and Microparticles are superior to duplex ultrasound in the diagnosis of DVT

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D-dimer, P-selectin and Microparticles are superior to duplex ultrasound in the diagnosis of DVT.

Background: Diagnosis of DVT is based upon clinical suspicion in patients at risk and confirmatory duplex ultrasound imaging of the deep venous system of the affected extremity. Currently, no single blood test exists alone to diagnose DVT and the most widely used test, D-dimer, is useful to exclude the diagnosis of DVT due to its high sensitivity, but cannot establish the diagnosis due to its poor specificity.

Aim: This study was performed to determine the significance of D-dimer, P-selectin and microparticles biomarkers in the diagnosis of DVT.

Methods: Three groups of individuals were examined: 50 normal individuals (Group I); 50 symptomatic patients for DVT whose duplex ultrasound was positive (Group II) and 50 symptomatic patients with normal duplex ultrasound (Group III). Measurment of D-dimer by immunoturbidimetric assay, P-selectin by flowcytometry and microparticles by ELISA was performed for all individuals.

Results: D-dimer, P-selectin and microparticles were significantly higher in groups II and III compared to group I individuals. Group II vs. Group III individuals had D-dimer values of 4.41 ± 2.07 vs. 2.1 ± 1.37 mg/L ($P = 0.000$); P-selectin values of 31.91 ± 14.9 vs. 22.96 ± 10.7%($P = 0.021$) and microparticles levels of 42.12 ± 14.1 vs. 21.08 ± 14.61 nM ($P = 0.000$). Using ROC curves, we determined D-dimer cut-off level of 0.92 mg/L, P-selectin value of 17.8% and microparticles level of 16.5 nM that can accurately differentiate normals from duplex positive patients for DVT. In Group III, 14 patients developed thrombosis in a period of time ranging from 3 to 7 days which necessitates the establishment of new cut-off levels for each of the three studied markers to differentiate duplex negative patients for DVT without thrombosis from those of the same group who developed thrombosis being 2.81 mg/L for D-dimer (57% sensitivity and 94% specificity), 30.2% for P-selectin (57% sensitivity and 94% specificity) and 26 nM for microparticles (43% sensitivity and 100% specificity).

Conclusion: D-dimer, P-selectin and microparticles can be used to diagnose DVT. In addition, newly estimated cut-off values for the three studied biomarkers can confirm the presence of DVT in patients

and thus they can start anticoagulation therapy even before duplex ultrasound turn positive or when it is unavailable.
Keywords: D-dimer, P-selectin, microparticles, DVT.

PO 070

Normalized activated protein C sensitivity ratio and protein S-specific activity are useful predictive markers for venous thromboembolism

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Background/Aims: Predicting venous thromboembolism (VTE) is very difficult and effective markers have yet to be established. This study aimed to determine whether the normalized activated protein C sensitivity ratio (APC-sr) and protein S (PS) activity/antigen ratio (PS-specific activity) are useful predictive markers for VTE.
Patients and Methods: APC-sr and PS-specific activity were examined in scheduled surgical patients and in patients with VTE onset before anticoagulation in Hamamatsu Medical Center between January 2010 and March 2012. Citrated plasma was obtained perioperatively from surgical patients. APC-sr was determined by an endogenous thrombin potential (ETP)-based assay with computer-assisted calibrated automated thrombography. The total PS activity assay is comprised of three solutions, Reagent 1 (APC), Reagent 2 (Factor Va) and Reagent 3 (Factor Xa, prothrombin and S-2238), and is readily applicable to automated analyzers. To measure total PS activity, the PS-C4bBP complex in plasma was first dissociated by diluting samples and adding liposomes with high affinity for PS. PS antigen was assayed by latex agglutination by an anti-PS monoclonal antibody. Then, PS-specific activity was calculated. Pulmonary embolism (PE) and deep venous thrombosis (DVT) were diagnosed and then confirmed by CT scanning or Doppler blood flow scanning. Approval for this study was granted by the Hamamatsu Medical Center Review Board in 2009. Informed consent was obtained from all participants prior to blood collection.
Results: Twenty orthopedic patients, 30 patients with abdominal malignancies, six pregnant women who had cesarean deliveries, and 22 symptomatic VTE patients (16 PE, 6 DVT alone) were enrolled in this study. Among 56 surgical patients, one with a gynecological malignancy developed DVT postoperatively. The mean (± SD) preoperative APC-sr was 1.27 (± 0.69) in orthopedic patients, 1.27 (± 0.68) in abdominal malignancy patients, and 1.82 (± 0.45) in pregnant women. These values were somewhat high, and increased through the 3rd to 4th days postoperatively. APC-sr in patients with VTE at the onset before anticoagulation was 2.92 (± 1.47) ($P < 0.01$, Games-Howell test). These data indicate a reduction of sensitivity to APC in these patients. PS-specific activity was less than 0.7 (−3 SD) in 7 of 19 VTE patients (36.8%) and in 6 of 16 PE patients (37.5%). Total PS activity was less than 60% in 5 of 19 VTE patients (26.3%) and in 4 of 16 PE patients (25%). Moreover, APC-sr and PS-specific activity were 2.76 and 0.61, respectively, in a patient with postoperative DVT. There was an inverse correlation between APC-sr and total PS activity. Cut-off values calculated employing a receiver operating characteristic curve were 2.0 (sensitivity 71%, specificity 88%) for APC-sr, and 0.72 (sensitivity 37%, specificity 94%) for PS-specific activity.
Conclusion: These data suggest APC-sr (> 2.0) and PS-specific activity (< 0.72) to potentially be useful predictive markers in patients at high risk for VTE.

PO 071

DVT to VTE: a nursing evolution or revolution?

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As part of the Emergency Department at the Belfast City Hospital, an outpatient deep vein thrombosis (DVT) service, a nurse led clinic was setup to triage all patients presenting with suspected DVT. Eighty-five percent of all patients reviewed did not have a DVT whilst all suspected pulmonary embolus were admitted directly.
The Belfast City Hospital ED has faced a period of temporary closure. This posed a challenge to the nurse led service. An options appraisal review took place and it concluded to only manage those patients with confirmed DVT, pulmonary embolus with a PESI score of 0 and superfical thrombphlebitis.
We have collated our data and present the findings of the new challenging patient cohort.
At a total of 120 patients have been managed with an average of 8.5 per week. The age range was 18–99 years with 45% male. Of 90 patients diagnosed with a DVT two-thirds were in proximal lower limb. Risk factors for VTE included 31 with a previous personal history of VTE, 17 with a positive family history, 8 of the females were on OCP/HRT. One in every four patients was diagnosed with a hospital acquired thrombosis (HAT), 3 post partun, 13 general medical/surgical, and 17 related to trauma/orthopaedics. Of these patients just 14 had received chemical thromboprophylixis, 3 were risk assessed but not prescribed.
Whilst the change in service has been challenging it has also been rewarding, as we have been motivated into developing new care pathways for patients and information leaflets relevant to the many questions which we are most frequently asked.

PO 072

Thromboses of unusual localization and pregnancy

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Background: Thromboses of atypical localizations are life-threatening complications in pregnant woman.
Materials and Methods: From 2001 to 2012 we observed 27 cases of thromboses of unusual localization at pregnant women: splanchnic system thromboses (Badd-Kiari syndrome, mesenterial, splanchnic veins and portal vein), thromboses of renal veins and retinal veins, thromboses of the upper extremities, a catheter – associated thromboses which haven't been associated with oncology, thromboses of brain venous sinus. Among them 18 cases were associated with pregnancy, 6 – with oral contraceptives, 3 – with IVF.
Analyses for the genetic and acquired defects of a hemostasis were conducted in all patients. At 24 of 27 pts thrombophilia was detected: the phrothrombin mutation (geterozygous)- at 6 pts, a homozygous mutation of factor V Leiden – at 1 pts, heterozygous – at 6, hyperhomocysteinemia – at 50%, AFA circulation- at 67%, at one patient we detected a combination of prothrombin mutation and F V Leiden. In almost all cases the combination of the genetic and acquired forms of thrombophilia was detected.
Twelve from these patients in the subsequent were observed for pregnancy-course. Anticoagulant treatment was carried out since the pregnancy beginning, during all pregnancy and within 6 weeks of the postoperative or postnatal period with enoksaparin sodium (from 0.6 to 2 ml daily). There were no repetitions of thromboses of common and unusual localization, as far as there were no hemorrhagic complications.

PO 073

A comparative study of two automated assays for D-Dimer used in patients admitted in the Emergency Department in a university hospital

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Background: D-dimers are the products of degradation of fibrin by plasmin. Their detection is evidence of the activation of fibrinolysis. When a patient is admitted for suspected venous thromboembolism (VTE), a clinical score determine the probability, low, medium, high for this diagnosis. In case of low or moderate probability, D-dimer test is useful with a sensitive test: if the D-dimer result is below the positive threshold, this result can rule out the diagnosis of VTE.

Aims: The aim of our study was to compare the results between two automated quantitative assays used in our laboratory.

Methods:

- 548 plasma samples from 541 adult patients admitted to the emergency department were tested from July to December 2012. Seven patients had two samples at 6 months, or during the same hospital stay or during two successive hospital stays.
- Exclusion criteria included pregnancy and age over 60 years.
- 249 samples (45%) were collected from men and 299 (55%) from women.
- The median age was 38 years in both sexes (range 16–60 years).
- The 548 samples were tested by simple determination within 2 h after the admission of patient to the emergency department. Two methods were used: a quantitative automated turbidimetric immunoassay (STA-Liatest D-DI on STA-R analyzer, Diagnostica Stago) and a quantitative automated enzyme immunoassay automated (VIDAS D-Dimer Exclusion, BioMérieux SA).
- The detection limit was different between the two assays: 0.220 microg/ml (FEU) for STA-Liatest D-DI and 0.045 microg/ml (FEU) for the Vidas method.
- The clinical cut-off used for subjects aged less than 60 years was 0.500 microg/ml (FEU).

Results:

- 493 patients (90.0%) had normal results and 12 (2.2%) had abnormal results with the two tests.
- 40 patients (7.3%) had a normal result with the STA-D-DI Liatest assay and an abnormal result with Vidas assay. The median value of the differences was 0.190 microg/ml (range 0.060–0.390). Nineteen samples were slightly above the upper cut off level used in the Vidas D-dimer assay. The collection of clinical data (Wells score, diagnosis, radiology) is in progress.
- three patients (0.5%) had an abnormal result with the STA-D-DI Liatest assay and a normal result with the Vidas D-Dimer assay. There were small variations in either side of the clinical cut-off. Neither the rheumatoid factor, nor monoclonal gammapathy were looked for.

Summary/Conclusions: These two methods of determination of D-Dimer well give concordant results for 92.2% of plasmas tested.

But the rate of discordant results (7.3%) seems high (although two tests have different sensitivity) and a study of the clinical features of patients is underway to understand.

PO 074

Inferior vena cava agenesis as cause of deep venous thrombosis in a young patient: case report and literature review

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Background: Inferior vena cava agenesis (IVCA) is a rare anomaly with an estimated prevalence at 0.5% in the general population. Deep venous thrombosis (DVT) is the main clinical feature associated with this condition.

Aims: We report clinical date of DVT associated IVCA in a young patient, together with a literature review surrounding this association.

Methods: We performed a systematic literature review of published reports based on Pub Med and Medline up to December 2012.

Results: The 13 years old male teenager was admitted to our teaching hospital complaining of 2 day of severe lower abdominal pain. The abdominal ultrasound showed chronic inferior vena cava thrombosis with extensive collaterals and a partial thrombosis in mesenteric vein and an image suggesting ileoileal intussusception. He underwent laparotomy with successfully correction of the problem. Due the chronic nature of thrombosis, none anticoagulant was initiated. The patient recovered and was discharged. Four days after, he returned to hospital with severe back and groin pain. The CT scan revealed an infrahepatic AVCI with venous drainage through dilated lumbar venous collaterals into the azygous system in addition an extensive bilateral iliofemoral-popliteal venous thrombosis. The thrombophilia screening was negative. The patient was put on lifelong oral anticoagulation and remains well at 2 years follow-up. The literature has reported 86 cases of IVCA associated DVT, with a median age of 30 years (8.5–67), a male sex predominance (64 patients/74%) and in 40% of the cases the site of DVT was the bilateral proximal veins of the legs. An interesting finding is that, due the aberrant collateral venous drainage in rare cases the thrombosis mimicked ureteric colic or lumbar disc herniation. The majority of the cases (88%) the treatment consisted solely of long term anticoagulation.

Conclusion: This is the first case of IVCA associated with bowel obstruction. IVCA should be regarded in young patients (predominantly males) with spontaneous bilateral DVT.

PO 075

Role of D-dimer monitoring for the diagnosis of deep vein thrombosis in patients undergoing neurosurgery for brain tumor

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Background: Patients undergoing neurosurgery for brain tumor remain at risk for deep vein thrombosis (DVT) despite the use of multimodality prophylaxis. Clinical suspicion may be challenging in this setting due to consciousness alteration, sensitivity and/or motor deficits in the post-operative period. Lower limb venous ultrasound (US) monitoring would be useful, but it is often not feasible in clinical practice. D-dimer measurement may help early detection of DVT, but its transient increase after surgery may lead to misinterpretation.

Aims: To evaluate the behaviour of D-dimer levels before and after surgery in patients operated for brain tumor, with or without DVT confirmed by US.

Methods: Consecutive patients undergoing craniotomy for high grade glioma (GBM) or meningioma were tested for D-dimer (normal value < 225 μg/L) before (T0), 1 day (T1) and 7 days (T2) after neurosurgery. Each patient received complete compression US of lower limbs at T0 and T2 to verify the presence or absence of DVT. During the hospital stay, all patients received multimodality antithrombotic prophylaxis with elastic stockings, intermittent pneumatic compression and low-dose LMWH. Informed consent according to Helsinki Declaration was obtained by each patient.

Methods: Forty-two patients, 27 males and 15 females with a mean age of 61.0 ± 11.5 years, were enrolled; 25 patients had GBM and 17 had meningioma. Two patients had DVT before surgery and were excluded; six patients developed DVT within 7 days after surgery. Median (IQR) D-dimer levels in patients with and in those without

DVT were 144.5 (133–839) μg/L and 110 (61–167) μg/L at T0, 909 (537–1486) μg/L and 560.5 (308–1023) μg/L at T1, 1994.5 (1733–2063) μg/L and 110.5 (61–167) at T2, respectively. The differences were significant by ANOVA between patients (F 9.9, p 0.0032) and within patients at T2 (F 9.18, $P < 0.001$). Age and gender adjusted analysis did not change the results. In each patient the T2-T1 difference (delta T2-T1) in D-dimer values was calculated. If a delta T2-T1 cut-off level of 116 μg/L was used, D-dimer demonstrated a sensitivity of 100% (95% CI 0.46–1.00), a specificity of 87% (95% CI 0.69–0.95), and a negative predictive value of 75% (CI 0.57–0.87) for DVT.

Conclusions: In patients undergoing craniotomy for brain tumor, a close monitoring of D-dimer during the hospital stay might be useful to exclude a suspicion of DVT, avoiding to perform US, when a decrease in D-dimer levels is observed after the transient rise related to the surgical intervention.

PO 076

Prevalence of perioperative asymptomatic proximal deep vein thrombosis following gynecological cancer surgery in Thai patients

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Background: The long belief that acute deep vein thrombosis is rare in Asian patients is challenged by many recent studies. Incidence of thromboembolism among Asian patients had increased. However, thromboprophylaxis for perioperative cancer surgery in Asian countries is not routinely performed. Gynecologic malignancies were the most common cancer which associated with acute deep vein thrombosis among Thai patients.

Aims: In this study, we reported the prevalence of perioperative asymptomatic proximal deep vein thrombosis of 100 patients with gynecologic cancer.

Methods: Duplex ultrasonography of proximal vein of legs was performed in each patient before and 7–14 days after gynecologic cancer surgery. Demographic data were collected including: age, sex, height, weight, body mass index (BMI), previous diagnosis of treatment of DVT and PE, history of irradiation and chemotherapy, family history of VTE, recent immobilization of lower extremities (more than 3 days), recent surgery within 3 month, recent trauma within 3 month, recent admission of severe medical illness, other associated cancers, varicose vein, thrombophilia, use of contraceptive pills, hormonal therapy, number of pregnancy, cardiac diseases and cerebrovascular disease. Hospital charts were reviewed for the following information: site of cancer, type of cancer, staging of cancer, type of operation, patient position, type of anesthesia, duration of anesthesia, duration of surgery, duration of postoperative immobilization, blood loss and blood replacement, complication, duration of ICU stay and duration of hospital stay.

Results: The results showed that preoperative prevalence of asymptomatic proximal DVT of legs was 5% and postoperative incidence of asymptomatic proximal DVT of legs was 2.11%. We found only 1 case of symptomatic pulmonary embolism associated with asymptomatic proximal DVT of lower extremities.

All DVT cases had adenocarcinoma of ovary or uterus, but not cervical cancer. Risk factors of venous thromboembolism were comparable between DVT and non DVT group except number of children which was higher in DVT group.

Conclusion: So the patients with adenocarcinoma of ovary and uterus especially in patients who have high number of pregnancy seem to be the greatest risk of perioperative VTE. Our result might make the physicians concern the high prevalence of VTE in perioperative period of gynecologic cancer of Asian patients.

PO 077

Evaluating the effectiveness of warfarin therapy in patients with deep vein thrombosis of the lower limbs

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Aim: Patients with DVT to anticoagulation vitamin K antogonist (VKA) observed the inefficiency applied therapy. The aim of our study was to investigate the risk of ineffective therapy.

Methods: We tested 60 patients with DVT (aged 56 ± 15 year-old, M/F 34/26). Patients were treated with unfractioned heparin (UFH) in dosage of 450 U/kg 3 times per day starting from day 1 for 5 days and with UFH combined with Warfarin starting from day 6 till day 10. From day 11 patients were treated with Warfarin only. Tests for D-dimer, international normalized ratio (INR) and Trombodynamics were performed at day 0, 10 and 15. Data are presented as median (25%, 75%).

Trombodynamics (HemaCore LLC, Russia) – a new global coagulation test based on video-microscopy of a fibrin clot growing from an imitated damaged vessel wall (surfaces with immobilized tissue factor) in space, the growth rate of the fibrin clot V was measured (norm 20–30 μm/min).

Results: INR from day 10 till day 15 increased from 1.4 (1.1; 2.1) to 2.6 (1.7; 3.6). As a result of anticoagulant therapy on 10 day, V decreased significantly from 23.0 (21.7; 27.5) to 9.1 (6.1; 14.1) μm/min ($P < 0.05$). By day 15, patients were divided into two groups. In the Group 1 ($n = 48$), the growth rate of the fibrin clot V where remained low 12.2 (8.2; 15.6), in Group 2 ($n = 12$) the level of V increased to 22, 3 (20.6;23.5) μm/min.

D-dimer level on day 10 of anticoagulant therapy decreased from 792 (422; 1161) to 276 (167; 370). On 15 day in the Group 1 D-dimer level was 192 (116; 244) μg/L. In the Group 2, there is a significant increase in D-dimer level to 530 (164; 826) μg/L, compared with the Group 1 ($P < 0.05$). Four of the six patients in the Group 2, which had D-dimer level higher than 400 μg/L having INR within the therapeutic range 2–3.

Conclusion: The increase of the growth rate of the fibrin clot V to normal levels in the patients with DVT on VKA therapy may indicate a risk group with elevated D-dimer.

PO 078

How we understand DIC today in Russia

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Background: Today there are many discussions on the definition of acute and chronic DIC. For the practical physicians it makes many problems in their work.

Aims: How to recognize it and what to do. In Russia we discussed this problem many years and came to this decision.

Methods: Guideline of the Russion Association on Thrombosis, Haemostasis and Vascular Patholy. Constant Intravascular Microcoagulation(im) and DIC-Syndrom.

Constant presence of the markers of IM in plasma of healthy and sick people give us the basis to think, that intravascular blood clotting is going constantly, and there is need to underline its existence by the term 'Constant intravascular microcoagulation'- CIMC.

The intensity of CIMC may be different. The level of the markers of IC of blood, which is measured in plasma of the healthy people must be adopted as 'normal'. The increase in intensity of IC may be discovered in some transient disorders and following physical stress. After those situations have passed, the intensity of intravascular clotting can

return back to the 'normal' level. Certain chronic diseases exhibit a constant increase of the intensity of IC, which does not exhibit any special traits except of the clinical picture of the main disease. Previously these states were named 'Chronic intravascular microcoagulation'. It is possible, that special regulation of this stage of the CIMC can improve the prognosis of the disease.
Levels of the CIMC intensity. 1-st grade NORMAL. 2-nd grade-Transient increase CIMC 3-d grade: Sustaned increased CIMC. 4th grade DIC-SYNDROM.
DIC-syndrome is only the stage of CIMC, where the increase of its intensity is an independent cause of the damage of body-organs and body-tissues, such as bleeding, multiple organ damage, hypotony, micro- and macrothrombosis and its different combinations.
Diagnostic criteria for CIMC, grade 4:-bleeding, or-low blood pressure, or- acute heart, lung, kidney, liver insufficiency without acute local ischemia Together with: fibrinogen level lower than normal +platelet level is lower than normal. Fibrinogen level and platelets numbers may be formally at the normal level, if they were increased earlier. Progressive reduction of fibrinogen level and platelet numbers must be the basis for DIC diagnosis and for the beginning of the treatment.
Results: Successful treatment of the main disease and good constant control of the coagulation tests-(fibrinogen and platelets- obligatory, D-dimer, and fibrin-monomer(TPP)-desirable) are the best way of DIC-prevention.
Conclusion: Inspite of the existing reports on the successful treatment of DIC-syndrome there is very high mortality of those patients with DIC. This fact is the basis for the future special research, which may answer the question on the importance of prevention DIC-syndrome by anticoagulant therapy. Further information is needed to determine the influence of difficult ways of increasing coagulation- fibrin formation, platelets and fibrinolitic activity is the mechanismus of the peculiar 'DIC-syndrom'.

PO 079

Normal prothrombinase activity, systemic thrombin activation, and lower antithrombin levels in patients with DIC at an early phase of trauma: comparison with Acute Coagulopathy of Trauma-Shock (ACOTS)

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Background: We tested the hypotheses that increase in systemic thrombin activation occurs both in disseminated intravascular coagulation (DIC) with the fibrinolytic phenotype and acute coagulopathy of trauma shock (ACOTS), and that the patients diagnosed as having ACOTS overlap or are identical to those with DIC.
Methods: With the approval of the Institutional Review Board and after written informed consent was obtained from either the patients or their next of kin, 57 trauma patients, including 30 patients with DIC and 27 patients without DIC were prospectively studied. Patients with ACOTS, defined as a prothrombin time ratio > 1.2, were also investigated. Twelve healthy volunteers served as control subjects. The levels of soluble fibrin, antithrombin, prothrombinase activity, soluble thrombomodulin, and markers of fibrin(ogen)olysis were measured on days 1 and 3 following trauma. Systemic inflammatory response syndrome (SIRS) and the Sequential Organ Failure Assessment (SOFA) were scored to evaluate the extent of inflammation and organ dysfunction.
Results: DIC patients showed more systemic inflammation and higher SOFA scores, and were transfused with more blood products than the non-DIC patients. On day 1, normal prothrombinase activity, increased soluble fibrin, lower levels of antithrombin, and increased soluble thrombomodulin were observed in DIC patients in comparison with control subjects and non-DIC patients. These changes were more prominent in DIC patients who met the International Society on Thrombosis and Haemostasis overt DIC criteria. The multiple regression analysis showed that antithrombin is an independent predictor of high soluble fibrin in DIC patients. Higher levels of fibrin and fibrinogen degradation products, D-dimer and the fibrin and fibrinogen degradation products/D-dimer ratio indicated increased fibrin(ogen)olysis in DIC patients. Almost all ACOTS patients overlapped with the DIC patients. Therefore, the changes in the measured variables in ACOTS patients coincided with those in DIC patients.
Conclusions: Normal prothrombinase activity and insufficient coagulation control give rise to systemic increase in thrombin generation and its activity in patients with DIC with the fibrinolytic phenotype at an early phase of trauma. The same is true in patients with ACOTS, and shutoff of thrombin generation was not observed.

PO 080

New diagnostic strategy of sepsis induced disseminated intravascular coagulation (SEDIC); a validation study

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Background: The majority ill patients a systemic inflammatory response have coagulation abnormalities. In the pathogenesis of sepsis, inflammation and coagulation play a pivotal role. Evidence of an extensive cross-talk between these two systems is increased recently. However, there are different diagnostic criteria in sepsis and disseminated intravascular coagulation (DIC). So we defined the new diagnostic criteria of sepsis induced DIC (SEDIC) as follow; Presepsin (PSEP) level > 900 pg/mL and Protein C (PC) activity < 45%.
Aims: In this study, we attempted to validate this scoring system.
Methods: A single center, prospective, observational study was carried out. Patients who had one or more systemic inflammatory response syndrome (SIRS) criteria were included in this study. The blood samples were collected at the time of admission. The patients were classified into the three groups. SIRS and sepsis were diagnosed according to the American College of Chest Physicians/Society of Critical Care Medicine guidelines. The scoring system for Japanese Association for Acute Medicine (JAAM) DIC was used for diagnosis of DIC. We examined the severity of illness of the patients was evaluated according the APACHE II score and organ failure was assessed by the SOFA score. All patients were followed up for 28 days after enrollment in the study, and 28-day all-cause mortality was assessed.
Results: A hundred fifteen patients were enrolled for this prospective study from July 2011 to December 2012. Twenty nine patients (19.2%;29/151) were SEDIC, sixty-one were pre-SEDIC (40.4%; 61/151) and sixty-one were non-SEDIC (40.4%; 61/151). SEDIC scoring system significantly reflected the positive rate of sepsis ($P < 0.0001$) and JAAM DIC ($P < 0.0001$), SOFA score ($P = 0.0015$), APACHE II score ($P = 0.0316$) and JAAM DIC score ($P < 0.0001$). The 28-day mortality rate was significantly worsened, depending on the cutoff points in the respective criteria (SEDIC 31.0%; pre-CEDIC 19.7%; non-SEDIC 1.64%, $P < 0.0001$).
Conclusions: From these results, we strongly believed that the SEDIC scoring system has an acceptable property for the diagnosis of sepsis induced DIC. Moreover, PSEP and PC were able to be measured with very simply and quickly. So we strongly suggest that this scoring system can be useful for early treatment in a critical care setting.

PO 081

Serum des-R prothrombin activation peptide fragment 2: a novel prognostic marker for disseminated intravascular coagulation

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Background: Disseminated intravascular coagulation (DIC) is diagnosed based on the combination of predisposing underlying conditions and laboratory tests for plasma coagulation markers. Because the collection of blood plasma samples is a fastidious procedure, the serum sample method may be preferred for measurement of coagulation markers when feasible.
Materials and Methods: The novel serum marker des-R prothrombin activation peptide fragment 2 (des-R F2) was measured using a sandwich enzyme-linked immunosorbent assay in 181 patients suspected of having DIC. Thrombin generation potential was estimated with a calibrated automated thrombogram.
Results: Serum des-R F2 was generated with an *in vitro* clotting process within a serum separation tube after blood collection. Low levels of prothrombin and thrombin generation potential resulted in low serum des-R F2 levels. Serum des-R F2 was significantly decreased in overt DIC. Levels of des-R F2 correlated with DIC severity and other coagulation markers. Of note, the decrease in serum des-R F2 levels was a significant marker for predicting mortality.
Conclusions: The serum marker, des-R F2, can be used for the investigation of DIC severity and prognosis. It should be considered a useful marker, especially when only serum samples are available.

PO 082

Kasabach Merritt Syndrome (KMS) in infant with hepatoblastoma

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Hepatoblastoma, the most common liver tumor in children, rarely present in association with consumptive coagulopathy, thrombocytopenia and microangiopathic hemolytic anemia. A 39 week gestation G2P1 infant was uneventful pregnancy born by vaginal delivery weighing 2750 g. His APGAR score at 1, 5 min were 9, 10 respectively. His physical examination showed huge liver mass extending to iliac crest without bruit and dyspnea. At day 2 of his age, he developed respiratory distress with cardiopulmonary failure requiring endotracheal intubation, mechanical ventilation and inotropic drugs. Echocardiogram showed patent ductus arteriosus 3–5 mm. with left to right shunt and good left ventricular contractility. He also developed early neonatal jaundice from hemolytic anemia requiring double phototherapy. Exchange transfusion was performed three times according to microbilirubin level nevertheless extensive phototherapy. Blood morphology was compatible with disseminated intravascular coagulopathy. Computerized tomography of abdomen showed marked hepatomegaly with huge heterogenous mass occupying in right liver size $10.1 \times 8.1 \times 9.5$ cm, neither calcification nor capsular refraction. Alpha fetoprotein was > 400,000 IU/mL. His abdominal circumference was increased from 34 to 38 cm within 9 days. Differential diagnosis between infantile hemangioendothelioma and hepatoblastoma was made. Due to KMS, treatment including dexamethasone, propranolol and alpha interferon for infantile hemangioendothelioma was commenced without success. Hepatic embolization was not done due to limitation of small catheter. Pediatric surgery team for tissue diagnosis and hepatic artery ligation was consulted but not done due to poor prognosis. He was expired from respiratory failure. An autopsy of liver was compatible with hepatoblastoma. KMS could rarely occur in association with hepatoblastoma.

PO 083

Coagulation abnormalities in adult hemophagocytic lymphohistiocytosis

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Background: Hemophagocytic lymphohistiocytosis (HLH) in adults is a rare and serious multi-system illness with a grave prognosis. While associated with several malignant, inflammatory and infectious processes, it often has a genetic basis involving genes controlling the immune response. (1) Delays in diagnosis and the non-specific nature of clinical findings as well as laboratory tests often contribute to the high mortality associated with this disease process. Detailed diagnostic criteria have been proposed to make the diagnosis of HLH more uniform and predictable. (2) These include several abnormalities associated with the coagulation cascade including thrombocytopenia and hypofibrinogenemia. However, there are few descriptions of the potential origin or clinical course of patients who present with significant abnormalities in these variables. Several publications suggest consumptive disseminated intravascular coagulation (DIC) or severe liver dysfunction as being etiologically involved in causing severe hypofibrinogenemia. (3).
Aim: To further elucidate the factors associated with thrombocytopenia and hypofibrinogenemia in adult onset HLH
Methods: We performed a retrospective chart review on 7 consecutive adult patients with a diagnosis of HLH seen over an 18-month period at the Indiana university hospital system. The diagnosis of HLH was confirmed in all patients using the latest diagnostic criteria. (2) We evaluated the data with regression and correlation analysis utilizing non-parametric methods. SPSS software (US) was used for data-analysis.
Results: The age range of patients was 32–76 years, with three men and four women. A malignancy was diagnosed on presentation in 3 of 7 patients, a documented infection was present in 3 of 7 and 3 of 7 cases were idiopathic. Laboratory tests included platelet count, fibrinogen, ferritin, prothrombin-time, d-dimer, ALT and serum bilirubin. DIC was graded according to ISTH 2007 criteria. Fibrinogen was found to be profoundly diminished (< 50 mg/dL) in 2/7 patients, one with malignancy associated HLH and the other with an idiopathic illness. An elevated prothrombin time or depressed platelet count were not associated with low fibrinogen levels. Liver malfunction reflected by increasing bilirubin and ALT values were also not associated with low fibrinogen. Presence or absence of overt DIC was not associated with low fibrinogen (Spearman rho associated $P = 0.47$). Appropriate non-parametric analysis was done in all cases.
Conclusion: Hypofibrinogenemia and thrombocytopenia have been used as diagnostic markers for hemophagocytic lymphohistiocytosis. In our preliminary study of seven patients, two of whom had severe hypofibrinogenemia, we found no correlation between hyprofibrinogenemia and thrombocytopenia, elevated prothrombin time, liver function abnormalities, age or diagnosis of DIC. While several patients had markedly diminished platelet counts, these did not correlate with low fibrinogen levels. Our data suggest that low fibrinogen values in this disease process may need an alternate explanation other than DIC or liver malfunction. We plan to extend our study to more patients with HLH to further elucidate the origins of low fibrinogen levels found in this disease.
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PO 084

Catastrophic evolution of disseminated intravascular coagulation as initial manifestation of recurrent prostate cancer

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Background: Disseminated intravascular coagulation (DIC) has been extensively documented in prostatic cancer, with an incidence of 10%.in advanced stages. In the majority of the cases the coagulopathy is subclinical and overt bleeding is rare. Moreover, DIC as an initial manifestation of disease is unusual.

Aims and Methods: We describe clinical and laboratorial characteristics of a patient in whom DIC was the presenting sign of recurrence in prostate cancer.

Results: A 76-years- old man was admitted to emergency department of our teaching hospital with a 7 days history of diffuse spontaneous ecchymoses and an extensive hematoma in left upper limb. His past medical history included successfully treatment for prostate cancer 2 years ago. He referred that in the last follow-up visit 3 months ago the disease was in remission. Laboratory evaluation revealed: platelets count $89 \times 10^3/\mu L$ (normal: $150–400 \times 10^3/\mu L$); the prothrombin time (PT): 30 s, INR: 1.8 (normal: < 1.25), the activated partial thromboplastin time (APTT): 22.8 s, R: 1.2 (normal: < 1.25), fibrinogen level: 66.9 mg% (normal:200–400 mg%), D- dimer: 4.4 mg/L (normal: < 0.55 mg/L). A test for serum prostate-specific antigens was 9932 ng/ml (normal: < 4 ng/ml). Other parameters of blood count, as well as renal and liver functions tests were within normal values. Bone marrow biopsy showed prostatic adenocarcinoma, and the patient was referred to oncology. After 10 days of onset of symptoms he returned to the hospital with loss of consciousness. The brain CT scan demonstrated an extensive intraparenchymal hemorrhage and the patient died. The treatment for cancer had not yet started.

Conclusions: DIC without evident cause should always prompt a search for a neoplastic disease, especially in eldery. The delay in diagnosis and treatment can be fatal.

PO 085

Evaluation of two automated soluble fibrin assays for use in the routine hospital laboratory

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Background: Fibrin monomer (FM) (which alone or in complex with fibrinogen circulates as soluble fibrin [SF]) is an early product formed after the initial action of thrombin on fibrinogen before the formation of cross-linked fibrin. It is a much earlier produced fragment than D-dimer which only forms after plasmin cleavage of cross linked fibrin. FM could be considered as an earlier thrombotic marker potentially expressing better specificity and sensitivity than D-dimer, especially in disseminated intravascular coagulation (DIC). The manual semi quantitative method SF method (FS Test) is both time consuming and the evaluation of the result is often complicated and operator dependent.

Aim: To evaluate two automated soluble FM assays and compare them with FS Test.

Methods: Fifty-one consecutive samples referred for SF testing with positive D-dimer (Roche) and 30 samples from emergency unit (patients expected to have coagulation activation) were tested with both the STA-Liatest FM (Stago) and the LPIA-Iatro SF (Mitsubishi) and compared with the FS Test method.

Results: All samples testing positive with the semi quantitative SF method were also positive with both of the automated methods. Slightly more than half of the samples positive for D-dimer and negative with the old semi quantitative method were also positive with both automated methods. Eight samples positive for D-dimer were negative with all three SF/FM methods. Of the 30 samples obtained from the emergency unit only one was positive with all four methods. Fifty-five samples negative in the manual SF test were positive by D-dimer. Of these, 30 were also positive by the STA-Liatest FM test and 33 were positive with the LPIA-Iatro SF test. Kappa coefficient between F.S. Test and STA-Liatest FM was 0.41 (moderate agreement) and between STA-Liatest FM and LPIA-Iatro SF was 0.66 (good agreement) (Fisher's exact test $P < 0.0001$). Intra- and inter-assay CV's for both low and high controls were < 3% for the STA-Liatest FM and around 10% for the LPIA-Iatro SF.

Conclusion: Both STA-Liatest FM and LPIA-Iatro SF showed a good correlation with the old SF method. All samples positive with the old method were also positive with new methods, thus excluding the risk of obtaining increased false negative results. Probably, as a consequence of the increased assay sensitivity, both of the automated quantitative tests gave more positive results than the semi quantitative SF test. The STA-Liatest FM gave better precision and the assay was easier to perform than the LPIA-Iatro SF.

PO 086

The expression and secretion of ADAMTS13 in human microvascular endothelial cells

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Background: Like other vascular endothelial cells,our study has confirmed that ADAMTS13 also express in human microvascular endothelial cells. However, could human microvascular endothelial cells secret ADAMTS13 protein? Does the secretion of ADAMTS13 from human microvascular endothelial cells be regulated? These questions are still unknown.

Aims: our study is aim to investigate that whether ADAMTS13 is expressed and secreted by human microvascular endothelial cells, and whether the secretion is to be regulated.

Methods: We used different concentrations of TNF-alpha (from 0 to 10 ng/ml) to stimulate human microvascular endothelial cells, Collected and concentrated cell supernatants, and directly detected and observed the expression and secretion of ADAMTS13 in human microvascular endothelial cells, by using real-time quantitative PCR, Western blot technology and immunofluorescence microscopy methods. We also detected the proteolytic activity of ADAMTS13 by using GST-VWF73-His peptide as a specific substrate.

Results: We found that ADAMTS13 expression did exist in microvascular endothelial cells and its supernatant, and by a dose-dependent manner, 10 ng/ml TNF-alpha inhibited ADAMTS13mRNA expression which could reduce 59% comparison with the control group, The results were confirmed from the levels of ADAMTS13 protein. And 10 ng/ml TNF-alpha inhibited ADAMTS13 activity which could drop to 38% comparison with the control group in human microvascular endothelial cells' supernatant.

Conclusions: The study suggests that human microvascular endothelial cells could also express and secret ADAMTS13, which may be a very relevant compartment for plasma ADAMTS13 level. And the expression and secretion of ADAMTS13 in human microvascular endothelial cells also be regulated by TNF-alpha.

PO 087

Twin pregnancy with a very low ADAMTS13 activity and existence of schistocytes together with thrombocytopenia

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Background: Twenty-nine years old woman with 18 weeks twin pregnancy was admitted to hospital with complaint about ecchymosis. Hemoglobin and platelet values were 11.4 g/dL, 4.000/μL, respectively. There were 3–4 schistocytes in per microscopic field at 100× magnification in her peripheral blood smear. After this observation we checked hemolysis laboratory. Direct and indirect coombs were negative. Other tests were such as: LDH 176 U/L, total/direct bilirubin 0.5/0.13 mg/dL, haptoglobulin 52 mg/dL (30–200). In urine analysis, urobilinogen and erythrocyte hemoglobin were 0.2 mg/dL and 200 erythrocyte/μL, respectively. There were 6–8 erythrocytes in urine sample. DIC parameters were such as: PT: 9.59 s, PTT 23.9 s, Antithrombin III 104%, D-Dimer 1.3 mg/L (0.1–4.4), fibrinogen 2.7 g/L, Thrombin time 19.35 s. Creatinine, AST and ALT were normal.
Her blood pressure was always within normal limits.
We sent sample for ADAMTS13 antigen and activity because of schistocytes were observed in serial peripheral smears.
Corrected reticulocyte was normal (1.24%). The reason for normal values of reticulocyte, LDH, bilirubine, we diagnosed patient as ITP not TTP despite schistocytes. Steroid of 1 mg/kg and 2 g/kg of IVIG were started. Platelet was increased to 54,000 at the end of 1 week however decreased to 20,000 in 13th days of treatment. We repeated to IVIG and continued to steroid. Platelet increased to 164,000 and dropped to 117,000 in 3th days and 10th days of second IVIG treatment, respectively. At that day; fifteen days later after sample sending; results for ADAMTS13 came. ADAMTS13 antigen was 1.08 (0.5–1.6 μ/mL); ADAMTS13 activity was < 0.2 (40–130%), ADAMTS13 inhibitor was 13.6 (< 15 U/mL). Patient's LDH and bilirubin were still normal, so that very low ADAMTS13 activity (< 0.2) level was inconsistent with patient's other laboratories except schistocytes. She treated 1 month steroid and then dose decreased. After 3 months, patient's hemoglobin and platelet are still normal. Now, she is at 31 weeks of her twin pregnancy. Her platelet 262,000 and health of babies are good.
Conclusion: We wanted to share this twin pregnancy patient because of that: despite the existence of schistocytes and very low ADAMTS13 activity there was not obvious laboratory for hemolysis.

PO 088

Alterations of plasma VWF and ADAMTS13 activity in the patients receiving bone marrow transplantation

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Background: Thrombotic complications are common and potentially fatal in patients undergoing bone marrow transplantation (BMT). Plasma ADAMTS13, a von willebrand factor (VWF)-cleaving protease, may play a role in the pathogenesis of transplantation related thrombosis by cleaving the prothrombotic ultralarge VWF into less active VWF multimers.
Aims: To investigate the alterations of plasma ADAMTS13 and VWF in BMT recipients during the course of transplantation, and to evaluate their clinical significance in the transplantation-related thrombotic complications.
Methods: A total of 113 hematologic patients receiving allogeneic-BMT were enrolled in this study, the sodium citrate anticoagulated plasma was collected in −2, 0, 2 and 4 weeks following transplantation. Fluorescence resonance energy transfer substrate VWF73 (FRETS-VWF73) assay was established to detect the plasma ADAMTS13 activity while VWF antigen was measured by ELISA. Of all the patients recruited for his study, eight patients were diagnosed to have the thrombotic disorders and 49 patients were classified to have acute graft-vs.-host disease (aGVHD). The alterations of ADAMTS13 activity and VWF level in the plasma of patients were analyzed during transplantation, and the correlation between ADAMTS13/VWF and transplantation-related thrombosis was evaluated using the SAS program (version 9.3).
Results: The average plasma ADAMTS13 activity in 113 cases following transplantation at each period were less than the healthy controls ($P < 0.01$), while the VWF antigen level in each period were higher than the controls ($P < 0.05$). Among all the patients after pretreatment, 69 showed decreased plasma ADAMTS13 activities (69/113, 593%), including nine patients with more than 60% (9/113, 8.0%) decrease, while the average plasma VWF antigen level of this 69 patients was significantly increased in patients after pretreatment ($P < 0.05$). Considering thrombotic complications, the data showed that eight patients with thrombotic complications had decreased plasma ADAMTS13 activity ($P < 0.01$) and increased VWF antigen level after pretreatment ($P < 0.01$) as compared with the non-thrombotic patients; three out of 8 (37.5%) showed more than 60% decrease in plasma ADAMTS13 activity. The level of ADAMTS13 activity dropped in the 49 patients with aGVHD as compared with healthy controls ($P < 0.01$), but there was no significant difference between patients with and without aGVHD. Twenty-five patients showed decreased plasma ADAMTS13 activities only at the onset of aGVHD occurrence ($P < 0.01$), in which two of them decreased more than 60% (6%). Logistic regression analysis showed that the ADAMTS13 activity declined by more than 60% was the risk of thrombosis ($P < 0.01$), while decreased ADAMTS13 activity after pretreatment was not the risk factor for aGVHD.
Conclusions: We observed decreased plasma ADAMTS13 activity and increased plasma level of VWF antigen in patients following transplantation after pretreatment, especially in the patients with thrombotic complications. Regression analysis showed a decrease more than 60% in plasma ADAMTS13 activity is the risk factor of thrombotic complications, which was not correlated to the development of aGVHD although ADAMTS13 activity reduced in the patients when aGVHD occurred. Therefore, the plasma ADAMTS13 activity could be an important parameter for the development of vascular disorder, which has a potential role for the early diagnosis and therapy of thrombotic complications.

PO 089

Von Willebrand Factor, ADAMTS13 activity, inflammatory marker and their relationships with risk factors of coronary artery disease

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Background: Coronary artery disease (CAD) is associated with endothelial dysfunction and inflam−mation which in turn accelerated thrombosis in individuals with CAD risk.
Aims: This study aimed to assess the relationships among von Willebrand factor (vWF) antigen, a disintegrin-like and metallo−protease with thrombospondin type 1 repeats 13 (ADAMTS13) activity and levels of high sensitive-C reactive protein (hs-CRP) as an inflammatory marker with the risk factors of CAD.
Methods: The plasma levels of vWF antigen (vWF:Ag), ADAMTS13 activity and plasma hs-CRP were determined in 16 patients with CAD and 11 individuals without CAD. vWF:Ag levels and ADAMTS13 activity were measured by an in-house enzyme-linked immuosorbent assay (ELISA) and collagen binding based on ELISA method respectively, whilst hs-CRP was assessed by using nephelometry method. The ratio of the vWF:Ag level to ADAMTS13 activity was calculated.

Results: The vWF:Ag levels, ADAMTS13 activity and hs-CRP levels were decreased in CAD group compared to those of non-CAD group. However, the differences were not significant ($P = 0.629$, 0.445 and 0.445, respectively). The ADAMTS13 activity was significantly lower and the hs-CRP levels were significantly higher in males than those in females (65.88 ± 20.13 vs. $86.40 \pm 15.28\%$, $P = 0.016$ and 1.92 ± 5.84 vs. 0.89 ± 0.52 mg/L, $P = 0.047$). The vWF:Ag levels were significantly increased in smokers compared to the non-smoker group (0.85 ± 0.32 vs. 0.57 ± 0.28 IU/mL, $P = 0.031$). Individuals who have risk factors for CAD including age ≥ 60 years old, hypertension, smoking and obesity showed tendency of an increase in hs-CRP, vWF:Ag levels and vWF:Ag/ADAMTS13 activity ratio, whereas the ADAMTS13 activity was decreased when compared to those without risk factors. However, no significant differences of these parameters between both groups were found ($P > 0.05$). The inflammatory marker, hs-CRP levels were negatively correlated with ADAMTS13 activity ($r = -0.383$, $P = 0.049$).
Conclusions: Alterations of ADAMTS13 activity as well as hs-CRP levels in male and increased vWF in smokers were demonstrated in this study. This might suggest the association of these parameters with the risk factors for CAD and might be applied for the prognostic markers of poor clinical outcome of the patients. However, to confirm this association, a larger number of subjects should be recruited in further study.

PO 090
Fibrinogen concentration and tensile strength of clots

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The thromboembolism can be the cause of death and invalidity. Destruction of an initial blood clot or thrombus leading to embolus formation depends on many factors. Fibrinogen seems to be one of them and, probably, the most important one. As fibrin net is the strength base of a clot its concentration must determinate the firmness of a whole thrombus. There is no information about it in publications.
The aim of our work was to study if the tensile strength of blood and plasma clots depended on fibrinogen concentration.
Thirty samples of blood were obtained from volunteers. Centrifugation was used to get plasma. Clotting took place inside the cell of the special measure stretching equipment. After coagulation had completed cylindrical blood or plasma clot (diameter 6 mm) was stretched before rupture. The critical rupture force and the fibrinogen concentration were measured. The last equaled 9.26 + 2.23 mcM/L.
The equation of linear regression of dependence between the rupture force and the fibrinogen concentration was calculated.
For blood clots it was: F = 0.13 C + 32.6
(correlation coefficient 0.5, correlation relation 0.66).
For plasma clots it was: F = 0.51 C + 43.1
(correlation coefficient 0.61, correlation relation 0.69).
Where: F – critical rupture force, mN; C – fibrinogen concentration, mcM/L.
Thus the linear statistical dependence was shown to be between fibrinogen concentration and tensile strength of blood and plasma clots.

PO 091
Elimination of coagulation factor XIII from fibrinogen preparations

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Background: Activated factor XIII (FXIIIa) catalyses cross-linking between alpha- and gamma-chains of fibrin during clot formation and also cross-links the major plasmin inhibitor, alpha 2-antiplasmin to fibrin. Contamination of FXIII can be found in commercially available sources of fibrinogen purified from human blood.
Aims: To elucidate FXIII contamination in different commercially available fibrinogens purified from human blood, and to characterise two main techniques to remove FXIII from these fibrinogen preparations.
Methods and Results: Commercial fibrinogen was prepared according to the manufacturers' instructions, and further purified to remove any FXIII contamination by one of two methods (i) ammonium sulphate precipitation wherein ammonium sulphate was added to 20% (w/v) with 10 mM $CaCl_2$ and the sample was centrifuged to remove precipitated material, including FXIII; (ii) affinity chromatography employing an IF-1 antibody (Kamiya Biomedical, Seattle, WA, USA, a calcium dependent antibody that binds to the D-region of fibrinogen), which includes a high salt wash that aids the dissociation of other proteins from column bound fibrinogen. The ammonium sulphate precipitation method allows large scale purification, whereas the affinity chromatography is more suitable for small volumes due to the maximum fibrinogen binding capacity of the column (typically < 5 mg dependent on amount of antibody immobilised). All commercial fibrinogen preparations that had not been specifically depleted of FXIII showed FXIII-A subunit was present in a western blot. Additionally, they showed varying degrees of FXIII activity as shown by an in-house FXIII activity assay by incorporation of biotin-pentylamine into either casein or fibrin substrates. Both the in-house (ammonium sulphate precipition or IF-1 antibody affinity) and commercially FXIII depleted samples showed no activity of FXIII in the fibrinogen preparations. However, batch to batch variation of the degree of FXIII-A subunit antigen was observed in the ammonium sulphate method although this was inactivate.
Summary and Conclusion: Fibrinogen from commercial sources is highly contaminated with FXIII antigen and shows significant cross-linking activity. In instances where it is key that fibrinogen is free of FXIII contamination we recommend that either ammonium sulphate precipitation is employed where large amounts of fibrinogen are required. However, where small amounts of fibrinogen are required we recommend that the fibrinogen is applied to affinity purification with an IF-1 antibody column.

PO 092
Fibrinogen deficiency and surgical hemostasis-our first experience

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Aim: Fibrinogen is a blood plasma protein produced by liver who plays essential role in coagulation. It is converted into fibrin, catalyzed by thrombin and also induces platelet activation and aggregation by binding to the platelet reception GP2a/3b.
Case report: We report a case of a 9 years old boy who has to be prepared for surgical intervention with Dg: Hernia inguinal lat.dex. Prior to get ready him, we determined its blood group and Rhesus factor, also and screening coagulation tests. PT and aPTT were normal, but TT was abnormally prolonged (referenced value for that day reagent was 22,7″, received value was 55,7″). We used equal parts of normal plasma and patient plasma and elaborated a correction test-the result was corrected to 26,5″. Our first suspicion was that there is deficit of coagulation factor or eventually some undiagnosed liver disease. In our biochemistry laboratory immediately was worked out the level of fibrinogen and liver tests. Liver tests were normal, but Fibrinogen level was decreased to 0.9 g/L, without any symptoms of bleeding before, as the mother said to us. We suspected that this is probably isolated factor deficiency (for which no factor concentrate is available in our country). We also do not produce cryoprecipitate in our transfusion service, because of technical reasons. So we decided to consult transfusiologist and hematologist in the reference centre in our capital town.

The results were confirmed in their laboratory that the case is hypofibrinogenemia with instructions the boy to be prepared for surgery with fresh frozen plasma – 20 mL/kg tt. That was done, although we know the limitation of plasma-low fibrinogen level, large volume and risk of viral transmition. After the plasma treatment, fibrinogen level was increased to 3, 6 g/L, TT was normalized and the surgery was successfully finished.

Conclusion: This is the first case of fibrinogen deficiency in our transfusion centre. There were different opinions between us, specialists of transfusion medicine and surgeons about relationships between plasma fibrinogen level and preoperative hemostasis. We agreed together in one-if fibrinogen level is depleted, the supplementation is the key for the maintenance of hemostasis function-to 2 g/L for surgeon hemostasis. The following is this case is to determinate exactly is this is an inherited or some aquired hypofibrinogenemija.

PO 093

Detection of alpha-2-antiplasmin heterogeneity in plasma by immunoprecipitation and SDS-PAGE/ western blotting

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Background: Alpha-2-antiplasmin (a2AP), the main natural inhibitor of the fibrinolytic system, occurs in blood in different molecular forms: a plasminogen-binding (PB-a2AP) form and a nonplasminogen-binding (NPB-a2AP) form. These molecular forms can be distinguished from each other by the presence or absence of an intact *C*-terminus. This *C*-terminus is crucial for the initial interaction with plasmin and is a key component of a2AP's rapid and efficient inhibitory mechanism. NPB-a2AP has functionally been characterized via a modified crossed immunoelectrophoresis technique. However, efficient methods to isolate and biochemically characterize the NPB-a2AP have not yet been described.

Aims: We aimed to develop an efficient method to detect and biochemically characterize the different forms of a2AP, especially NPB-a2AP in plasma by immunoprecipitation (IP) and SDS-PAGE followed by Western blotting.

Methods: An IP method was set up to precipitate the different forms of a2AP present in human plasma using a2AP-specific antibodies. Magnetic protein G Dynabeads were labeled with a polyclonal total-a2AP antibody to capture all the molecular forms of a2AP. Covalent binding of the antibody to the protein G at the surface of the Dynabeads was ensured by chemical crosslinking. Human normal pooled plasma, depleted from IgGs present in plasma, was added to the bead-antibody complex for a2AP antigen capture. A2AP was eluted from the bead-antibody-complex by heating for 10 min at 95 °C in the presence of SDS. Lastly, the eluted a2AP was reduced at 95 °C and run on a 10% SDS polyacryl amide gel followed by Western blotting. The molecular forms of a2AP were detected using a polyclonal total a2AP antibody, and a monoclonal antibody specifically directed to the *C*-terminus of a2AP to distinguish PB-a2AP and NPB-a2AP. Fluorescently-labeled secondary antibodies were used to visualize the different protein bands with an Odyssey scanner.

Results: With our developed method we were able to detect different a2AP forms present in plasma on a Western blot. Using a polyclonal total a2AP antibody as well as a specific antibody directed to the *C*-terminus of a2AP, we confirmed the presence of NPB-a2AP in plasma. PB-a2AP had an estimated molecular mass of 70 kDa, confirming the known molecular mass of a2AP. NPB-a2AP had an estimated molecular mass of 60 kDa, which indicated an approximately 10 kDa molecular mass difference between PB-a2AP and NPB-a2AP.

Summary/Conclusions: In summary, we have developed a fast and efficient method to isolate and biochemically characterize the PB-a2AP and NPB-a2AP from plasma using immunoprecipitation and SDS-PAGE followed by Western blotting. This method will be used to identify the *C*-terminal cleavage site of a2AP by mass spectrometry.

PO 094

T-PA and PAI-1 levels in Tunisian Behçet patients

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Background: Behçet's disease (BD) is a systemic vasculitis. Endothelial damage is one of the ethiopathogenic mechanisms which can be associated with defects in coagulation and/or fibrinolysis.

Objectives: Assess the fibrinolytic activity in Tunisian patient with BD through the determination of tissue type plasminogen activator (t-PA), and plasminogen activator inhibitor 1 (PAI-1) levels.

Investigate the link of these findings to patients'clinical features, activity and severity.

Methods: A-44-BD patients were enrolled according to the criteria of the international study group on Behçet's disease. According to the Yosipovitch severity scale, patients were divided into three subgroups: mild, moderate and severe disease. The activity index was elaborated according to the Yazici scale. Concentration of t-PA was determined by enzyme linked immunoabsorbent assay (ELISA) in patients and compared to 46 healthy controls. Because of financial considerations, PAI-1 levels were determined in only 12 patients.

Results: Mean age was 40 years (sex ratio M/F: 42/15).Mean level of t-PA was 4.85 $\pm$ 7.36 ng/mL in patients. No difference was found compared to controls (5.62 $\pm$ 8.49 ng/mL). Among patients, there was no correlation between t-PA levels and clinical manifestations. No association was found with severity index (6.5 vs. 4.5 ng/mL). T-PA levels were higher in severe and moderate subgroups than in mild one. Mean PAI-1 level was 10.7 ng/mL. It was significantly decreased in patients with ocular involvement (1.66 vs. 19.75 ng/mL; $P = 0.03$). PAI-1 levels were higher in active form than in inactive one and in severe and moderate subgroups. There was no correlation between t-PA and PAI-1 variations.

Conclusions: PAI-1 and t-PA vary in Behçet's disease. These findings reflect endothelial dysfunction which is the cornerstone of the etiopathogenesis in BD. Further studies with larger groups are needed to elucidate the impact of fibrinolytic parameters in BD.

PO 095

Functional characterization of metalloproteinases (colombienases) with fibrinolytic activity from Bothrops colombiensis venom

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The hemostatic system is designed to maintain the blood fluidity by inhibiting blood coagulation and platelet aggregation and promoting fibrinolysis. Thrombosis is probably the most frequently symptom among cardiovascular diseases. At the present time, trying to improve the safety and efficacy of fibrinolytic therapy, several investigators have been tracking to find out fibrinolytic enzymes from different snake venoms.

Aims and Methods: Functional characterization [hemorragic, ADP-Platelet aggregation, fibrino(geno)lytic, proteolytic (extracellular matrix and basal membranes proteins- type IV collagen, laminin, gelatin and fibronectin), hemolytic, edematogenic and cytotoxic (cardiac myoblasts cultures) activities] of fibrinolytic enzymes isolated from *Bothrops colombiensis* venom.

Results and Discussion: Colombienases directly act on the fibrin, without activating the fibrinolytic system (plasminogen/plasmin), they degrade the fibrinogen Aα and Bβ chains, as well as α-polymers,

dimers γ-γ and α and β chains of fibrin and the fibronectin molecule; additionally Colombienase-2 degrade fibrinogen γ chains. Laminin and type IV collagen were resistant to the Colombienases action. Gelatin-zymogram showed in *B. colombiensis* crude venom several active bands (< 148 > 64 kDa) that may correspond to P-III class metalloproteinases. Additionally, Colombienase-2 presented an active band at approximately 22 kDa. In contrast Colombienase-1 did not show gelatinolytic activity. All these activities were abolished by metalloproteinases inhibitors. Colombienases have an insignificant edematogenic activity, and both enzymes lacked hemorrhagic, hemolytic, cytotoxic, plasminogen activator or coagulant activities, and no effect on platelet aggregation induced by collagen or ADP. All these characteristics let us to classify these enzymes as P-I class metalloproteinases that lack the disintegrin domains and/or rich-in-cysteine, which appear to be important for the induction of apoptosis and hemorrhagic activity. Additionally, Colombienase-1 was recognized by a polyvalent anti snake venom but Colombienase-2 was not recognized by this antivenom, suggesting that this protein presents low antigenic determinants or that their presence in crude venom is low, unable to induce an antibody response. Knowing the fibrinogenolytic activity of Colombienases, this toxin should be administered in a high dose to induce an adequate neutralizing immune response in animals.
Conclusions: The direct fibrino(geno)lytic activity of the Colombienases, besides its poorly toxic effect, could be considered potential thrombolytic agents, given they property of dissolving fibrin clots or preventing their formation.

PO 096

Plasminogen activity in patients with ligneous conjunctivitis and hydrocephalus in Indonesia

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Background: Plasminogen is plasmin precursor, an important enzyme needed in fibrinolytic system which is also important in wound healing. It was reported that type 1 plasminogen deficiency results in pseudomembranous diseases which manifested as ligneous conjunctivitis. Besides ligneous conjunctivitis, it is also associated with occlusive hydrocephalus.
Aim of study: The aim of our study was to investigate plasminogen activity in patients with ligneous conjunctivitis and hydrocephalus.
Methods: Seventy seven subjects were enrolled in this study, consisted of one patient with ligneous conjunctivitis and his parents, 21 patients with hydrocephalus, 20 normal children, and 33 normal adults. The plasminogen activity of normal subjects was used as reference value. Citrated plasma was obtained from each subject, and plasminogen activity was measured by chromogenic substrate using reagents from Sysmex company, Japan. This study had been approved by the ethical committee of the Faculty of Medicine University of Indonesia and informed consent for each subject had been obtained. Our study was conducted at Cipto Mangunkusumo Hospital, Jakarta, Indonesia in September 2012 until January 2013.
Results: Within run coefficient of variation (CV) plasminogen activity in normal range was 3.1%, between day was 5.4%. In pathological range between day CV was 9.6%. Plasminogen activity in patient with ligneous conjunctivitis was 11.9%, very low compared with normal children whose median was 116.8%(70.9–133.5%). His parents plasminogen activity was 68.45% and 80.65% which were quite low compared with the activity in normal women 118.65%(112.48–124.43%) and the activity in normal men 115.91% (110.60–121.24%).. Among hydrocephalus patients, 5 out of 21 showed plasminogen activity < 80%, 2 of them were 79.4%, one patient was 79.8%, two of them neonates whose plasminogen activity were 57.1% and 55.4%.
Conclusion: This study suggests the possibility of heterozygote defect in a patient with plasminogen activity lower than 80% and the reference range for plasminogen activity should not be as low as 75% as believed today. This needs further study about chromosome defect in subjects with plasminogen level < 80%, including hydrocephalus patients.

PO 097

Computational study of Textilinin as an anti bleeding agent to improve the stability and activity

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Background: textilinin-1, a serine protease inhibitor from Pseudonaja textilis venom binds to and blocks the activity of a range of serine proteases, including plasmin and trypsin. This compound is a more specific plasmin inhibitor than aprotinin while the side effects are much fewer. So, it could be the possible drug in surgery. Although a number of plasmin inhibitors are introduced, they are suffered from poor potency and/or specificity of inhibition that either results in reduced efficacy and decreased clinical application. Consequently, there is a need for further development of high-affinity plasmin inhibitors that maintain selectivity over other serine proteases.
Aims: The generation of new mutants by using the computational study to improve the activity and stability of textilinin-1 and decrease the side effects.
Methods: Based on the structural, evolutional and functional information, new mutants were designed. Then, to predict the three dimensional structure of each mutant, 10,000 model were generated by MODELLER package. The Ramachandran plot, verify 3D and Z-score were used to confirm the quality of the best model. The Gromacs 4.5.5 was employed to energy minimization of the selected models by which the models were put in a water box for 5 nano second molecular dynamic simulation. Finally, the binding affinity was investigated using ClusPro software.
Results: Most mutants (ten ones) were stable after modeling and 5 nano second simulation. A better binding to substrates were observed for four mutants including A18V, R15L, V16A and E34I. The Canonical loop (17–21), which is important for binding to serine proteases, was conserved in all mutants but the structural shift toward beta-sheet structure were found in two mutants: PCRVRF (13–18) and RV (15–16). Replacement of Ala 18 substitution with Val lead to more conformational space for interaction with substrates. Moreover, the mutants interaction with water is probably accelerated by the surface charge reduction.
Conclusion: To find the improved mutant with higher stability and activity, the computational study would be a hopeful method.

PO 098

Circumcision in patients with haemophilia: a single centre experience

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Background: Circumcision is the oldest and most frequent surgical procedure in the world and especially in Turkey as is seen in the other Islamic countries because of religious and traditional pressures.
Aims: In this study, we aim to report the experience of circumcision at our clinic in a total of 58 patients with hemophilia (Haemophilia A = 49, Haemophilia B = 9) between 1990 and 2013.
Methods: We retrospectively reviewed medical records of 56 haemophilia patients without inhibitors and two haemophilia A patients with inhibitors who had been circumcised. Factor concentrates were given before and after circumcision for 5–7 days. By-passing agents were used for circumcision in haemophilia patients with inhibitors. In all patients antifibrinolytic agents were used also. Fibrin glue was not

used during circumcision procedures. Circumcisions performed with an open method, with dorsal slit technique.
Results: In 27 (47%) patients factor activities were < 1%, 14 (24%) were between 1 and 5% and 17 (29%) were > 5%. Their ages of circumcision were ranged from 3 months to 14 years (median 8 years). Ten of 58 patients without inhibitors were referred to our centre with bleeding after the circumcision before diagnosis of haemophilia. One of these ten patients had moderate bleeding and nine of them had mild bleeding. Forthy-eight patients with haemophilia were circumcised in our centre under general anaesthesia. In three (6.25%) patients without inhibitors had moderate bleeding, despite adequate factor replacement. Transfusion was not needed. Thrombotic events were not observed during factor replacement therapy and antibody occurrence was not detected in these patients.
Conclusion: Examination of hemostasis tests carefully before circumcision leads to the recognition of patients with bleeding diathesis. Our experience showed that circumcision for patients with haemophilia should be carefully performed by surgeons together with paediatric haematologist, under appropriate conditions in haemophilia centres which has comprehensive coagulation laboratory.

PO 099

Iliopsoas hemorrhage in congenital factor deficiencies: the experience of Çukurova University, Adana, Turkey

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Background: Iliopsoas hemorrhage is a serious complication of bleeding disorders that occurs most commonly in patients with severe hemophilia and less commonly von Willebrand disease and Factor V deficiency and is considered as potentially life threatening condition and significantly associated with morbidity. Despite its clinical importance, there are few reports on the mode of presentation, treatment and sequela. In this study, we presented our experience about iliopsoas hemorrhage in patients with severe bleeding disorders.
Aim: In this study, we evaluated our patients with iliopsoas hemorrhage and compared with literature.
Patients and Methods: From 1995 to 2012, 19 patients with iliopsoas hemorrhage were evaluated inÇukurovaUniversity, Hemophilia center. The findings of physical examination, pain locations, complications were assessed. Iliopsoas hematomas were confirmed by ultrasonography, CT or MR scan. The treatment strategies were noted.
Results: We evaluated 29 episodes of iliopsoas bleeding from 19 patients with congenital factor deficiencies (CFD). There were 13 with hemophilia A, 4 with hemophilia B, 1 with factor V deficiency, and 1 with von Willebrand disease. Age range was from 45 days to 19 years. Fourteen patients had one episode, three had two episodes, one had three episodes, one patient with severe hemophilia A with inhibitor had six episodes. Three patients had a high titer inhibitor against factor VIII. Iliopsoas hematomas were confirmed by ultrasonography in all patients. Five patients needed erythrocytes transfusion. The mean duration of therapy was 11.55 ± 3.54 days, and the duration of hospitalization was 10.50 ± 3.54 days. Patients with inhibitors were treated with by-passing agents. In six bleeding episodes of one severe hemophilia A patient with inhibitor treated with recombinant factor VIIa (rFVIIa). Activated prothombin complex concentrates (aPCC) was given in two episode in two severe hemophilia A patients with inhibitor. Long term complications included paresthesia in five patients in the distribution of femoral nerve and quadriceps atrophy in four patients.
Conclusion: Iliopsoas hematoma is a serious bleeding event in patients with hereditary clotting disorders. In this report, we present our experience and treatment modality in 28 episodes of serious iliopsoas hematoma in hereditary congenital factor deficiencies.

PO 100

The effect of water exercise on atrophic muscles associated with limited range of motion in severe haemophilia A patients

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Aim: Haemophilia causes musculoskeletal problems over many years secondary to recurrent hemarthrosis. In this study, the effects of water exercises on the musculoskeletal system of severe haemophilia A patients with muscle and joint problems are described.
Materials and Methods: Eleven severe haemophilia A patients on prophylaxis treatment participated in the study.
Result: After completing a protocol of regular exercise, subjects displayed statistically significant increases in mid-thigh, upper thigh and calf circumference ($P < 0.05$). Compared to pre-exercise values, significantly increased strength leg extensor and flexor as well as range of motion were also observed ($P < 0.05$). In addition, serum levels of growth hormone were found to be significantly higher at the completion of all exercise protocols vs. pre-exercise levels ($P < 0.05$).
Conclusion: These results show that some easily performed exercise protocols such as water exercises can promote muscle development and increase range of motion of the joints. Water exercise is especially preferred, because it is not a contact sport and the likelihood of collision is very low. Thus, potential future deformities may be prevented if appropriate exercises are started without delay following an acute episode of bleeding.

PO 101

How well is haemophilia known among non-hematologists?

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Background: Haemophilia is a rare inherited bleeding disorder caused by the deficiency of the clotting factors VIII or IX.
Haemophilia A (FVIII deficiency) is the most common form of the disorder, present in about 1/5000–10,000 male births. Haemophilia B (FIX deficiency) occurs in around 1/20,000–34,000 male births. Haemophilia, once a disease of the childhood, now became a clinical problem extending into the adult ages with the advent of modern treatment tools. It is frequently observed that physicians facing with adult people with haemophilia (PwH) either exaggerate the bleeding risk and avoid offering treatment for non-haematological conditions or underestimate the bleeding tendency leading to increased morbidity and mortality. This lack of knowledge about haemophilia greatly hampers the management of both the bleeding episodes and other clinical conditions.
Aim: The aim of the present survey was to determine the level of awareness about haemophilia among non-haematologist physicians treating adult patients in Turkey.
Methods: Fellows and consultants who are not primarily taking part in the clinical management of the PwH were included in the study. They were asked to complete a questionnaire with 10 multiple choice and 14 true/false questions about the diagnosis and treatment of haemophilia. Physicians were also expected to report about their personal experience on haemophilia.
Results: A total of 133 physicians from aStateHospitaland aUniversityHospitalentered the study. All of the participants correctly identified haemophilia as an inherited bleeding disorder. However, 83.5% only knew that the manifest disease was primarily associated with male gender. Only 1/5 of the subjects were aware of acquired haemophilia. One hundred and six physicians (79.7%) could correctly recall the most frequent sites of bleeding. Seventy percent of the subjects chose aPTT as

the screening test for haemophilia. One in 10 physicians thinks that platelet transfusion is an integral part of haemophilia management. Almost 60% of the physicians had seen 1 or more PwH for a consultation. However, more than 70% of the included physician stated that they would preferably not encounter with PwH and refer them to a haematologist even for the treatment of non-haematological problems. Almost all physicians agree that emergency treatment of bleeding episodes should be known by all but regular follow-ups and the adulthood medical problems should be managed by the haematologist or the internist.

Summary/Conclusion: The results of this survey showed that the study group was partially informed about haemophilia. The most striking two outcomes were: (1) Eighty percent of the physicians had not heard of acquired haemophilia; (2) In the era of home replacement therapy more than 2/3 of the physicians still thought that joint bleeds should be treated in the hospitals. These findings suggest an obvious need for increasing the level of awareness about haemophilia among non-haematologists. This survey would help in redesigning the basic as well as continuing medical education programmes to defeat the fear against haemophilia.

PO 102

Hematuria in congenital coagulation factor deficiencies

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Background: Spontaneous gross hematuria or subclinic microscopic hematuria is not an uncommon manifestation in severe hemophiliacs and has been thought to reflect tubular damage.

Aims: In this retrospective study, we evaluated the prevalence of hematuria in hemophiliacs and other congenital factor deficiencies in a comprehensive care center.

Methods: All medical records of hemophiliacs who attended this comprehensive hemophilia care centre between 1990 and 2012 were evaluated. Six hundred and twenty-nine patients with congenital hemorrhagic diathesis (383 hemophilia A (HA), 77 hemophilia B (HB), 99 type-1, 16 type-2 and 14 type-3 vonWillebrand's Disease (VWD), 16 FVII deficiency, 8 FV deficiency, 5 FX deficiency and 11 rare factor deficiencies) were evaluated retrospectively in this study.

Results: Hematuria was determined in 39 of 383 HA patients, 12 of 77 HB patients, 5 of 129 VWD patients and 3 of 40 patients with the other factor deficiencies. The first attack of hematuria occurred at the age of 3–34 years (mean 15.5 $\pm$ 6.9 years). Hematuria was seen in 59 of all hemophilia patients. Thirty four of these hemophiliacs had only one hematuria episode, 8 had two, 4 had three, 3 had four episodes and 10 had five and more episodes. Hematuria was seen in 29 severe, 20 moderate and 2 mild FVIII and FIX deficiencies. Inhibitors were positive in 7 of these patients.

While there were no significant findings in ultrasonography in 41 of 59 patients (70%) who had hematuria, nephrolithiasis was observed in seven patients (12%), pyelocaliectasis in one patient (1.7%) and renal cyst in one patient (1.7%). Ultrasonography was not performed in nine patients.

While eight patients (13.6%) received only hydration therapy, 43 patients (72.9%) received both hydration and factor replacement therapy. Five patients (8.5%) received hydration and factor replacement therapy. Two patients (3.4%) received fresh frozen plasma and 1 patient (1.7%) received fresh frozen plasma with combined prednisone therapy.

No cause for hematuria was identified in 46 of these patients (78%). Nine patients (15.3%) had nephrolithiasis, 1 (1.7%) had poststreptococcal glomerulonephritis, 2 (3.4%) had urinary tract infection, 1 (1.7%) had renal cyst.

Conclusion: Our results are consistent with the other investigations which rarely demonstrate urinary system abnormalities in these patients. The long-term outcome of hematuria is largely unknown.

PO 103

SNC bleeding: experience on a single haemophilia center (1990–2013)

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Background: Central nervous system (CNS) bleeding is one of the most severe manifestations that occur in patients with Hemophilia, with a high morbimortality.

Aims: The objective of this study was to evaluate retrospectively 110 patients affected with Hemophilia A (HA) and Hemophilia B (HB) who had CNS bleeding, on a single center from 1990 to 2013, inorder to establish incidence of recurrence, death rate, neurological impairment, most frequent location, and efficacy of treatments.

Design and Methods: Of one thousand and nine hundred ambulatory visits, we enrolled 110 patients: 93 patients with HA (78 patients with HAs, eight patients with HAm and seven patients with HAl), 17 with HB (14 patients has HBs, 2 has HBm and 1 with HBl).

They were presented 156 (12 events per year) central nervous bleeding episodes, 27 patients (17, 2%) (21 HA, 6 HB) experienced a recurrence and 10 (6%) (HAs 8, HAm 1, HAl 1) had more than one recurrence. Twenty two (19, 8%) patients had an early onset of CNS bleeding before the first 2 years of life, (15 HAs, 1HAm, 4HBs) others 88 (80.18%) later in life (62 HAs, 7 HAm,7 HAl and 9 HBs, 2 HBm, 1HBl). In 58 (36 HAs, 5 HAm, 3 HAl and 10 HBs, 3 HBm, 1 HBl) of the episodes, CNS bleeding was spontaneous.

Results: Neurosurgery was performed in 27 patients: 20 (74%) with HA (18 HAs, 1 HAm, 1HAl) and 6 (22.2%) with HB (5 HBs, 1 HBm), in association with factor replacement therapy. Four (1%) patients presented seizures. Psychomotor impairment were seen in 9(2.4%) patients. Thirty nine deaths were recorded. Sixteen events occur in patients that presented a discontinuation in prophylaxis.

Conclusion: Prophylaxis with clotting factor concentrates is considered optimal care for patients with severe haemophilia A or B.

In our experience, CNS is the first cause of mortality .According with the high proportion of patients (around 20%) that had, recurrence of CNS bleeding, mostly patients with severe hemophilia. It would be wise to optimize the indication of prophylaxis and intensive education of patients and family in order to prevent CNS bleeding.

PO 104

Primary prophylaxis in Venezuelan cohort: 5-year experience

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Introduction: Primary prophylaxis (PP) has been used with the purpose of preventing recurrent hemarthroses and serious bleeding. In Venezuela, once we achieved safe treatment of all people with hemophilia (PWH) we started PP in children.

Patients and Methods: We enrolled boys with severe or moderate hemophilia (FVIII or FIX $\leq$ 2.5UI/dL) and without inhibitors (< 0.65Bethesda Units). The inhibitors were detected every 3 months, an annual musculoskeletal evaluation in HJHS scale was done and education regarding prophylaxis was given to parents. We used modified Canadian dose-escalating protocol, making the following changes: lower dosage in < 1 year and skipping to the next phase when one spontaneous hemarthroses appeared.

Results: Fifty-seven patients (severe HA: 43, moderate HA: 9, severe HB: 4, moderate HB: 1) since January 2007 to December 2012. Average age of initiation in PP: 19 months (7–39), average duration: 25 months (2–60), average IU/kg/day:33.7(17–56), average hemarthroses in last year: 1.7 (1–5), average soft-tissue bleeding episodes in last year: 2.3 (1–7). Twelve children were dismissed because of high-response inhibitor development and 3 were dismissed for lack of

adherence. No serious bleeding happened during the period under analysis. One boy had one target joint, clinically evaluated.
Conclusions: It is key to keep educating parents continuously, explaining the importance of complying with instructions and recording the treatment in written form.

PO 105
Incidence of reduced bone mineral density among patients with hemophilia

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Background: Osteoporotic fractures bear a high morbidity and mortality rate, particularly in hemophilia patients. Reduced bone mineral density (BMD) has been recently recognized in hemophilia patients, but the pathology of bone metabolism is still unclear.
Aim: The aim of our study was to evaluate the impact of severity of hemophilia, number of target joints and presence of inhibitors and hepatitis C infection on bone mineral density in hemophilia patients.
Methods: We evaluated BMD in 58 patient with hemophilia A and hemophilia B. Thirty-eight patients had severe hemophilia, defined by FVIII of FIX activity < 1%, and 19 patients had mild and moderate hemophilia, defined by activity of FVIII or FIX < 5%. Among patients with severe hemophilia nine patients (23%) had positive inhibitors to FVIII. Eighty-five patients were hepatitis C positive. Number of target joints was higher in patients with severe hemophilia when compared to patients with mild and moderate hemophilia, median 3.82 and 1.70 respectively. Lumbar spine and hip BMD was determined by dual-energy X-ray absorptiometry. Osteoporosis was considered when T-value was < -2.0 and osteopenia when T-value was < -1.0.
Results: Out of 58 patients with hemophilia 43% had normal bone mineral density, 48% osteopenia and 9% osteoporosis. BMD was significantly lower in hemophilia patients than in controls. The severity of osteoporosis correlated with severity of hemophilia, but not with number of target joints. There was no difference between groups regarding presence of inhibitors or infection with hepatitis C.
Conclusion: Our results showed that patients with hemophilia have high risk of developing osteoporosis and osteopenia, which is dependent on severity of hemophilia, but not on presence of FVIII inhibitors or hepatitis C infection.

PO 106
Feasibility study of a randomized control trial to evaluate an internet-based self-management program for adolescents with hemophilia: preliminary results and observations

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Background: As adolescents with hemophilia approach adulthood, they must assume responsibility for their disease management. An innovative bilingual (English and French) Internet-based self-management program, 'Teens Taking Charge: Managing Hemophilia Online', was developed to support adolescents during this transition.
Aim: To determine the feasibility of an 8-week online educational program using a randomized control trial design in terms of study accrual and attrition rates; participants' willingness to be randomized, compliance with program and completion of outcome measures, and satisfaction; as well as preliminary estimates of treatment effectiveness.
Methods: A non-blind pilot randomized control trial (NCT01477437) was designed to test the feasibility the planned study of 'Teens Taking Charge: Managing Hemophilia Online'. Adolescents ($N = 31$), ages 12–18, were selected from three tertiary care centres in Canada. After a research assistant (RA) had explained the study in detail and obtained informed consent, adolescents were randomized to the intervention ($n = 15$) or the control arm ($n = 16$). Adolescents in both arms of the study were asked to complete pre- and post-outcome measures online and received weekly telephone support from a trained RA. Following completion of the 8-week program, one-on-one semi-structured interviews were conducted to assess user satisfaction with the intervention. This study was approved by research ethics boards at each site.
Results: At present, nine participants in the intervention arm have completed the study in an average of 14.4 weeks (SD = 5.8) and three are active in the program. Among those randomized to the intervention, two participants dropped out of the study and 2 were lost to follow-up, resulting in an attrition rate of 25% (4/16). Six participants in the control group have completed the study. Despite the plan to give the control participants access to the website after the study's completion, the control arm has a higher attrition rate with five participants being lost to follow-up and four participants dropping out or a rate of 56% (9/16). Reasons for dropping out included: disappointment in not being assigned to the intervention, a lack of interest and being 'too busy' to complete the program. Post-intervention interviews are ongoing, with three participants interviewed to date. Overall, these teens found the website to be informative, comprehensive and easy to use and were satisfied with the program.
Summary: This pilot randomized control trial is ongoing. Interim findings indicate an acceptable attrition rate in the intervention group as compared to other online interventions. Higher than expected attrition rate in the control group, suggests the need to improve the strategies for maintaining participant engagement in the design of this web-based intervention prior to a larger randomized control trial to assess efficacy. Collection of qualitative interview data is ongoing and further analysis is needed to assess study feasibility and alter design, if deemed necessary.

PO 107
Ischemic stroke and haemophilia A: what therapy?

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Background: Advances in haemophilia care have led to an increase in the overall life expectancy of patients with haemophilia such that it is approaching that of the general male population. Although data on the protective effect of haemophilia on cardiovascular diseases are conflicting, their longevity exposes this group of patients to the same cardiovascular risk factors (hypertension, smoking, diabetes mellitus, hyperlipidemia, obesity) as the general population. There are limited data on the prevalence and management of such risk factors in patients with haemophilia when compared to the general population. In addition, evidence-based guidelines on management of patients with cardiovascular diseases are lacking.
Aim: The purpose of our report was to describe three cases brought to our attention from our adult haemophilia population. We also looked at outcomes of our management of haemophilia patients with overt cerebral ischemic events.
Methods/Methods: A 45 years patient, with severe haemophilia A treated on-demand, high blood pressure, HBV-HCV hepatitis, is hospitalized for subjective vertigo, postural instability, nausea, vomiting and diplopia, right ptosis, right emivolto hypoesthesia. The TSA and transcranial Doppler showed right vertebral artery with dissection of the wall and occlusion of intracranial tract of left vertebral artery. The Cerebral MRI showed ischemic lesion in the left lateral medulla and inferior cerebellar peduncle (in the Posterior Inferior Cerebellar Artery

territory); dissection of both vertebral arteries and left vertebral artery occlusion; intramural hematoma along the course of the cervical right vertebral artery. At follow up, the MRI described complete resumption of the hematoma, persistence of left vertebral artery occlusion. To date (44 months of follow-up) are not described further ischemic events. A 53 years patient, with moderate haemophilia A treated on-demand, HCV hepatitis, smoker, is hospitalized for right hemisoma weakness. The Cerebral MRI showed acute ischemic lesion in the right corona radiata in the territory of the perforating branches of middle cerebral artery. He had a progressive recovery and to date (48 months of follow-up) did not relapse. A 77 years patient, with moderate haemophilia A treated on-demand, overweight, hypertension, diabetes mellitus, smoker, is hospitalized for diplopia, dysarthria, and instability in the standing. The cerebral TC highlighted some areola of clear hypo-density bilaterally in nucleus-capsular as recent vascular outcomes. All symptoms progressively regressed. To date (24 months of follow-up) did not relapse. The antiplatelet therapy has not been administered to any patient because of the protective effect of the haemophilia. In the first patient it was administrated prophilaxis dose of FVIII, because of the intramural hematoma along the right vertebral artery. In all cases we have acted in the correction of cardiovascular risk factors.

Conclusions: The cases are among the first described in the literature and confirm the management difficulties and the uncertainty on the correct treatment. Given the increased risk, haemophilia patients should be screened regularly for hypertension, tobacco use, diabetes, cholesterol, obesity especially during their periodic haemophilia evaluation, and managed appropriately.

PO 108

Physical and mental quality-of-life in patients with haemophilia in Belgium: the impact of financial issues and of patients' understanding of their condition

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Background: Haemophilia affects 988 people in Belgium. However, there are very few studies evaluating patients' quality of life (QoL) in the country.

Aim: This project aimed to collect such data and assess the influence of both well-identified factors (eg. arthropathies) on QoL, and of other less studied, modifiable variables (eg. patients' understanding of haemophilia-related issues, financial concerns).

Methods: This study involved adult haemophilia A (HA) and B (HB) patients, without current inhibitors, on replacement therapy, regularly followed-up at our comprehensive treatment centre. After Ethics Committee approval, patients meeting the inclusion criteria (n = 84) were informed and invited to complete a questionnaire, including the SF36v2 QoL forms. Seventy one (n = 59 HA, n = 12 HB) responded. Analysed variables included factors known to have an impact on QoL (age, haemophilia type, treatment type [secondary prophylaxis vs. on-demand], severity, number of arthropathies, sport activities, HCV/HIV status) and others (occupation, self-assessed compliance, concerns about haemophilia-related personal/public financial issues, understanding of general haemophilia-related issues [transmission, identifying emergencies, holiday planning, identifying sources of information, latest treatments available, insurance coverage, meaning of 'inhibitor']). Data analysis was performed using the QualityMetric® software for SF36v2 data, and a regression tree-based model (RT) to evaluate the relative impact of the aforementioned variables on QoL scores (norm-based). RT-based models are non-linear, non-parametric alternatives to linear models for regression problems. All significant variables appearing in the trees are clearly mentioned.

Results: QoL scores in our population (mean ± SD age: 45.2 ± 14.7 years old) were similar to those of other countries: the physical QoL score (Physical Component Summary, PCS) was lower compared to the healthy population (PCS = 46.3), whereas the mental QoL score (Mental Component Summary, MCS) was within normal values (MCS = 51.1). Scores did not depend on haemophilia type.

The PCS was primarily influenced by the number of arthropathies (RT showed a PCS = 55.4 without arthropathies vs. 42.1 with ≥ 1 arthropathy, $P < 0.001$), followed by the patients' understanding of inhibitors, haemophilia severity, and eventually patients' compliance.

The MCS was primarily affected by patients' concern regarding personal financial issues (spending) related to haemophilia (RT showed a MCS = 54.2 when not concerned vs. 37.9 when concerned. Within this last group, those with poor knowledge of insurance coverage had lower MCS scores than their peers, $P < 0.001$). Age was the second factor in the whole population, closely followed by patients' understanding of insurance coverage, ability to identify sources of information, haemophilia severity, HIV status, and ability to plan for holiday. All other variables had little or no impact on these scores.

Summary/Conclusion: Haemophilia patients' QoL in Belgium is comparable to that of other countries. Originally, our study highlights modifiable variables influencing QoL, such as patients' compliance and their understanding of haemophilia-related issues, including financial issues. Indeed, these played a major role in mental QoL – a most interesting finding in a time where the cost of clotting factor use challenges most countries' healthcare systems.

We believe that addressing these questions by providing patients with better focused information could significantly improve their QoL.

PO 109

Treatment of acute abdomen resulting from hematoma of the jejunum in severe Haemophilia A

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Background: Adequate haemophilia care requires well-coordinated specialist input when diagnosing and treating bleeding episodes.

Aims: We illustrate this with a case presenting with a bleeding episode in an unusual location, easily diagnostically mistaken.

Methods: Presentation: A male patient, 55 years old, weight 54 kg, with a history of tuberculosis with family and personal history of severe Hemophilia A without inhibitors to Factor VIII, and previous episodes of hematuria, hemoptysis and multiple episodes of bleeding in both hip joints leading to musculoskeletal grade IV hemophilic arthropathy, uninfected with blood borne viruses. The patient was admitted to the Emergency Service with epigastric pain conformant with diagnosis of gastritis for which he was treated. Readmitted 12 h after the patient complaining of burning epigastric pain, anorexia, nausea, vomiting, signs of peritoneal irritation and intestinal bleeding. At this stage a Transfusion Medicine specialist consultation was requested. A history of an episode of hematoma of the jejunum, confirmed by imaging studies 2 years previously, was revealed a presumptive clinical diagnosis of hematoma of the bowel loop was subject to confirmation by investigation.

Investigations: Ultrasound revealed a thickened mass in the small bowel loop, with diffuse and regular contours, approximately 11 cm by 3 cm longitudinal axis cross, suggesting a hypogastrium hematoma and moderate amount free fluid in pelvis. Coagulation Tests: APTT: 79. 8 s (VR 24–35) Prothrombin Time Activity 86%, Fibrinogen 816 mg/dl. Complete Blood Count: Hb 14.6 g/dl, Hematocrit 48.6%, WBC 15,000 per mm^3, platelets 213 000 per mm^3.

Treatment: Commercial Factor VIII (Octanate®, Octapharma) loading dose 1000 IU IV bolus and 2000 IU/given by continuous infusion (CI) in the first day was followed by 2000 IU on the second day and 1000 IU every 24 h for a further 5 days.

Results: Clinical remission and CT scan resolution of hematoma. Evolution:

The patient was readmitted with the same symptoms after a week. The treatment course outlined above was repeated with full resolution. He was discharged for further complete investigation of intestinal tract and was kept on a prophylaxis regimen of 1000 IU every 2 days to

minimize the risk of bleeding until perform the invasive study that revealed no evidence of underlying lesions and confirm full resolution. The patient has had no recurrence since 2 years.
Conclusions: Specialist input for haemophilia is crucial if misdiagnosis with possible negative effects is to be avoided. The interface between the admitting ward and the Transfusion Medicine specialist requires improvement and formalization. Although a rare manifestation of hemophilia, jejuminal hematoma has been described (Int J Hematol. 2006 Aug;84(2):166–9, Arch Klin Chir 1908;87: 542 -551) and should be suspected with cases running a course similar to our report.

PO 110

Hemophilia children receiving immune tolerance induction with adjuvant rituximab: 5-year long-term follow-up

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Background: Immune tolerance induction (ITI) is essentially required for hemophilia patients with inhibitor.
Aims: To evaluate the long-term outcome of hemophilia children receiving low dose ITI.
Methods: Four hemophilia boys with inversion of intron 22 (A 2 cases), large deletion (A 1 case) and stop codon at exon 2 (B 1 case) exhibiting inhibitors were enrolled in the study. Inhibitor was found at 1 year of age in three patients except for one hemophilia A was referred to us at 10 years of age. All patients had high inhibitor titer ranging from 30 to 3400 Bethesda units (BU). The hemophilia B patient had anaphylaxis to prothrombin complex concentrate and factor IX concentrate. ITI was initiated at 10 years of age except for one hemophilia A at 2 years and 10 months. The levels of inhibitor at initiating ITI were 4.5, 16, 17 and 336 BU. Rituximab at the dose of 375 mg/m^2/week for 4–6 doses were given simultaneously with factor concentrate. For hemophilia A patients, factor VIII concentrate at the dose of 100 unit/kg three times per week was given for 6 months followed by 50 unit/kg three times per week for 6 months and then tapered to 50 unit/kg twice per week continuously. For hemophilia B patient, he initially received desensitization to factor IX concentrate in the intensive care unit. However, factor IX concentrate at the dose of 40 unit/kg three times per week was given for 1 month followed by 40 unit/kg twice per week continuously. All patients were monitored for 5 years.
Results: At the first year of ITI, the occurrence of spontaneous bleeding episodes were seldom found. They were responsive to the administration of recombinant activated factor VII (rFVIIa). The anamnestic response of inhibitor was found during the first 4–6 weeks of ITI ranging from 164 to 3000 BU. Three hemophilia A and B patients' inhibitors were gradually decreased to 3, 25 and 20 BU after completing ITI for 1 year. During the follow-up, they exhibited 6.1, 8.5 and 2.7 BU in the second year; 0.3, 0.9 and 9.6 BU in the third year; 0.87, 0 and 6.8 BU in the fourth year; and 0.1, 0 and 7.2 BU in the fifth year of ITI. Although the success of ITI was achieved in two hemophilia A patients with inversion of intron 22 at 3 and 4 years, respectively, the episodes of bleeding were infrequent and additional rFVIIa was seldom required. The hemophilia B patient did not have nephrotic syndrome. One hemophilia A patient with large deletion failed to ITI since he exhibited high inhibitor of 1040 BU at 6 months of ITI. Therefore, ITI was stopped and he was switched to prophylaxis treatment of rFVIIa at the dose of 90 mcg/kg three times per week for 1 year and two times per week for the subsequent 4 years. He tolerated well with prophylaxis and seldom exhibited spontaneous bleeding episodes.
Conclusion: Low dose ITI with the simultaneous adjuvant rituximab administration was helpful in hemophilia children with inhibitor.

PO 111

Challenging diagnosis of haemophilia

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Background: Haemophilia A (HA) is a rare X-linked recessive bleeding disorder caused by deficiency or functional defects in coagulation factor VIII (FVIII).
Aims: Here we report two cases of challenging diagnosis of HA because of unusual presentation.
Results: The first case is a 10-month-old female, admitted to our hospital because of a neck mass appeared within the previous 24 h, who had a past medical history consistent with recurrent spontaneous haematomas but no family history of bleeding disorders. During hospitalization, blood tests revealed aPTT prolongation (61.5 s) and a FVIII level below 1%. The levels of von Willebrand factor antigen and ristocetin cofactor activity were within the normal range. Further laboratory tests showed absence of FVIII inhibitors and lupus anticoagulant antibodies. Ultrasonography and contrast enhanced computer tomography were performed to investigate the neck mass and revealed a solid lesion (approximately 44 × 47 × 23 mm) extended to the infraclavicular and pectoral regions. In the suspicion of a lymphoproliferative disease, the patient underwent surgical excision of the lesion. Histology of the lesion surprisingly revealed a haematoma. Chromosomal analysis showed a normal female karyotype and a mutation into the FVIII intron 22 associated with a skewed X chromosome inactivation. This mutation, present in the proposita and undetected in the peripheral blood DNA of her mother and her father, could be the result of a *de novo* mutation. The patient started prophylactic treatment with intravenous recombinant factor VIII, with progressive normalisation of aPTT.
The second case is a male neonate, born by vacuum-assisted vaginal delivery, whose mother had a thrombophilic condition and was prophylactically treated during pregnancy with antiplatelet agents and low molecular weight heparin. Family history was otherwise non significant. On the first day of life, the child presented with petechiae and gastric bleeding and received plasma and platelet transfusion. On the third day, he developed generalized seizures. The brain ultrasonography was suspicious of an aneurysm of vein of Galen and hydrocephalus. Brain MRI showed an anomaly, suspicious of a vascular malformation, localised on the quadrigeminal cistern that caused compression of the cerebellum, anterior displacement of the brainstem and consequent hydrocephalus. Blood tests revealed severe anaemia and prolonged aPTT. Due to worsening neurological symptoms, diagnostic imaging was repeated and showed initial herniation of the cerebellar tonsils into the foramen magnum. The dosage of coagulation factors revealed a severe deficiency of FVIII. Given the suspicion of haemorrhagic diathesis, radiological images were re-evaluated; vascular anomalies were excluded and the diagnosis of haematoma was made. Molecular studies were performed as described before, and showed a rearrangement of the FVIII gene involving intron 22. Because of the obstructive hydrocephalus, the child underwent surgical placement of a ventriculo-peritoneal shunt and replacement therapy with intravenous recombinant factor VIII was started. In the following days, his general and neurological conditions have significantly improved.
Conclusion: The HA diagnosis can be challenging. Lack of family history, difficulties in detecting haematomas by imaging techniques, female gender and neonatal age represent misleading factors that can delay the diagnosis.

PO 112

Management of dental invasive procedures in hemophilia A/B (HA/HB) and von willebrand disease (VWD) outpatients: experience of a single center

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Background: Dental procedures in Hereditary Bleeding Disorders (HBD) patients can be complicated by excessive bleeding. Replacement prophylactic therapy, and local measures, dramatically reduce the risk of complications in these surgeries. Moreover, self-infusion and home-treatment reduce therapy cost. Aim. To describe the dental procedures performed in a group of HA/B and VWD outpatients utilizing systemic prophylactic therapy and local measures.
Methods: During the last 6 years, in our Dental Department, we performed 154 surgeries on 49 patients (41M, 8F; median age 48 years, range 11–82) affected by HBD: 15 severe, 7 moderate, 10 mild HA; 2 severe, 2 mild HB; 6 type 1, 1 type 2A, 3 type 2B, 3 type 3 VWD. TheHemophiliaCenterprovided personalized therapeutic schemes, on the basis of coagulopathy type/severity and type of surgery. Factor VIII concentrates were administered in severe, moderate and few cases of mild HA; FIX concentrates in severe and mild HB; desmopressin in mild HA, VWD type 1 and type 2A; FVIII/VWF concentrates in VWD type 1, in case of low response or contraindication to Desmopressin, type 2B and type 3. One hundred and twenty one dental and roots extractions, 10 third molar surgical extractions, 1 excisional biopsy, 2 cysts enucleation, 18 scaling and roots planing, 1 gingival graft, 1 hypertrophic gingival tissue removal were performed under local and loco-regional anesthesia. Local hemostasis was ensured applying gelatine packing, fibrin glue, absorbable suture, 15-min compression with tranexamic acid saturated gauze. In the post-operative period patients were treated with antibiotics and continued the self-infusion of concentrates/desmopressin for an average period of 5 days (3–7). Tranexamic acid mouthwashes (three times a day for 3 days) were prescribed; acetaminophen was used as the only pain-relief treatment.
We didn't observe any hemorrhagic or infectious complications. All patients completed the post-surgery home-treatment.
Conclusions: A tailored prophylactic treatment ensures a good hemostasis in HBD patients undergoing dental procedures. Multidisciplinary approach allow to manage coagulopathic patients without bleeding complications; home-treatment reduces management cost.

PO 113

Rituximab plus prednisolone for managing hemophilia A with FVIII-inhibitor

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Backgrand: The Hemophilia A patients with FVIII inhibitor often experienced recurrent episodes of deadly bleeding and treating them is difficult.
Aim: to explore the treatment for the Hemophilia A patients with inhibitor with Immunosuppressive therapy.
Methods: Case: An 23-year-old Chinese male was diagnosed with severe hemophilia A with high title anti-FVIII inhibitor. He had Hematuria and soft tissue hemorrhage in his left psoas. A blood examination showed neutrophilia with a white blood cell count of 11,200/μL (77.1% neutrophils), an activated partial thromboplastin time of 126.90 s, coagulation factor VIII (FVIII) < 1.0%, and anti-FVIII inhibitor, 5 BU/mL. Anti-FVIII inhibitor title, FVIII level and the percent of CD20+ cells were detected after rituximab used.
Results: The bleeding episodes were controlled with intravenous high-dose FVIII followed by fresh frozen plasma and PCC. In addition, oral prednisolone (30 mg/day) plus four low-dose of rituximab (100 mg/week × 4) effectively suppressed anti-FVIII inhibitor levels while simultaneously reducing the CD20+ cells count (0%). No other drug side effect occurs.
Conclusion: This report describes the effectiveness of a combination of prednisolone and rituximab followed by immune tolerance therapy in managing hemophilia A with high title anti-FVIII inhibitor.

PO 114

Evaluation of quality of life in adult patients with hemophilia in Northwest of Iran

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Background and Aims: Several studies have been published addressing quality of life (QoL) issues in people with hemophilia, and nowadays several hemophilia Health Related QoL (HRQOL) measures are available. The purpose of HRQoL evaluation is to go beyond the presence and severity of symptoms of disease or side effects of treatment, examining how patient perceive and experience these manifestations in their daily lives.'A36 Hemofilia-QoL' is a disease specific questionnaire for the assessment of the HRQoL in adults living with hemophilia. This questionnaire is a self-report measure that comprises 36 items, and is quick and simple to administer. Higher Score is better QoL in these patients.
Aims: The main aim of this study is evaluation of Quality of Life in adult patients with hemophilia in northwest of Iran, Tabriz Hemophilia Treatment Center.
Methods: A total of 100 Adults with Hemophilia were recruited assessed in Hemophilia Treatment Center of Tabriz. Inclusion criteria were age over 17 years, mild to severe hemophilia A or B (HA and HB), and informed consent from the participants. All statistical analyses were performed using the SPSS statistical package (version 13.0).
Results: The Mean age of the hemophilia patients was 32.16 years (range 17–70, SD 11.68). Eighty tree patients were HA, and 17 cases were HB, and the average of the Qol were 71.70 and 72.75 respectively. There was statistical difference in patients whit different severity of diseases, as severe hemophiliacs had less score: Mild = 104.53, Moderate = 68.56, and Severe = 64.31.The QoL was very poor in urban area in contrast to patients who lived in the city (54.45 vs. 74.21, respectively).Single patients have a better QoL than married patients (76.56 vs. 68.50, respectively).
Conclusion: Using items from A36Hemofilia QoL instruments proved to be a useful approaches to the assessment of Physical Health and Health Related criteria in patients with Hemophilia.

PO 115

A possibility of relation between weather to joint bleedings in haemophic patients

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Background: It has been known well that weather has some relevance to joint pain in persons with rheumatism, metal plates by the treatment of bone fractures and some trouble in their joints. Haemophilic patients also have arthritis as well as them and some complain that there are more joint bleeds in bad weather than those in fine weather.

Aims: To clarify whether there is the relation between weather to joint bleedings or not in haemophic patients.
Methods: Hand-held patient diaries have been collected from the patients who have home infusion treatment in Hiroshima University Hospital once a year. The days and parts of joint bleedings in respective patients were identified on their patient diaries. And then, we investigated the weather, minimum temperatures and atmospheric pressures at patients' residential area on the day and the day before of respective bleedings on the basis of database from Hiroshima Local Meteorological Observatory.
Results: The data from 23 haemphilic patients in our hospital was collected and investigated. The total number of joint bleeding episodes amounted to 2391 in the total 71 years' data. There was no difference of bleeding counts among the seasons. The mean temperature difference when bleedings episodes occurred was 1.61 degree C and the mean atmospheric pressure difference was 2.83 hectpascal on the day when bleeding episodes occured. Nine of 11 patients who experienced more bleeding episodes at the change of weather than at invariable weather had bleedings when the weather became worse. All of them except for one case had some severe target joints. On the other hand, a half of 12 patients who had no relation to the change of weather on bleedings had bleedings when the weather became worse.
Summary/Conclusions: It could not be clarified statistically whether there is the relation between weather to joint bleedings or not in haemophic patients. As the reason, so many factors concern to joint bleedings as exercises, unusual motions, existence of target joints or prophylaxis and so on. However, the bleeding episodes may more frequently occur when the weather became worse. It is possible that change of weather could concern to irritation or occult pain of target joints at least.

PO 116

A prospective post-authorization safety surveillance study in 384 hemophilia A patients with antihemophilic factor (recombinant) plasma/albumin free method demonstrates safety and efficacy in Japan

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Background: Post authorization surveillance studies are essential for the evaluation of safety in the real life clinical practice settings. Japanese Post-Authorization Safety Surveillance Study (J-PASS) of ADVATE [Antihemophilic Factor (Recombinant) Plasma/Albumin Free Method rAHF-PFM] was initiated in February 2007 under Japanese ordinance Good Post-Marketing Study Practice (GPSP).
Aims: To investigate safety, i.e. adverse events, namely inhibitor development, and efficacy in hemophilia A patients who are using rAHF-PFM in routine clinical practices, which are including on-demand and prophylaxis treatments.
Methods: The prospective, multicenter, open-label, observational surveillance was conducted at 101 sites during the period from February 2007 to June 2012 to investigate hemophilia A in patients who were prescribed rAHF-PFM under treatment regimen for 2 years, with more than three exposure days experience of previous FVIII products (EDs), and regardless of presence or absence of inhibitors, using electrical data capture system (EDC).
Results: Of 384 subjects receiving the rAHF-PFM more than once, 341 (89%) completed 2 years of observation and 43 subjects were withdrawn: lost to follow-up (15), move to other hospitals (7), unable to complete during the study period (7), subject request (6), adverse events (3) or unsatisfied therapeutic response (1), and others (6). The severity of hemophilia were as follows: < 1%:288 (75%), 1–2%: 45 (12%), > 2–5%:28(7%) and > 5%:21(6%). The mean age at the time of enrollment was 25 years (range:0–81). EDs at study entry were as follows: ≤ 50EDs (n = 42), 51–150EDs (n = 16), to ≥ 151EDs (n = 326). As the worst record on efficacy evaluation during study period, Excellent/good rating was 92.7% for prophylaxis and 95.3% for on-demand therapy. Thirty-six subjects had inhibitor history, and 6 among them had positive titer at entry. Of the 30 subjects with a prior history of inhibitor at entry, no one developed recurrent inhibitors. One of the 6 subjects with inhibitor at entry dropped out, because of an increase in inhibitor titer from 5BU/ml to 33BU/ml after ITI. Three of 39 subjects with ≤ 50EDs and no inhibitor history at entry developed low titer inhibitor (7.69%, 95%CI:1.62, 20.87). No *de novo* inhibitor developed in 309 subjects with ≥ 51ED (0%, 95%CI:0, 0.96). No inhibitor was detected in subjects who switched from pd-FVIII or 2nd generation rFVIII to rAHF-PFM. Of 236 subjects who started or were on prophylaxis at entry, 210 continued on the prophylaxis regimen and their median annual bleeding rate (ABR) was 3.96 times/y. The prophylaxis regimens were 3 times per week (50%), two times per week (30%), one time per week (12%) and other. Of 148 who started on-demand therapy at entry, 32 moved to prophylaxis. Their median ABR was improved from 23.52 to 1.98.
Conclusions: This result further supports the safety profile of rAHF-PFM with both prophylaxis and on demand therapy in a large Japanese Hemophilia A population.

PO 117

Treatment of hemophiliacs with inhibitors Experiences of mascara-ALGERIA-N.GAID MEHALHAL, F .ARBAOUI, H.CHALABI, N .YAKHOU. E.P.H YESSAD KHALED. MASCARA.- ALGERIA-

Gaid Mehalhal N

EPH Yessaad Khaled, Mascara, Algeria

The appearance of alloantibody inhibitor or anti FVIII or FIX side effect is the largest and most formidable replacement therapy concentrates.
The incidence of inhibitors is higher in severe HA, 20% and 30%, it is only 4% and 5% in HB.
Paptients and Methods: We report in this observation a series of 37 hemophiliacs with 33 HA (89%) and 4 HB (10%) including 3 from the same sibling.
The median age was 21 years (range 2–40 years).
Hemophilia is severe in 19 cases (51%), moderate in 11 cases (29%), Minor in seven patients (18%).
Between 2005 and 2012, and 19 severe hemophiliacs 5 of them have developed inhibitors with a hemophiliac B.
The median age at the time of the discovery of the ACC is 8 years (range 2–14 years old).
The diagnosis was read 4 times a periodic review and once to the inefficiency of replacement therapy.
The treatment was administered the FVII available in our center since 2007.
A hemophiliac A 10 year old received prophylaxis for 2 years. due to an injection of Novoseven. due to 90 μg/kg per week and self-treatment.
Control of the ACC as was done in about every 6 months depending on the availability of reagents. We found a rate stabilization ACC or a regression to an advanced 0.6 UB.
Conclusion: The occurrence of inhibitors in hemophiliacs is the most serious complications making replacement therapy ineffective and support difficult due to irregular and insufficient amounts of specific treatments in our country and irregular systematic research specific treatments in our country.

PO 118

Retrospective study of sporadic haemophilia in the region of Murcia, Spain

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Background: Classically, the incidence of sporadic haemophilia is about a third of total cases, but in more recent publications there is an incidence of 30–50% depending on several series, predominantly in severe haemophilia.
Aims: Our goal was to study the distribution of sporadic haemophilia in our population and the gene mutation type.
Methods: In our region there are 80 hemophiliac patients, 65 HA and 15 HB, distributed in 53 unrelated families. We have studied the family pedigree of 46 families in the years 2007–2011. We study the genetic mutation causing hemophilia by long range polymerase chain reaction (PCR).
Results: We obtained 24 sporadic families (52.2%). In this group the incidence of severe haemophilia (A or B) was 79.1% (severe haemophilia A 54.2% and severe haemophilia B 25%. The type of gene mutation found were: Intron 22 inversion (5 families), point mutations (14 families), splicing mutations (2 families), large deletions (1 family), partial deletions (1 family), duplication (1 family). Five familes had mutations not described in the Haemophilia A Data Base (HAMSTeRS). Eight families had mutations with high risk of developing inhibitors.
Conclusions: The sporadic haemophilia is a signifiant group in the hemophilic population. In our series, the incidence is similar to the recently data published with the special importance of new mutations not previously described and the mutations with high risk of inhibitor development.

PO 119

Physiotherapy is an important element of the modern haemophilia treatment

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Introduction: Patients with haemophilia suffer from joint bleedings which can leed to athropathia.
The administration of factor concentrate is primary to prevent of these bleedings.
Thus we like to investigate if there is an additional benefit by subsidiary measures. We offered these patients physiotherapy in our centre and investigated them systematically.
Methods: Forty-four patients (age 4–62 years) were interviewed to quality of life and their pain sensation before and after 1 year of physiotherapy. We used also the substitution calendar as a data source. Twenty-nine patients (66%) were treated prophylactically and 15 patients (34%) were treated on demand.
For the data analysis we used SPSS (version 21.0). With algebraic sign test by the scaled sizes and CHI2-test by the dichotomous sizes.
Under physiotherapy the well being has increased. Pain could be diminished 2.6 scale points in the mean. Analgesics use was reduced 1 scale point in the mean. The number of bleedings decreased and additional concentrate administration could be reduced. During the physiotherapy all kind of injuries were less frequent as before.
Conclusions: An individualized physiotherapy can raise the quality of life of a patient with haemophilia through increase of the physical function, prevention of bleedings and injuries and also pain reduction. Besides this costs can be reduced as there is less coverage of factor concentrate.
These results can only be achieved if the physiotherapy is adjusted on the status of the individual joint.

PO 120

Assessment of QoL in Korean hemophiliacs: the impact of health- related factors, social state and a treatment factor on QoL of Korean hemophiliacs

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Background: Health-related factors, treatment factors and social state of hemophilia patients may have impact on their QoL. However, the impact of these factors, especially social state and treatment factors, may be different among countries with different social, cultural backgrounds and treatment approach. Therefore, identification of those factors in each country is vital to establishing potential strategies to intervene in hemophilia patients.
Aims: The impact of health-related factors, a treatment factor and social state on QoL of Korean hemophilia patients were evaluated.
Methods: Data were collected by questionnaires and reviews of the medical records of Korean hemophilia patients over 17 years of age. QoL was evaluated with the standardized questionnaires of SF36. The disease- and health-related factors such as the severity and type of disease, presence of inhibitor, HCV and HIV infection history, disability, and analgesic use history were obtained from medical records. Arthropathy was evaluated by a patient's perception of involved major joints, both knee, ankle and elbow joints. Sociodemographic data such as marital state, occupational state and education years were obtained by questionnaires. Self-injection ability, as a treatment factor, was evaluated by self-injection diaries and questionnaires. Age-adjusted partial correlation analysis and multiple regression analysis were conducted to elucidate the impact of theses observed data on QoL of the patients.
Results: Similar to results of other studies, the severity of hemophilia, inhibitor presence and other disease- and health-related factors were correlated only with several physical health scales of SF36. Marital state, the only social state, correlated with QoL of hemophilia patients in Korea, also had impact only on the physical health scale. However, self-injection score, the only treatment factor surveyed in the study, was significantly associated not only with physical health but also with mental health scales of Korean hemophilia patients.
Summary/Conclusion: Health-related factors and social state had impact only on the physical health of Korean hemophilia patients. A treatment factor, self-injection ability, had impact on both the physical and mental health of these patients.

PO 121

Participation for innovation: survey of motivation for clinical studies evaluating new treatments for haemophilia

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Background: Over the last 50 years, clinical research into new treatments has contributed to transform the care of individuals with haemophilia. This has been largely possible through the commitment of people with haemophilia to take an active part in clinical research. While much has been achieved, much remains to be accomplished such as the development and validation of longer acting clotting factor concentrates, new bypassing agents and the validation of treatment strategies aiming at preventing or reducing the risk of inhibitor development. Although many clinical trials are currently ongoing around the world, most of them are facing difficulties in recruiting candidate

patients, mainly in developed countries. In this context, there is a major need to improve the patients' awareness of the importance of clinical research in haemophilia and highlight the opportunities of an active participation in clinical trials.

Aims: A survey was launched to evaluate the motivation of patients with haemophilia for clinical research and identify factors which might influence their willingness to participate.

Methods: A specific questionnaire was sent to 136 adults with haemophilia (≥ 18 years) regularly attending the haemophilia treatment Center of the Cliniques Universitaires Saint-Luc, Brussels, Belgium. Different aspects such as socio-demographic status, familial situation, haemophilia treatment, knowledge about principles and perceived benefits and risks of clinical studies, positive and negative factors for motivation were extensively addressed. The classification tree (CT) was used to identify predictors of willingness to participate. CT-based models are non-linear and non-parametric alternatives to linear models for regression problems.

Results: A total of 60 patients returned the completed questionnaire (n = 60/136, 44.1%). Results show that most patients have a limited knowledge about the general principles of clinical research. Among the 60 patients, 49 would agree to participate in a clinical trial but are mainly concerned by possible side effects of new treatments (n = 25/49, 51.7%) or time lost from school or work (n = 26/49, 53.1%). Moreover, few patients are ready to take part in phase 1 trial fearing unknown reactions or side-effects (n = 10/49, 20.4%). Predictors of willingness to participate in a clinical study were evaluated using CT and three groups were created. Group 1 is compounded by patients who report no knowledge of clinical research modalities (Rate of willingness to participate: 28.6%, n = 2/7). The 2 other groups are compounded by patients who report a good knowledge of clinical research modalities and who are not interested in having more information (Group 2, Rate of willingness to participate: 0.0%, n = 0/2) or in contrary who want more information (Group 3, Rate of willingness to participate: 92.2%, n = 47/51).

Summary/Conclusions: In conclusion, the rate of willingness to participate in clinical studies is significantly lower in patients who report no knowledge of clinical studies modalities and in patients who are not interested in having more information on it. This survey highlights the importance to raise awareness and provide better knowledge about the modalities, risks and benefits of clinical research in order to increase the number of potential participants in clinical trials in haemophilia and probably other rare diseases.

PO 122

Do inhibitors, treatment regimen and bleed frequency impact parent-directed haemophilia treatment centre and provider utilisation: An analysis of parents of children with haemophilia in the HERO study

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Background: Congenital haemophilia is primarily treated through comprehensive care at a haemophilia treatment centre (HTC) by a variety of healthcare professionals. Annual or semiannual comprehensive care visits support home treatment in most developed countries. However, little is known about the relationship amongst the presence of inhibitors, on-demand vs. prophylactic treatment or bleed frequency, and HTC utilisation of the comprehensive team members involved in care.

Aims: To describe the relationship between resource utilisation, inhibitor status, treatment regimen and bleed frequency as reported by parents of children with haemophilia (children) in the Haemophilia Experiences, Results and Opportunities (HERO) Study.

Methods: A post-hoc descriptive analysis of data from parents of children < 18 years in eight countries with home treatment (Argentina, Canada, Germany, France, Italy, Spain, UK and United States). Annual bleed frequency was categorised for analysis (0, 1–5, 6–10, 10–20, > 20 bleeds).

Results: Of 453 parents, most reported their oldest children < 18 years had no inhibitors (411), and most children were treated with prophylaxis (without inhibitors: 80 on-demand, 311 prophylaxis; with inhibitors: 7 on-demand, 24 prophylaxis). The distribution of children among each annual bleed frequency category (0/1–5/6–10/10–20/> 20) was 65/197/78/27/24. Mean/median ages (years) were similar across treatment regimens among children without inhibitors (on-demand 9.5/9, prophylaxis 9.7/9) and among children with inhibitors (on-demand 11.7/11, prophylaxis 11.3/13). Median age was higher with increased annual bleed rates (9/9/10/11/12). Most parents responding were female (without inhibitors81%, with inhibitors 71%). Rates of full-time, part-time or self-employment were similar in parents of children without inhibitors (on-demand 33%, prophylaxis 36%), in parents of children with inhibitors (on-demand 29%, prophylaxis 32%) and with increasing bleed frequency (38%/36%/32%/32%/30%). Activity risk was similar across children with and without inhibitors, irrespective of treatment or annual bleed frequency. Mean/median annual bleeding frequency was higher in children without inhibitors on on-demand (7.5/3 vs. 5.2/3) and in children with inhibitors on prophylaxis (12.1/8) and on-demand (9.2/6). Mean/median annual HTC visits were higher in children on prophylaxis (without inhibitors 5.5/2, without inhibitors 7.3/4) than on-demand (without inhibitors 4.8/3, with inhibitors 4.8/3). Median visits were higher with increased bleed frequency (2/2/3/3/5). The healthcare professionals reported as involved in haemophilia management were largely similar across groups, including by annual bleed frequency. Nurses were more frequently involved in the care of children with inhibitors on prophylaxis (71%) than on-demand (57%), whereas social workers were less frequently involved (8% vs. 29%). Physiotherapist involvement was more frequently reported for children without inhibitors on prophylaxis (34%) than on-demand (23%).

Conclusions: Parents responding to the HERO survey provided interesting insights into differences between care of children and adult with haemophlia. While children with higher bleed frequencies were older, parental employment rates were similar irrespective of bleed frequency, the presence of inhibitors and treatment regimen. HTC utilisation was higher in children on prophylaxis vs. on-demand, suggesting perhaps closer monitoring; utilisation also increased in children with higher bleed frequency. Involvement of physiotherapy and social workers in comprehensive care was less than expected.

PO 123

Potential relationships between negative impacts on employment or relationships with haemophilia centre/provider utilisation: An analysis of parents of children with haemophilia from the HERO study

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Background: Psychosocial issues affecting children with haemophilia, their parents and unaffected siblings include negative parental experiences with employment and, for both parents and patients, difficulties with relationships with friends and siblings. The relationship amongst these issues and healthcare utilisation is not well known.

Aims: To describe the relationship of utilisation of haemophilia treatment centres (HTCs) and healthcare professionals with parent-reported negative experiences with employment and parent/children/sibling relationships in the Haemophilia Experiences, Results and Opportunities (HERO) Study.
Methods: A post-hoc descriptive analysis of data from parents of children with haemophilia < 18 years from 8 countries with home treatment (Argentina, Canada, Germany, France, Italy, Spain, UK and United States).
Results: Of 453 parents (80% female), 268 reported a negative experience with work, 175 reported a negative experience telling friends about their son's haemophilia and 38 reported a negative impact on unaffected siblings. There was no difference in employment rate amongst parents who did/did not report negative experiences with work (73%). The majority (55%) of parents who reported a negative impact described it as small. Parents who reported negative experiences telling friends were less likely to be employed (63%) than those who did not (78%) and were more likely to report a considerable or a little negative impact on siblings (28% vs. 18%). Parents with negative experiences at work reported higher mean/median HTC visits/year (6.3/3 vs. 4.1/2 without). Social workers were involved in the management of 26% of children whose parents reported a negative impact on siblings. Social workers were more frequently involved with children whose parents reported negative experiences with work (30% vs. 24%) and telling friends (33% vs. 25%). Nurse involvement was higher in those reporting negative experiences with work (71% vs. 64%) and telling friends (74% vs. 64%). The frequency of counsellor/psychologist involvement was similar amongst parents who did/did not report negative experiences with work or telling friends. More parents with negative experiences with work (22% vs. 12%) or telling friends (25% vs. 13%) reported treatment for psychological conditions for themselves, their partners or their sons in the previous 5 years. Psychological support was more frequent for their sons (work: 24% vs. 14%; friends: 25% vs. 17%) and for the parent or partner (work: 31% vs. 24%; friends: 33% vs. 24%). Ratings of helpfulness of support were similar across those with/without negative experiences.
Conclusions: For parents, there was no difference in the employment rate of those with or without negative impact on work, and the majority who reported a negative impact described it as small. Those with negative experiences telling friends were more likely to report negative impacts on unaffected siblings. HTC visits were higher when parents had negative experiences with work. Social workers were slightly more frequently involved in management of children whose parents reported negative experiences with work or telling friends. Slightly more parents with negative experiences reported treatment for psychological conditions, including support for their son or themselves/partners. Further studies might assess negative experiences and their temporal relationships with HTC visits and feedback.

PO 124

Potential relationships between negative impacts on employment or relationships and haemophilia treatment centre/provider utilisation: an analysis of adults with haemophilia from the HERO study

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Background: Psychosocial issues facing people with haemophilia include dealing with negative experiences with employment, relationships and intimacy, and with other adults. The relationship amongst these issues to healthcare utilisation is not well known.
Aims: To describe the relationship of haemophilia treatment centres (HTCs) and healthcare professional utilisation with negative experiences with employment, relationships and intimacy amongst adults with haemophilia in the Haemophilia Experiences, Results and Opportunities (HERO) Study.
Methods: A post-hoc descriptive analysis of data from adults ≥ 18 years from 8 countries with home treatment (Argentina, Canada, Germany, France, Italy, Spain, UK and United States).
Results: Of 515 adults, 393 a reported negative impact on work, 158 reported a negative impact on relationships and 123 reported a negative experience telling friends about their haemophilia. Of the 265 adults in long-term relationships, 135 reported impacts on intimacy. Mean/median ages were similar amongst adults with and without negative experiences. Adults who reported negative impacts on work were less likely to be employed (59% vs. 80%); of the 393 adults reporting a negative impact, the impact was reported as very large (25%), moderate (34%) and small (41%). More adults reporting negative impact on work (32% vs. 25%) sought HTC advice on employment. Adults reported HTC advice on employment as helpful (mean/median 4 on a 1-to-5-point scale), although this was lower for those reporting a negative impact on work (mean 3.8 vs. 4.6 without). Adults with negative experiences with relationships and telling friends were more likely to have sought advice on employment. Adults reporting negative impacts on relationships were likely to have also had negative impacts on intimacy (30% vs. 12%) and telling friends (39% vs. 27%). Adults reporting negative experiences telling friends were likely to report negative impacts on relationships (33% vs. 22%) and intimacy (32% vs. 18%). Adults reported HTC advice on intimacy as helpful (mean/median 3.9/4). Adults reporting impacts on intimacy were more likely to have had discussions on intimacy with the HTC (37% vs. 20%), but were less likely to report this discussion as very helpful (28% vs. 48%). Median number of HTC visits per year was 2, regardless of negative experiences. Nurses were more frequently involved in haemophilia management for adults with negative experiences with work (56% vs. 41%), relationships (55% vs. 52%), telling friends (61% vs. 50%) and intimacy (70% vs. 44%); social worker involvement was more frequently reported by those with negative experiences (work, 24% vs. 8%; relationships, 23% vs. 19%; telling friends, 33% vs. 16%; intimacy, 29% vs. 11%). Counsellor/psychologist involvement was infrequently reported (15%), including in those with negative experiences. Slightly more adults with negative impacts on relationships (34% vs. 22%) and negative experiences telling friends (32% vs. 24%) reported being treated for psychological conditions.
Conclusions: Most adults found advice from HTCs helpful, and those with negative experiences were more likely to have seen an HTC nurse, social worker or counsellor/psychologist. More specific studies assessing the temporal relationship with HTC visits/advice and negative experiences may guide better resource utilisation.

PO 125

Relationship of quality of life, pain and self-reported arthritis with activity, bleed rate and haemophilia treatment centre/provider utilisation: results from the HERO study

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Background: Haemophilic arthropathy results in varying degrees of pain and disability. The Haemophilia Results, Experiences and Opportunities (HERO) study examined health-related and psychosocial issues impacting adults with haemophilia (adults) and associated effects on quality of life (QoL).

Aims: To examine potential relationships amongst QoL measures, pain interference and self-reported arthritis and age, employment, activity, bleed frequency, and haemophilia treatment centre (HTC) and healthcare professional utilisation.
Methods: Adults ≥ 18 years completed a 5-point Likert scale on pain interference over the prior 4 weeks, a health-related visual analog scale (VAS, 0–100, coded as an 11-point categorical response) and a standard QoL assessment (EQ-5D-3L mobility, usual activities, self-care and pain/discomfort). Data from 8 countries with home treatment (Argentina, Germany, France, Italy, Spain, UK and United States) are presented.
Results: Overall, 515 adults (236 with self-reported arthritis) responded. Adults with self-reported arthritis were older (median 41 vs. 35 years); median age increased with progressive disability and worsening pain. The percentages reporting full-time, part-time or self-employment declined with increasing disability (EQ-5D self-care none/moderate/unable: 73%/53%/36%) and pain interference (none or a little/moderate/a lot or extreme: 73%/67%/40%); among adults with arthritis, the percentage employed was lower (57% vs. 69% unemployed). The percentage self -reporting arthritis incompletely correlated with 'moderate' functional disability on EQ5D (self-care 61%, usual activities 70%, mobility 63%, pain/discomfort 49%) and pain interference (a lot/extreme 66%). The percentage reporting 'good' EQ-5D VAS scores of 80–90–100 declined with increasing pain/disability as indicated by the EQ-5D mobility (none/moderate/unable: 56%/17%/50%), usual activities (39%/8%/0%), self-care (52%/10%/7%) and pain/discomfort (none/moderate/extreme: 63%/26%/14%), pain interference (54%/19%/10%) and presence of arthritis (no/yes: 43%/22%). Median annual bleed rates increased with increasing pain/disability on EQ-5D mobility (2/7/203), usual activities (5/13/7), self-care (3/8/38) and pain/discomfort (2/6/18), pain interference (3/6/10) and self-reported arthritis (3/10). Adults with disability/pain on EQ-5D reported a higher mean but only slightly higher median HTC visits per year; there was little difference even in mean visits across pain interference categories and between those with and without arthritis. The percentage of adults reporting a lot/extreme pain interference was generally higher in those with more disability as reported by all 4 EQ-5D domains, and in those with arthritis. Adults reporting difficulties with self-care and pain/discomfort, pain interference or arthritis were more likely to report nurse involvement (self-care: 45%/60%/79%; pain/discomfort: 49%/51%/67%; pain interference: 48%/52%/61%; arthritis: 45%/61%) as well as social worker involvement (self-care: 16%/23%/50%; pain/discomfort: 14%/20%/32%; pain interference: 14%/25%/28%; arthritis: 14%/27%) in their haemophilia management. In contrast, physiotherapist utilisation was only moderate and did not change much with disability (self-care 32%/44%/43%), pain (pain/discomfort 39%/35%/44%) or arthritis (34%/41%).
Conclusions: Increased disability and pain were associated with increased age, lower employment, more bleeds and slightly more HTC visits. Whereas nurse and social worker involvement in haemophilia management increased with disability/pain and the presence of arthritis, physiotherapist utilisation was moderate and fairly similar regardless of the extent of disability/pain or presence of arthritis. Additional physiotherapy involvement or follow-up might be helpful in those with haemophilic arthropathy; this could be evaluated in a prospective study.

PO 126

Does higher risk activity change bleed frequency and haemophilia treatment center/provider utilisation in children with haemophilia: an analysis from the HERO study

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Background: Low- to moderate-risk physical activity is recommended in people with haemophilia; however, relationships amongst activity risk level, treatment regimen, presence of inhibitors, and haemophilia treatment center (HTC) and healthcare professional utilisation have not been investigated.
Aims: To examine associations between activity risk level and HTC and healthcare professional utilisation in children with haemophilia (children), either with (WI) or without (WOI) inhibitors, taking treatment (on-demand vs. prophylactic) regimen into account.
Methods: A post-hoc descriptive analysis of data from parents participating in the Hemophilia Experience, Results and Opportunities (HERO) Study from 8 countries with home treatment responding about their oldest affected children < 18 years old.
Results: Overall, 453 parents responded; mothers accounted for 81% of parents of children WOI and 71% of parents of WI. Most (n = 391) children described had no inhibitors. Overall, 311 children WOI and 24 WI were on prophylaxis. For children WOI, most reported participating in medium-risk activities for both on-demand (lower/medium/higher: 26%/44%/30%) and prophylaxis (21%/45%/34%). Amongst the smaller number of children WI, activity was mostly reported as lower or medium-risk (on-demand: 57%/29%/14%; prophylaxis: 42%/46%/13%). Median ages (years) of children WOI and WI were 10 and 12 years, respectively. Median age generally increased with increasing activity risk amongst children, both WOI (on-demand: 2/12/11; prophylaxis: 4/11/9) and WI (on-demand: 12/10/17; prophylaxis: 13/11/13). The median number of bleeds/year was similar amongst children WOI with increasing activity risk levels (on-demand: 2/5/3; prophylaxis: 3/3/3); in WI, median annual bleed frequency decreased with increasing activity in those receiving on-demand (15/7/2) but increased in WI on prophylaxis (1/10/12). Median HTC visits/year were generally lower with higher-risk activity amongst children WOI (on-demand: 3/3/2; prophylaxis: 4/2/2) but varied for WI (on-demand: 3/5/2; prophylaxis: 6/4/4). Amongst children WOI, nurse involvement in haemophilia management increased with higher activity risk in those on prophylaxis (65%/78%) but decreased in those on on-demand (81%/67%); the same was seen for social work involvement (prophylaxis: 24%/41%; on-demand: 38%/25%). Physiotherapy involvement increased with increasing activity risk in children WOI on prophylaxis (30%/30%/42%) but not in those on on-demand (24%/23%/21%); physiotherapist visit frequency was similar. Median perceived disease control (0 = not at all to 10 = well controlled) was similar across activity risk levels for children both WOI (on-demand: 8/9/8; prophylaxis: 9/9/9) and WI (on-demand: 6/10/7; prophylaxis: 8/8/8).
Conclusions: More common use of prophylaxis treatment in the children with or without inhibitors in HERO allows for broader assessment of relationship of activity risk level to bleed frequency and HTC and healthcare professional utilisation at the expense of describing the on-demand population. As expected, increasing activity risk was reported with older children. In children WOI, bleed frequency was generally unchanged and HTC visits generally reduced with higher-risk activities; data were more variable in the small group with inhibitors. Irrespective of treatment regimen and presence of inhibitors, parents perceived good disease control regardless of activity risk.

Prospective studies are needed to better understand the relationships between activity, bleeding and HTC monitoring, and the potential impact of prophylaxis compliance on bleed frequency.

PO 127

Do bleed rates and haemophilia treatment centre/provider utilisation vary with physical activity risk in adults with haemophilia: An analysis from the HERO study

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Background: Lower- to moderate-risk physical activity is recommended in adults with haemophilia; however, relationships amongst activity risk level (accounting for treatment regimen and inhibitors) and haemophilia treatment centre (HTC) and healthcare professional utilisation have not been investigated.

Aims: To examine associations between activity risk (lower/medium/higher) and HTC and healthcare professional utilisation in adults, taking absence (WOI) or presence (WI) of inhibitors and treatment (on-demand vs. prophylactic) regimen into account.

Methods: A post-hoc descriptive analysis of data from adults ≥ 18 years participating in the Hemophilia Experiences, Results and Opportunities (HERO) Study from eight countries with home treatment.

Results: Overall, 515 adult (431 without inhibitors) responded, with treatment mainly divided between on-demand (n = 180) and prophylaxis (n = 203). The distribution of activity risk was shifted toward higher-risk activities for adults WOI on prophylaxis (lower/medium/higher: 44%/40%/16%) vs. on-demand (50%/40%/10%). While there were few total numbers of adults WI reporting moderate- to higher-risk activities, a similar shift was noted for those on prophylaxis (72%/17%/10%) vs. on-demand (82%/15%/3%). Adults reporting higher-risk activities tended to be younger (median age: 41/36/31 years), except for adults WI on on-demand (median age: 36/31/41 years). Employment rates were higher in those reporting higher-risk activities (57%/68%/77%), regardless of treatment regimen or inhibitor status. For adults WOI on prophylaxis, mean bleeds/year were greater with higher-risk activity (10/14/29); however, median values and interquartile ranges were similar across risk groups. Data were more variable for adults WOI on on-demand and for adults WI. Regardless of treatment regimen, adults WOI reporting higher-risk activities were more likely to report no/little pain interference in the prior 4 weeks (68% prophylaxis, 71% on-demand) than those reporting lower-risk activity (45% prophylaxis, 47% on-demand). Mean/median number of HTC visits/year did not differ by activity risk level. In adults WOI on prophylaxis, nurse and social worker involvement in haemophilia management increased with higher-risk activity (42%/64%/68% and 13%/27%/21%, respectively); in contrast, physiotherapist involvement was similar regardless of activity risk level (37%/39%/43%). For adults WOI on on-demand, nurse/physiotherapist involvement declined and social worker involvement increased with increasing activity risk. Data were more variable for adults WI.

Conclusions: Activity is encouraged for adults as a means to maintain joint function and overall health. However, the interaction between activity risk level, treatment regimens and inhibitors is complex, even when assessed in large global data sets like HERO. While the HERO data suggest adults WOI engaged in higher-risk activities are younger, more often employed and more often on prophylaxis, the associations are more difficult to study in those WI even in this large dataset. While activity risk did not seem to significantly impact HTC visit frequency, we observed that those WOI on prophylaxis doing higher risk activities may have higher bleeding rates and may be more likely to report nursing and social work support to management of their disease. More complex multivariate analyses of the HERO data and additional focused prospective research may help to further investigate these interrelationships between activity, bleeding and HTC monitoring.

PO 128

Association of treatment regimen and location with bleed frequency, quality of life and comorbidities in adults and children with haemophilia from the HERO study

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Background: Congenital haemophilia with inhibitors or without inhibitors is primarily treated with on-demand factor replacement or prophylaxis, administered at home or at a haemophilia treatment centre (HTC). Little is known about the relationships of treatment regimen and location with bleed frequency, quality of life (QoL) or comorbidities in haemophilia.

Aims: To examine the associations of treatment regimen, treatment location, bleed frequency, QoL indicators and comorbidities in adults with haemophilia (adults) and bleed frequency in children with haemophilia (children) from the Haemophilia Experiences, Results and Opportunities (HERO) Study.

Methods: A post-hoc analysis of data from adults ≥ 18 years and parents of children < 18 years. QoL in adults was assessed using EQ-5D-3L.

Results: Overall, 648 adults responded and were treated with factor concentrates. Most were treated with either on-demand (n = 289) or prophylaxis (n = 207), with fewer reporting on-demand plus situational prophylaxis (n = 146); 6 responded they did not know or declined to answer. Of 79 adults with inhibitors who responded on treatment, most were treated with either on-demand (n = 43) or prophylaxis (n = 29), with few reporting on-demand plus situational prophylaxis (n = 7). Most adults were treated primarily at home (76%), with 50% treated 'always at home' and 26% treated 'mostly at home but sometimes at the HTC'. Overall, mean/median annual bleed frequency did not differ amongst adults receiving on-demand (13.9/5.0) vs. prophylaxis (14.8/5.0); for those treated on-demand plus situational prophylaxis, mean/median was 28.9/20.0. Mean/median annual bleed frequency was higher for adults treated at home (20.0/8.0) than at the HTC (10.8/4.0). More adults treated at home reported any comorbidity (home: 85.9%; HTC: 73.9%) and, specifically, arthritis (home: 55.0%; HTC: 28.8%). Likewise, more adults with inhibitors treated at home reported any comorbidity (home: 100%; HTC: 81.5%) and arthritis (home: 59.6%; HTC: 14.8%). Overall, fewer adults treated with prophylaxis (prophylaxis: 36.2%; on-demand: 47.8) or at home (home: 37.5%; HTC: 54.9%) reported no issues with EQ-5D mobility, as did adults with inhibitors treated at home (home: 32.7%; HTC: 74.1%). More adults receiving on-demand reported no pain/discomfort (on-demand: 32.9%; prophylaxis: 20.8%); there was no difference attributable to treatment location. Overall, there were no differences in mean/median EQ-5D index by treatment regimen (on-demand: 0.750/0.800; prophylaxis: 0.747/0.778) or location (home: 0.736/0.778; HTC: 0.753/0.800). However, adults with inhibitors treated at home had a lower mean/medianEQ-5D index (home: 0.687/0.764; HTC: 0.760/0.816). Overall, 547 parents of children treated with factor concentrates rsponded; most children were treated with prophylaxis (prophylaxis: n = 356; on-demand: n = 143) and at home (home: n = 397; HTC: n = 146). Mean/median annual bleed frequency was lower for children treated with prophylaxis (6.2/3.0) than on-demand (12.0/6.0). Bleed frequency did not differ by treatment location in children.

Conclusions: Whereas bleed frequency did not differ by treatment regimen in adults, children on prophylaxis reported a lower annual bleed rate than for children on on-demand. Amongst this nonhomogenous patient population, adults who were treated at home and received prophylaxis were the most frequent bleeders and more difficult to treat. In HERO, patients and parents reported treatment modalities and HERO is not designed to evaluate the different treatment regimens.

PO 129

Association between treatment regimen and quality of life assessed by EQ-5D-3L and pain interference in adults with haemophilia with and without inhibitors in the HERO study

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Background: Treatment options for haemophilia A (HA) and B (HB), with or without inhibitors, include on-demand and prophylactic factor replacement. Little is known about the relationship between treatment regimen and quality of life in adults with haemophilia, with and without inhibitors.
Aims: To examine the association between treatment regimen and quality of life in adults with and without inhibitors from the Haemophilia Experiences, Results and Opportunities (HERO) Study.
Methods: A post-hoc analysis of data from adults ≥ 18 years from 10 countries participating in the HERO Study. Adults completed a standard EQ-5D-3L assessment; EQ-5D index was calculated based on Shaw et al, 2005.
Results: In total, 675 adults completed qualitative and quantitative questions on quality of life and well-being. Most adults reported HA (498), 86 reported HB and 91 reported HA/HB with inhibitors. Half (n = 45) of those reporting inhibitors were from the US. Adults with inhibitors were younger (median/maximum age 34/69 years) than those without inhibitors (36/86 years). At a global level, treatment was split between on-demand (289, 43%) and prophylaxis (207, 31%); fewer reported on-demand plus situational prophylaxis (146, 22%). This was similar for adults with inhibitors (on-demand 43 [47%], including 20 US; prophylaxis 29 [32%], including 22 US; on-demand with situational prophylaxis 7 [8%]). Most adults (598, 89%) and adults with inhibitors (86, 95%) reported pain had interfered with their daily life in the past 4 weeks; of those, 301 (50%) reported constant pain (38 [44%] with inhibitors). Mean EQ-5D index for adults with inhibitors was 0.707; it was 0.745 for HA and 0.741 for HB. Median EQ-5D index was the same for HA, HB and adults with inhibitors (0.778). Mean EQ-5D for adults on prophylaxis was 0.707, 0.712 for adults on-demand and 0.731 for adults treated on-demand with situational prophylaxis. Almost half reported no issues with mobility (HA: 41%; HB: 38%; inhibitors: 44%). Most adults reported no issues with usual activity (HA: 81%; HB: 79%; inhibitors: 74%). For adults with inhibitors, the percentage reporting no issues with usual activity was similar across treatment regimens (on-demand: 72%; prophylaxis: 79%; on-demand with situational prophylaxis: 71%). Approximately half reported no issues with self-care (HA: 56%; HB, 49%; inhibitors: 43%) or anxiety/depression (HA: 54%; HB: 53%; inhibitors: 54%). Few denied any pain/discomfort (HA: 26%; HB: 28%; inhibitors: 19%). Self-care is the only index that shows a significant difference amongst the types of haemophilia. Statistical comparison for mobility and usual activity was not possible due to the small number who responded to each question.
Conclusions: Globally, adults with inhibitors in HERO had a lower mean EQ-5D index and more frequently reported problems in individual EQ-5D domains than those without inhibitors. While differences in age and treatment regimen across participating countries limit descriptive analysis, multivariate analyses may yield further insights. The underlying reasons for selecting treatment regimens (e.g. pain vs. bleed control, desired activity participation, employment) likely also impact quality of life and need to be factored into future studies.

PO 130

Patient survey – Pain therapy in haemophilia in Germany

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Background: Pain management for patients with haemophilia is assumed to be highly relevant. As a result from the disease and its complications, patients with haemophilia often suffer from pain, which has to be treated adequately. One challenge, among others, is the selection and application of appropriate analgesics.
Aims: We have conducted a patient survey on 'pain therapy in haemophilia', which aims to evaluate the current situation of pain management for patients with haemophilia and to identify potential areas for improvement.
Methods: A pain management survey was developed in a joint effort by physicians, patient representatives and Bayer. In cooperation with two major patient organisations in Germany, the Deutsche Hämophiliegesellschaft (DHG) and the Interessengemeinschaft Hämophiler (IGH), about 1.960 copies were sent to patients with haemophilia A and B. Parents of children with haemophilia were requested to fill in the questionnaire on their behalf. Questions concern the types of pain, frequency and severity, medical specialists consulted, types of pain therapies, and the patients' level of satisfaction with the pain management.
Results: The survey started in September 2012 and was completed by the end of October. Six hundred and eighty-five questionnaires were filled in and sent back, i. e. a 35% response rate. Preliminary results show that 86% of respondents experienced episodes of pain. About half of the patients have used physiotherapy as a measure of pain management. Joint pain is the most common type of pain, remarkably so even in young patients. The comprehensive evaluation of the patients' view is underway. It is expected to contribute important insights on the current pain management situation in haemophilia, thus helping to identify factors for improvement.
Conclusions: Patient and physician surveys are powerful tools, which we have used for the assessment of the current situation in pain management for patients with haemophilia. Following the complete evaluation of results, measures for optimization will be developed in order to improve the long-term care of patients with haemophilia.

PO 131

An experience of use of Traumastem P in control of spontaneous mucosal bleeding in patients with inherited bleeding disorders in Southern Iran

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Background: Bleeding complications are the most important complications in patients with inherited bleeding disorders. Use of local haemostatic agents could help to decrease blood loss, need to blood transfusion, systemic coagulation therapy and consequently duration of hospital stay especially in patients with inherited bleeding disorders due to more susceptibility of bleeding.

Aim: We evaluated the safety and efficacy of Traumastem P for the management of spontaneous mucosal bleeding in patients with congenital bleeding disorders.
Methods: In this case series, 12 patients with inherited bleeding disorders who were complicated by nose or mouth bleeding were investigated from May to August 2012 in southern Iran. Our participants selected from the patients who were registered at the Hemophilia center of Shiraz University of Medical Sciences. Traumastem P (Emoxicel Polvere)(Bioster company, Czech Republic) was used in all patients as hemostat agent without factor infusion. Response to this haemostatic powder was evaluated in patients. Complete stop bleeding in a 1–4 min period was considered as good response, stop bleeding in a 4–30 min period was considered as fair response and not stop bleeding as no response. Patients were followed for any adverse reactions till 72 h after administration. The study protocol was approved by the Ethics Committee of Shiraz University of Medical Sciences. Informed consent was obtained from all patients or the parents of children.
Results: Participants consisted of five female and seven male; six patients with hemophilia type A, two Factor VII deficiency, two von Willebrand disease (vWD) type III, one vWD type I, and one glanzmann's thrombasthenia. Mean age of the patients was 13.4 ± 9.9 years with median age of 10.5, and range of 1–34 years. From eight hemophilia patients, five had severe (< 1%) factor deficiency. The Level of factor deficiency in other patients was ranged between 3.5% and 26.8%. Age at diagnosis of the patients ranged from at birth to 20 years old. All patients came with nose bleeding except one with mouth bleeding. All patients showed good response to Traumastem P except one with fair response; a 34-year-old man with severe hemophilia A and complicated by mouth bleeding who had been diagnosed at birth.
Conclusion: Traumastem P can use as a safe and effective local hemostatic agent for control of nose bleeding in patients with inherited bleeding disorders. Subsequently it could be an economically valuable hemostatic agent in these patients. Further studies are needed to evaluate the effect of Traumastem P for control of other site of bleeding in these patients.

PO 132

Areas of concern for caregivers of children with hemophilia A and B: results of a cross-sectional survey in the US

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Background: Hemophilia is a bleeding disorder resulting from reduced or absent clotting factors. It is a life-long condition characterized by recurring joint bleeds that can lead to chronic pain. Treatment of bleeds and pain in children are typically managed at home by parents or other relatives (i.e. caregivers), who may be affected by the management of the disorder. Previous research with hemophilia caregivers suggested that caregivers are affected by emotional stress, financial issues, personal sacrifice, medical management, child's pain and transportation issues when caring for children with hemophilia.
Aims: To quantify the overall burden of hemophilia on caregivers and to identify specific areas of concerns.
Methods: A caregiver opt-in-research database was used to invite 681 caregivers to complete an on-line survey. The survey included 64 questions on caregiver demographics, six burden domains (emotional stress, financial, sacrifice, medical management, child's pain, and transportation), and three visual analogue scales (VAS). The three VAS scales (0–10) measured overall burden, current child's pain and burden when seeing child in pain. For each survey question, respondents were asked to rate their burden on a scale from 1 to 5 where '1' represented 'never burdened' and '5' represented 'nearly always' burdened. Survey questions were reviewed with three caregivers prior to implementation. IRB approval was obtained.
Results: Of the 681 caregivers invited, 310 (45.5%) caregivers completed the on-line survey. Most respondents were mothers (88%) between 18 and 54 years; 73.9% of respondents reported taking care of 1 child with hemophilia. Most children (86.45%) had been diagnosed with hemophilia A and (85.81%) had been diagnosed more than 2 years ago. On a scale from 0 to 10, the mean level of burden felt by caregivers when a child is in pain was 7.54 (SD 2.62) and the most common response (29.7%) was a '10'. Of the six survey domains, 'Child's pain' was the most burdensome (mean = 3.54 out of 5). Fifty-nine percent and 77% of caregivers reported they were 'quite frequently to nearly always' burdened with worrying about the child's joint and bleeding pain and wishing they can take their child's pain away, respectively. Emotional stress was the second most burdensome domain (mean = 2.79); 51% reported they 'quite frequently' or 'nearly always' are concerned with the child's future when they are not around. Other areas of concern identified by those reporting they were 'quite frequently' or 'nearly always' burdened included: extra planning involved when traveling (42%), rising co-payments over time (33.5%), accessing child's vein or port (24.5%), and the expectation that life would be different at this age (22.6%) .
Conclusions: Hemophilia caregivers are impacted by the burden of their child's pain. Parents also report concerns with the child's future, venous access, travel planning, and rising co-payments. Recognizing these factors is important when considering educational programs to lessen caregiver burden. Future research that incorporates these findings into the formal validation of an instrument to assess caregiver burden should be explored.

PO 133

Immune tolerance induction in children with severe hemophilia A and inhibitor in Poland

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Background: The development of factor VIII inhibitors (FVIII) is the most serious complication in patients with haemophilia today. The highest risk of developing inhibitors is during the first 20 exposure days. First-line treatment for patients with haemophilia A and inhibitors is to attempt to induce immune tolerance by regular infusion of FVIII.
Aims: The results of immune tolerance induction (ITI) were presented in this study.
Methods: In 1993–2012 ITI were used in 31 children with severe hemophilia A and high titer of FVIII inhibitor in Poland. Inhibitors were measured according to Bethesda method in Nijmegen modification.
Results: The median age of patients was 18 months (ranged from 5 to 81 months) in the time of inhibitor detection. Inhibitor was detected within 3 to 100 exposure days to FVIII concentrates (median 19 exposure days). Median of maximal titer of FVIII inhibitors found before ITI start was 40 and ranged from 3.8 to 1024 BU/ml. Titers of FVIII inhibitors ranged from 3.1 to 151 BU/ml (median 16 BU/ml) at the initiation of ITI regimen. Most patients (23) were treated with plasma derived FVIII concentrates, medium or high-purity, seven patients with recombinant FVIII concentrates and one with cryoprecipitate before inhibitors were detected. In ITI usually the same type of concentrate was used. In 23 children ITI was started with single daily dose

of ≥ 100 IU/kg, in two patient daily dose of 50 IU/ml was given and in four children FVIII concentrate in a dose of 100 IU/kg was administered twice a day. In the 2 remaining patients doses of 50 IU/kg 2–3 times a week were repeated. Total elimination of factor VIII inhibitor was achieved in 22 patients. In the other two patients the ITI was partially effective and the recurrences of low titer inhibitor, below 1 BU/ml was observed. In another two patients immune tolerance was not achieved and ITI regimen was discontinued after 15 and 18 months. ITI is ongoing in five patients. Duration of ITI ranged from 2 to 83 months (median 12 months). In patients with positive results the median of ITI was 10.5 months. One boy started second procedure of ITI in 9 months after failure of first one. It was partially effective after 42 months but several doses of rituximab were added. The other patient with ITI evaluated as partially effective was treated with rituximab as well. After achievement of complete ITI, prophylaxis with FVIII concentrates was continued 2 or 3 times a week in dose of 20–50 IU/kg. Only 1 boy was treated on demand.

Conclusion: High efficacy of ITI was found in young children with severe haemophilia A.

PO 134

A systematic review of treatment patterns in Japanese haemophilia patients

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Background: Literature detailing the clinical patterns of haemophilia treatment and the economic and humanistic burden in Japan is scarce. This is the first review attempting to address the aforementioned issues.

Aims: To conduct a systematic review of epidemiology, clinical practice, economic and humanistic burden of hemophilia in Japan.

Methods: A systematic literature search was conducted to identify existing literature with clinical patterns of haemophilia and the economic and humanistic burden of haemophilia in Japan. We searched MEDLINE and JDream II using 'haemophilia' and 'Japan' as key words. The search included publications from January 2002 to October 2012 with both English and Japanese. JDream II was searched in Japanese. We also searched reports and presentations from professional associations and government agencies for grey literature.

Results: We identified 318 publications at the first round, and after removing duplicates and irrelevant articles, 50 were included in this systematic review. In a population-based report by the Japan Foundation of AIDS Prevention demonstrates that the prevalence of haemophilia in Japan is low with 4475 hemophilia A cases and 971 hemophilia B cases in 2011 (approximately 0.004% overall). Population-based studies found that 43–59% of patients with haemophilia are treated with prophylaxis. Of patients under 20 years old with severe and moderate haemophilia, 60–76.7% were on prophylaxis. Reported home treatment rate was high (66.3–83.5%), administered by either by patients themselves or parents, but poor adherence based on short dosing interval was reported among adolescents (aged 13–19 years). While prophylaxis and home-infusion are accepted as standard of care in most young and severe patients, patients preferred on-demand vs. prophylaxis. Search results revealed very little research focusing on humanistic and economic aspects for haemophilia.

Summary/Conclusions: This is the first systemic review on clinical patterns and utilization of haemophilia treatment in Japan. Clinical practice patterns are found to be very similar to the West with regard to recommending prophylaxis and offering convenient home treatment to patients. Nonetheless, adherence to prophylaxis regimens among adolescents and young adults is relatively low. Patient education and the options for new therapeutic regimens offering shorter dosing-interval should be explored further as they may aid in deterring long term consequences such as the development of target joints, arthropathy, and poor quality of life.

PO 135

Treatment strategy and outcomes among US haemophilia patients: results of a patient survey

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Background: Haemophilia can be treated on an as-needed basis with episodic (on-demand) therapy or routinely with prophylaxis. Those treating on-demand may have more bleeding episodes relative to those on prophylaxis, poorer health outcomes, and greater use of healthcare resources.

Aims: To assess the relationship between use of on-demand vs. prophylactic treatment with patient-reported outcomes and adherence in adult US haemophilia patients.

Methods: Adults with haemophilia were identified through a panel of patients originally recruited from haemophilia treatment centers and associations. The study protocol received central institutional review board approval and all patients reported consent to be included in the study. Panelists reporting moderate or severe haemophilia were invited to complete an on-line questionnaire assessing experience with bleeding and joint problems, personal characteristics, and validated scales. The revised Short Form-12 Health Survey (SF-12v2) was included to assess health status, and the Validated Haemophilia Regimen Treatment Adherence Scale-Prophylaxis (VERITAS-Pro) was included to assess adherence to prophylactic treatment. Those using prophylactic treatment were compared to those treating on-demand using *t*-tests for continuous measures and chi-square for categorical variables. Comparisons were first conducted on a combined sample of A & B patients, and repeated among only those with haemophilia A. Limited sample sizes prevented separate analysis of patients with haemophilia B.

Results: A total of 76 patients with haemophilia (74 male, 2 female; 62 with haemophilia A, 14 with haemophilia B) completed the survey, with a mean age of 35.9 years, with most (62%) using prophylactic treatment for haemophilia. Relative to patients using prophylactic treatment, those treating on-demand were older (40.8 vs. 32.5 years, $P < 0.05$) and had lower physical component summary (PCS) scores (41.5 vs. 48.6, $P < 0.05$). These patients also reported more bleeding episodes in the past year 23.2 vs. 10.7, $P < 0.05$). Analyses limited to patients with haemophilia A (60 male, 2 female) yielded similar results mean age was 34 years, with most (68%) using prophylactic treatment. Haemophilia A patients using on-demand treatment were older (39.9 vs. 31.1 years, $P < 0.05$), had lower physical component summary (PCS) scores (39.1 vs. 48.9, $P < 0.05$), and more bleeding episodes in the past year (24.2 vs. 11.0, $P < 0.05$) vs. haemophilia A patients on prophylaxis. Only a minority of patients reported 'Always' doing prophylaxis infusions on scheduled days (38%), infusing the recommended number of times per week (38%), or adhering to the schedule provided by the treatment center (28%), though most (70%) reported always using the doctor-recommended dose.

Summary/Conclusions: Health status was worse and bleeding more common among moderate and severe adult haemophilia patients treated on-demand relative to those treated with prophylaxis, though most patients reported some non-adherence to the recommended treatment schedule. These conclusions support current clinical guidelines advocating prophylaxis as the current standard of care for haemophilia, though further efforts to improve patient adherence may be warranted.

PO 136

Regional factors influencing participation in clinical trials in hemophilia in the United States of America and South Africa

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Background: Clinical trials are the gold standard for evaluating medical interventions and developing best practices in clinical care. Multicenter, multinational trials are common, especially in rare bleeding disorders such as haemophilia. Regional differences in perceptions about health research and decision making when engaging in such research will affect enrolment. We hypothesized that there are regional cultural differences in health research participation in the bleeding disorders communities in the USA and South Africa (SA).

Aim: To elicit factors influencing participation in clinical trials in patients with haemophilia and other congenital bleeding disorders in the USA compared to SA.

Methods: This was a collaborative cohort study conducted in the USA (Comprehensive Louisiana Hemophilia Care Center) and SA (Comprehensive Haemophilia Care Centre, Johannesburg). Both sites obtained ethics approval. Adult patients and parents of pediatric patient with congenital bleeding disorders at both participating hemophilia centers were invited to participate in a one-time, anonymous, on-line survey (implied consent).

Results: Seventy four (53 patients, 21 parents) participants in SA and 64 (28 patients, 35 parents) in the USA completed the survey. More participants in SA classified their disease as severe (78 vs. 59%). There was a comparable family history of a bleeding disorder (57 vs. 59%). Slightly fewer subjects in SA had participated in clinical trials before (41% vs. 52%). Both sites felt that the doctor had the highest level of responsibility in clinical studies, but both also felt high levels of responsibly lay with the patient. When asked how they were protected during clinical trials, most subjects in SA felt it was themselves (80% vs. 50% in USA, $P = 0.0002$) and at both sites not all patient felt that they were protected by their physician (68% in SA and 55% in USA felt protected by their physician). At both sites the most important reason to participate in clinical studies was to contribute to research in the field, a free gift/money was least important. More subjects in SA thought that participation in research was safe/very safe (88% in SA vs. 69% in USA, $P = 0.0067$). At both sites, the subject was the primary decision maker (over the doctor, nurse and family members) to participate in study. While family and health care team played a major role in decision making, spiritual/religious considerations, friends and co-workers did less so. Significantly more subjects in SA were willing to engage in clinical studies: phase I (26% vs. 8%, $P = 0.0039$) and phase II (86% vs. 52%, $P < 0.0005$), phase III (88% vs. 50%, $P < 0.0005$), phase IV (94% vs. 69%, $P = 0.0001$)

Summary/Conclusions: While the motivation to participate in clinical studies was similar in both SA and USA, it appeared that there was less trust and likelihood for participation at the USA site. This site has a higher percentage of African American patients than many other US sites. Knowing the sensitive history of human protection of African American subjects in the US, this raised the question whether there could be racial factors influencing decision making. We are collecting further data to make this analysis.

PO 137

The use of central venous catheters in children with haemophilia in Poland

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Introduction: Central venous access devices (CVaDs) are essential in the management of haemophilia patients who need repeated urgent administration of coagulation factor concentrates. The use of a facilitates treatment of young children with difficult peripheral venous access.

Methods: Between 1997 and 2012 CVaDs were used in our department. Seventy-two CVaDs (port-a-cath) have been inserted in 53 patients aged 3 months to 18 years.

Results: Fifty-three patients had haemophilia A and 2 had haemophilia B. Twenty-one out of 53 had haemophilia A complicated by high-titre inhibitors and immune tolerance was used. In two patients CVaDs were inserted three times, in 11 patients these catheters were inserted twice and the remaining patients (44) they were inserted once. The period of vascuport use ranged from 2 to 103 months. CVaDs infections were observed 19 times in 12 children. All infected ports were successfully treated with antibiotics, thrombotic complications occurred in 7 boys 11 times. In 2 cases the ends of the CVaDs got torn and moved to the heart and in 2 other cases the reservoir stated leaking.

Discussion/Conclusion: Our investigations show that CVaDs are convenient in use but complications can occur. Central venous access devices are recommended for these children.

PO 138

Is venous or arterial thrombosis a clinical problem in patients with haemophilia? Experience from a Danish haemophilia centre

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Background: Theoretically, haemophilia patients are to some degree protected from arterial and venous thrombosis due to a hypocoagulable state. However, as life expectancy rises for these patients due to more effective treatment, with older age they can be faced with an increased risk of atherosclerosis, surgical procedures and cancer, which are all risk factors for thrombotic events. It could be feared that the risk of thrombosis in haemophilia patients is an underestimated and probably sometimes overlooked, and therefore the magnitude of the problem and the clinical implications are difficult to estimate. Thromboprophylaxis and treatmentof haemophilia patients with thrombosis is also a field where experience is limited, and approaches to treatment vary considerably.

Aim: The aim of the study was to examine the prevalence of arterial and venous thrombosis in the populations of haemophilia A and B patients. We furthermore reviewed how patients were treated medically in case of a thrombotic event and whether any bleeding tendency was seen in those patients.

Methods: We conducted a retrospective study, where medical records of all patients with haemophilia A and B registered at the Haemophilia and Thrombosis Centre, Aarhus University Hospital, Denmark from 1985 to 2011, were reviewed thoroughly and all the thrombotic events noted in the records were registered.

Results: Of 169 haemophilia A patients, 5 cases of arterial thrombosis, all in a coronary artery (3%), 3 cases of venous thrombosis (2%) and 2 cases of unclassified (eye vessel) thrombosis (1%) were found. Of 30 haemophilia B patients, 1 case of coronary artery thrombosis (3%), 1

case of cerebral artery thrombosis (3%) and no cases of venous thrombosis were found.
The antithrombotic treatment received differed considerably between cases, with acetylsalicylic acid and low molecular weight heparin as the most commonly used drugs. In 1 case, increased bleeding tendency was reported.
Summary/Conclusion: Arterial and venous thrombosis does occur and can be a clinical problem in hæmophilia patients, especially with co-existing risk factors. We found a prevalence of coronary artery thrombosis lower than the Danish male background population and comparable to that reported in the literature. Due to the small patient populations and the scarcity of literature in this field, however, it is not possible to characterize the magnitude of this problem. Further multicentre studies are needed, preferably prospective and cross-national.

PO 139

The importance of taking the *in vivo* recovery test as a routine evaluation for Hemophilia patients

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Background: As the replacement therapy evolves, the general management of hemophilia is getting more important nowadays. Having regular standardized evaluations including musculoskeletal issues, inhibitors, and transfusion transmitted infections, every 12 months, is mainly recommended by WFH guidelines for the management of hemophilia. However, through our precedent study, Individualized replacement of FVIII based on *in vivo* recovery (IVR) in hemophilia A patients during surgery, which was announced in the 17th congress of EHA, 2012, we found that it is very important to check IVR on all occasions and supply the coagulation factor VIII concentrates according to the result to maximize the therapeautic effect. Since then, we have done IVR test as a routine evaluation for all the hemophilia patients who are managed in the Department of Pediatrics, Kyungpook National University Hospital, Daegu, Korea.
Aims: Through this study, we analyzed the results of IVR tests to emphasize the necessity of IVR test.
Methods: Ten hemophilia A patients who had IVR test as a routine evaluation from January 2011 to December 2012 were reviewed. All patients were checked coagulation factor VIII activity four times respectively, before, 15 min, 30 min, and 60 min after the injection of factor concentrates. Green mono® or Advate® was used. Coagulation factor VIII activity was measured using the STA-Unicalibrator analyzed by dilution with STA-Owren-Koller® buffer based on the parameter entered in the instrument (DIAGNSTICA STAGO, France).
Results: Two patients were severe (FVIII:C < 1 IU/dL), five patients were moderate (FVIII:C 1–5 IU/dL), and the remainder were mild (FVIII:C > 5 IU/dL) hemophilia A. They were all males. Their mean age at the time of IVR test was 21 (range from 0 to 41) years old. The peak coagulation factor VIII activity level was median 72.0% (range from 43.4–138.0%), and the time at peak coagulation factor VIII activity level was median 25, six were over 80%, one was under 60% and the other three were between 60 and 80%. Five patients (50%) had under expected peak coagulation factor VIII activity level.
Conclusions: This study shows that all the patients has his own patterns on recovery test. Therefore, having regular IVR tests should be included to the routine evaluations for all the hemophilia patients to produce the better result in the treatment process.

PO 140

Clinical study to investigate the immunogenicity, efficacy and safety of treatment with human-cl rhFVIII in previously untreated patients with severe haemophilia A

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Background: *Human-cl rhFVIII* is a B-domain deleted, human cell-line derived recombinant FVIII concentrate for intravenous use, which will be provided in single use vials containing a nominal potency of 250, 500, 1000 or 2000 IU each of freeze-dried rFVIII concentrate to be reconstituted in 2.5 mL of WFI. Due to the absence of immunogenic epitopes seen in recombinant FVIII concentrates from hamster cell lines *Human-cl rhFVIII* is thought to be potentially less immunogenic.
Five prospective GCP studies with *Human-cl rhFVIII* were conducted in 135 adults and children with severe haemophilia A. In these studies pharmacokinetics, efficacy and safety of the product was evaluated. Observational period per patient was at least 6 months and at least 50 exposure days. The data indicate that Human-cl rhFVIII is bioequivalent to Kogenate FS (both analyzed by the one-stage and the chromogenic assay) and effective in the treatment and prophylaxis of bleeding episodes. There was no product related serious adverse event and none of the PTPs treated with *Human-cl rhFVIII* developed an inhibitor.
Aim: To assess the immunogenicity in previously untreated patients (PUPs), a prospective, multicentre, multinational, open-label, non-controlled clinical trial is planned in around 45 clinical centres worldwide, starting by the early 2013.
Methods: Hundred patients with severe haemophilia A without previous exposure to any FVIII concentrate or FVIII containing products will be enrolled.
An inhibitor assay according to modified Bethesda method will be performed pre-treatment, every 3–4 exposure days (ED 1–20) and every 10–12 EDs (ED 21–100) but at least every 3 months.
Secondary objectives are the assessment of the efficacy of *Human-cl rhFVIII* during prophylactic treatment (based on the frequency of spontaneous break-through bleeds), the assessment of the efficacy during treatment of bleeds, and in surgical prophylaxis. Also the safety and tolerability of *Human-cl rhFVIII* will be assessed. Furthermore pharmacooeconomic aspects of treatment with *Human-cl rhFVIII* will be assessed for the first time. The optional assessment of predictive factors for the development of inhibitors is included in the study protocol.
Results: The study is planned to start in Q1 2013.
Conclusion: Based on available data in PTPs *Human-cl rhFVIII* seems to be bioequivalent to a licensed full-length rFVIII and safe and effective in the treatment and prevention of bleeding episodes with no occurrence of an inhibitor. A global GCP-study is currently started in order to investigate whether the reduced immunogenic profile of Human-cl rhFVIII will translate into a lowered inhibitor incidence in PUPs.

PO 141

A new board game to assess coping and perception of children with hemophilia: validation and evaluation

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Background: How children cope with and perceive their disease is an important predictor of self-management and Health Related Quality Of Life (HRQOL). The Hemophilia Coping and Perception Test (HCPT) for children is a new tool (a board game) to assess coping,

perception and knowledge of children with hemophilia. The HCPT is based on the Asthma Coping and Perception Test (ACPT).

Aims: The aim of this study is to evaluate and validate the HCPT for boys with hemophilia between 8 and 12 years old in the The Netherlands.

Methods: The HCPT is a board game containing a comprehensive set of 33 questions about hemophilia, including knowledge, self-management, coping, anxiety and perception. Additional questions concern fun subjects or contain fun assignments. In this study, all boys from five hemophilia treatment centers in theNetherlands (Amsterdam, Groningen, Eindhoven, LeidenandRotterdam) with mild, moderate and severe hemophilia between 8 and 12 years old were eligible. To validate the HCPT, children and parents complete the HaemoQoL (Haemophilia Quality of Life questionnaire – short version), two coping questionnaires (CODI; Coping with a Disease and the Op Koers questionnaire) as well as the State-Trait Anxiety Inventory for Children (STAIC). After finishing the HCPT, children and parents respond to an evaluation questionnaire about the HCPT.

Preliminary results and hypotheses: So far, a total of 32 boys (response rate 50%) played the HCPT (mean age 9.8 ± 1.7). Both parents and children report the HCPT to be informative and entertaining. We expect the HCPT to be a valid and effective tool to detect problems regarding coping, disease management, anxiety and the perception of children with hemophilia and facilitate communication with children with hemophilia.

Summary: To be able to provide tailored health care, it is important to get insight in children's knowledge, coping and perception of their disease. With the use of this new board game it is possible to get insight in these aspects in a playful, child friendly way.

PO 142

Satisfaction with self-reported annual bleed rates within a severe hemophilia a population

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Background: Prospective clinical trials have demonstrated that Factor VIII prophylaxis can help patients with severe hemophilia A achieve or approach zero bleeding episodes per year. It is important to understand current bleeding rates outside of these clinical trials and to measure patient satisfaction with how frequently they are bleeding to identify opportunities to improve patient outcomes.

Aims: The objective was to compare patient self-reported ABR with their level of satisfaction with their current ABR.

Methods: Adult patients and parents of children (< 18 years old) with a self-reported physician diagnosis of severe hemophilia A in the United States were recruited and completed a web-based survey. The survey included questions regarding patient demographics, treatment regimen, ABR, and satisfaction with their ABR using a five-item Likert scale. ABR responses were sorted into five categories (0–1, 2–5, 6–11, 12–23, ≥ 24). Chi-square tests were used to test for statistically significant differences between ABR and satisfaction levels. All respondents provided online informed consent and the study complied with the Declaration of Helsinki, receiving ethics board approval.

Results: The final sample included 164 respondents. The average patient was 19.2 years old. One hundred and thirty-nine (85.8%) respondents reported that they currently receive prophylaxis (defined as infusions administered on an ongoing, regular schedule to prevent bleeds). The most frequently (57.1%) reported reason that the on-demand patients gave for not receiving prophylaxis was 'not bleeding enough to justify prophylaxis' and the mean ABR reported by these respondents was 10.4 (SD = 13.4). The mean reported ABR for all respondents was 7.8 (SD = 13.4). There were 44 (26.8%), 61 (37.2%), 29 (17.7%), 21 (12.8%) and 9 (5.5%) respondents who reported 0–1, 2–5, 6–11, 12–23 and ≥ 24 ABRs, respecitvely. Overall, 60 (36.6%) respondents were not satisfied with their self-reported ABR. Satisfaction with ABR differed significantly by ABR category ($P < 0.0001$). While 88.6% of those who reported zero to one ABR were 'very satisfied' with their bleeding rate, the percentage of respondents reporting they were 'very satisfied' significantly declined as ABRs increased (with 50.8%, 6.9%, 9.5% and 0% reporting 'very satisfied' with ABRs of 2–5, 6–11, 12–23 and ≥ 24, respectively). There was a statistically significant difference in satisfaction levels between the 0–1 and 2–5 ABR categories (each $P < 0.0001$). In those who reported having two bleeds or more per month (≥ 24 ABR), 66.7% reported 'neither satisfied nor dissatisfied' with the remaining third reporting either 'somewhat dissatisfied' or 'very dissatisfied'.

Conclusions: As would be expected, severe hemophilia A patients who reported zero to one ABR were highly satisfied with their bleeding frequency. Healthcare professionals should be aware that patient satisfaction was significantly lower for patients reporting ABRs between 2 and 5, implying that many of these patients may envision having fewer bleeds. Additionally, the majority of patients reporting an ABR ≥ 24 were not dissatisfied with their ABR. Healthcare professionals may consider engaging these patients regarding the consequences of a high ABR and ensure that these patients are familiar with recent clinical trial results that demonstrate the effectiveness of prophylaxis when patients are adherent to the regimen.

PO 143

Immune tolerance induction (ITI) according to the Bonn protocol in haemophilia A patients with inhibitors using a plasma-derived VWF-containing factor VIII concentrate: two paediatric case reports

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Background: ITI is the treatment of choice in haemophilia A patients with high titre factor VIII (FVIII) inhibitors. Whether the source of F VIII (plasma-derived-pd or recombinant-r) used for ITI has any impact on success is discussed controversially. We report on two paediatric haemophilia A patients with high titre inhibitors who underwent successful ITI using VWF containing F VIII for first line as well as a second line ITI.

Case 1: Due to a positive family history a severe haemophilia A was diagnosed in the first patient after birth. Genetic analysis revealed a splice site mutation which is associated with a low risk to develop inhibitors. Since the age of 7 months the boy had several exposures to rFVIII due to bleeds and surgery (herniotomy). After 23 FVIII exposures he developed a high-titre inhibitor (maximal 31 BU). ITI was started with the same rFVIII product (2 × 100 U/kg/d) leading to a decrease of the inhibitor titre. After 5 months of treatment the inhibitor titre rose again to 13 BU despite any changes of treatment or any detectable triggers. The patient was switched to a VWF-containing FVIII concentrate. *In vitro* testing showed a lower inhibitory activity against VWF-containing F VIII concentrate compared to r FVIII. After 8 weeks the inhibitor was not measurable any more. FVIII recovery and half-life became normal.

Case 2: The second child presented with an extended soft tissue bleeding after vein puncture at the age of 7 months leading to the diagnosis of a severe haemophilia A caused by a large deletion in exons 24 and 25 of FVIII gene which is associated with a high risk to develop inhibitors. Prophylaxis with r FVIII was started at the age of 26 months. After 7 exposure days the child developed a high-titre inhibitor (maximal 12 BU). Again *in vitro* testing showed a lower inhibitory activity against VWF-containing concentrates. ITI according to the Bonn protocol was started using a pd VWF-containing FVIII product leading to a complete elimination of the inhibitor within 7 months.

Conclusions: Case 1 supports the fact that intensive treatment, such as a surgical procedure within the first 20 exposure days increases the risk of inhibitor development in severe haemophilia A patients markedly.

As reported by others, both cases indicate that patients may benefit from the use of a plasma-derived VWF-containing FVIII concentrate in the frame of ITI. The relevance of *in vitro* testing in patients undergoing ITI is investigated in the OBSITI study in which both patients were included.

PO 144

Identification of carriers of mutations in the factor VIII gene that cause severe hemophilia A: first study in Venezuela

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Haemophilia A (HA) is a genetic, recessive and X-linked hemorrhagic disease caused by mutations in the coagulation factor VIII (F8) gene. Severe HA is characterized by the lowest levels of F8 and is caused primarily by the intron 22 inversion and by the intron 1 inversion; the rest of the causative mutations are greatly diverse. Since this is an X-linked disease, half of the male offspring of a HA carrier woman will probably inherit the disease. This makes the identification of carriers and genetic counseling very important to the control of HA. Therefore, the aim of this study was to determine whether eleven mothers of severe HA patients have the mutations in the F8 gene previously found in their sons (either in this study or in a previous one). First, in twelve severe HA patients and then in potential carriers, the presence of the intron 22 inversion and of the intron 1 inversion was investigated, using an inverse and a regular PCR, respectively. There were also diagnosed potential carriers of large deletions through real-time PCR, and potential carriers of point mutations through sequencing of the involved region of the F8 gene. The intron 22 inversion was found in three out of twelve severe HA patients (frequency: 27.3%) and it was found in all five mothers of severe HA patients who had it. Intron 1 inversion was found in none of the patients analyzed. None of the two mothers of patients with large deletions are carriers and all four mothers of patients with point mutations are carriers. The frequency of severe HA carriers was 81.8%. This is the first study conducted in Venezuela and it will allow to establish the detection of carriers in our country.

PO 145

Implementation of a Mobile Haemophilia Outpatient's Care in Germany – results of the HomeMHA project

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Background: Several haemophilia patients are facing obstacles visiting their Haemophilia Treatment Centre (HTC) which is due to poor infrastructure in some rural regions inGermany or patients' impaired health condition. Those patients would benefit from a mobile health care centre.

Aim: The major goal was to build up a mobile haemophilia outpatient's care implementing a more extensive social and medical care by getting knowledge about disease-specific problems in the direct private environment of haemophilia patients (e.g. at kindergarten, school, job, or within the family home).

Methods: The 3-years longitudinal project into the implementation of a Mobile Haemophilia Outpatient's Care (Homburger Mobile Haemophilie Ambulanz = HomeMHA) started in 2010 at the HTC of theSaarlandUniversityHospital. Haemophilia patients and their families were visited by a social education worker and/or a physician of the HTC with a project car. The project included home visits and interviews to measure patients' health-related quality of life (HRQoL) and treatment satisfaction (TS) by means of validated questionnaires (HRQoL: paediatric Haemo-QoL, adult Haem-A-QoL; TS: Hemo-Sat); adult patients and parents of paediatric patients were asked as well about their needs and wishes for a HomeMHA. In addition sociodemographic and clinical data were collected.

Results: In total 56 adult haemophilia patients (78.6% haemophilia A, 60.7% severely affected, 48.2% on prophylaxis) with a mean age of 37.4 ± 16.4 years and 23 paediatric patients (82.6% haemophilia A, 69.6% severely affected, 60.9% on prophylaxis) with a mean age of 9.8 ± 4.2 years and their parents were interviewed. The median distance from patients' home to the HTC was 45 km (range 3–200 km) and travel time was in median 40 min (10–120). More than 90% of the haemophilia patients and parents considered an intense binding to the HTC and the implementation of the HomeMHA as 'rather/very important'. A 35.7% of haemophilia patients and parents would use the services of the HomeMHA, especially for counselling and due to their immobility. Adult patients reported a good HRQoL (Haem-A-QoL total score 23.09 ± 17.1) with highest impairments in the domain 'sport & leisure' as did young children aged 4–7 years (M = 54.76 ± 28.4), while children aged 8–12 years reported highest impairments in the domain 'friends' (M = 57.64 ± 17.4) and adolescents in the domain 'dealing' (M = 38.93 ± 28.4). Adult patients were highly satisfied with their haemophilia treatment (M = 16.9 ± 14.6) and were mainly unsatisfied in the domain 'ease & convenience' (Hemo-Sat); by contrast parents of haemophilic children reported most impairments in the domain 'burden' (M = 33.85 ± 20.7).

Conclusions: Haemophilia patients were generally very satisfied with the implementation of the HomeMHA and felt quite supported by their haemophilia care which was as well reflected in their HRQoL and TS.

PO 146

Sub-visible particles in recombinant FVIII products: increased risk for immunogenicity?

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Background: The development of neutralizing antibodies against FVIII is the most challenging complication in current therapy for hemophilia A patients. Despite progress in explaining the regulation of anti-FVIII immune responses, the root cause for development of FVIII inhibitors is still unknown. Studies with other protein therapeutics have shown that certain types of protein aggregates, e.g. sub-visible particles, are associated with an increased risk for immunogenicity. The formation of such protein aggregates can be caused by physical stress factors such as elevated temperature, freeze/thaw cycles or shear stress. The sensitivity of different recombinant FVIII (rFVIII) products to form sub-visible particles when exposed to physical stress has not been previously investigated.

Aim: To assess quantities and properties of sub-visible (0.75–70 μm) particles in different rFVIII products including full-length and B-domain deleted products, and to analyze the sensitivity of different rFVIII products to form sub-visible particles under conditions of physical stress.

Methods: Two full-length rFVIII products and two B-domain deleted rFVIII products were analyzed. Sub-visible particles were assessed (1) immediately after reconstitution of the products and (2) after exposure of reconstituted products to physical stresses. Three different lots were included for each product.

Samples were analyzed using a flow cytometry-based method based on a combination of size calibration beads, counting beads and a number

of different fluorescent probes that bind to distinct structural elements of sub-visible particles. This method allows assessment of the amount, size and nature (e.g. protein and non-protein particles) of sub-visible particles. Moreover, we used the fluorescent dye 4-(dicyanovinyl)-julolidine (DCVJ) to obtain structural information of the sub-visible particles. DCVJ binds to cross-beta-sheet structures in amyloid-like protein particles (Hawe at al. Pharm Research 2008). Amyloid-like protein particles could be associated with an activation of the innate immune system as was demonstrated for Amyloid beta peptides. For comparison, we used Micro-Flow Imaging, a state of the art technology for the quantification of sub-visible particles.

Results: Immediately after reconstitution, the concentration of sub-visible particles varied significantly between the different rFVIII products analyzed. The structural properties of the detected sub-visible particles were similar in all freshly reconstituted products.

After exposure of freshly reconstituted rFVIII products to physical stress, the total number of sub-visible particles increased significantly in products containing B-domain deleted rFVIII, but remained constant in products containing full length rFVIII. In addition, an increased concentration of cross-beta-sheet containing sub-visible particles was found in B-domain deleted rFVIII products but not in full length rFVIII products.

Conclusions: We conclude that rFVIII products containing full-length rFVIII and products containing B-domain deleted rFVIII differ in their intrinsic propensity to form sub-visible particles when exposed to physical stress. Furthermore, products containing B-domain deleted rFVIII seem prone towards formation of cross-beta-sheet containing sub-visible particles when exposed to the physical stress conditions tested in our studies. This could be due to the lack of glycosylation sites contained in the B domain of FVIII. Whether these cross-beta-sheet containing sub-visible particles are associated with increased immunogenicity of FVIII remains to be investigated.

PO 147

Evaluation causes of deaths among individuals with inherited haemophilia A & B in North-eastern Iran

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Introduction: Inherited haemophilia A & B (HA & HB) are worldwide bleeding disorders that affect all ethnic groups. It is estimated that about 1,000,000 affected peoples with haemophilia exist in worldwide. Determination of causes of death in haemophilia may be useful for healthcare officials to program for elimination causes terminate to death in haemophilia.

Patients and Methods: We know 552 cases with inherited bleeding disorders in North-eastern Iran. Among them, there are 287 cases with HA and 92 with HB. To determine number of death and causes of deaths among them, a call phone established to each affected ones to extract data and record in questionnaire form. The rough data entered to software Prism version 5 and descriptive methods of analysis presented here.

Results: Overall among 379 cases with HA & HB, there were 43 deaths as follow:

HA: Among 287 cases, there were 143 (49.8%) with severe type, 59 (20.6%) with moderate and 85 (29.6%) with mild. We could find 38 deaths among them youngest one was 3 months year old and oldest one was 70 years old and mean of age among dead ones was 25.84 year. The oldest death was happened 52 years ago and newest one has happened 2 years ago and mean of time passed of death time was 15.6 years. The dead one included 29 severe type (76.31%), 5 moderate (13.15%) and 4 mild type (10.5%). The death's causes included 10 episodes of central nervous system (CNS) bleeding (26.3%), 6 cases with post trauma bleeding (15.7%), 6 cases with various cancers (15.7%), 4 cases with cirrhosis of liver (10.5%), 4 cases due to various uncontrolled bleeding (10.5%), 3 cases due to HIV infection (7.8%), 2 cases due to unknown cause (5.2%), and a case due to heart problem (2.6%) and another a case due to uncontrolled post surgery bleeding (2.6%).

HB: Among 92 cases, there were 43 (46.7%) with severe type, 33 (35.9%) with moderate and 16 (17.4%) with mild. We could find 5 deaths among them youngest one was 28 year old and oldest one was 76 years old with mean of 46.2 year. The oldest death was happened 50 years ago and newest one has happened 6 years ago and mean of time passed of death time was 16.2 years. The dead one included 2 severe type, a moderate and 2 mild type. The death's causes included 2 episodes of CNS bleeding (40%), 2 cases of liver cirrhosis (40%), and 1 case with severe nose bleeding.

Discussion: The most common cause of death in the group under survey was spontaneous or post trauma bleeding in CNS. As most of patients in the current group are on-demand infusion of relevant coagulation factors, it seems prophylaxis regimens may be useful in prevention at least part of deaths due to CNS bleeding.

PO 148

Meta-analysis of Post Authorization Safety Studies: worldwide postmarking surveillance of hemophilia A patients treated with antihemophilic factor recombinant plasma/albumin-free method rAHF-PFM

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Background: A rigorously designed Post-Authorization Safety Surveillance (PASS) program on rAHF-PFM use in hemophilia A patients has been conducted in Europe, US, Australia, Korea, Taiwan and Japan to identify adverse events (AEs), strengthen inhibitor risk estimates obtained from pivotal trials, and explore product effectiveness.

Aims: To synthesize evidence on rAHF-PFM safety in a standard clinical setting by analyzing available data collected from registered PASS studies conducted from 2004 to 2012.

Methods: Summary data was extracted from clinical study reports for hemophilia A patients enrolled in rAHF-PFM-PASS in Europe (11 countries plus Italy as a stand-alone data collection), US, Taiwan and Australia. Outcomes included: AEs (total, serious [SAEs] and treatment-related), including inhibitors (total, high responding [HR], *de novo*). Incidence rates were calculated using exact binomial method and then pooled using random effects model, with heterogeneity measured using I^2. Subgroup analyses consisted of the following: disease severity, previous exposure to FVIII concentrates at enrollment (< or ≥ 50 exposure days [EDs]), inhibitor history, and on-demand/prophylaxis regimen.

Results: Overall 883 patients were included, followed up for over 1 year, mean EDs from 85 to 103 across 5 studies; 611 (69.2%) had severe disease (FVIII:C < 1%), mean age ranged from 12.1 to 31.4 years. 524 of 596 (11.6%) patients reporting the number of previous EDs at enrollment were treated for ≥ 50 EDs. During the study, 440/883 patients (49.8%) were treated only on-demand, 353 (40.0%) only according to a prophylaxis regimen. About 741 total AEs and 36 rAHF-PFM-related AEs (22 of which were inhibitors) occurred at a rate of 2.2 (95% CI 1.2–3.3, I^2 99%) AEs every 100 infusions and 3 (95% CI 0.7–6, I^2 70%) rAHF-PFM-related AEs every 10,000 infusions. Total SAEs (n = 79) and rAHF-PFM-related SAEs (n = 24, 22 of which were inhibitors) occurred at a rate of 10 (95%CI 5–20, I^2 79%) SAEs and 2 (95%CI 0.3–3%, I^2 57%) rAHF-PFM-related SAEs every 10,000 infusions. The proportion of patients with at least one rAHF-PFM-related AE was not significantly different among those initially treated on-demand and those initially receiving prophylaxis (2.8% vs. 4.0%, OR [95%CI] = 0.65 [0.19, 2.20]). Among the 22 patients positive for inhibitor (2.0%, 95%CI 0.3–3.6%, I^2 39%), 10

were *de novo* (i.e. without history of inhibitor; data on inhibitor titer at enrollment was not available for every patient), 9 HR, and 6 were in severe patients. Six of 524 (1.1%, 95%CI 0.05–2.2%) patients with ≥ 50 previous EDs developed an inhibitor (4 *de novo*, 1 HR, 3 in severe patients).

Summary: Meta-analyzing data from rAHF-PFM-PASS conducted in different countries confirmed in the routine clinical setting the overall favorable safety profile of rAHF-PFM. Effectiveness and sources of heterogeneity will be explored.

PO 149

Mortality and cardiovascular disease in patients with haemophilia: a pilot study in a single centre

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Improvements in haemophilia care and antiviral treatments led to increasing longevity for persons with congenital haemophilia. Older patients face not only challenges related to haemophilia but also to general comorbidity associated with aging such as cardiovascular disease (CVD) because the risk factors are expected to increase. The paucity of data is evident and there are no guidelines to help treaters to manage these patients.

Aims:

1 To analyze the morbidity and mortality of patients with haemophilia along the last 25 years in a single centre.
2 To investigate the cardiovascular risk factors and the actions taken according to them.

Methods: This is a retrospective study of patients with haemophilia A and B attending our hospital for the past 25 years (n = 144). The medical records were reviewed and separated in two groups: Group I – Dead patients until data analysis (n = 33) Group II – Patients attending regularly to our hospital (n = 81). A descriptive data analysis was made due to heterogeneity of population included.

Results: In Group I we observed that mean of age at time of death was 29.53 years and 75.8% (25/33) were HIV positive. Causes of death in HIV positive patients was in 30.3% (10/25) related to AIDS, 18.2% (6/25) related to hemorrhage, 12.2% (4/25) related to hepatitis C virus (HCV) infection and 5 other causes. Causes of death in HIV negative patients (8/33) were: 2 haemorrhages, 1 related to HCV, 1 lung cancer, 1 sepsis, 2 other causes and only 1 cardiovascular disease that suffered a intracraneal bleeding during a hypertensive crises.

In Group II (n = 81), 33.3%(n = 25) had severe haemophilia, 4%(n = 3) moderate and 70.6% (n = 53) mild. The mean age was 36.8 years. A 28.4% (n = 23) had less than 21 years old and 71.6% (n = 58) were older than 21 years. Comorbidities in adult's population was: 24.7% (20/81) had VHC infection being all adults, 8.6% (7/81) wereHIV positive, 4.9% (4/81) had malignancies. A 56% (32/58) of patients with more than 21 years had CVD risk factors (RF) (hypercholesterolemia, hypertrigliceridemia, diabetes, hypertension, smoker, over weight or obesity); 53.3% had 1RF, 31.2% had 2RF, 9.3% had 3RF and 6.2% had 4RF. Seven patients presented major cardiovascular complications. There were 3 venous thrombosis, one mesenteric thrombosis that needed a shunt portal-cava, 1 deep vein thrombosis treated with vitamin K antagonist for 6 months and 1 central catheter related thrombosis that needed anticoagulation with heparin. There was also 1 critic carotid stenosis, 1 mitral valve stenosis submitted to surgery biological prosthesis valvular and 1 ischemic cardiopathy, all of them treated with chronic antiagregation therapy. Finally 1 supraventricular tachycardia that needed a catheterism with heparin.

Conclusions: Hemophilia patients have higher life expentancy and morbid-mortality is changing to CVD and risk factors similar to the general population. These findings suggest the need of CVD prevention measures and establishment of guidelines to treat these patients.

PO 150

Effectiveness and safety of long-term treatment with recombinant factor VIII formulated with sucrose for hemophilia A in clinical practice of emerging-market countries

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Background: Full-length recombinant factor VIII formulated with sucrose (rFVIII-FS) was one of the first rFVIII products introduced for patients with hemophilia A in many emerging-market countries. In these countries, plasma-derived FVIII products are still used in the large majority of patients with hemophilia A. Therefore, clinical evidence on rFVIII products from these countries is still scarce.

Aims: This study aims to analyze the effectiveness of rFVIII-FS for the treatment of patients with mild, moderate, and severe hemophilia A under real-life conditions in countries from North Africa, the Middle East area, and Eastern Europe and to gain further insight into the safety profile of rFVIII-FS in daily practice.

Methods: Patients diagnosed with mild, moderate, or severe hemophilia A, treated according to standard clinical practice with rFVIII-FS as their only source of FVIII, were enrolled in this study. Informed consent was obtained by all study participants or their guardians. No additional limitations on inclusion criteria were made. Patient characteristics, treatment characteristics, joint status, laboratory tests including inhibitor measurements, and adverse events (AE) were documented on paper case report forms at enrollment, month 12, and month 24 by the treating physician. The patients recorded their infusions and bleeds in patient diaries. The study was approved by the relevant ethics committees or authorities.

Results: One hundred and eighty-six patients were included from 51 centers in 11 countries. Mean age was 12.8 years (range, 0–55). One hundred and seven (58%), 65 (35%), and 14 (8%) patients had severe (< 1% FVIII), moderate (1–5% FVIII), or mild (> 5% FVIII) hemophilia A, respectively. About half of the sample (54.3%) was treated with > 150 exposure days (EDs) before enrollment. One hundred and eight patients (58.1%) were on prophylaxis with 1, 2, or 3 injections per week from enrollment until study end, and 59 patients (31.7%) were treated only on demand. An additional 15 patients (8.1%) switched between prophylaxis and on-demand treatment during the study, and four patients (2.2%) received inhibitor-adapted therapy. About half of the patients (47.8%) had a target joint. Patient diaries were available for 155 patients (83.3%). Patients with severe hemophilia A on prophylaxis reported a median of 0.0 (range, 0–23.8) bleeds per year, whereas those treated on demand had a median of 14.6 (range, 2.1–50.9) bleeds per year. A 91.1% of bleeds in the whole sample were treated with 1–2 infusions. For 27 of the 186 patients (15%), a positive inhibitor test before enrollment was reported. 9/186 patients showed positive inhibitor test results during the study; of these, 5 tested positive before enrollment, and 4 (2.1%)- all with < 150 EDs – had no positive inhibitor test documented before study inclusion. Thirty-one patients (16.7%) reported 74 AEs, of which the 9 mentioned inhibitor cases were the only drug-related AEs.

Summary/Conclusions: This study reconfirms the evidence for rFVIII-FS in effectively treating acute bleeds in patients with mild, moderate, and severe hemophilia A as well as preventing bleeds via weekly prophylactic injections. The safety profile is as expected in hemophilia A.

PO 151

Utility of thrombin generation test in laboratory management of hemophilia A patients

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Background: Based on FVIII activity, hemophilia A is classified as severe, moderate or mild. Large heterogeneity between FVIII activity and clinical severity has been observed among patients with hemophilia A, indicating that the residual level of FVIII is not the only determinant of the clinical phenotype. Nowadays, new laboratory methods that assess overall hemostatic potential have been developed, with intention to better diagnose and monitor hemophilia A patients.
Aims: The aim of this study was to compare new commercially available thrombin generation test (TGT) with laboratory methods routinely used for monitoring hemophilia A patients. Obtained results were correlated with known clinical parameters – age at first joint bleed, number of joints with hemophilic arthropathy, number of annual joint bleeds and annual FVIII consumption.
Methods: Total of 81 hemophilia A patients (all hemophilia A patients) were included in the study and divided in severe hemophilia A patients ($N = 37$) and non-severe hemophilia A patients ($N = 44$). TGT was performed with Endogenous Thrombin Potential Test, setting ETP-C for hemophilia patients on BCS-XP (Siemens Medical Solutions Diagnostics, Germany). Four ETP parameters were measured: area under the thrombin generation curve (AUC), peak thrombin concentration (Cmax), time to peak thrombin concentration (t-max) and time to signal beginning (t-lag). FVIII clotting activity (FVIIIclot) and FVIII chromogenic activity (FVIIIch) was performed on BCS (Siemens, Marburg, Germany).
Results: Weak correlation between AUC/Cmax and FVIIIclot/FVIIIch was found in all hemophilia A patients and non-severe hemophilia A patients (r = from 0.306 to 0.596). No correlation was found in all three groups of patients for t-lag and t-max parameters. Similarly, no correlation was found between ETP parameters and clinical parameters, except weak correlation between AUC/Cmax and age at first joint bleed ($r = 0.248$ and 0.291, respectively). The cut-off value of ≤ 25.6 mA for AUC, obtained by ROC analysis, allowed the discrimination of patients in two groups that statistically differ in all clinical parameters, with sensitivity and specificity of 86.5% and 47.7%, respectively.
Summary/Conclusions: Although the AUC was the best discriminating parameter in TGT, based on the obtained results, the used ETP-C method did not fulfill, for the time being, the goals expected for a routine laboratory method. It seems that weak thrombin signal obtained in hemophilia patients makes this method more sensitive to different analytical and preanalytical factors, that need to be resolved in the future.

PO 152

Progress report on the experience of immune tolerance induction with a VWF/FVIII concentrate in haemophilia a patients in Colombia

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Background: The development of inhibitors to FVIII in up to 40% of patients is currently a major medical obstacle affecting the quality of life of treated individuals and is associated with increased treatment costs. Immune Tolerance Induction (ITI) is the only therapy capable of eliminating inhibitors. This therapy has longer been considered that could not be done in developing countries because of its high cost, but today there are several pharmacoeconomic studies have shown that ITI is cost-effective compared to on-demand or prophylactic therapy with bypassing agents.
Since the Hemolife program establishment in 2006, has sought to improve the management of haemophilia patients in Colombia. In order to provide the best treatment for haemophilia patients with inhibitors, the Hemolife physician group decided to enrol their patients into the Observational Immune Tolerance Induction study (ObsITI).
Objectives: Evaluate and document data on the success rate of ITI in haemophilia A patients with FVIII inhibitors treated according to the Bonn protocol.
Compare the costs of ITI therapy with the costs of the prophylactic treatment with bypassing agents.
Methods: Since September 2009, 20 patients with inhibitors and poor-prognosis for ITI success have been treated with octanate for ITI according to the Bonn protocol. The outcome was assessed according to the stringent success criteria defined in the ObsITI study: Inhibitor elimination (< 0.6BU), FVIII *in vitro* recovery of > 80%, and FVIII half-life of > 7 h.
Results: Eight patients out of 20 were unfortunately excluded from the research program: one because of difficult venous access, three changes the treatment center, and 4 due to their insurance decision. The results from 12 patients will be presented. Seven of 12 patients have achieved negative inhibitor in a mean time of 3.7 months (range 2–8). Four of these 12 patients have continued ITI without achieving negative inhibitor in a mean time of 10.7 months (range 2–22) and the other patient stopped ITI after 24 months and inhibitor title > 480BU.
Five (41.6% of 12) out of seven patients that have achieved negative inhibitor title, have reached three success criteria in a mean time of 11.8 months (range 5–22). Of these, one patient has been on follow up prophylactic treatment with octanate for 12 months without relapse, and four patients are still in ITI and tailing-off the FVIII dose.
Cost-of-care analysis was performed for seven patients undergoing ITI with octanate, and the results were compared to prophylactic treatment with activated prothrombin complex concentrate (aPCC) and rFVIIa. Average savings of ITI over 10 years per patient were estimated at US$8.1 million compared with prophylactic treatment with aPCC, and US$12.4 million compared with prophylactic treatment with rFVIIa.
Conclusions: ITI with octanate has proven to be effective in inhibitor elimination. ITI therapy is cost-effective and allows major savings to the health system in comparison to the prophylactic treatment with aPCC and rFVIIa, so every time more patients, even from developing countries, should get the benefit of this therapy.

PO 153

Rapid immune tolerance induction following primary immunologic prophylaxis in a hemophilia A patient with high-titre inhibitor

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Background: Observational studies suggest that primary immunologic prophylaxis (PIP) – starting factor VIII (FVIII) exposure early and in the absence of immunologic 'danger signals'- may decrease the incidence of FVIII inhibitors in hemophilia A patients. It is unknown whether PIP might have other effects, such as altering the ability to achieve tolerance in patients who do develop inhibitors or altering the biologic parameters of their anti-FVIII immune response. The longitudinal behaviour of anti-FVIII IgG1 and IgG4 levels during the early phase of inhibitor development and immune tolerance induction (ITI) has not previously been described. We report a case of rapidly successful ITI in a boy with severe hemophilia A who developed an inhibitor after PIP, along with analysis of his anti-FVIII IgG1 and IgG4 levels over time.

Methods: A boy with severe hemophilia A caused by a five base pair insertion into exon 14 of the *F8* gene started PIP with recombinant FVIII (Advate) 10 IU/kg once weekly at 11 months of age, without prior bleeding events or FVIII exposures. ITI, when started, was with FVIII (Advate) 100 IU/kg once daily. Patient plasma collected at various times was assayed for FVIII inhibitory activity with the Nijmegen modification of the Bethesda assay. Plasma was assayed by ELISA for anti-FVIII total IgG, IgG1 and IgG4. For total IgG and IgG1, positivity was defined as optical density at 490 nm (OD) more than 3 SD above the mean OD of six normal plasma samples. For IgG4, OD above blank values were obtained, without a positivity criterion.
Results: An inhibitor was detected after 5 exposure days (ED) with a titre of 1.5 Bethesda units (BU). FVIII exposure continued until 19 ED. Nine days after last FVIII exposure, the inhibitor peaked at 19 BU. ITI started 8 weeks after last FVIII exposure at a titre of 9 BU. No inhibitor was detectable 6 weeks after initiating ITI. Levels of anti-FVIII IgG1 and total IgG paralleled the Bethesda titres, with both being below positivity at the same time that inhibitory activity became undetectable. Levels of anti-FVIII IgG4 became detectable later than IgG1 and FVIII inhibitory activity were found, and continued to increase after these other parameters had plateaued. IgG4 had decreased significantly when the other parameters became undetectable.
Conclusions: Our patient underwent successful ITI in less than 6 weeks. Given that his Bethesda titre peaked at 19 BU, it should not be assumed that this was a transient inhibitor. It may be that although PIP did not prevent an inhibitor, it predisposed him toward rapid tolerance induction. The sequential generation of anti-FVIII IgG1 and IgG4 is a novel finding, but the significance and generality of these as prognostic biomarkers of the anti-FVIII immune response must be established. Similar investigations should be undertaken in more patients, with and without PIP.

PO 154

Frequency of FVIII inhibitor in patients treated with plasma derived FVIII concentrates

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Background: Development of the inhibitors against FVIII is the most challenging complication of the treatment of hemophilia. In the era of using high purity FVIII concentrates we are witnessing the increasing frequency of inhibitors in severere hemophilia. The results of studies comparing inhibitor incidence in patients treated with plasma derived FVIII (pdFVIII) and recombinant products (rFVIII) are inconclusive. Most recently Gouw et al. (NEJM 2013) demonstrated the cumulative incidence of clinically relevant inhibitors in 33.1% (high titer inhibitors 25.7%) of patients treated with pdFVIII, the frequency similar to that, observed after rFVIII products. In Slovakia so far the majority of hemophilia patients have been treated with pdFVIII concentrates, so we evaluated the incidence of inhibitors in this patients group.
Methods: Since 1997 all severe hemophilia patients have been followed systematically for the development of inhibitor, which was tested after each 5, 10 and 20 exposure doses (ED) until the first 20, 50 and 80 exposures to FVIII, later on every 6 months. Inhibitors above 0.7 BU or 0.5 Nijmegen BU confirmed by three consecutive tests were considered positive.
Results: Cumulative incidence of inhibitors was evaluated in 45 previously untreated patients (PUP′s) with severe hemophilia A (FVIII < 1 IU/dL) born between 1997 and 2011 and followed at our two centres. Thirty nine PUP′s have been treated with pdFVIII, out of them 33 PUP′s reached more than 80 ED of pdFVIII. Eight and one patient switched for rFVIII after reaching 80–100 and 4 ED of pdFVIII, respectively. Inhibitors developed in 5/38 (13.2%) pdFVIII treated patients after a mean 22 ED (10–27). The mean age at the inhibitor detection was of 39 months (22–68). One, two and two inhibitors were transient, low titre and high titre, respectively. No inhibitor was observed in eight patients who switched for rFVIII after 80–100 ED of pdFVIII, however, a patient with four exposures to pdFVIII developed low titre inhibitor after consecutive 21 ED of rFVIII. Yet rFVIII was used from the beginning of replacement therapy in only four PUP′ s. Two of them developed high titre inhibitor at age of 14 and 18 months, after 20 and 40 ED of rFVIII, respectively, in absence of risk factors such as major bleed, vaccination, infection, surgery and/or intensive treatment.
All but one patient, who had transient inhibitor, have been treated with immune tolerance induction (ITI) with a succesful outcome in 2 low and 3 high responders, respectively. The high dose ITI is still ongoing in two high rersponders developing inhibitor after rFVIII.
Conclusion: Cumulative incidence of inhibitors in our severe hemophilia patients treated with pdFVIII was 13.2% (high titer-inhibitors 5.3%), much lower than that reported most recently in the literature. Our group of patients is too small to evaluate the role of other confoundig inhibitor risk factors. However, the development of high-titer inhibitors in 2/4 PUP ′s treated exclusively with rFVIII resulting in a challenging management of this complication, is worying.

PO 155

Situation with haemophilia in Ukraine – the first results of work of regional haemophilia center

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Organization of care for patients with hereditary diseases is an extremely difficult problem. In historical terms, the model of care for patients with haemophilia varied depending on the availability of drugs clotting factors for substitution therapy. But the greatest positive effect on the dynamics of care to patients with impaired blood clotting was observed in those countries that are centres for comprehensive health services for patients with haemophilia. This model of care was one of the most successful public health programs in the developed world. In Ukraine, mainly the organization of care for patients with haemophilia was sent for treatment of complications of the disease. This model of care for patients with haemophilia in the absence of clotting factor concentrates, and then in a large deficit drugs had the right to life, because doctors could only provide treatment of complications of haemophilia in haematology departments.
Today Ukraine is gradually increasing funding and provision of patient clotting drugs. In 2011–2012 pp. centralized purchasing and procurement through regional budgets have been significantly increased. In 2011, only for children had purchased 14 million IU, in 2012 – 16 million IU through centralized procurement for state programs. Additionally, each region held purchase factors for patients in their region. Therefore, the provision of children in 2012 averaged 39,000 IU per one patient. The further increase in funding the purchase of drugs, approved clinical protocols dramatically increase the possibility of selecting certain types of treatment. The most effective way to solve these problems is to create a multidisciplinary team of specialists (haemophilia centers) in each region of Ukraine, which can provide medical qualified service.
In 2012 regional haemophilia center for patients of Kyiv region was established. For diagnostic coagulation studies and laboratory monitoring of therapy was purchased coagulometer and reagents. Under the supervision of specialists regional center for haemophilia and other disorders of haemostasis are 35 patients with severe haemophilia, including – 23 patients with severe haemophilia A (13 – adults and 10 children), 11 patients with severe haemophilia B (8 – adults and 3 children), and one patient with severe von Willebrand disease. Today Regional Hemophilia Center provides diagnosis haemophilia,

conducts qualitative and quantitative determination of inhibitors, clinical and laboratory monitoring of treatment.
Thus, the regional haemophilia of Kyiv region commenced the implementation of modern principles of care for patients with haemophilia and other disorders of haemostasis.

PO 156

Moba-Roku – a new internet assisted monitoring system of home infusion program for hemophilia care in Japan

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Background: In Japan, home infusion program for hemophilia patients has become very popular. Many hemophilia patients have been treated with home infusions of FXI or FVIII products by themselves or by their parents. In this program, complete monitoring of infusion records is priority of good care management for patients and for their health care providers. We have two way of recording system of this program, one is classical printed form type, another is new one – an internet assisted infusion recording system, which is through a mobile phone and/or a personal computer (PC). With this system, patients, their parents and health care providers can easily access the graphical collections of data which have contained of bleeding sites, date of bleedings occur, number of infusions, type of products, and etc., via internet. We call this in Japanese 'Moba-Roku' which means mobile phone assisted infusion recording system, provided by Baxter Japan, and it is coming into wide use. This is the first presentation of the internet assisted infusion monitoring system 'Moba-Roku'.
Aims: In this report, we have evaluated the quality of this new monitoring system of home infusion program for hemophilia care.
Methods: We conducted two surveys aimed to evaluate this new monitoring system in August 2011 and April 2012. The questionnaires were distributed among registered 'Moba-Roku' users who were patients and their parents by email. Thirty-seven users responded to 1st survey and 14 users responded to 2nd survey.
Results: In Japan, before introducing 'Moba-Roku', when hemophilia patients had underwent infusion therapies, they had recorded home infusion data through classical printed form type only, always recording; 62.2%, sometime; 10.8%, seldom; 27.0%, respectively. But almost of those records were kept left at their home, very few were seen by their health care providers, therefore they might have not enough medical consultation of disease status based on bleeding and infusion records. After introducing 'Moba-Roku', they had recorded home infusion data, always; 71.5%, sometime; 14.3%, seldom; 14.3%, respectively. When they were going to record their infusion data with this internet assisted monitoring system, through a mobile phone; 57.2%, through a PC; 35.7%, through either; 7.1%, respectively. There no difference of frequency of infusion recording between the classical printed form type and the new internet assisted one. But after introducing 'Moba-Roku', 57.1% of patients and their parents had consulted their health care providers with these graphical collections of infusion data, when they had visited their clinics. Since 2010, our facility also has introduced 'Moba-Roku' to 10 patients and it enhances our daily practice communication between patients and our hemophilia care team (physicians, nurses, pharmacists, etc.). We have regarded it contributes to the good care of hemophilia patients.
Conclusion: The new internet assisted infusion monitoring system 'Moba-Roku' might enhance the quality of care management for hemophilia patients themselves and also for their health care providers.

PO 157

Causes of death in a cohort of patients with haemophilia in a Haemophilia Care Center in the last 5 years

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Background: The increased life expectancy of the hemophilia population, as a result of advances in factor replacement therapy and the introduction of highly active antiretroviral therapy (HAART) has enabled hemophiliacs to reach an older age. Since the 1970s, mortality in the hemophilia population has been dominated by human immunodeficiency virus (HIV) and hepatitis C virus (HCV)-associated complications. However, age-related diseases such as cardiovascular disorders and cancer, are being increasingly recognized in such patients.
Aims: This abstract describes causas of death in a cohorte of patient with haemophilia in a haemophilia care center in the last 5 years.
Method: A causes of death analysis was conducted among 257 patients with haemophilia A and B (68 severe, 39 moderate and 152 mild haemophiliacs), who were treated at the haemophilia care center in Sevilla. Information on mortality in our patient cohort was obtained from the medical records.
We analized data on age, type and severity of haemophilia, modality of treatment, inhibitor development, HIV and HCV status and cause of death.
Results: Among the 257 haemophiliacs patients treated at our haemophilia center, eight deaths were observedbetween January 2008 and December 2012. Their average age was 45.8 years (range 40–60). Hemophilia type was A in all cases and severe in six cases, moderate and mild in the other two patients, respectively. All patients were on demand treatment, 4 with plasma-derived and 3 with recombinant factor VIII concentrates. One patient presented a high-titer inhibitor, treated with bypass agent on demand. Six patients were HIV-positive, all of them well controlled withHAARTand five of then were coinfected with HCV. Two patients presented chronic HCV infection. One patient died of lung cancer, smoker as a risk factor. One severe haemophiliac died of a spontaneous intracranial haemorrhage. Two patients died of HCV-associated complications; one of them, coinfected with HIV and cirrhosis status and previus failure of conventional treatment of chronic HCV infection, developed fatal hydropic decompensation in relation to a new antiviral treatment. The other patient died of endstage liver disease. Four patients died of cardiovascular disease. A haemophiliac A severe suffered an acute myocardial infarction after administration of recombinant FVIII and died of a fatal ventricular arrhythmias after apercutaneous coronary intervention. The second patient, a haemophiliac with inhibitor, had a first coronary event after infusion of a single dose of bypass agent; after 2 years in relation to a surgery episode, he suffered a massive acute myocardial infarction. The third patient, a moderate haemophiliac with several aging diseases (atrial fibrillation, cardiac valvulopathy and cirrhosis), died of an acute myocardial infarction. The last patient died of a ruptured abdominal aorta aneurysm.
Conclusion: Infection with HIV and HCV continue to play dominant roles in the older hemophilia population. However, after introduction of HAART the causes of mortality are changing in the last years. The results of this study show that cardiovascular diseases have become a new challenge for physicians working in hemophilia centers and underline the need to optimize the management of ischemic heart disease in hemophiliacs.

PO 158

Filling the gap on long-term joint health and HRQoL outcomes data in hemophilia A using a single product (rAHF-PFM), non-interventional registry, AHEAD

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Background/Aims: Hemophilic arthropathy resulting from recurrent joint bleeds leading to joint damage – is a major cause of disability in patients with hemophilia A. Most post-approval safety surveillance studies in hemophilia A however only monitor safety and effectiveness for 6–12 months leaving a gap in our understanding of longer-term joint health outcomes and health-related quality of life (HRQoL). The ADVATE (rAHF-PFM) Hemophilia A Database (AHEAD) study is designed to capture long-term outcomes data on patients with hemophilia A in Germany and other European countries receiving treatment under routine practice setting including on-demand therapy, primary or secondary prophylaxis, or immune tolerance induction therapy. Data is being collected from patients prescribed rAHF-PFM using standard dosing treatment regimens as well as emerging treatment practices including individualized pharmacokinetic (PK)-guided prophylaxis dosing. Study endpoints include long-term joint health outcome and other hemophilia-related co-morbidities, HRQoL, effectiveness and safety.

Materials and Methods: Eight hundred and fifty subjects with hemophilia A (FVIII ≤ 5%) are to be enrolled (500 in Germany; 350 from other European countries). Informed Consent is required; study to comply with Declaration of Helsinki. Joint health will be assessed using physical examination as well as routinely used imaging technologies. HRQoL will be measured using validated questionnaires such as SF-10 or SF-12, hemophilia-related co-morbidities and safety and effectiveness will be measured using standard methods. The protocol has been amended to capture additional data on the determination of individual PK parameters and dose calculations used in PK-guided prophylaxis dosing. Study enrollment began in 2010 in Germany and 2011 in other European countries. Subjects will be observed for 4 years; enrollment continues through 2015.

Results: As of January 2013, 469 patients (318 in Germany and 151 in other European countries) have been enrolled at 43 initiated sites in Belgium, Czech Republic, Denmark, Greece, Hungary, Italy, Portugal, Spain, Sweden, Switzerland, and the UK, and from 30 sites in Germany.

The mean age (range) for the 'European' and German cohorts is 20.8 (0–68) and 28.6 (1–80) years, respectively. In both study arms, most patients had severe hemophilia A (FVIII < 1%): 52% in 'Europe' (n = 78) and 76% in Germany (n = 242), and were receiving prophylaxis: 66% in 'Europe' (n = 100) and 74% in Germany (n = 236). At baseline, 35% of patients in the 'European' cohort showed at least one joint with arthropathy. In the German cohort, 40% of patients exhibited at least one joint with arthropathy at baseline. Of the 469 enrolled patients, 19 patients in Germany have been followed for 2 years, and 123 for 1 year; 38 patients in 'Europe' have been followed for 1 year.

Conclusions: The AHEAD study will capture important long-term outcomes data on a broad patient population using rAHF-PFM in routine clinical practice. The amended protocol will also facilitate collection of outcomes data on new, emerging treatment practices such as individualized PK-guided dosing. These data will fill an important gap in the current literature and may provide insights on best treatment practices and patient variables related to good long-term outcomes.

PO 159

A study of risk factors for the development of FVIII inhibitors in Indian severe haemophilia A patients

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Background: Development of 'FVIII Inhibitors' or alloantibodies to Factor VIII in patients with congenital haemophilia A is a serious complication of FVIII replacement therapy. It prevents the efficient clinical management of these patients, particularly those with severe haemophilia A, and leads to a substantial increase in mortality, as well as in the frequency and cost of management of bleeds among these patients, especially post-operatively. Inhibitor development in haemophilia A patients is thought to be complex and influenced by various genetic and non-genetic risk factors. Associations with various risk factors have been suggested in different populations. In earlier studies in Indian haemophilia A patients, *IL10* haplotypes and *TNFA rs1799724* promoter polymorphisms have been found to be significantly associated with inhibitor development, while four exonic non-synonymous *FVIII* SNPs, G1679A [R484H], A2554G [R776G], C3951G [D1241E], and A6940G [M2238V], whose haplotypes encode six wild-type FVIII proteins (H1 – H6) have not been found significant.

Aim: The aim of this study was to analyse the association of other genetic (HLA) and non-genetic (treatment-related) risk factors with inhibitor development in Indian severe haemophilia A patients.

Methods: One Hundred Indian severe hemophilia A patients, (40 consecutive inhibitor positive patients and 20 concordant/discordant family members, as well as 40 consecutive inhibitor negative control patients (over 10 years of age with > 10 exposures to treatment products) were included in the study after their informed consent. The study was approved by the Institutional Ethics Committee for Research on Human Subjects of the National Institute of Immunohaematology (ICMR), Mumbai. A clinical proforma was designed to record patient details. *HLA- DRB1* and *DQB1* alleles were genotyped by PCR with Sequence Specific Primers (SSP), using the AllSet^{+}™ Gold SSP HLA-DQ and HLA-DR Low Resolution kits (Invitrogen, USA). The results were analysed for statistical significance by Fisher's exact test.

Results: The mean age of the inhibitor positive patients was 24.57 (6–55 years) and mean lifetime exposures to treatment products was 19.22 (4–43); and the mean age of the inhibitor negative patients was 28.30 (12–65 years) with 43.625 (15–220) mean lifetime exposures. Among the inhibitor positive patients, 31/40 (77.5%) patients were high-responders (> 5 BU/ml). The HLA-DRB1*13 allele (P: 0.048) and the HLA-DQB1*05/*06 (P: 0.025) genotype were found to be significantly higher in the inhibitor positive samples as compared to the controls, in the samples studied so far. Treatment-related risk factors such as type of treatment product and exposure to treatment products before the first year of life (22.5% inhibitor positive patients vs. 12.5% inhibitor negative patients) were not significantly different.

Conclusion: The association of these factors with other risk factors such as FVIII mutations also, could provide useful insights into the FVIII immune response, and possibly influence timely prediction, prevention and treatment of FVIII inhibitors.

PO 160

Impact of rAHF-PFM prophylactic treatment on annual bleeding rate and health-related quality of life of adults with severe hemophilia A

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Background: Limited prospective data exists comparing on-demand and secondary prophylaxis in an adult-only population.
Aims: Assess the annual bleeding rate (ABR) and health-related quality of life (HRQOL) for adults who switched from on-demand to secondary prophylaxis in the rAHF-PFM Prophylaxis Study.
Methods: Patients previously treated on-demand with FVIII levels ≤ 2% received 6 months of on-demand treatment and were then randomized to 12 months of either standard (20–40 IU/kg every other day) or pharmacokinetic (PK)-tailored (20–80 IU/kg every third day) prophylaxis. All adults (≥ 18 years) from this study were selected for this post-hoc, subgroup analysis. ABR and HRQOL (measured using the SF-36) were compared between the end of the on-demand and prophylaxis phases. Bleeding events and HRQOL were also assessed longitudinally to understand how quickly prophylaxis impacted these outcomes. Informed patient consent and ethics board approval was received for this study.
Results: The intent-to-treat adult subset analysis included 55 subjects. The median (range) patient age was 30 (18–59) years. The median (IQR) ABR during on-demand treatment was significantly reduced from 43.7 (26.1) to 1.0 (4.1) using either prophylaxis regimen ($P < 0.0001$). During on-demand therapy, the median interval between bleeding episodes was 7.1 days, however, after initiating prophylaxis, patients experienced a median of 155.3 consecutive bleed-free days ($P < 0.0001$). Statistically and clinically significant improvements in physical HRQOL were reported following 12 months of prophylaxis ($P = 0.0035$). This improvement was explained by significant improvement ($P < 0.001$) in Bodily Pain after 3 months of prophylaxis which approached average pain levels reported by the general US population and was sustained for the entire 12-month period.
Summary/Conclusions: Adults patients who initiated secondary prophylaxis quickly saw a significant reduction in their ABR and subsequent improvement in their physical HRQOL.

PO 161

Prophylaxis in adults hemophiliac patients with severe arthropathy

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Primary Prophylaxis is the standard management for patients with haemophilia. Prophylaxis started in adulthood improves orthopedic scores, decreases hemarthrosis frequency, physical disability, hospitalization rates, school/work absenteeism and has a positive impact on quality of life. However, prophylaxis in adults has many barriers and involves very high cost. Scientific evidence is limited. We described a cohort of patients treated in a developing country.
Methods and Results: Twenty-four adult hemophliac patientes, treated in a hemophlia center in Bogotá, Colombia has been followed during 18 months. Age: 32.years (-range 18–63). Eighty percent with Hemophilia A, 20% hemophilia B. Seventy-six percent received plasma-derived factor, 22% is given recombinant factor. One patient with inhibitors is receiving FEIBA. All patients have severe arthropaty, 32% refers permanent or most of the time joint pain and 24% are complety disabled. Prophylaxis infusion frequency (number/week) 2.28 (2–3), prophylaxis dose (IU/kg week): 15.6 (7.8–23). Mean number of bleeds in 18 months was 3.8. During following there were no life-threatening bleedings. There were only two hospitalizations due to hemorrhage. Quality of life was assessed with SF-36. Emotional role, mental health, social function and livelness were score above 70.
Conclusions: Prophylaxis in adults with hemophilia and artropathy is feasible in a developing country. Our patients has very low bleeding and hospitalization rates, prophylaxis factor dose is relative low in comparison with established doses for other series. Prophylaxis has a positive impact on quality of life.

PO 162

Immune tolerance induction in adult severe haemophilia a patients with a single FVIII/VWF product: the UK experience

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Background: In haemophilia A patients, the development of neutralising antibodies against their replaced exogenous Factor VIII treatment greatly increases the risk of developing major bleeding complications. Immune tolerance induction (ITI) treatment protocols based on the regular infusion of FVIII are the currently recommended intervention for this serious complication, with the goal to eradicate the inhibitor and to return to effective treatment with FVIII.
Aim: To evaluate the outcome of primary and rescue ITI in adult severe haemophilia A patients with a single plasma derived FVIII/VWF product (Fanhdi®, Grifols, Barcelona, Spain).
Methods: Retrospective data were analyzed from severe hemophilia A patients (FVIII < 1%) with inhibitors from threeUKcenters. Complete success was assessed as clearance of the inhibitor, partial success when the inhibitor was reduced to less than 5 BU and the patient responds effectively to Factor VIII replacement without needing bypassing therapy. Results:Results are presented for nine adult patients (ages 28–74) who underwent primary ITI (8 patients) or rescue ITI (1 patient) with FVIII/VWF concentrate. Treatment regimens ranged from 40 IU/kg three times a week to 200 IU/kg per day. In the primary ITI patients, mean peak inhibitor was 30.5 BU (range 0.9–93), mean titre at start of ITI was 6.1 BU (range 0.6–22.9) and mean ITI duration was 6.9 months (range 1–16). In the rescue ITI patient, peak inhibitor was 1000 BU, titre at start of ITI was 16 BU and ITI duration was 12 months. Immune tolerance was achieved in all eight primary ITI patients, of which 7/8 achieved complete success. Partial success was achieved in one patient. ITI failed in the one and only rescue patient. The overall rate of complete success was 77.7%. No patients were observed to relapse in a mean follow-up period of 5 years (range 1.3–6.7).
Conclusion: The observed outcomes in ITI with Fanhdi® confirm other recently published data which demonstrate high success rates with this type of product.

PO 163

Intracranial hemorrhage in hereditary bleeding disorders: the experience of Çukurova University Hemophilia Center

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Background: Intracranial hemorrhage (ICH) is a life threatening complication of hereditary bleeding disorders in childhood resulting in high rates of mortality and disabling sequelae.

Aim: In this study, we evaluated our patients with intracranial hemorrhage and compared with literature.
Patients and Methods: From 1995 to 2012, 21 patients with intracranial hemorrhage were diagnosed and evaluated in Çukurova University Hemophilia Center. ICH episodes, the findings of physical examination, CT scan or MRI and treatment strategies including surgical interventions were reviewed retrospectively.
Results: We evaluated 22 episodes of ICH from 21 patients with hereditary congenital factor deficiencies (CFD). Age range was from 9 days to 12 years. There were 15 patients with hemophilia A, 2 patients with hemophilia B, 2 patients with factor VII deficiency, 1 patient with factor X deficiency, 1 patient with von Willebrand disease. Two patients with factor VII deficiency, 1 patient with factor X deficiency and 1 patient with von Willebrand disease were female. Except one patient with factor X deficiency, all patients had one bleeding episode. Two patients had a high titer inhibitor against factor VIII. The most important factor was trauma. A history of recent trauma was documented in 10 patients. Intracerebral and subdural hematoma were more frequently seen. The most frequent symptoms were seizure and headache. The diagnosis of hemophilia was established in five patients after intracranial hemorrhage who referred to our center with ICH. In nine patients, hematoma was evacuated. Three hemophilia patients died due to ICH and four patients presented late sequelae.
Conclusion: Intracranial hemorrhage is the most serious complication in childhood, especially for hereditary bleeding disorders. Urgent establishing diagnosis and treatment with prompt doses of factors to initially maintain a normal concentration of circulating factor is mandatory. Despite the prompt treatment, death and late sequelae can be seen in this patients group.

PO 164

Treatment of outpatient dental extractions in persons with inherited bleeding disorders

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Background: Excessive bleeding after tooth extraction is a common risk for patients with Hemophilia A, Hemophilia B and von Willebrand disease. Current treatment uses clotting factors or desmopressin acetate and oral antifibrinolytics to prevent prolonged bleeding; however, there are few well-defined protocols based on prospective studies available for the prevention of post-dental extraction bleeding.
Aims: We completed a retrospective review of our treatment center's experience in the management of patients undergoing dental extraction to evaluate the effectiveness of our protocol in preventing delayed bleeding. We evaluated use of additional clotting factor, hospitalization or patients who had delayed bleeding.
Methods: Four hundred and fifty charts of persons with bleeding disorders were reviewed and 27 patients who had undergone dental extractions were identified. The protocol included collaboration with community dentists and local hematologists. For patients with moderate or severe Hemophilia A or B, and von Willebrand Type II or III, a factor infusion was administered to raise factor levels to at least a predicted 80% correction. In some patients, this peak was measured by appropriate laboratory tests. Patients with von Willebrand disease, type I were treated with a dose of desmopressin acetate nasal spray one h prior to procedure. All patients were treated with oral aminocaproic acid, 100 mg/kg every 6 h for 5–7 days. A phone call was made immediately after the procedure to assess bleeding complications. Further follow up was determined as needed.
Results: Twenty-seven patients with hemophilia A ($n = 12$) or Hemophilia B ($n = 8$) and von Willebrand disease (5 = type 1, 2 = type 2 or 3) underwent single extractions, third molar extractions or multiple extractions in outpatient dental offices. Twenty-two patients with uncomplicated bleeding disorders did not experience delayed bleeding nor did they require treatment with additional doses of factor or admission to the hospital. All delayed bleeding episodes occurred in patients with complicated bleeding disorders. One patient with an active high titer inhibitor required additional treatment with bypassing agents for 3 weeks; one patient stopped his oral antifibrinolytics at day 2, experienced delayed gingival oozing and required five additional doses of factor daily; one patient with a psychiatric history was treated with factor infusions daily for 1 week for complete dental extraction. Two patients with complicated medical histories were admitted to the hospital and not treated with the usual protocol: one with a history of intracranial hemorrhage and transportation difficulties and one with HIV and Hepatitis C-related thrombocytopenia.
Summary/Conclusion: Our data indicate that the treatment protocol we use for routine dental extraction is safe and effective in preventing excessive bleeding after tooth extraction in persons with uncomplicated Hemophilia A, B and von Willebrand disease. Our experience suggests that persons with complicated bleeding disorders should be considered for additional treatment. Our protocol, when used in collaboration with community dentists, could be used as a guide for those treating patients with inherited bleeding disorders, and as a protocol for prospective studies of treatment regimens for dental procedures.

PO 165

Surgery in hemophilia – a real therapeutic challenge for a low-resource country

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Introduction: Surgery is a big challenge in the developing world because of low accessibility and availability to an adequate substitution.
Objectives: We aimed at evaluating the outcomes of surgical procedures in our conditions of substitution, of obstacles and difficulties we met during surgery, assessing the risks and complications we were confronted with.
Patients and Methods: Our descriptive observational retrospective study included 171 surgical procedures, undertaken on 101 patients (83- hemophilia A, 13 -hemophilia B and 5- von Willebrand disease, 92.07% of them with severe form) over a period of 11 years. In 15.84% of cases-2, in other 10.89% of cases – 3 and in 14.85% more than 3 interventions have been undertaken, respectively.
Methods: Invasive elective procedures were predominant (84.80%) vs. emergency measures (15.20%). We considered that 35.67% of surgical interventions were major, 21.63% intermediate and 42.69% minor. By the type of surgery, the orthopedic interventions prevailed (69%), followed by complex stomatological (17.54%) and thoraco-abdominal surgery (11.69%). The rate of complications was highest in major surgery (67.79%), compared with moderate (20.33%) and minor (11.86%) interventions. Their proportion was more important in patients who underwent emergency (63, 63%) vs. programmed interventions (46.83%). The main obstacles were: low dosage (10.89%), short duration of substitution (21.78%) or/and usage of native blood products (6.93%), leading to increased postsurgical bleeding (38.61%) and post-hemorrhagic anemia (25.74%); local infections complicated the evolution in 8.91% of cases; the main threatening complication was the development or increase of low (3.96%) or high (2.97%) titer inhibitors; the risk of blood borne infection or thrombotic accident was low (0.99%). The general outcome was encouraging: life-saving in 32.67%, improving the quality of life with assurance of professional and social insertion in 67.32%.
Conclusion: Surgery is a high demanding intervention in hemophilia, which cannot be ignored in a low resource country. It acts as lifesav-

ing, life prolonging and quality of life improving measure. An emergency depot is needed in each Hemophilia Treatment Center, and a latent therapeutic demand of factor concentrates has to be calculated in order to avoid the use of native blood products and complications.

PO 166

Quality of life and well-being of haemophilia patients and parents in China: subgroup analysis of the HERO study

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Background: Haemophilia A (HA) and B (HB) are serious congenital bleeding disorders, whose prognosis and outcomes are improved by comprehensive care and access to treatment. Bleeding and complications have negative influence on patients' and parents' daily life and social relationships, and can induce psychosocial problems. The Haemophilia Experiences, Results and Opportunities (HERO) study's primary objective was to quantify the impact of key psychosocial factors affecting people with haemophilia (PWH). Questionnaires targeting PWH $\geq$ 18 years and parents of patients < 18 years were used to collect information on demography, social relationships and treatment in this survey involving 10 countries. EuroQoL-5D (EQ-5D) Index was used to assess quality of life (QoL).

Aims: To present and compare data on QoL and well-being in the HERO study between China and other countries.

Methods: A descriptive comparative analysis of Chinese patients' and parents' subgroups.

Results: Overall, 675 patients and 561 parents were recruited, of which 110 (16.3%) patients and 58 (10.3%) parents were from China. There were 40.0% and 89.7% of Chinese patients and parents married or in a long-term relationship (60.0%, $P < 0.01$ and 83.5%, $P = 0.22$ for non-Chinese). For Chinese PWH: The overall mean EQ-5D score (healthy subject score = 1.00) was 0.71 (0.75 for non-China, $P = 0.07$). Mean scores for subgroups by disease were 0.72 (HA) and 0.68 (HB), and mean scores for subgroups by treatment were 0.70 (on-demand), 0.87 (prophylaxis) and 0.73 (on-demand with situational prophylaxis). Issues with mobility (71.8%), self-care (27.3%), usual activities (58.2%), pain/discomfort (65.5%) and anxiety/depression (60.0%) were noted in higher percentage than those reported in non-China except pain/discomfort. Overall, 36.4% (28.7% for non-China, $P = 0.11$) reported self-assessing 'health' scores $\leq$ 50 using a 100-point scale. And 13.6% (7.4% for non-China, $P < 0.05$) thought that pain interfered 'extremely' with their daily life, while 32.7% (14.9% for non-China, $P < 0.0001$) rated this as 'quite a lot'. Only 7.3% received psychological treatment in the past year (25.3% for non-China, $P < 0.01$). For Chinese parents: The percentages of Chinese vs. non-Chinese parents hiding haemophilia from others (60.3% vs. 13.3%, $P < 0.0001$), feeling too much pressure (37.9% vs. 17.1%, $P < 0.01$), considering that the close relationship with partner was hampered (27.6% vs. 16.7%, $P < 0.05$) and preventing patients from 'too dangerous' sports (89.7% vs. 50.9%, $P < 0.0001$) were significantly higher. Significantly less Chinese parents thought that haemophilia 'did not prevent desired holidays' (37.9% vs. 69.6%, $P < 0.0001$) or 'did not influence their son's school relationships' (36.2% vs. 56.1%, $P < 0.01$). Altogether, 25.9% received psychological treatment in the past year (24.3% for non-China, $P = 0.79$). On a 7-point scale (where a higher score indicated more optimistic attitude) for patient/parent future outlook, the mean score in China was 4.22/3.96 (5.13/5.64 for non-China, $P < 0.0001/P < 0.0001$), with a significantly lower percentage scoring 7 point (very optimistic) than their counterparts in non-China (11.8%/0.0% vs. 23.5%/30.6%, $P < 0.01/P < 0.0001$).

Conclusion: Haemophilia impacted QoL of patients and families more in China than in other countries in the study. A few of Chinese patients and parents received psychological support and treatment. Chinese patients and parents were not as optimistic about future outlook as their counterparts from other countries.

PO 167

Adult onset high titer inhibitor disappeared in a patient with congenital hemophilia A after immune tolerance induction therapy

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Background: Factor VIII (FVIII) inhibitors most frequently develop in patients with severe hemophilia A during relatively young ages and in the early stage of their replacement therapy.

Objective: We report the case of a 38 year-old male patient with severe hemophilia A in whom recently developed FVIII inhibitor was eradicated after immune tolerance induction therapy (ITI).

Clinical course: The patient was diagnosed at 3 months of age because of his bleeding tendency, which was not present in any of his family members. The patient was treated with plasma-derived FVIII concentrate until 2003 and recombinant product (rFVIII) thereafter. On first detection in 2002, his FVIII inhibitor titer was 1.6Bethesda Units (BU)/ml. Thereafter, the inhibitor titer increased gradually to 2.4 BU/ml in 2004 and 5 BU/ml in 2007. The patient and his general practitioner did not attempt to rectify the low inhibitor titer because the FVIII concentrate continued to be effective for his subcutaneous and joint bleeding. The frequency of his subcutaneous hemorrhages increased from around 2004 and increased doses of FVIII concentrate ceased to provide an adequate hemostatic effect. Thus, he visited another general hospital. In 2008, his inhibitor titer rose to 8 BU/ml. In 2009, when he moved to the hemophilia treatment center in our university hospital to plan his therapeutic options, his inhibitor titer was 17.9 BU/ml. His screening examination showed no significant findings with respect to autoimmune diseases. Episodic on-demand treatment with FVIII concentrates was discontinued and treatment with a low-dose protocol of ITI, 50 U/kg of rFVIII, Advate® (Baxter Inc.), 3 times a week, was introduced.

Result: After starting ITI, his inhibitor titer reached a maximum 36.8 BU/ml in the third week of treatment. However the titer declined slowly and episodic bleedings decreased. After 1 year of ITI treatment, the inhibitor was eradicated (< 0.6 BU/ml, more than 70% of the recovery). At present the patient has been receiving the same regimen as a regular replacement and there has been no relapse. At the time of writing, only a few results regarding the usage of ITI in adult patients with congenital hemophilia A has been reported.

Conclusion: ITI treatment was thought to be worth considering for inhibitor patients who have not yet received it, and even for older patients or patients in whom ITI treatment failed without the patient receiving sufficient dose or completing the full course of treatment.

Informed consent was obtained from the patient concerned and the authors state that they have no conflict of interest in this study.

PO 168

Surgery in FVIII inhibitor patients: single center experience of 13 procedures in 8 patients

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Background: Surgery in congenital and acquired hemophilia A patients with factor VIII inhibitors is problematic. There are no widely accepted consensus guidelines for managing surgery in these patients. As inhibitor patients are generally refractory to replacement therapy with factor VIII concentrates, achieving hemostasis requires the use of the inhibitor bypassing agents activated prothrombin complex concentrate (APCC) or recombinant activated factor VII (rFVIIa). Porcine plasma-derived factor VIII was used as a bypassing agent in the past, but is no longer available. Bypassing agents are less reliably effective than replacement therapy, and there are no widely available or validated laboratory assays to monitor their efficacy.

Aims: Our aim is to share our experience of consecutive major and minor surgical procedures in inhibitor patients done in a single adult hemophilia treatment center over a period of 14 years.

Methods: Between 1998 and 2012 we performed 13 surgical procedures in eight inhibitor patients using all three inhibitor bypassing agents. The nature of the procedures, hemostatic management protocols, and outcomes of these consecutive, unselected cases will be reviewed.

Results: Six patients had severe and one had mild congenital hemophilia A; one had acquired hemophilia. All had longstanding factor VIII inhibitors. Patient ages at the time of surgery ranged from 20 to 74 years. The surgeries included: 4 major orthopedic procedures in three patients (2 total knee arthroplasties, 1 ankle arthroplasty, 1 ankle fusion); four dental surgeries in two patients; one pacemaker implantation (in the acquired hemophilia patient); two nasopharyngoscopies with biopsies and cauterization (in the same patient); one craniotomy (bilateral burr holes); and 1 tracheostomy. The surgeries were done under cover of treatment with APCC, rFVIIa, or porcine factor VIII concentrate. One procedure, a tooth extraction done with APCC in a congenital hemophilia patient, had a poor outcome; he had severe bleeding which required intensive care admission, sequential rFVIIa and APCC, and lingual artery embolization. One knee arthroplasty done with APCC had a fair outcome; early re-bleeding occurred, which was felt to be due to excessively rapid tapering of APCC, and which responded to more intensive APCC administration. All other surgeries had good or excellent outcomes. For major procedures, coagulation parameters were followed in part to confirm perturbation of the hemostatic system but mainly as surveillance for consumption coagulopathy. Thromboprophylaxis was not given to those treated as hospital in-patients.

Summary and Conclusions: We observed excellent or good outcomes in 11 of 13 surgical procedures in inhibitor patients, a fair outcome in 1, and a poor outcome in 1. The use of global hemostasis assays might have proven helpful for monitoring therapy but the value of these modalities in surgery needs to be validated. Necessary surgery should not be withheld in hemophilia A patients because of the presence of a factor VIII inhibitor. Although access to a specialized coagulation laboratory is not necessary to guide therapy, these procedures should only be performed in hemophilia treatment centers, under the care of physicians and surgeons experienced in managing patients with complex bleeding disorders.

PO 169

Use of factor VIII after inhibitor clearance in patients with moderate hemophilia

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Background: Anti-factor VIII (FVIII) inhibitory antibodies (inhibitor) are a major complication of hemophilia A (HA) and can be seen in up to 30% of persons with severe HA and about 0.9–7% of those with mild or moderate HA. Inhibitors can be eradicated through the use of immune tolerance induction (ITI), rituximab, or other immune modulating treatments. It is unknown whether persons with moderate HA (MHA) who develop an inhibitor can restart factor replacement therapy after resolution of their inhibitor in the absence of ITI. To our knowledge, there are no published reports describing the re-initiation of FVIII replacement therapy in persons with MHA complicated by an inhibitor.

Aims: To review a single institution's experience with re-initiation of FVIII treatment in patients with MHA after clearance of their inhibitor without ITI.

Methods: Persons with MHA with inhibitor were identified from a database of current patients treated at the Emory/Children's Hospital of Atlanta (CHOA) Hemophilia Treatment Center (HTC). Their medical record was reviewed. Information collected includes baseline FVIII levels, *f8* gene mutation, the occurrence of intensive FVIII exposure (> 5 days) prior to inhibitor development, the peak inhibitor titer, inhibitor eradication treatment received, and when the patient restarted FVIII replacement.

Results: At the Emory/CHOA HTC, 67 patients with MHA are followed routinely. Among this cohort, five were identified to have their course complicated by an inhibitor; 4/5 had high-titer inhibitor; 4/5 had prior intensive exposure; and 3/5 (Case #2, #4 and #5) had the N1922S mutation. One patient (Case #1) underwent ITI and was unsuccessful. Case #2 received rituximab without inhibitor clearance until 13 months after treatment. One month after inhibitor resolution, he restarted FVIII replacement therapy. At his first re-exposure to FVIII, his FVIII recovery was > 100% and half-life was 10.8 h. He continued to treat with FVIII (25 IU/kg) thrice weekly to prevent recurrent ankle bleeds. After 3 months of treatment, he had similar FVIII recovery and half-life and negative Bethesda assay and has continued on the same regimen for an additional 5 months of follow-up. Three patients did not receive any treatment and had resolution of their inhibitor after 36 months (Case #3), 23 months (Case #4), and 9 months (Case #5). Of these three that had spontaneous resolution, 2 are now receiving FVIII replacement therapy on-demand. Case #3 was re-exposed to FVIII, following 10 years of negative inhibitor titers, for treatment of persistent hematuria despite recombinant factor VIIa (rFVIIa) requiring hospitalization. After 3 days (6 doses) of FVIII treatment, he did not have an anamnestic response. He has received 1 FVIII infusion since re-initiation of FVIII replacement therapy. Case #4 had 3 months between undetectable inhibitor and re-exposure and has been treated on-demand for 17 years without recurrence. His current annual exposure is approximately 30 infusions. The third patient (Case #5) with spontaneous resolution has elected to continue to use rFVIIa to treat bleeding episodes.

Summary/Conclusion: Persons with moderate hemophilia A who clear their inhibitors without ITI may be successfully treated with FVIII without recurrence of inhibitors.

PO 170

Outcome of liver transplantation haemophilia patients in the Nordic countries

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Background: Until the mid-1980s, the vast majority of persons with haemophilia who had received plasma-derived non-heat-treated coagulation factor concentrates became infected with hepatitis C virus, HIV or both. Of those, about 10–15% developed end stage liver disease (ESLD) or hepatocellular carcinoma and required liver transplantation. Liver transplantation (LTX) in haemophilia patients has the potential to cure haemophilia as the donor liver is able to synthesise the deficient coagulation factor. However, the coagulopathy associated with ESLD may carry an additional bleeding risk affecting the outcome of this surgical intervention. Previous reports from different LTX centers worldwide have in general concluded that the peri- and postoperative outcomes in haemophilia patients were similar to non-haemophilia patients but the recurrence of HCV infection remains the main challenge for longterm outcome. Nevertheless, little is known about the outcome of LTX in haemophilia patients in the Nordic countries.
Aims: The objective of this study was to evaluate the outcome of LTX in haemophilia patients in the Nordic countries.
Materials and Methods: Scandiatransplant is a Nordic organ exchange organization established for more than 40 years ago with a close collaboration between the transplant centers in the five Nordic countries (Norway, Iceland, Denmark, Sweden and Finland). The study included all haemophilia patients who have undergone liver transplantation in the involved countries. Data from the Nordic Liver Transplant Registry supplemented by clinical information obtained from medical records provided by each transplant centre were used for analysis. The Nordic Liver Transplant Registry is a prospective database of all patients undergoing LTX where data are updated regularly, at least once a year.
Results: From 1993 to 2011, a total number of nine patients with haemophilia underwent LTX (2 from Norway, 4 from Sweden, 1 from Finland and 2 from Denmark). The mean age at LTX was 59 (range 45–64) and the main indication for LTX was HCV related liver disease. Three are still alive while 6 have died. The mean survival time post LTX for those who died were 11 months (range 3–20 months). The longest survival among the patients who are still alive is 15 years. The causes of death were renal failure, gastrointestinal haemorrhage, non-anastomotic biliary complication, recurrence of original tumor, cerebrovascular event and bacterial infection. For those undergoing LTX between 2001 and 2008 only 20% in the haemophilia patient population had a 5 years survival compared to 83% in the non-haemophilia patients.. Among the three longterm survivers the mean age at LTX was 48 vs. 56 years for those who have died.
Conclusion: The study showed that LTX in haemophilia patients under the coverage of coagulation factor concentrates in the Nordic countries was safe and consistent with previous reports from other centers. However, the 5 years survival was substantially reduced in the haemophilia patients. The age at transplantation seemed to be the main factor associated with reduced survival in patients with haemophilia.

PO 171

Experience of home prophylactic treatment of inhibitory form of haemophilia A in children using recombinant activated FVII

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Background: Prophylactic treatment of children with Haemophilia A and FVIII inhibitor by recombinant activated factor VII (rFVIIa) has wide dose range from 90 to 300 mkg/kg for obtaining effective hemostasis.
Aims: To assess the efficiency of rFVIIa – Koagil-VII ('GENERIUM', Russia) which was used in individual doses and schemes for reduction of hemartrosis and severe bleedings frequency, also for prevention of complications (chronic synoviitis, arthropathy etc.).
Methods: Four patients obtained home prophylactic treatment by Koagil-VII within 1 year (since June 2011 to June 2012) with the following doses: from 141 to 257 mkg/kg with interval of 12 or 24 h. The quantity of bleedings and conditions of target joints were compared with results of prior year treatment (since May 2010 to May 2011). Treatment was 'on demand' and after serious bleeding patients got short courses of prophylactic treatment during up to 3 months with the following doses: from 90 to 120 mkg/kg with interval of 48 h.
Results: Patient 1: 2.6 years old. Weight – 14 kg. Inhibitor > 40 BE. Treatment scheme: 171.4 mkg/kg with interval of 12 h during 4 months, further 257.1 mkg/kg in the morning and 171.4 mkg/kg in the evening during 8 months. Bleedings amount before prophylactic was 29 (16 hemarthrosis), bleedings amount during prophylactic – 9 (2 hemarthrosis). Patient 2: 6 years old. Weight – 25 kg. Inhibitor – 27 BE. Treatment scheme: 120 mkg/kg with interval of 12 h during 12 months. Bleedings amount before prophylactic – 25 (20 hemarthrosis), bleedings amount during prophylactic – 4 (3 hemarthrosis). Patient 3: 6.3 years old. Weight – 30 kg. Inhibitor – 36 BE. Treatment scheme: 160 mkg/kg with interval of 48 h during 1 month, subsequently 160 mkg/kg with interval of 24 h during 11 months. Bleedings amount before prophylactic – 28 (17 hemarthrosis), bleedings amount during prophylactic – 13 (7 hemarthrosis). Patient 4: 14 years old. Weight – 34 kg. Inhibitor > 40 BE. Treatment scheme: 141.1 mkg/kg with interval of 24 h during 162 days, further 141.1 mkg/kg with interval of 12 h during 187 days. Bleedings amount before prophylactic – 8 (3 hemarthrosis, 1 extensive hematoma of thigh, 3 retroperitoneal hematomas, 1 renal bleeding), bleedings amount during prophylactic – 6 (2 hemarthrosis, 3 small hematomas, 1 renal bleeding). Regression of chronic synoviitis in the target joints took place in all patients. During the prophylactic period all identified hemartrosis and hematomas occur after injuries. No side effects of Koagil-VII was identified.
Summary/Conclusion: Prophylactic treatment of children with FVIII inhibitor by recombinant activated factor VII (rFVIIa) – Koagil-VII is efficient when individual high doses and treatment schemes are determined. It resulted in reduction of bleedings in 1.5–6 times and stopped of arthropathy progress.

PO 172

Diagnosis of inherited coagulation disorders – when and why?

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Background: The testing of inherited coagulation disorders has been carried out in our institution for more than 35 years. The total number of patients with coagulation disorder was 107, as follows: 39 with hemophilia A, 12 with hemophilia B, 8 with FXI deficiency, 15 with von Willebrand's disease, and 33 patients had rare coagulation disorder.

Aim: Analysis of the age of diagnosis and the reason why coagulation disorder has been diagnosed.
Method: Analysis and statistics elaboration of medical and laboratory data.
Results: Hemophilia A has been diagnosed in 39 patients. Severe hemophilia A has been diagnosed in 18% (7/39), which is 3/7 (43%) in the first year of life while the other 57% (4/7) till the third year of life. All the mentioned above have been tested for the same reason, which is bleeding. Moderate hemophilia A has been diagnosed in 23% (9/39), within the ranging of diagnosis from 1 till 40 years of age. Reason for testing was bleeding in 67% (6/9), preoperative screening in11% (1/9) and family screening in 22% (2/9). Mild hemophilia A has been diagnosed in 23 cases (59%), in the range from 2 months till 50 years of age. The reason for testing was bleeding in 43% (10/23), family screening in 26% (6/23) and preoperative testing in 30% (7/23) of cases.
Hemophilia B was found in 12 patients. One patient (8%) had severe hemophilia B, the reason for testing was bleeding in the child's first year of life. Moderate hemophilia B has been diagnosed in 50% (6/12) of cases in the range from the 1st year of age till they were 40 years old, and the reason for testing was bleeding in 66% (4/6) of cases, preoperative screening as well as family screening was reason in 17% (1/6). Mild hemophilia B has been diagnosed in five cases (42%) from the first year of age till the age of 60. The bleeding was reason for 2/5 (40%) of patients to get tested, preoperative screening diagnosed 2/5 (40%), while family screening diagnosed 1/5 (20%) of cases.
The factor XI deficiency was proved in eight patients, ranging from 20 years till 70 years of age. In 7/8 (87%) of cases the reason for being tested was preoperative screening while in 1/8 (13%) of cases was bleeding. Von Willebrand's disease was diagnosed in 15 patients from 3 months of age till 40 years of age. The reason for being tested was bleeding in 67% (10/15), preoperative testing in 13% (2/15) and family screening in 20% (3/15) of cases.
Other rare coagulation disorders, as the FI, FV, FVII, FX, FXIII deficiency or combined deficiency have been found in 33 patients.
Conclusion: severe cases of hemophilia and severe hereditary coagulation disorders have been diagnosed at an early stage of life, mostly due to bleeding. Milder cases of hemophilia and coagulation disorders have been diagnosed at a rather wider range of age span and it was mainly due to preoperative testing or family screening.

PO 173

Successful anticoagulation with concomitant factor VIII replacement in a severe haemophilia A patient suffering a life threatening thrombotic event

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Background: Venous thromboembolic events (VTE) in severe hemophilic patients are rare, with spontaneous life-threatening thrombosis even scarcer. Literature is limited and clear guidelines regarding the management of anticoagulation (ACO) in hemophilic patients including factor replacement therapy, ACO agent, treatment duration, intensity, and safety of ACO are not available.
Aims: A teenage male with severe haemophilia A (HA) suffered a spontaneous life-threatening thrombosis requiring both FVIII replacement and ACO. The patient received 6 weeks of ACO comprised of initial unfractionated (UFH) then subsequent low-molecular-weight heparin (LMWH), and FVIII with complete resolution without recurrence (4.5 months follow-up). This case demonstrates the use of safe ACO in haemophilia.
Methods: An 11 year-old male with severe (< 1%) HA without inhibitor who was maintained on prophylaxis with recombinant FVIII (Xyntha®) via peripheral venous access (dosing tailored by pharmacokinetics (PK solutions®- SummitPK.com) with every other day infusion), presented to his local emergency room with acute incapacitating abdominal pain. A presumptive diagnosis of appendicitis was made and the patient infused with his standard FVIII dose (67 IU/kg) and transferred to our institution. Further evaluation revealed ischemic bowel syndrome due to a thrombosis with total occlusion of the superior mesenteric vein documented by Doppler ultrasonography and CT venogram. ACO was initiated within 6 h from acute pain onset. Protrhombotic evaluations performed at diagnosis, and as outpatient were negative for underlying risk factors (protein C, S, antithrombin deficiency; factor V Leiden mutation; protrhombin gene 20210A mutation; Lupus Anticoagulant/Phospholipid dependent antibodies; lipoprotein-a; homocystein; paroxysmal nocturnal hemoglobinuria, fibrinogen).
Results: Following 48 h of ACO with UFH (targeted anti-Xa 0.35–0.70 units/mL) and recombinant FVIII (rFVIII; Xyntha®) continuous infusion targeting FVIII > 80%, total clot resolution was observed via US Doppler. ACO was continued with UFH for 5 days with concurrent rFVIII (levels between > 80% and < 200%). Pain improved following 24 h, and resolved after 48 h of ACO. A transition to LMWH was performed 1 week after documentation of clot resolution (1 mg/kg/dose enoxaparin SC BID; targeted anti-Xa 0.5–1 units/ml). Following discharge on once daily bolus rFVIII the patient was unable to maintain trough FVIII activity > 10% despite a calculated 120% correction, therefore twice daily calculated 60% correction with rFVIII was initiated and resulted in troughs of 30–50% activity. Anatomical evaluations with MRI/MR venography revealed no underlying structural abnormality that contributed to the thrombotic event. The patient was maintained on ACO and twice daily rFVIII for 6 weeks without recurrence or bleeding complications.
Summary/Conclusion: Here we report successful and safe ACO covered with factor VIII replacement in a patient with severe HA suffering from a life threatening spontaneous VTE event. While occurrences of myocardial infarction and cerebrovascular accident in patients with HA have been documented, in contrast, little literature exists regarding venous thrombosis in these patients. It is extremely important that clear guidelines concerning the safety and duration of ACO following VTE be established in this specific patient population.

PO 174

Clinical experience with new third generation recombinant B-domain deleted factor VIII concentrate (beroctocog alpha) in a single center

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Background: Third generation recombinant factor VIII concentrates have become the prevailing drug for hemophilia A patients. By 2010, 2 types of the concentrates, 1 full-length recombinant factor VIII (octocog alfa) and 1 B-domain deleted factor VIII (moroctocog alfa), were available across the world. Recently new B-domain deleted factor VIII (beroctocog alfa) has been introduced in Korea.
Aims: We aimed to evaluate the efficacy and safety of the new drug. As for efficacy, we conducted *in vivo* recovery (IVR) and efficacy rating. Regarding safety, inhibitor titer and adverse events were monitored.
Methods: We reviewed 89 hemophilia A patients' medical records and surveyed for efficacy rating through telephone. Two previously untreated patients were included. Phenotype was severe in 63, moderate in 18, and mild in eight patient. Exposure days (ED) were available in 82 patients. IVR with dosage of 25 U/kg of the concentrate was carried out in 29 patients using one-stage assay. Inhibitor assay using Bethesda assay were available in 31 patients. At the time of inhibitor assay, their mean ED reached 17, and ED was 50 or more in 13

patients. In terms of efficacy rating, 45 patients responded as 4 point scale.

Results: IVR was 2.4%/u/kg and inhibitor titer 0.1 BU/ml with no development of inhibitor. Eighty-four percent of the patients rated the efficacy as excellent (42%) and good (42%). Sixteen percent of patient rated as fair. Six of 82 patients (8%) reported eight minor adverse events: posterior neck flushing sense (3), nausea (2), chest thightness (1), buring heart (1), dizziness (1).

Summary: The new third generation B-domain deleted factor VIII showed satisfactory IVR (2.4%/u/kg) and efficacy rating (84%). There was no inhibitor development in 31 patients with mean 17 ED. Only minor adverse events were observed.

PO 175

Spectrum of molecular events encountered in Hemophilia B in a referral hospital in North India

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Background: Hemophilia B (MIM# 306900; HB) is the second most common severe inherited bleeding disorder encountered in our population. Due to a wide range of molecular heterogeneity, identification of the mutation is necessary to offer carrier detection and prenatal diagnosis.

Aims: We characterized the molecular events in eighteen patients (17 families) and performed carrier screening in family members to offer appropriate counselling.

Methods: Identification of cases of HB was carried out at our Institute by the coagulation screening tests followed by Factor IX assays. Genomic DNA was extracted from peripheral blood leucocytes using standard phenol-chloroform method. Coding regions including the splice junctions were amplified by PCR using specific sequences. Automated sequencing was performed on the ABI Prism 310 sequencer (Applied Biosystems, Foster City, CA, USA).The software Sequencher was used for evaluating the sequencing results, followed by base to base evaluation of results. Whenever possible the mother's sample was sequenced to identify the inheritance and sisters were evaluated for carrier status.

Results: Eighteen patients of HB in 17 families were analysed. Ten different point mutations were identified of which nine were missense mutation and one was an acceptor splice (b) Ex 2 (CAG→CGG). Three of the missense mutations resulted in a stop codon termination. Seven of the mutations involved the amino acid arginine and consequently a CpG island. Exon 2 was the commonest exon to be involved in a mutation/deletion (11 cases). The commonest recurring mutation was *c*. 6364 Cd -4(CGG→TGG) R-W in exon 2. Three cases showed deletions of which one case showed deletion of exons 1–3. Four novel mutations found were splice (b) Ex 2 (CAG→CGG), c.6436 Cd 20 (GAA→TAA) E-X, Cd 121 (CAG→CTG) Q-L with 26 nucleotide deletion (including beginning of intron 6) and Cd 169 (del actc) Fs X 28/29. Four mutations were found to be reported earlier in the Haemophilia B mutation database (version 13, 2004) http://www.kcl.ac.uk/ip/petergreen/haemBdatabase.html, but not from India. The Malmo polymorphism (rs6048:G>A; Ala194Thr in the primary translation product and Ala148Thr in the mature protein) in the activation peptide of the protein encoded by exon 6 was detected in 2 of 10 alleles.

Twelve mothers were tested for the specific mutation detected and all were found to be heterozygous. Carrier screening for twelve sisters showed six each to be carriers and normal. Prenatal diagnosis was carried out successfully in one family.

Summary/Conclusions: This study has characterized seventeen hemophiliac families from Northern India and identified four novel mutations/deletions. Additional four mutations not yet described from India were also found. Except for two mutations which were recurrent, all were single cases. No sporadic case has been identified as yet. Thirteen cases (72%) involved the PCR product amplifying exons 2 and 3 together and this can therefore be tested for initially. The analysis is useful to carry out carrier screening and prenatal diagnosis for our population.

PO 176

Real-life use of activated recombinant Factor VII (rFVIIa) in patients with haemophilia B with inhibitors – data from the UKHCDO/NHD registry

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Background: Activated recombinant Factor VII (rFVIIa, NovoSeven®, Novo Nordisk, Denmark) is established as a well-tolerated and effective bypassing agent for the treatment of patients with haemophilia with inhibitors, and is approved for use at standard (3 × 90 μg/kg) and high (1 × 270 μg/kg) doses in the United Kingdom (UK). Data on the use of rFVIIa in patients with haemophilia B (HB) is limited because of the rarity of the condition; however, anecdotal reports suggest rFVIIa may be less effective in HB than haemophilia A (HA).

Aims: To assess the real-world use and safety of rFVIIa in patients with haemophilia with inhibitors in a post-marketing surveillance study, requested byEMA and conducted by the UK National Haemophilia Database (NHD) (1 Jan 2008 to30 June 2011).

Methods: The NHD collects treatment and adverse event data quarterly from all UK haemophilia centres, in accordance with the Declaration of Helsinki. Anonymised data on rFVIIa dosing and adverse event occurrence were reported from patients with haemophilia treated with rFVIIa. Dose regimens were determined by the managing clinician.

Results: Sixty-seven patients (median age 23.2 years) from 25 centres were included, involving 1057 treatment episodes with rFVIIa. Sixty HA patients reported 976 treatment episodes (42.1% in patients < 18 years old; 8.1% in those ≥ 65 years old) and seven HB patients (11%; median age 25 years) reported 81 episodes (40.7% in patients < 18 years old; none in those ≥ 40 years old). Most HB treatment episodes (76/81, 93.8%) involved acute, non-surgical bleeds, while two (2.5%) were treated prophylactically and three (3.7%) were for surgery. Patients with HB received a lower initial rFVIIa dose compared with HA (mean 139.5 μg/kg vs. 180.7 μg/kg; median 119.0 μg/kg vs. 148.1 μg/kg; range 23.6–291.6 vs. 11.0–491.8 μg/kg). Higher initial rFVIIa doses (≥ 180 μg/kg) were administered more frequently to patients with HA than HB (40.5% vs. 13.5%), although more patients had missing data in the HB group (32.1% vs. 8.4% for HA). Patients with HB received a higher mean number of rFVIIa doses than HA (5.2 vs. 4.5), had a longer mean duration of treatment (47.0 vs. 41.6 h) and received a higher mean total dose of rFVIIa (759.6 vs. 604.7 μg/kg). Single-dose regimens (a dose spaced > 26 h from previous and following doses) were used in 57.1% of patients with HB for treatment of 35.8% of episodes; this is substantially lower than the proportions of patients and episodes treated with single-dose regimens in the subgroup with HA (71.7% and 52.3%, respectively). There were no adverse events judged to be related to rFVIIa. No thromboembolic events or episodes of anti-rFVIIa antibody formation were reported.

Conclusions: These data reveal differences in the rFVIIa dose regimens used in patients with HB vs. HA in theUK. Patients with HB are treated with lower initial rFVIIa doses, and are more likely to receive multiple dosing than patients with HA. Treatment of HB may be improved by administering higher initial rFVIIa doses. There were no reports of adverse drug reactions and no thromboembolic events in HA or HB patients.

PO 177

RFVIIa prophylaxis for hemophilia B with recurrent high-titer inhibitors: a single patient experience

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Many patients with severe hemophilia develop inhibitors to coagulation factors, the presence of inhibitors makes the treatment very challenging for both patients and doctors. Hemostatic cover for bleeding episodes is costly and thus has a substantial economic impact on healthcare budget. Life quality of these patients usually deteriorates along with progression of arthropathies that lead to permanent disability.

We present a case of a male patient with severe hemophilia B born in 1997. The diagnosis was made at the age of 8 months (factor IX < 0.1%), deletion at Ex6 was detected. During first years of life he received *on demand* treatment with plasma-derived factor IX concentrate. In pre-school age two major target joints (right knee and left ankle) developed, followed later by other knee and ankle. In-hospital treatment usually was required 2–3 times per year due to recurrent severe bleeding episodes. In 2006 we performed Rifampicin synoviorthesis procedures for target joints, covered by factor IX. Between the procedures an allergic skin reaction (diffuse urticaria) to an unidentified agent (probably, FIX or rifampicin) was observed. Two weeks after synoviorthesis the patient developed severe hemorrhage after tooth extraction that was refractory to high-dose FIX, inhibitors > 250 BU were detected. Therapy was switched to rFVIIa *on-demand* treatment; still, frequent in-hospital treatments occurred. In July 2008 an episode of resistance to rFVIIa was detected when continuous bleeding after tooth extraction with hemostatic covering (rFVIIa) lasted for 4 days. Local treatment (surgical revision, orthodontic plate) and tranexamic acid were applied in combination with rFVIIa for 5 days, IVIG and Methylprednosolone. In 5 days clinical response was achieved, inhibitor titer at this time was 5 BU. Parents refused ITI that time, in 6 months during routine follow-up the inhibitors disappeared. Frequent hemorrhages occurred during next 3 years, leading to arthropathies in major leg joints. Twenty-seven bleeding episodes of different localizations were observed in January-November 2011 (mean rate – 2.5 bleeds per month), with monthly consumption of rFVIIa 516–2459 μg/kg.

The decision to start prophylactic treatment with rFVIIa was made in adolescence (14 years) due to progressively deteriorating quality of life. Low-dose empirical approach (90 μg/kg 3 times per week) was chosen. The prophylactic treatment was started in the end of November 2011. Only 5 bleeds occurred during 9 months of prophylaxis, with monthly consumption 800–1575 μg/kg. In August 2012 a massive spontaneous left leg soft tissues hematoma occurred that turned refractory. FIX was added with good clinical effect at the beginning, but the loss of hemostatic control occurred in 5–6 days, while titer of inhibitors rose > 16BU. After this episode we changed the prophylaxis to the current 230 μg/kg 3 times per week. With this regime only one nose bleeding was detected in 5 months period, monthly consumption of rFVIIa during the last months was 1900–3500 μg/kg. Thus, the number of bleeding diminished dramatically during the last year of prophylaxis, the compliance to therapy was very good. The optimal dosage remains unclear, repeated episodes of resistance to rFVIIa are still unpredictable.

PO 178

Treatment strategy and outcomes among US haemophilia B patients: results of a patient survey

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Background: Information on patient-reported outcomes and adherence is scant in haemophilia B.

Aims: To describe the treatment characteristics and outcomes of US haemophilia B patients.

Methods: Adults and caregivers of children with haemophilia were identified through a panel of patients originally recruited from haemophilia treatment centers and associations. The study protocol received central institutional review board approval and all patients reported consent to be included in the study. Panelists reporting moderate or severe haemophilia were invited to complete an on-line questionnaire assessing experience with bleeding and joint problems, personal characteristics, and validated scales. The revised Short Form-12 Health Survey (SF-12v2) was included to assess adult health status, and the Short Form 10 Health Survey (SF-10) was used to assess health status in children. Adherence to treatment was measured with the VERITAS-Pro for patients treating prophylactically. The current analysis was limited to patients with haemophilia B.

Results: A total of 24 patients with haemophilia B (14 patients & 10 parents) completed the survey; more than half (14/24) were on prophylactic treatment. All patients/parents surveyed considered themselves/their child to have severe haemophilia. Relative to the average of US adults, adult haemophilia B patients had physical health status slightly below the average (PCS = 46.4) and mental health status slightly above the average (MCS = 53.8). The pattern among children was similar, with mean physical health status below population norms (PHS = 48.5) and psychosocial health slightly above the norm (PSS = 55.8). Over 70% of the patients (17/24) had experienced at least one bleed requiring replacement factor in the past year, and almost half of these patients (8/24) experienced 10 or more bleeds requiring replacement factor over the same period of time. Two thirds (16/24) had experienced joint pain in the past month, and almost 80% (19/24) had experienced joint pain in the past year. Two patients reported having at least one hospital stay due to bleeding and five patients at least one emergency room visit due to a bleeding episode in the past year. Approximately one-third of those using prophylactic treatment (5/14) were considered non-compliant based on the VERITAS-Pro (total score > 57.7 as suggested by literature), an instrument utilized to measure compliance within the haemophilia population.

Summary/Conclusions: Physical health among haemophilia B patients was low, and complications were common. One-third of those on prophylactic treatment were considered non-compliant to regimen. Further research is needed to characterize the relationship between adherence to therapy, health status, and clinical outcomes.

PO 179

Identification of mutations in the Factor IX gene in patients with Hemophilia B in Venezuela

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Hemophilia B (HB) is an X-linked disorder caused by genetic abnormalities in the coagulation factor IX (F9) gene. Severe HB (SHB) patients generally experience prolonged bleeding episodes, which, if not treated, could result in serious life-threatening complications. Mutations causing HB are entirely restricted to the F9 gene, which is located at Xq27, and the specific genetic lesion determines the severity of the disorder. The aim of this study was to determine the genetic alteration causing HB in a population from Venezuela and evaluate the possible effect of the mutation on the structure and function of the protein. Blood samples were obtained from the Banco Municipal de Sangre de Caracas and informed consents were obtained from all patients. In the Human Molecular Genetics Laboratory of the Universidad Simón Bolívar, we extracted the genomic DNA and detected the mutations present in the F9 gene using Conformation-Sensitive Gel Electrophoresis (CSGE) as a mutation screening method. Fifty

percent of the variations were found on the region corresponding to exon 8 (serine protease catalytic region), 13% to exon 6 and 5 (activation peptide and EFG-2 domain), and 6% to exons 4, 2 and 1 (corresponding to EFG-1, Gla domain, and signal peptide, respectively). The possible effect on the function of the protein of each of the mutations identified was analyzed, and its relationship with the severity of the disease was evaluated. This study allowed the identification of 11 new mutations non previously described, including 4 missense, 1 del/ins, 3 deletions, 1 nonsense and 2 mutations within critical splicing sites. This is the first time that molecular studies in the *F9* gene from patients with HB were performed in Venezuela, leading to the initiation of new studies where carrier identification in families with hemophilia B patients could be performed.

PO 180

Mitroaortic valve replacement in a haemophilic B patient with an intracardiac abscess and cerebral septic embolism. Case report

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Background: Life expectancy of persons with haemophilia has improved markedly. Around 1 in 30 patients with hemophilia will have intracranial hemorrhage at least once during their lives. When cerebral hemorrhage is associated with septic embolism, the scenario becomes more critical. Cardiac surgery constitutes a major hemostatic challenge because of sternotomy, heparinization, extracorporal circulation, hypothermia and cardiac arrest.
Case Report: A 28 years-old patient, with known mild haemophilia B (FIXC: 30%), entered in emergency room (ER) with a motor deficit in his right side and sphincter incontinence with 12 h evolution. His wife referred a 3 month history of headache and dizziness, with febrile evening peaks. Due to these symptoms, he went to a local hospital 3 weeks before, and was discharged with a diagnosis of a respiratory infection and 7 days of antibiotherapy. When he finished antibiotherapy, started to feel pain on his lower limbs, without limitations, that got worse. On admission on ER he was awaked and cooperating with no dizziness or headache at that time. Physical examination revealed a grade II mesosystolic murmur over aortic valve and a grade IV systolic murmur over mitral valve, tachycardia, normal blood pressure and afebrile. On neurologic evaluation he presented a Glasgow Score of 15, right side hemiparesis (involving face) and hypoesthesia without visual involvement. He was diagnosed a multifocal cerebral haemorrhage after computed tomography (CT). He was admitted on the stroke unit for conservative treatment. Transthoracic echocardiogram (TTE) detected moderate aortic insufficiency (bicuspid valve) and moderate to severe mitral insufficiency, slight thickening of mitral valve, with adherent vegetation on the anterior leaflet that extended to interatrial septum. Transesophageal echocardiography confirmed those findings. The abscess (38 mm length/11 mm thickness) was adjacent to anterior aortic leaflet, interatrial septum, and slightly on right atrium, continuing to anterior mitral leaflet. *Streptococcus mitis* was found in blood cultures and a diagnosis of cerebral haemorrhage as a result of a septic embolism from intracardiac abscess was made. Looking for the cause of infection, a dental abcess was found and three teeth extractions were performed.
Due to high risk of septicaemia and haemorrhage, he underwent cardiac surgery 1 month later. He needed aortic and mitral valve replacement with bioprosthesis and reconstruction of mitral aortic curtain (bovine graft). All the procedure was made under factor replacement. He was discharged 1 week after surgery and entered an intensive physical rehabilitation programme at a local hospital. Presently, 2 years after, the patient is on anticoagulation therapy with warfarin, without physiotherapy although lightly disabled, nevertheless he regained his drive license.
Conclusions: This report is a 'happy end' of a critical scenario in a haemophilic patient only achieved due to excellence clinical care in a haemophilia reference center, within a central hospital and a high experienced multidisciplinary team.

PO 181

Linkage analysis coupled with direct mutational screening in carrier detection and prenatal diagnosis of hemophilia B

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Background: Hemophilia B is an X-linked recessively inherited bleeding disorder characterized by deficiency of procoagulant factor IX (FIX). The treatment is expensive, hence to step down the incidence of this disease in developing countries, the method of choice is carrier detection followed by prenatal diagnosis.
Aim: The aim of the study was to find out potential carriers and affected fetus.
Methods: Fifty one (30 with family history & 21 sporadic) unrelated hemophilia B patients and their family members were the subjects of this study. Carrier detection by linkage analysis using the polymorphic markers Mnl I, Dde I, Hha I and Sal-I was performed in families with history of Hemophilia B. In sporadic families, the carrier status of female subjects was ascertained by conformation sensitive gel electrophoresis (CSGE) followed by sequencing. Prenatal diagnosis was performed in 28 fetus samples using chorionic villus sampling in 22 cases and 6 with cordocentesis depending on the gestational age and informativity of the linkage markers.
Results: Of the 30 families with positive family history, the defective X chromosome was tracked in 25 mothers. CSGE helped us to diagnose 21/26 (21 sporadic hemophilic and 5 uninformative families from linkage analysis) mothers. Fetuses affected with Hemophilia B were diagnosed in 8 (50%) by chorionic villus sampling whereas cord blood factor IX assay diagnosed 2 to be affected. Six of the fetus from the C vs. was diagnosed as a female child.
Conclusion: Carrier detection followed by prenatal diagnosis serves as an effective way to curb Hemophilia B in an Indian setting. We found that the choice of technique, chorionic villus or cordocentesis, is not really an alternative, but rather dependent on the gestational age of presentation.

PO 182

The efficacy and safety of Benefix in severe and moderate Hemophilia children in China: a signal centre observation study

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Background: BendfiX as the only recombinant product for hemophilia B has been well comfirmed its effects, but not yet in Chinese pediatric hemophilia B.
Aims: To explore the efficacy and safety of the application of Benefix in Chinese severe and moderate Hemophilia B children for low-dose prophylaxis.
Methods: Benefix for low-dose prophylaxis 20 UI/kg, once a week for severe and moderate Hemophilia B children in Beijing Children's Hospital, observed (1) efficacy:Reduction of bleeding, improvement of daily life participation; (2) safety: the side-effect during the Benefix intervention and monitor the inhibitor during the prophylaxis period.

Results: There were 11 analyzable cases, the duration was mean 25 weeks (17–27 weeks); mean age 7 year and 2 month(range 9 months to 15 year-old); nine cases were severe and two cases moderated hemophilia B; six cases received on-demand, 4 received prophylaxis intervention methods and 1 case without any treatment before the observation, median exposure day was 68ED (range 0–> 150ED). (i) Efficacy: the total reduction of bleeding was 67.7%(78.6% reduction compared to original on-demand treatment and 47.8% for original prophylaxis cases; 8/9cases daily life participation improved (5/6 for original on-demand or without treatment and 3/3 for original prophylaxis treatment); (ii) Safety: there was no anaphylaxis during the Benefix intervention and only one person and one time = 0.6 BU/ml transient inhibitor presented and low-downed to 0 BU/ml in the end of observation for all cases.
Conclusions: There were the efficacy and safety for the Benefix application in Chinese severe and moderate Hemophilia B children for low-dose prophylaxis.

PO 183

Persistence of circulating heparin levels during maintenance hemodialysis in end stage renal disease patients

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Background and Aims: Despite the development of newer oral anticoagulants, heparin has remained the anticoagulant of choice for hemodialysis patients. Wide variations in the heparinization responses have been observed in hemodialysis patients. The purpose of this investigation was to measure circulating heparin levels in patients prior to and after hemodialysis.
Methods: This study included 119 End Stage Renal Disease (ESRD) patients undergoing maintenance hemodialysis. For the 3–4 h hemodialysis duration, a heparin-loading dose of 1000 Units followed by two dosages of 500 Units/h were administered. Blood samples were collected prior to and immediately after the dialysis. Citrated plasma was frozen at −70 °C and analyzed utilizing Activated Partial Thromboplastin Time (APTT), Heptest and Prothrombinase Induced Clotting Time (PiCT). Circulating Anti-Xa levels were measured using a chromogenic substrate method. Thromboplastin induced thrombin generation (TGA) was also measured using a fluorogenic method. The Antithrombin (AT) levels were measured using functional assay. Heparin levels were determined using a calibration curve constructed from the heparin used in the dialysis unit. TFPI antigen levels were measured using an ELISA method.
Results: In the APTT and Heptest, no significant differences between pre and post plasma samples were noted. The circulating levels of heparin were from 0 to 1.08 U/ml with a mean of 0.07 ± 0.11 for the APTT and a range of 0 to 1.98 for the Heptest with a mean of 0.09 ± 0.26 U/ml. In the PiCT test the range was from 14.0 to 300 s for the pre dialysis samples with a mean of 32.0 ± 38.2, whereas for the post samples the range was from 15.2 to 110 with a mean of 29.6 ± 14.0. The circulating Anti-Xa levels in the pre dialysis samples ranged from 0 to 0.77 with a mean of 0.10 ± 0.14, whereas the post level ranged from 0 to 0.51 with a mean of 0.13 ± 0.11. For the thrombin generation, the% inhibition levels ranged from 0 to 100% pre dialysis with a mean of 34.2 ± 34% and ranged 0 to 100% post dialysis with a mean of 44.5 ± 34.4%. The Antithrombin levels ranged from 28 to 130% with a mean of 86.6 ± 9.5% in the pre dialysis samples. The TFPI levels were 88 ± 12 ng/ml in the pre-dialysis and increased to 106 ± 18 ng/ml after dialysis.
There was no significant difference between pre and post dialysis samples using APTT, Heptest, PiCT and TFPI, whereas the Thrombin generation and Anti-Xa resulted in a statistically significant P value < 0.05 when comparing the two groups.
Summary/Conclusion: Wide variations in circulating heparin levels are noted in maintenance hemodialysis patients at pre dialysis and post dialysis time periods. Some patients exhibit higher levels of heparin due to a vascular access flush. These results also suggest that the use of heparin in maintenance hemodialysis patients in repeated regimen results in a steady state hypocoagulation as evidenced by the inhibition of thrombin generation, circulating Anti-Xa level and the prolongation of various clotting times.

PO 184

The effect of empiric systemic anticoagulation prior to imaging for pulmonary embolism on mortality

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Background: Current evidence is conflicting about the impact of administering heparin prior to results of imaging, or empiric systemic anticoagulation (ESA), for pulmonary embolism (PE). Prior work shows that delay in anticoagulation increases mortality, but patients selected for ESA are more ill and have increased overall mortality.
Aims: To create a propensity matching score which corrects for illness acuity in patients given ESA, and then retest the association of ESA with mortality.
Methods: Using an existing multicenter sample of 7940 emergency department patients who underwent testing for acute PE (n = 481), we used logistic regression to create a propensity score (propscore). The score was tested for accuracy at predicting ESA using receiver operating characteristic curve (ROC) analysis. The independent predictive effect of ESA was tested using conditional logistic regression, with cases (deaths, 1.3% of sample) and controls (survivors) matching by the propscore, and predictors: ESA, the pulmonary embolism severity index (PESI), respiratory distress, and end-stage condition.
Summary/Conclusions: The six propscore predictors for ESA (350/7940 or 4.4%) were: PE the most likely diagnosis, unilateral leg swelling, history of PE, active malignancy, O2 < 94%, and HR > 100. The area under the ROC for the propscore was 0.78 (95% CI 0.73–0.80) indicating fair accuracy for predicting ESA. Conditional logistic regression revealed odds ratios (95% CIs) for the prediction of death were as follows: PESI 1.04 (1.03–1.05), ESA 1.2 (0.6–2.4), Respiratory distress 1.2 (0.4–3.8), End stage disease 5.0 (2.8–8.9). After propensity matching, the use of ESA has no effect on mortality. The appearance of equipoise justifies the need for a randomized trial.

PO 185

Comparison of adherence to three times a day low dose unfractionated heparin for venous thromboembolism prophylaxis between surgical and medical ward patients

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Background: Low dose unfractionated heparin (LDUH) received a Grade 1A recommendation for venous thromboembolism (VTE) prophylaxis. While the optimal daily LDUH dose is unclear, many institutions and guidelines recommend 5000 units subcutaneously three times per day. Adherence with three times daily administrations is challenging, and if suboptimal prophylaxis administration occurs, an increased risk for hospital acquired VTE may exist.
Aims: The purpose of this evaluation was to assess differences in adherence with three times daily LDUH VTE prophylaxis between

inpatient surgical and medically ill patients. The study also identified barriers to LDUH adherence.

Methods: After IRB approval, patient demographic and LDUH administration data was extracted from medical charts and pharmacy records of a random sample of hospitalized patients admitted for > 24 h and with active orders for three times per day LDUH VTE prophylaxis. The patients were grouped in two cohorts, surgical (patients in non orthopedic and intensive care surgical wards) and medical (i.e. patients in medical wards). The unit of analysis was the observation (i.e. hospital stay requiring LDUH VTE prophylaxis). Patients receiving therapeutic anticoagulation or LDUH < 24 h were excluded from analysis. Descriptive analysis, *t*-test and chi-square tests were performed in the analysis. The level of statistical significance was set *a priori* at 0.05.

Results: The study included 586 observations in the medical cohort and 141 observations in the surgical cohort corresponding to 554 and 125 patients, respectively. The average age of patients was higher in the medical cohort (63.5 ± 17.5) than in the surgical cohort (56.7 ± 16.9) ($P < 0.001$). The proportion of women was 51.9% and 50.5% in the medical and surgical cohorts, respectively (NS).

The surgical cohort had higher proportion of all potential doses received than the medical cohort (70.2% vs. 27.2%; $P < 0.001$) and lower average number of total given doses (7.74 ± 6.41 vs. 9.18 ± 9.79; $P < 0.05$). The average number of missing dosages was lower in the surgical than in the medical cohorts (0.44 ± 0.84 vs. 3.13 ± 5.49; $P < 0.001$). The average of the percentage of the total number of dosages received was higher in the surgical than in the medical cohort (94.30 ± 13.07 vs. 71.86 ± 31.69; $P < 0.001$).

The main documented reasons for missed doses were: patients off-floor ($n = 7$, 16.9% of 41 cases with missing doses), patient refusal ($n = 5$; 12.2%) and ambulation ($n = 1$; 2.4%) in the surgical cohort; and ambulation ($n = 119$; 28.0% of 425 cases with missing doses), patient refusal ($n = 89$, 20.9%) and patient off-floor ($n = 72$; 16.9%) in the medical cohorts.

Conclusions: Adherence to LDUH three times a day dosing is significantly greater in patients admitted to surgical wards vs. medical wards, even though the total potential doses for both cohorts were numerically similar. Barriers to adherence appeared to be higher in medical ward patients. The impact of lack of adherence on the risk of developing in-hospital VTE requires further investigation.

PO 186

Effects of novel oral anticoagulants on venous thrombosis model and bleeding time assay

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Background: Heparin has been used for more than 50 years to treat and prevent thrombosis. Although heparin is the second most frequently used natural drug, its source is very limited since it is only obtained from pig intestine or bovine lung. Therefore, due to the increasing use of heparin there is an urgent need for new anticoagulants or alternative sources of heparin.

Objectives: In the present work, we compare the effect of novel oral anticoagulants such as dabigatran etexilate, rivaroxaban and apixaban using venous thrombosis model and bleeding time assays. Coagulation parameters were also evaluated and compared with fucosylated chondroitin sulfate, a potent anticoagulant polysaccharide extracted from sea cucumber.

Methods: Wistar were randomly divided into several groups. Antithrombotic activity was investigated in rats with the vena cava model using thromboplastin as the thrombogenic stimulus. Bleeding tendency was evaluated using the bleeding time model.

Results: Thrombus formation was completely inhibited at 20 mg/kg of apixaban and dabigatran etexilate, while rivaroxaban had a great variation effect in thrombus weight at this dose. Total inhibition of thrombus formation using fucosylated chondroitin sulfate was achieved only at 50 mg/kg. All novel anticoagulants caused intense blood loss, while fucosylated chondroitin sulfate and low molecular weight heparin had no effect on bleeding time assay.

Conclusion: The approach to study novel anticoagulants involves testing compounds with well-defined structures in different assays to define the multitude of their haemostatic effects. The complexity of the regulatory mechanisms involved in the action of these compounds makes it difficult to predict the *in vivo* effect exclusively using *in vitro* assays.

PO 187

No clinically relevant interaction between sugammadex and heparin (enoxaparin and unfractionated heparin) on coagulation

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Background: The selective relaxant binding agent sugammadex (Bridion®) is associated with limited and transient prolongation of activated partial thromboplastin time (APTT) and prothrombin time (PT) in healthy male volunteers. *In vitro* experiments indicated these effects may be explained by a transient decrease in Factor Xa formation and activity induced by sugammadex. This study assessed the potential interaction between sugammadex and heparin (enoxaparin and unfractionated heparin [UFH]).

Methods: A randomized, double-blind, placebo-controlled, 4-period, cross-over design was conducted in healthy male volunteers for enoxaparin (Part 1, 40 mg subcutaneously) and UFH (Part 2). In Part 1, 13 subjects (23.2 ± 3.7 years, BMI 22.6 ± 2.4 kg/m^2) received one of the following treatment sequences: placebo-sugammadex (4 mg/kg), enoxaparin-placebo, enoxaparin-sugammadex (4 mg/kg), or enoxaparin-sugammadex (16 mg/kg). Sugammadex/placebo was administered intravenously 3 h after enoxaparin/placebo administration. In Part 2, 43 subjects (24.3 ± 5.4 years, BMI 23.0 ± 2.8 kg/m^2) received similar treatments as in Part 1, however, 5000 units UFH replaced enoxaparin and the placebo-sugammadex combination was performed at 16 mg/kg. Anti-Xa activity, APTT, sugammadex plasma concentrations, and safety were assessed. Evaluation of clinically meaningful interaction was based on true geometric mean ratios (GMRs) and corresponding 2-sided 90% confidence intervals (CI) for different $AUEC_{3–30\ min}$ treatment comparisons. The pre-defined clinically meaningful effect margin for APTT and anti-Xa activity was 1.50.

Results: Enoxaparin treatment induced anti-Xa activity up to 0.35–0.37 IU/mL within 3 h after administration in all 3 treatments arms. Anti-Xa activity was not affected by additional sugammadex administration; the GMR and corresponding upper limit of CI for sugammadex co-administered with enoxaparin vs. enoxaparin alone excluded the pre-defined clinically meaningful effect margin of 1.50 (1.02–1.07 and 1.04–1.08, 4 and 16 mg/kg sugammadex, respectively). Enoxaparin prolonged APTT by approximately 5–6 s (2h55 min after enoxaparin: 36.3–37.4 s). Sugammadex induced an additional immediate limited prolongation of approximately 2 and 5 s (4 and 16 mg/kg, respectively), which disappeared within 1 h post-dose (GMR and upper limit CI for 16 kg/mg sugammadex with enoxaparin vs. enoxaparin alone: 1.09–1.13).

UFH prolonged APTT up to 1–2 s (2h55 min after UFH: 33.6–34.3). Sugammadex induced an immediate additional prolongation of approximately 2 and 7 s (4 and 16 mg/kg sugammadex, respectively), which gradually declined within 2–3 h. The GMR and corresponding upper limit of CI for sugammadex co-administered with UFH vs. UFH alone were below the pre-defined clinically meaningful effect margin of 1.50 (1.04–1.06 and 1.13–1.15, 4 and 16 mg/kg sugammadex, respectively). Anti-Xa activity was 0.05–0.06 IU/mL after UFH

treatment and did not differ between the treatment arms. Subsequent administration of sugammadex/placebo did not affect anti-Xa activity (GMR and upper limit CI for 16 kg/mg sugammadex with UFH vs. UFH alone: 1.05–1.09). The plasma concentration profiles of sugammadex were comparable in presence and absence of heparins. Sugammadex was well tolerated by all treated subjects (with and without heparin).

Conclusion: No clinically relevant interaction between sugammadex and heparin (enoxaparin and UFH): no additional effect of sugammadex on anti-Xa activity was observed, and the additional effect of sugammadex on APTT was limited and transient.

PO 188

Biossimilar enoxaparins available for clinical use in Brazil

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Background: Patent protections for Low Molecular Weight Heparins (LMWHs) expired or will expire soon. As a consequence biossimilar drugs are trying to obtain approval for clinical use. Five biossimilar versions of enoxaparin are now available for clinical use in Brazil. However, there still skepticism about the possibility to obtain preparations of LMWHs similar to the reference drug because of the complexity involved in the process to generate LMWHs, starting from unfractionated heparin. Here, we performed a careful analysis of biossimilar *vs*. reference enoxaparin available for clinical use in Brazil.

Aim: We intend to use a detailed protocol to investigate the similarity of enoxaparins available for clinical used in Brazil. We employed a detailed structural analysis of the biossimilar *vs*. reference enoxaparins, test of their *in vitro* and *in vivo* anticoagulant and antithrombotic activities and finally their effects after continuous administrations to rats during a period of 30 days. We intend to assure the efficacy and safety of the biossimilar enoxaparins available for clinical use in Brazil.

Materials and Methods: We compared biossimilar *vs*. reference enoxaparins using a variety of methods. Their structures were investigated using high field nuclear magnetic resonance (NMR), including one dimensional and two dimensional spectra (^{1}H-^{1}H TOCSY and ^{13}C- ^{1}H HSQC), the molecular size distribution was determined by gel permeation chromatography and their anticoagulant activities were established based on the anti-Xa anti-IIa assays and on direct binding to antithrombin. Approximately 100 batches of biossimilar enoxaparins were included in this initial investigation. On a second stage selected bathes were tested on animal models of experimental thrombosis and bleeding, associated with determination of their pharmacodinamic. Finally, enoxaparins were administered continuous to rats over a period of 30 days in order to investigate possible toxic effects after a long period of administrations.

Results: The biossimilar and reference batches of enoxaparin available for clinical use in Brazil have similar structures and anticoagulant activities. They also showed equivalent responses in animal models of experimental thrombosis and bleeding, achieved similar plasma concentrations, showed no toxic effect after continuous administration over a period of 30 days.

Conclusions: Our results indicate that biossimilar enoxaparins available for clinical use in Brazil are similar to the reference drug. Of course, biossimilar enoxaparins are now produced by an increasing numbers of companies. Each preparation requires a carefully analysis to assure their efficacy and safety. We believe the protocol of our study may help to define a guide line for analysis of biossimilar LMWHs.

PO 189

A comprehensive evaluation of factors effecting range and precision for heparin Anti-IIa and Anti-Xa chromogenic assays

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In response to the heparin contamination crisis, regulatory agencies in the US, Europe, and Japan have begun implementing anti-factor IIa (Anti-IIa) and anti-factor Xa (Anti-Xa) testing requirements for manufacturers of unfractionated heparin. The present and proposed pharmacopeial assay protocols confine heparin concentrations to a relatively narrow range, which limits the general utility of these assays. In the present studies, we sought to optimize these Anti-IIa and Anti-Xa heparin assay protocols to allow for measurement of an expanded range of heparin concentrations while maintaining precision of the assay. Based upon the biochemistry and enzymology underlying these assays, we focused our study on two variables, namely, the initial concentration of antithrombin (AT), as well as the time of incubation allowed for AT-heparin complex formation. We hypothesized that decreasing either the AT concentration or the incubation time would result in a corresponding increase of the heparin measurement range of the assay. Due to the critical nature of precision for these heparin pharmacopeial assays, our studies sought to determine the test precision for each test (Anti-IIa and Anti-Xa) and for each parameter varied. Using three different automated hemostasis analyzers, our results demonstrated that it was possible to expand the heparin range at least 4-fold over the original pharmacopeial test concentrations using decreases in either AT concentration or incubation time, without a significant negative impact on test precision. These studies demonstrate that the Anti-IIa and Anti-Xa pharmacopeial assays can be made more versatile and widely applicable without sacrificing test precision.

PO 190

The assessment of patients with heparin-PF4 antibody positivity: the importance of expert haematological input remains

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Background: Heparin-induced thrombocytopenia (HIT) is an antibody-mediated reaction to heparin characterised by thrombocytopenia and increased thrombotic risk. Increased awareness and the need for early diagnosis have led to the requesting of HIT screens by physicians unfamiliar with their interpretation. As a tertiary referral centre with a large cardiothoracic practice, thrombocytopenia and suspected HIT occurs frequently.

Aims: To critically evaluate the indications, management and outcomes of patients with positive HIT screens over a 3-year period and address the need for a diagnostic algorithm involving haematologists.

Method: A single centre retrospective analysis of all positive HIT screens between 2010 and 2012 was undertaken. Paper and electronic records were reviewed.

Results: One hundred and eighty-one HIT screens using a GTI-PF4 ELISA were performed. Twenty-nine positive results were identified involving 27 evaluable patients – 6 females and 21 males with a median age of 70 years (range 3–89). Eleven patients had undergone cardiothoracic surgery in the preceding 30 days. Others were renal (5), surgical (5), orthopaedic (1), medical (4) and paediatric (1).

No patients had a high pre-test probability (4T score 6–8). The pre-test probability score was intermediate (4T score 4–5) in eight patients and low (4T score 0–3) in 19. In the latter group eight received an alternative anticoagulant (danaparoid). Eight continued heparin and in three, heparin was stopped but no alternative anticoagulant given during the episode of thrombocytopenia. No thrombotic events occurred in this group. One patient in the low pre-test probability group developed

skin necrosis whilst receiving unfractionated heparin on haemofiltration. Although HIT was felt to be clinically unlikely, in view of his strongly positive HIT ELISA (OD 4.1585) he was converted to danaparoid which continued until his death.

Seven of the eight patients with an intermediate 4T score were switched to danaparoid. Three of these had thrombotic events.

In five of the patients with positive HIT screens there was bleeding of WHO grade 2 or more. Two of these were patients in the intermediate score group.

Sixteen of the screen positive patients received platelet transfusions during the inpatient episode. These were administered therapeutically for haemorrhage and prophylactically before invasive procedures.

On review it was felt that five of the 27 patients with positive HIT screens were likely to have had HIT. Three of these experienced thrombotic complications. All were managed with danaparoid. The mean OD in this group was 2.55 (95% CI 1.18–3.92) compared to a mean OD in the others of 1.32 (95% CI 0.93–1.71).

Summary: Our local experience reveals that diagnosis of HIT remains complex. Recently at our centre there has been a reduction in the involvement of haematologists in suspected HIT. Our audit identified the overdiagnosis of a rare condition, with implications of inappropriate care. Despite the increased awareness of HIT, expert haematological involvement remains paramount. In response to these findings, we have developed a diagnostic algorithm involving haematological advice from the outset. We suggest that the accurate diagnosis of HIT depends on the involvement of the haematology service.

PO 191

Serotonin Release Assay (SRA) results for confirmation of Heparin Induced Thrombotic Thrombocytopenia (HITT)

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Background: Heparin induced thrombocytopenia (HIT) is an immune mediated disorder associated with heparin therapy with clinically significant HIT defined when patients have both thrombocytopenia and thrombosis. Washed platelet assays such as the serotonin release assay (SRA) are the most useful in the detection of clinically significant HIT antibodies.

Aim: The aim was to assess the effectiveness of SRA in the confirmation of clinically significant HIT.

Method: The Haematology Department, South Eastern Area Laboratory Services (SEALS) located at the Prince of Wales Hospital, Sydney, Australia has been performing the SRA for HIT confirmation since the 1990's. Samples are received from laboratories around Australia and New Zealand. The serotonin release assay performed is the original assay described by Sheridan (Blood Vol 67, 1986). A review was carried out on all positive SRA samples tested in the last 2 years to determine whether the positive result correlated with a clinical picture of HIT in the patient.

Results: A total of 249 samples were tested for SRA in 2010 and of these 42 samples were positive. In 2011a total of 206 samples were tested for SRA and of these 37 samples tested positive.

The HIT screening test was positive for all cases in 2010 and for all cases except one in 2011, where the HIT screen was negative and the doctor insisted on the SRA due to the clinical history of the patient. The majority of the positive SRA tests that had clinical information provided were determined on the basis of this information to have clinically significant HIT, due to a low platelet count whilst on heparin therapy and a thrombotic event.

Negative SRA tests that had clinical information provided were also reviewed with the majority of the negative SRA tests only having thrombocytopenia whilst on heparin therapy with very few having both thrombocytopenia and thrombosis.

Conclusion: As thrombocytopenia on heparin is the trigger for HIT screening both negative and positive SRA patients were found to have thrombocytopenia. Thrombosis a serious complication of HIT along with thrombocytopenia occurred more commonly with SRA positive patients. Our review confirms the importance of SRA testing in patients suspected of HITT due to the high proportion of patients with positive SRA with both thrombocytopenia and thrombosis.

PO 192

Detection of of heparin-induced thrombocytopenia antibodies by functional and immunological methods and comparison with genetic risk factor (polymorphism of Fc? receptor)

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Aim: In our study we investigated group of 100 plasma samples from patients with suspected HIT. The patients were sent for testing of HIT on basis 4 T's score. The aim of our study was to compare the results of the functional test (whole blood impedance aggregometry – WBIA) and immunoassay (ELISA) well as to determine the risk of a genetic predisposition for the development of HIT. From literature findings, was as genetic predisposition risk factor of developing HIT determined polymorphism of platelet receptor Fcγ (FCGR2A gene). The FCGR2A gene polymorphism is manifested as substitution amino acid arginine for histidine in position 131.

Method: The samples were tested by functional and immunological assay. We used WBIA as functional assay and ELISA as immunological assay.

The WBIA method uses determine the degree of activation of donor platelets in the presence of patient's plasma by heparin at concentration 0.5 IU/ml. The WBIA test was performed: 300 μL of citrated whole blood with donor platelets, 150 μL of heparin (final concentration of 0.5 resp. 100 IU/ml) and 300 μL of patient platelet-poor plasma (PPP). Changes in the aggregation was monitored for 20 min. The cut-off for screening assay was upper than 20 AUC. The confirmation with higher dose of heparin must provide negative results.

ELISA test were performed in IgG, IgA and IgM class and results were classified as negative for OD < 0.300, suspected (0.300–0.500) and positive OD > 0.500.

Genetic analysis for presence FCGR2A polymorphism was performed by real-time PCR on LightCycler. The assay was be performed on basis melting curve analysis. The temperature melting was determined for amino acid arginine 62 °C and histidine 52 °C.

Results and Conclusion: Method of WBIA were positive 10 patients and 90 negative patients in comparison to ELISA where were positive only four patients mostly in IgG class antibodies. The WBIA is easy examination with rapid turn-around time and warrants a multi-laboratory trial to complete its validation as a confirmatory assay for platelet activation. The measurement by WBIA provides in group of patients higher specificity and sensitivity than ELISA assay.

The genetics predisposition was detected significantly higher in group with positive results by WBIA than in patients with negative results by WBIA. The representation of normal alleles in the whole cohort was 20.2% and mutational state was 79.8%.

Supported by a grant LF-2013–04.

PO 193

A rheological study of incipient clots formed by heparinized plasma: assessing the health risk associated with HIT and exploring the use of Gel Point detection for therapeutic management

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Background and Aim: Unfractionated heparin (UFH) is often administered to cardiac and orthopedic patients undergoing surgery in order to prevent thrombosis. Occasionally, a fatal reaction to UFH, termed Heparin-Induced Thrombocytopenia (HIT), may occur. Rapid and accurate diagnosis of HIT is very important, as any delay in the cessation of UFH has been associated with an initial 5–10% daily risk of thrombosis, amputation, or death. Misdiagnosis of HIT can result in exposing thrombocytopenic patients to alternative anticoagulants, contributing to major hemorrhage.

Up to 5% of patients who receive UFH develop HIT-associated antibodies, causing platelet activation, leading to thrombocytopenia and thrombosis. Diagnostic tests such as the ELISA assay have limited specificity, alternatively, the SAR assay is highly specific but doesn't allow for real time results.[1] The purpose of the present work is to investigate the potential application of rheometrical measurements of incipient clots, as a novel diagnostic tool for detecting the onset of HIT. The work reported herein involves measuring changing gelation times and fractal dimensions,(d_f), of plasma samples at different concentrations of UFH (*Heparin sodium salt from porcine intestinal mucosa: H3149 Sigma-Aldrich)*.

Method: Platelet poor plasma, (PPP), represents an ideal model system, wherein the number of immeasurable variables associated with the coagulation process can be controlled, leaving only a quantifiable number of platelets and the remaining enzymes and proteins.

The rheometrical detection of the gel point (GP) of coagulating blood provides a method of determining the structural characteristics of incipient clots, which are dominated by a fibrin network. Thrombi with 'dense' fibrin networks are more difficult to lyse and are potentially more dangerous than those with 'more open' networks. Fractal analysis of the GP of coagulating blood provides a method of quantifying clot structure in terms of a d_f, as well as providing a normal clotting time, (CT). Previous rheometrical experiments on blood show that the CT and d_f of incipient clots formed in healthy volunteers are significantly altered by the *in-vitro* addition of heparin. Showing an increase in CT and a progressive decreases in d_f with increasing levels of heparin.

Results: Pooled PPP from healthy individuals, with the addition of 0.15NIH/ml of thrombin and 0.005 mg/ml of tissue factor, at 37 °C, have a normal CT = 147 s, ± 36.09 s. With a normal d_f = 1.92°, ± 0.12°. When 0.2 U/ml of Heparin is added to the PPP there is an increase in CT = 404 s, ± 156.82 s.

Current experiments include the addition of different concentrations of PF4, to optimize the ratio of PF4: UFH, neutralizing the UFH effect on the PPP. The last step will include the addition of anti-bodies to the model system and the measurement of any deviation of CT and d_f from normal heparinized PPP values.

Summary: This study is important to fully understand and elucidate the mechanisms of HIT and to explore the potential use of rheometry as a tool for monitoring *in-vivo* heparin therapy, in patients most at risk of developing HIT post-surgery.

PO 194

The utility of ELISA optical density as a prognostic tool in patients with confirmed heparin-induced thrombocytopenia

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Background: Heparin-induced thrombocytopenia (HIT) is associated with anti-platelet factor-4 (PF4)/heparin antibodies that activate platelets, resulting in thrombocytopenia and a pro-thrombotic state. ELISA-based methods are commonly used to screen for the presence of these antibodies; however, only functional assays confirm their platelet-activating activity. At our institution, antibody-mediated platelet activation is demonstrated by lumi-aggregometry, a method validated previously against the gold standard (serotonin-release assay). It has been suggested that in ELISA positive patients higher optical density (OD) correlates with more frequent thrombotic manifestations. It is not well established whether this is because the magnitude of the OD correlates with the likelihood of HIT, or if the magnitude of the OD is also prognostic of thrombosis in patients with confirmed HIT.

Aims: The objective of this study was to evaluate the association of OD with thrombotic events in patients with HIT confirmed by lumi-aggregometry. The predictive value of the strength of the luminescence ratio was also evaluated.

Methods: Quantitative anti-PF4 detection was performed using an IgG-specific ELISA kit (Gen-Probe, San Diego) in patients with clinically suspected HIT. Then confirmatory testing by lumi-aggregometry was performed for samples with ELISA OD $\geq$ 0.400. HIT antibody-induced activation of washed healthy donor platelets was tested at therapeutic (0.1 and 0.5 U/mL) and high (100 U/mL) concentrations of porcine heparin. The degree of platelet activation was quantitated luminographically based on the light flash reaction of ATP (released from platelet dense-granules) with luciferin luciferase reagent. A ratio of therapeutic to high heparin luminescence amplitude of > 5.0 and platelet aggregation at therapeutic but not high concentration was considered a positive result. HIT assay results from September 2010 to July 2012 were reviewed to identify patients with positive HIT testing by lumi-aggregometry and to record ELISA OD and luminescence amplitude ratio data. Patient records were then reviewed to identify objectively confirmed new or recurrent venous or arterial thrombotic events occurring in the 5 days preceding or the 30 days following the date of positive HIT testing.

Results: Among 33 patients with confirmed HIT, 14 (42%) experienced HIT-associated thrombotic events. On average, patients with thromboembolic events had a significantly higher ELISA OD than those patients without (Mann-Whitney test, $P = 0.03$). However, the ratio of luminescence amplitude was not significantly different between the two groups ($P = 0.20$). There was no significant correlation between luminescence ratio and OD in patients with ($r_s = 0.40$, $P = 0.16$) or without thromboembolism ($r_s = 0.03$, $P = 0.92$).

Conclusion: In this large cohort of patients with HIT confirmed by lumi-aggregometry, OD, but not ratio of luminescence amplitude, is associated with thrombotic events. ELISA OD may be of value to prognosticate for thrombosis in patients with confirmed HIT.

PO 195

Recurrence of heparin-induced thrombocytopenia induced by the re-administration of heparin after negative conversion of HIT antibodies in a patient with hemodialysis

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The cause of heparin-induced thrombocytopenia (HIT) is the appearance of anti-PF4/heparin complex antibody (HIT antibodies), and the disease is characterized by thrombocytopenia and venous or arterial thrombosis. HIT antibodies are transient and disappear within about 100 days after the withdrawal of heparin. We have succeeded in use of heparin re-administered more than 100 days after the negative conversion of HIT antibodies in 14 patients who had developed HIT upon the introduction of dialysis. However, in the 15th patient HIT recurred upon heparin re-administration for dialysis.

The patient (69-year-old male) developed rapidly progressive glomerulonephritis and dialysis was introduced. Nafamostat mesilate (NM) was used as an anticoagulant for the initial dialysis. And then unfractionated heparin (UFH) was used from day 12. The platelet count was $291 \times 10^3/\mu L$ before the introduction of dialysis, but decreased to $95 \times 10^3/\mu L$ on day 12 and to $44 \times 10^3/\mu L$ on day 25. Suspecting HIT, heparin was withdrawn. Due to retinal bleeding, NM was used as an alternative anticoagulant, but was switched to argatroban on day 38. The platelet count rapidly recovered after the switch to argatroban. HIT antibodies were positive on both ELISA (PF-4 Enhanced®, GTI; Waukesha, WI, USA) and the ^{14}C-serotonin release assay (SRA). SRA and ELISA became negative on days 129 and 299, respectively. HIT antibody negativity (ELISA) was confirmed on day 402, and dialysis with heparin was initiated again on day 441. Dialysis with heparin was performed 4 times without any incident through day 448. Then blood coagulation was noted in the circuit on day 451 (10 days after restarting of heparin administration) and the platelet count decreased to $107 \times 10^3/\mu L$ on day 453. Suspecting HIT, administration of heparin was immediately discontinued, and argatroban was used as an anticoagulant. HIT antibodies became positive on both ELISA and SRA.

As an index of the level of enhanced coagulation, D-dimer (LPIA-ACE D-D dimer II; Mitsubishi Chemical Medience, Tokyo) was high (7.87 μg/ml) on day 66, but decreased along with a decrease of the HIT antibody level. The D-dimer level rose again (1.34 μg/ml) on day 479 (38 days after re-administration of heparin). The D-dimer level was higher during the initial episode of HIT than that of the second one, suggesting that the hypercoagulable state was more severe than that in the second episode of HIT.

Clinical symptoms of HIT including blood coagulation in the circuit and thrombocytopenia were observed after the fifth day from heparin administration in both episodes. In this patient, the conditions which induced the first HIT episode may have also been established in the second episode. It would seem logical to consider the second episode as a new development of HIT, rather than as a recurrence.

PO 196

Inherited thrombophilia and other risk factors in women with thrombotic complications of oral contraceptives

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Background: Venous thromboembolism (VTE), although rare, remains one of the serious adverse consequences of oral contraceptives (OC).

Aim and Methods: Clinical data, inherited thrombophilia and other thrombotic risk factors were evaluated in women with the contraceptives-related thrombosis referred to our centre for thrombophilia investigation.

Results: Out of seventy-one women with a median age of 32 years (19–45) 62 (87%) patients used oral contraceptives of the 3rd and 4th generation. Deep vein thrombosis (DVT), pulmonary embolism (PE), sinus vein thrombosis (SVT) and transitory ischemic attacks were diagnosed in 48/67.6%, 21/29.5%, 8/11.3% and 3/4% of the women, respectively. The source of embolisation was not revealed in 12/21 patients with pulmonary embolism. Even fifty-five (77.5%) women had at least one (28 pats) or a combination of more than two (27 pats) thrombotic risk factors in addition to the use of contraceptives (familial thrombosis 37, personal VTE history 2, obesity 5, trauma/surgery 6, smoking 5, inherited thrombophilia 25, lupus anticoagulant 2, mild hyperhomocysteinemia 1). Congenital thrombophilia was revealed in 25/71 (35.2%) women: heterozygous FVLeiden, heterozygous FIIG20210A and double heterozygous mutation in 20 (28%), 3 (4.2%) and 2 (2.8%) women, respectively. However, thrombophilia as the only additional risk factor of thombosis was present in 8/11.3% of all contraceptives users only. In seventeen of 25 (68.7%) thrombophilia patients an assotiation with other risk factor was present (familial thrombosis 13, DVT history 1, leg trauma 3). Eight patients developed sinus vein thrombosis, out of them 2, 2 and 1 had prothrombin mutation, lupus anticoagulant and mild hyperhomocysteinemia, respectively. In three SVT patients no other risk factor than the use of the OC was revealed.

Conclusion: Our study demonstrates a high prevalence of thrombotic risk factors in OC users who developed serious thrombotic complications. In fourty six (65%) patients the risk factors could have been identified and thrombotic complications avoided by thorough family/patient history and/or by providing adequate information to the women taking the pills. As the routine screening for inherited thrombophilia before prescribing the OC is not recommended, it is essential strictly to follow the medical eligibility criteria according to WHO recommendations for the use of oral contraceptives.

PO 197

Gynaecological problems in women with severe inherited platelet disorders – a single centre experience

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Background: We present a series of 3 teenage and 3 adult females with inherited platelet disorders (5 Glanzmanns thrombasthenia [GT], 1 Bernard Soulier Syndrome [BSS]) and describe their gynaecological management in a joint Gynaecology & Bleeding Disorders Clinic from March 2010.

Results: Case 1: Thirteen year-old (P0 + 0) with GT presented with menorrhagia and iron deficiency anaemia (IDA) since menarche. She was initially managed with norethisterone and tranexamic acid (TA). Five months later, norethisterone was substituted for a progesterone-only pill (POP), with good effect.

Case 2: Fourteen year-old (P0 + 0) with BSS, presented with menorrhagia at menarche reporting continuous bleeding for 4 weeks. Her haemoglobin was 8.3 g/dL and she was given HLA matched platelets,

TA and norethisterone. Thereafter she switched to a POP and TA, but continued to have irregular bleeding. She then started a combined oral contraceptive pill (COCP) plus TA during withdrawal bleed, with good effect.

Case 3: Sixteen year-old (P0 + 0) with GT and longstanding severe gingival bleeding, frequently requiring red cell and HLA matched platelet transfusions, presented with menorrhagia shortly after menarche. She was given norethisterone and TA which helped initially. However, her menses became heavier and due to concerns about compliance, was switched to a combined contraceptive transdermal patch, then to a higher dose COCP and thereafter to a POP, plus TA. With the POP she has lighter menses, however remains transfusion dependent.

Case 4: Twenty-three year-old (P0 + 0) with GT, presented with right iliac fossa pain, and a right sided ovarian cyst (8 × 8 × 8 cm) on ultrasound (US). A right ovarian cystectomy was performed with perioperative transfusion of HLA matched platelets. A 12 cm ovarian cyst was removed, histology showed an endometrioma. She had a progersterone-only implant inserted subdermally and currently has minimal bleeding and no dysmenorrhea. Her most recent US is normal.

Case 5: Twenty-five year-old (P0 + 0) with GT, presented with menorrhagia and IDA since menarche. She was managed with the COCP continuously for 3 months plus TA during the withdrawal bleed. Despite this, she had intermenstrual bleeding (IMB), and on one occasion was admitted with heavy PV bleeding and transfused red cells and HLA matched platelets. She declined alternative hormonal therapies. Her haemoglobin remains satisfactory on 3 monthly COCP.

Case 6: Thirty-nine year-old (P0 + 0) with GT and anti IIb/IIIa antibodies, presented with menorrhagia and IDA. MRI showed bilateral endometriomas and left tubuo-ovarian complex (3 × 5 × 3 cm). Ca 125 normal. She opted for the subdermal progesterone-only implant with HLA matched platelet and rVIIa cover. She bled post insertion and had further doses of HLA matched platelets and rVIIa. Since, she has had no PV bleeding and is not iron deficient. Her latest MRI showed the endometrioma has resolved.

Conclusion: These cases demonstrate the severe nature of gynaecological issues in women with GT and BSS. Menorrhagia since menarche is a prominent feature, but there are other complications for example haemorrhagic ovarian cysts. Joint management with a gynaecologist and haemophilia specialist is vital for the complex management of these high risk patients.

PO 198

Prenatal diagnosis in haemophilia A and B families: combine cordocentesis and gene diagnosis

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Background: Haemophilia A (HA) and B (HB) are the two most common X-linked recessive inherited bleeding disorders. In developing countries like China, the burden ofhaemophilia is heavy as there is a poor social support and lack of appropriate therapeutic measures. Reducing the incidence of haemophilia is thus one of the main objectives of any haemophilia care centre.

Aim: In this study, we offered prenatal diagnosis for females who from haemophilia A or B familiies with absence of affected members in the family. Twenty-nine females were investigated in this study in their second trimester.

Methods:Fetal sample was collected with the help of Ultrasound-guided puncture between 19 and 23 weeks. PT, APTT and fibrinogen testing were performed in the fetal blood. A battery of coagulation factor assays were assessed by one-stage clotting method detected on IL coagulation analyser, including all the coagulation factors except factor XIII.

Results: Eight boy fetus were diagnosed as haemophilia A patient and three were haemophilia B patient. Factor VIII activity mean value during 19 and 23 weeks gestation is 36.58% with the range from 12% to 58%, to factor IX which is 11.97% with the range from 5.4% to 16%. The blood samples of two HA fetus and one HB fetus were tested using gene diagnosis requested by their family. Genetic defection were all be detected and the results are consistent.

Conclusion: The results show that it is possible to offer prenatal diagnosis in index-case-absent haemophilia family by combine cordocentesis and genetic diagnosis. But for haemophlia B, it is always difficult to judge the result for fetus if the index case in this family is a mild to moderate patient. For the affected member absent family, if the fetus is a patient, maybe it is a good news for them so they can do the genetic diagnosis which is a high accuracy technology for prenatal diagnosis.

PO 199

Antiplatelet effect of Tyrame [N-(3-hydroxy-1:3:5(10)-estratrien-17β-yl)-4-hydroxy-phenethylamine]

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Background: Millions of women use oral contraceptives or estrogen replacement therapy even thought, it is known that estrogens increase the risk of venous thrombosis, myocardial infarction and stroke. To decrease dose pharmaceutical presentations has not completely offset this adverse effect. In the search for new compounds with estrogenic activity which do not favor a prothrombotic state were synthesized (amino-estrogens). We have previously communicated anticoagulant or antiplatelet effect of these compounds.

Aim: To design, synthesize and asses the antiplatelet aggregating effect of Tyrame, a new amino-estrogen.

Methods: Tyrame was synthesized by a condensation reaction between the corresponding amine and estrone. The compound was characterized by their physical constants and spectroscopic properties: melting point, infrared spectroscopy, mass spectrometry, FAB +, nuclear magnetic resonance and by X-ray crystallography.

Compounds were administered (1–4 mg/100 g weight, subcutaneously) to male mice, CD1, which are kept at a controlled temperature with food and water *ad libitum*, with light-dark cycles of 12–12 h. After 24 h, a blood sample was taken.

Whole blood platelet aggregation was performed using an aggregometer (Model 560 CA; software Model 810 AGGRO/LINK Chrono-log, Havertown, PA, USA). Briefly, 500 μL of saline and 500 μL of citrated blood were added into a test cell stirrer. After an incubation period of 5 min at 37 °C, the agonist was added and the recording started. During the following 6 min the ability of platelets to adhere and aggregate onto 2 metal wires was measured by the electrical resistance change between the sensor wires. The impedance change caused by the adhesion of the platelets onto the sensor surfaces was plotted against time and the top of the aggregation curve was quantified in arbitrary units (U).

The protocol was approved by the ethical committee of UNAM.

Results: The results show that Tyrame at 4 mg/100 g reduce 30% platelet aggregation induced by ADP.

Summary. Tyrame were designed and synthesized in the Institute of Chemistry of UNAM. It was characterized by their physical, spectroscopic constants and X-ray crystallography. Tyrame was administered subcutaneously and inhibit ADP-induced platelet aggregation in whole blood by 30%.

PO 200

Successful pregnancy outcome in a patient with factor XII deficiency and recurrent fetal loss

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Background: Factor XII (FXII) deficiency is a rare autosomal recessive disorder whose clinical implications are still undefined. It does not predispose towards a bleeding tendency and many reports have evidenced its association with an increased risk for both arterial and venous thrombosis. Its role in recurrent abortions is controversial.
We describe the successful outcome of a pregnancy in a woman with FXII deficiency and recurrent fetal loss.
Case Report: The patient is a 38-year old woman with an obstetric history of recurrent foetal loss (3 spontaneous miscarriages in the first trimester of pregnancy and a subsequent interruption of pregnancy due to a fetal abnormality). The endocrinological work-up revealed the presence of an autoimmune thyroiditis, treated initially with methimazole and subsequently with L-thyroxine. Familial and personal history was negative for thromboembolic and/or bleeding disorders. Coagulation tests evidenced a prolonged activated thromboplastin time, measured as ratio (R: 9.0) with a normal value of prothrombin time; clotting factors dosage showed the presence of a severe FXII deficiency (< 1%). Inherited trombophilia screening and antiphospholipid antibodies resulted negative; a moderate hyperhomocysteinemia was detected and treated with folate supplementation. At the age of 37 the patient planned a new pregnancy and started antithrombotic prophylaxis with enoxaparin (4000 IU/die) associated with folate therapy throughout the pregnancy. The antenatal period was uneventful and the woman underwent a caesarean section at term under general anaesthetic without any replacement therapy. No haemorragic or thrombotic complication were observed. Pharmacological thromboprophylaxis was continued in the post-partum period for a month.
Conclusion: Many reports have shown an association of FXII deficiency with recurrent miscarriages, even if such correlation has not been confirmed by other studies. In this patient with FXII deficiency the coexistence of other predisposing conditions, such as the hyperhomocysteinemia and the thyroid disease, may have contributed to the recurrent fetal loss. Thromboprophylaxis with enoxaparin resulted effective in venous thromboembolism prevention in absence of any bleeding event. Further data are needed to evaluate the possible role of low molecular weight heparin in supporting a positive outcome of pregnancy in FXII deficient women with recurrent foetal loss.

PO 201

Mutations within exon 8 of Protein Z gene of women with fetal loss: structural implications assessed by molecular dynamic simulations

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Background: Recently, several researches aimed at investigating the influence of Protein Z (PZ) on the pregnancy outcome. Women experienced fetal losses could present sporadic or polymorphic variants of PZ gene (PROZ), almost all with effect on the serine-protease (SP) domain. We previously reported a approximately 6-fold higher risk for fetal losses in women carrying mutations within PROZ exon 8. Crystal structures of PZ in complex with the PZ-dependent inhibitor (PZI) have been recently obtained.
Aims: To investigate structural implications of PROZ mutations for fetal loss events.
Methods: In two different Thrombosis Centres (San Giovanni Rotondo andRome), 611 women with previous recurrent fetal losses were recruited. As controls, 546 women with healthy pregnancies randomly selected from a Southern Italian general population without a history of VTE were enrolled. PZ levels were measured in all the controls and in 137 (22.4%) women with fetal losses. Direct sequencing of the exon 8 was performed. Eight sporadic mutations and two polymorphisms were found. Molecular dynamics simulations (10 ns) have been performed for the wild type PROZ and for 10 PROZ mutants observed in Italian patients, to investigate their structure in aqueous solution. Simulation data have been processed by novel tools to analyze the residue-by-residue backbone flexibility, and correlated with the statistics of the observed mutations. The study was approved by the local ethics committee and complies with Declaration of Helsinki. Participants gave their written informed consent for present and future use of the clinical data.
Results: All the mutations observed are located in the region coding for SP domain. Sporadic mutations have been associated with anomalous flexibility of residues belonging to specific regions. Among them, the most important is a loop region which is in contact with the longest helix of PZI. Other regions have been identified, which hold anomalous flexibility associated with potentially protective gene variants.
Summary/Conclusions: A possible interpretation of effects associated with observed gene variants is provided. The exploration of PZ/PZI interactions seems essential in explaining these effects.

PO 203

Successful anticoagulation to prevent recurrent miscarriage in two women with severe thrombophilia and von Willebrand disease

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Background: We report about successful outcome of pregnancy of two patients with recurrent miscarriage and severe thrombophilia (one woman with homozygous factor V Leiden mutation, one woman with antiphospholipid syndrome) in addition to von Willebrand disease (VWD) type I. VWD is a common hereditary bleeding disorder. Affected women often show menorrhagia, bleeding complications occur in more than 50% of women after childbirth or pregnancy loss.
Aims: In spite of increased bleeding risk of these two women, anticoagulant treatment during pregnancy to prevent miscarriage was given. Patients were treated with low molecular weight heparin (LMWH) since beginning of gestation, one woman additionally received acetylsalicylic acid until gestational age of 36 weeks.
Methods: Women I (body weight 83 kg) had a history of two miscarriages (first trimester: < 13 weeks). No thromboembolism, no bleeding tendency in history. Homozygous factor V Leiden mutation and VWD type I (RisCo 39% of normal, VWF Ag 52% of normal) were diagnosed at the age of 30 years. Women II (body weight 76 kg) had a history of three miscarriages (first trimester). No thromboembolism, but bleeding symptoms since childhood. Antiphospholipid syndrome (ß2-glycoprotein type IgM was repeatedly increased: 32 U/mL, norm < 20 U/ml) and VWD type I (RisCo 47% of normal, VWF Ag 67% of normal) were diagnosed at the age of 29 years.
Results: With beginning of gestation both patients received LMWH (dalteparin 5000 IE/d). Patient I was treated during two pregnancies up to 6 weeks after delivery without any complications. No thromboembolism or bleeding complications occurred during both pregnancies and vaginal delivery. No substitution of VW concentrates was necessary. Patient II was additionally treated with acetylsalicylic acid 50 mg/d until gestational age of 36 weeks. Due to repeated vaginal bleeding and cervix hematoma treatment with acetylsalicylic acid was consistently interrupted for some days. VW parameters increased

during pregnancy up to normal levels (RisCo 115% of normal, VWF Ag 201% of normal). Vaginal delivery was performed without any complications, but 3 weeks postpartum curettage was needed because of bleeding.
Conclusions: Anticoagulant treatment (LMWH and acetylsalicylic acid, respectively) in women with VWD and severe thrombophilia to prevent miscarriage remains save and seems to be successful regarding live birth rate. Pregnancy requires close monitoring.

PO 204

Platelet hyperaggregability in patients with fetal loss: the selected gene polymorphisms

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Aims: To evaluate selected polymorphisms of GAS6, MRVI1 and PEAR1 genes in patients with Sticky platelet syndrome (SPS) and fetal loss.
Methods: Twenty-three patients with SPS, manifested as fetal loss, 42 controls without SPS and with no history of fetal loss and thrombosis. SPS was diagnosed by platelet aggregometry (PACKS-4 aggregometer, Helena Laboratories, USA). Four single nucleotide polymorphisms (SNPs) of the GAS6 gene (rs7400002, rs1803628, rs8191974, rs9550270), two SNPs of the PEAR1 (rs12041331, rs12566888) and MRVI1 (rs7940646, rs1874445) genes were evaluated.
Results: We observed a significant higher occurrence of homozygotes for the variant C compared to holders of T allele (according to recessive association model of inheritance) at the rs7400002 (GAS6 gene) in the group of SPS patients with fetal loss vs. control subjects ($P < 0.049$). In addition, non-significant but still important result is that the haplotype C/C is associated with 8-fold increase risk of fetal loss vs. holders of T allele (odds ratio [OR] 8.632, 95% confidence interval [CI] 0.903–82.540). In adaptive model of inheritance, the results indicate that women carrying the genotype C/C have a significant 11-fold increased risk of fetal loss vs. holders of genotype C/T (odds ratio [OR] 11.429, 95% confidence interval [CI] 1.085–120.355). According to dominant model of inheritance, we identified a significant higher occurrence of homozygotes for the variant G compared to holders of T allele at a SNP in PEAR1 gene (rs12566888) in the group of SPS patients with fetal loss vs. control subjects ($P < 0.046$).
Summary/Conclusions: Our results can support an idea that selected GAS6 and PEAR1 gene polymorphism may be associated with the platelet hyperaggregability – a possible cause of fetal loss. SPS is likely a multifactorial inheritance disorders. Acknowledgement: Study was supported by project APVV 222-11, Vega 1/0016/12, UK/319/2012 and CEPV II (ITMS 26220120036) which is cofinanced from EC sources.

PO 205

Thrombophilia in ethiopathogenesis of IVF failure

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Background: IVF failure has recently been associated not only with embryo quality or endometrial receptivity but with thrombophilia.
Materials and Methods: we examined 109 patients after IVF failure. Among them – 76 after repeated IVF, and 33 pregnant women after IVF who received anticoagulant therapy at the period of planning of pregnancy. All of them had complicated obstetric history and venous thrombosis. Control group consisted of 100 healthy pregnant women. All patients were tested for acquired and inherited thrombophilia and hemostasis parameters.
Results: The prevalence of thrombophilia was found in all 109 patients with IVF failure and consisted 92%. The incidence of genetic thrombophilia was 45%. MTHFR mutation was found in 43%, prothrombin gene mutation -11%, Factor V Leiden mutation – 9%, PAI-I gene polymorphism – 71%, t-PA – 37%. Forty-four percent had circulation of antiphospholipid antibodies, 48% – anti-b2-GP I antibodies, 25% – anti-annexin V antibodies, and 11% – antibodies to prothrombin. Hyperhomocysteinemia was found in 57%. Multigenic thrombophilia was revealed in 78%, combination of genetic thrombophilic mutations and circulation of APA was identified in 61%. All women with pregnancy after IVF who received therapy from the fertile cycle and during all pregnancy (LMWH, antioxidants, aspirin, folic acid, natural progesterone, vitamins group B) were delivered with healthy newborn.
Comments: These data suggest that thrombophilia may play a role in the genesis of IVF failure, especially in cases of multigenic and combined thrombophilia. Since thrombophilia has been diagnosed antithrombotic therapy should be evaluated in patients with thrombophilia and IVF failure.

PO 206

LMWH and natural progesterone in patients with multiple pregnancy and thrombophilia

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Background: thrombophilia play an important role in obstetric complications, especially in cases of multiple pregnancy. Normal pregnancy is a prothrombotic state and associated with profound alterations in the coagulation and fibrinolytic systems.
Materials and Methods: we examined 42 patients with multiple pregnancy, thrombophilia and complicated obstetric history. All pregnancies were dichorionic. Twenty-three women were after IVF. These patients were compared with group of 75 women examined retrospectively with multiple pregnancy, thrombophilia (78.5%) and obstetric complications (fetal loss syndrome 48%, antenatal fetal death 35%, IUGR 15%, neonatal death 13%, preeclampsia 64%, severe preeclampsia 10%, abruptio placentae 29%, preterm labor 28%) and did not receive anti-thrombotic therapy.
All patients had been examined for the genetic and acquired thrombophilia.
Results: Forty-seven percent of patients had circulation of antiphospholipid antibodies, 31% – hyperhomocysteinemia. The incidence of factor V Leiden mutation was 9%, prothrombin gene mutation – 13%. Eighty-four percent of patients had combined thrombophilia.
Anticoagulant therapy was started after first visit to a doctor: 21 women with IVF were cured from preparing to IVF, 12 – from the period of pregnancy planning, 9 – at the beginning of I trimester of pregnancy. All patients received LMWH, folic acid, vitamins B, Omega-3, micronized progesterone.
In 4 cases there was fetal reduction in woman pregnant with triplets. Mild preeclampsia was in five patients at term 30–34 weeks of pregnancy. Eleven women had Cesarean section as planned at 37 weeks. Twenty-two patients were delivered at term 34–36 weeks and 9 at 30–34 weeks. There were no cases of obstetrical complications.
Comments: Testing for thrombophilia should be performed in all patients with multiple pregnancy. Since thrombophilia has been diagnosed LMWH therapy and micronized progesterone should be started.

PO 207

Biosimilar LMWH in prevention of repeated thromboses at pregnant women with trombophilia

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Background: The biosimilar enoxaparin is effective and safe in prevention of repeated thromboses at pregnant women.

Materials and Methods: We examined 57 patients at the age of 29.4 ± 5.5 years with a history of venous tromboembolism and burdened obstetrical anamnesis. The control group consisted on 60 women with the physiological uncomplicated pregnancy course. Among the patients with tromboembolism 32 patients had deep veins thromboses, 14–thromboembolism of the pulmonary artery, 11 – thrombosis of atypical localizations (splenic, mesenterial, hepatic, ovarian veins). Eighty-two percent had burdened obstetrical history (fetal loss syndrome – in 57%, preeclampsia – in 40%, placental abruption – in 32%). We performed laboratory screening for genetic and acquired thrombophilia. We detected thrombophilic condition in 90% of all cases: in 23.5% – mutation of the factor V Leiden (heterozygous), a prothrombin mutation – in 13.7% (heterozygous). PAI-1 – in 41.2%(homozygous) and in 33.3% – heterozygous. Heterozygous form of MTHFR C677T was found in 52.9%) In 40% of cases hyperhomocysteinemia was detected. Circulation of antiphospholipid cofactors (beta-2 GP -1) was found in 27.5% of patients. *Polimorphismes of proinflammatory cytokines were detected in* 40% *of patients*. Both genetic and acquired thrombophilia were detected in 75% of all cases.

Results: there was no repeated trombotic complications in patients treated with Hemapaxan. Twenty-five (44%) women were undergone cesarean cection, 32(56%) women had uncomplicated vaginal delivery. Fifty-seven alive newborns were born.

Comment: biosimilar enoxaparin applied in women with repeated thromboses and thrombophilia allowed to prevent repeated thromboses in 100% of cases and improve perinatal outcomes.

PO 208

Pregnancy and arterial thrombosis

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Background: The incidence of ischemic stroke associated with pregnancy is unknown but is estimated to be about 4.2–210 per 100,000 deliveries. Ischemic stroke is estimated to occur in 12–35% of women of 15–45 years old in association with pregnancy. Events occur most frequently in the III trimester of pregnancy or postpartum. Pregnancy is a kind of an essential spontaneous screening test for the risk of early stroke in women. Pregnancy augments the risk f stroke in 3–13%.

Materials and Methods: We observed 71 patients with history of arterial thrombotic complications and 60 healthy pregnant women (control group). Among 71 women with arterial thrombosis 5 of them had stroke and venous thromboembolic complications (VTE), 4 had myocardial infarction, three patients – acute arterial cerebral thromboembolism and thrombosis of prosthetic heart valves. Some women had history of stroke and ileo-femoral thrombosis and splenic infarction and thrombosis occurred in different other localizations (n = 19, 26.7%) including combination of ischemic stroke and retinal thrombosis, deep vein thrombosis, Takayasu syndrome and renal thrombosis, subclavian artery thrombosis, avascular necrosis of the femoral head and reoccurred cerebral thromboembolism in patients with catastrophic antiphospholipid syndrome.

I subgroup consists of 27 patients with history of arterial thrombosis. All these women were enrolled into the study from the fertile cycle or from the beginning of pregnancy andd therapy was started early. II subgroup accepted 23 patients from II or III trimesters of pregnancy and therapy was started after their admission. Thrombosis was associated with pregnancy in 17 women (28.8%). The remaining 21 patients with arterial thrombosis had thromboembolic complications during current pregnancy.

All included patients had been examined for the hemostasis system.

Results: risk factors of arterial thrombosis included systemic diseases (SLE, vasculitis) 16.9%, metabolic syndrome 37.3%, hypertonic disease 27.1%, cardiac arrhythmia 5.1%, the presence of prosthetic heart valves 6.8%, inadequate anticoagulant therapy, open foramen ovale 5.1%, intake of oral contraceptives 3.4%, surgery interventions 6.8%, septic complications 1.7%, vertebrobasilar abnormalities 11.9%.

Different forms of thrombophilia were present in 88.2% of patients. Multigenic inherited thrombophilia was recorded in 84.3% of patients (including one or more homozygous mutations), combined fibrinolytic defects in 76.5% patients, APA circulation in 41.2%, and hyperhomocysteinemia in 19.6%. It is important to pay attention to high prevalence of pre-eclampsia in patients with history of ischemic stroke (41.2% vs. 8.3% in control group, $P < 0.05$) but in II subgroup debut of pre-eclampsia occurred much earlier and was more severe probably because of lately started therapy.

All patients were delivered by cesarean section. Absolute indication for surgery was history of cerebral thromboembolism.

Conclusion: the revelation of the multigenic thrombophilic predisposition (84.3%) and especially fibrinolytic defects (76.5%) and APA circulation indicates an essential role of thrombophilia in the genesis of thrombotic complications. Early pathogenic anticoagulant therapy-from the fertile cycle when a women start to plan a pregnancy allows prevent reoccurred thrombotic events, severe obstetric complications and improve perinatal outcomes.

PO 209

Different clinical manifestations of APS in obstetric practice

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Background: obstetric complications such as fetal death, premature delivery, pre-eclampsia, fetal growth retardation, placental insufficiency and recurrent abortions are the characteristic manifestations of APS. They can occur in patients with known APS with previous arterial or venous events in any tissue or organ, or be its first or only manifestation.

Objectives: We determined different manifestations in the APS and assessed whether these clinical situations are due to the presence of antiphospholipid antibodies.

Methods: during 2000–2012 we observed more than 850 patients with different obstetric and gynecological complications and APS (recurrent miscarriage, preeclampsia, placental thrombosis, fetal growth retardation, metabolic syndrome, infertility, venous and arterial thrombosis, hypopituitarism Sheehans syndrome, failure of IVF, HRT, oral contraceptives and others).

Results: our investigation demonstrated that the presence of autoantibodies to various phospholipids and phospholipid-binding proteins (antibodies to beta-2-glycoprotein I, annexin V, prothrombin and to the antiphospholipid subtypes) has been identified in 64% of cases. In women with history of obstetric complications and circulation of APA fetal loss was found in 33.5% of cases, pre-eclampsia – 17.2%, thrombosis and placenta abruptio – 44%, stroke – 2.2%, IVF – 35.7%. In 71.5% APS was associated with genetic thrombophilia. Complex interactions of acquired and genetic thrombophilia are the worst combination and contribute to the causation of thrombotic events in most cases.

Conclusions: APS is one of the few treatable causes of pregnancy loss, and successful pregnancy rates of 70% or more can be achieved with

appropriate treatment. So, laboratory testing for aPL and antibodies to phospholipid-binding proteins has routinely to be recommended in the patients with suspected thrombophilia and attentive clinical care is required for best outcomes.

PO 210

Antithrombotic prohylaxis in pregnant patients with prosthetic heart valves

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In patients with prosthetic heart valves pregnancy and labor are associated with high thrombotic risk. Physiological hypercoagulation during pregnancy can reveal aquired and inherited hemostasis abnormalities which were asymptomatic before pregnancy.

Aim: To determine genetic thrombophilia and antiphospholipid antibodies (APA) in pregnant women with prosthetic valve thrombosis.

Material and Methods: Since 2000–2012 we have examined 11 pregnant women (at mean age 20–30 years) with mechanical valve thrombosis (mitral valve – $n = 8$, aortic valve – $n = 2$, tricuspid valve – $n = 1$) from 59 pts with prosthetic valves. Analysis of thrombophilia and APA was performed in all patients.

Results: History of obstetric complications (recurrent fetal loss, intrauterine growth restriction, preeclampsia, antenatal death) was observed in six women. Thromboembolic complications were observed in seven women (stroke – $n = 4$, renal and spleen thrombosis $n = 2$, iliofemoral thrombosis after cesarean section $n = 1$). Before the admission in our hospital (in 8–28 weeks of gestation) six patients received warfarin without regular monitoring, four patients – LMWH in low doses. One patient did not receive any anticoagulants in pregnancy during 1 month. Fetal mortality was 50%. One patient died due to pulmonary embolism 48 h after the simultaneous cesarean section and valve replacement. Thrombosis must be due to inadequate anticoagulation monitoring. Multigenic thrombophilia (≥ 4 mutations concomitantly) and APA were detected in 100%. MTHFR C677T, PAI-1 675 4G/5G, t-PA I/D, F Hageman 46C/T, fibrinogen – 455G/A, FV Leiden, prothrombin G20210A were detected in 6, 6, 2, 2, 4, 2 and 2 cases respectively. Lupus anticoagulant, anti-b2-glycoprotein I, anticardiolipins, anti-annexin V antibodies were detected in 4, 6, 3 and 4 patients respectively.

Conclusion: Pregnancy in patients with PHV carries significant risk. The presence of severe pregnancy complications, thrombotic events in anamnesis is an indication for the screening for genetic thrombophilia and antiphospholipid syndrome.

PO 211

Multiple pregnancy, chorion and placental abruption and thrombophilia

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We examined 91 women with multiple pregnancy after IVF. Seventy percent of patients had ovarian hyperstimulation syndrome. All pregnancies were complicated: abruption placentae – in 51 cases, chorion detachement – in 40 cases. Sixty-two percent of patients had previous history of fetal loss syndrome, 78% – abruptio placentae associated with preeclampsia, 35% – burdened thrombotic anamnesis, 47% – burdened family thrombotic history.

Methods: Clinical and hemostasiological examination. Criteria of genetic thrombophilia: mutation of the factor V Leiden, mutations of prothrombin G20210A, antithrombin III deficiency, protein C deficiency, 3 homozygous thrombophilic polymorphisms, or 5 heterozygotic polymorphisms, moderate or heavy hyperhomocycteinemia and antiphospholipid syndrome.

Results: We detected thrombophilia in 83% of cases: mutation of factor V Leiden (±) in 20%, mutation of prothrombin G20210A (±)– in 13%, PAI-1 – in 72%, hyperhomocycteinemia – in 36%, antiphospholipid syndrome in 25%, polymorphisms of proinflammatory cytokines in 68%, inherited thrombophilia assotiated with antiphospholipid syndrome- in 45% of cases.

Conclusion: We suggest that thrombophilia plays important role in pathogenesis of complications of multiple pregnancy after IVF. We recommend to perform screening for genetic and aquired thrombophilias before IVF in patients with placental and chorion abruption and other pregnancy comlications in anamnesis. In case of detection of thrombophilic condition anticoagulant and antoxidant treatment and vitamins support is recommended in preparation to IVF, during gestation after IVF and in postnatal period.

PO 212

Massive obstetric bleedings and pathology of hemostasis system

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In spite of existing standards of hemorrhages prophylaxis maternal death from massive obstetric bleeding remains the main cause of maternal mortality in developing countries and occurs in developed countries. According to our 25 years independent experience massive obstetric bleeding can be divided into moderate and massive. Due to our experience since 1989 to 2012 (analyses of 200 cases massive of obstetric clinic) massive obstetric haemorrhage mostly is initially coagulopathic. At the same time coagulopathy and hemostasis insufficiency have different character.

Main risk factors that provoke massive obstetric bleedings are, besides obstetric reasons, hemostasiologic disturbances:

1. (a) acute forms of DIC; (b)conversion of the chronic form of DIC to the acute or sudden appearance of acute forms – placental abruption, chorioamnionitis, septic shock, heavy preeklampsia, anafilaktoid syndrome of pregnancy (amniotic embolism)
2. the hidden undiagnosed forms of hemorrhagic diathesis – Willebrand disease, thrombocytopathias, taking of medicines influencing on hemostasis, liver diseases (defect of synthesis of coagulation factors and their inhibitors), sudden appearance of inhibitors of coagulation in bloodstream (Factor VIII inhibitor). Analyzing 200 cases of maternal death from massive obstetric bleeding we found that 45% of medical reports these women consist of notes about hemorrhagic history. It indicates the absence of correct diagnostics of hemorrhages.
3. absence of adaptive hemostasiological changes that are typical in pregnancy.

We suggest that extend laboratory screening for latent coagulopathic disturbances is of high clinical value in correct diagnosis and treatment of massive obstetric.

During last 7 years we observed and examined 91 women, survived after massive obstetric bleeding. Blood loss was mire than 2.0 L. According to our data in patients with history of massive obstetric bleeding we revealed thrombophilic and hemorrhagic defects in hemostasis system including thrombophilia in 61% (genetic – 55, acquired – 28, multigenic – 14), platelet's defects in 31%, and Von Willebrand disease in 13%.

Dysplasia of connective tissue of vascular wall predispose to bleeding due to primary vascular platelets defects in vast wound surfaces during placenta separation in labor or abnormal placenta abruptio bleedings. Mechanical methods of bleeding arrest which are widely used recently can) In our practice since 1998 to 2012 we observed pregnancy and labor in 56 women with mesenchymal dysplasia (Marfan, Ehlers-Danlos,

Osler-Veber-Rendu syndromes) including subclinical forms of diseases in patients of ages 18–36 years.
Hemostasis abnormalities were discovered in 69.6%: in 45% – hypofunction of platelets and acquired von Willebrand disease.
All 56 were delivered by cesarean section. Vaginal delivery is contraindicated because of risk of uterus rupture and vessels rupture. There were no cases of bleeding because of fresh frozen plasma uses pre-, intra- and postoperatively.
We advised 1 case of sudden death of patient with undiagnosed earlier Marfan syndrome. Autopsy studies have found aortic rupture.
Hepatic disorders characterized by coagulopathy as well as thrombotic complications. In cases of diagnosed primarily coagulopathy massive obstetric hemorrages can be successfully prevented (before labor or cesarean).

PO 213

Successful pregnancy outcome in women with bad obstetric history and recurrent fetal loss due to thrombophilia: efficacy of combination of aspirin and heparin

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Backgound: Recent investigations highlight the potential role of thrombophilia for determining unfavorable pregnancy outcome including recurrent fetal loss. However, the results so far published furnish discordant results. There are differences of opinion whether these patients need to be treated with aspirin, unfractionated heparin, low' 'molecular weight heparin or corticosteroids.
Purpose: To evaluate the safety of anticoagulant agents, such as aspirin and heparin, in women with a history of at least two spontaneous miscarriages or one later intrauterine fetal death without apparent causes other than inherited thrombophilias.
Material and Methods: We studied, for the common tests for acquired and congenital thrombophilia, 108 women with previous adverse (i.e., preeclampsia, intrauterine growth restriction (IUGR), placental abruption, intrauterine fetal death and recurrent pregnancy loss). Low molecular weight heparin was given at 4000 IU subcutaneously once daily, started at positive pregnancy testing and followed until delivery. Aspirin, 100 mg daily, was given in addition to enoxaparin to women with and without antiphospholipid syndrome. The anticoagulation was continued 6 weeks in postpartum period.
Methods: All the women were positive either for a solitary or for a combination of acquired and heritable thrombophilia markers (FVLeiden, 13%, FII mutation 12%, MTHFR C677T 49% MTHFR A1298C 34.5% Combined defects 32.7% Fasting homocysteine levels 14.8%, LAC 22%); 52 out of 108 patients (48.1%) had subsequent pregnancies. They were treated with low' 'molecular weight heparin plus aspirin, and all of them had successful pregnancy outcome (live birth rate of 100%). None of the patients had any adverse reactions such as heparin-induced thrombocytopenia, thrombosis, or fracture. None of the patients had to interrupt the therapy for any adverse treatment-related complications.
Conclusions: Thromboprophylaxis with aspirin and heparin in women with bad obstetric history and recurrent fetal loss due to thrombophilia seems to be safe in prevention of pregnancy loss in women with inherited and acquired thrombophilia. Its efficacy should be tested in properly designed clinical trials.

PO 214

Excessive menstrual blood losses can cause severe iron deficient anemia in women with inherited or acquired coagulation disorder for oral anticoagulant treatment

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Background: Excessive menstrual blood losses (EMBL) are a frequent symptom of a inherited bleeding disorder (IBD) but they can also complicate treatment with oral anticoagulants (OAT). EMBL negatively affect the quality of life and can lead to severe iron deficient anemia, sometimes requiring red blood cells transfusion. The quantitative determination of menstrual blood losses can effectively support the management of this frequent complication through an early detection that can help to avoid excessive iron losses and maintain a positive balance of iron homeostasis.
Objective: To quantitatively determine in a prospective evaluation, menstrual blood and iron losses in women with a diagnosis of inherited or acquired coagulation disorder secondary to OAT referring to our Center for iron deficient anemia.
Methods: Alkaline Hematin Method (AHM) was applied for the quantitative determination of menstrual blood losses (MBL) in each enrolled woman. A personal and gynecological history was recorded and all other potential known causes of iron deficiency were excluded in each enrolled woman. Sanitary protection wears used during menses were carefully collected and analyzed in a central laboratory. Menses were studied from 1 cycle to 3 consecutive cycles. A group of healthy women with normal hemoglobin and ferritin levels was enrolled as control. Blood cells count and ferritin serum levels were determined before enrollment and at least 2 weeks after the last menses. EMBL were defined as MBL > 80 mL. Iron losses were determined from the hematin values.
Results: Seventeen women with a known diagnosis of inherited bleeding disorder or under OAT, with a therapeutic INR (range:2–3), were enrolled for iron deficient anemia: von Willebrand's Disease (vWD, $n = 3$), Factor VII deficiency ($n = 6$), OAT for Deep Vein Thrombosis or Pulmonary Embolism ($n = 8$). Mean age at enrollment was 30.2 years (SD:5.19). Mean Hemoglobin levels were: 9.7 g/dL (SD:2.4); mean serum ferritin levels: 5 ng/dL (SD:3.37); mean menses duration was 6 days (SD:2); mean MBL was 95 ml (SD:12.7). Median amount of iron lost was 5.2 mg/cycle. In the control group ($n = 25$), mean age at enrollment was 29.9 years (SD:6.4), mean Hemoglobin levels were: 13.5 g/dL (SD:0.9); mean serum ferritin levels: 36.2 ng/dL (SD:20); mean menses duration was 4.5 days (SD:1); mean MBL was 45 ml (SD:17.2). Median amount of iron lost was 0.8 mg/cycle.
Conclusion: Excessive menstrual blood losses can be the leading cause of moderate to severe iron deficient anemia in women with inherited bleeding disorder or under OAT. The quantitative determination of menstrual blood losses can help to clinically define the problem and take adequate measures to prevent iron deficient anaemia and improve quality of life

PO 215

D-Dimers and pregnancy

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Background: Elevated D-Dimers (D-D) levels are observed in all diseases and condition with increased coagulation activation especially during pregnancy which is common hypercoagulability state. Some of women have high values of D-D, don't have trombotic problems or problems with pregnancy, but some of them have low values of D-D and have problems with thrombosis or problems with pregnancy. D-D values are very useful tool to prevent thromboembolic complications, such as VTE – DVT or pulmonary embolism during pregnancy.
Aims: To show the values of D-D in pregnant women during trimesters in ITM for first 3 months in 2012 year.
Methods: Two hundred and sixty pregnant women in different months of pregnancy. Quantitative determination of D-D in plasma of pregnant women with Innovace D-D, immunoturbidimeric assay (normal value < 500 ng/mL) for use on BCS (Bechring – Siemens) analyzer.
Results: Two hundred and sixty pregnant women were admitted in Outpatient department in ITM in the first 3 months in 2012 year. The values of D-D show those results: In the first trimester in 34 women values of D-D was until 1.316 ng/mL. In second trimester 107 women had D-D values until 2.369 ng/mL but 50 women in VI th lunar month of pregnancy had higher D-D values instead those in IV th or V th lunar month. In last trimester in 167 women values was until 3.122 ng/mL. All those women have born healthy child.
Summary/Conclusions: Range of D-D not correlates with pregnancy problems. Values of D-D are increasing proportionally during each month of pregnancy, so question is which value of D-D would be upper border to start with prophylaxis therapy. Collaboration between gynecologist and coagulations is most important.

PO 216

Evaluation of protein C activity in pregnant Nigerians

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Introduction: Thromboembolic phenomenon is under reported among pregnant Nigerians. The paucity of data and hence low index of clinical suspicion among obstetricians and care givers are hugely contributory to this.
Aims and Objectives: This study is aimed at establishing mean levels of protein C activation (PCA) ratio, prothrombin time (PT), activated partial thromboplastin time (APTT) and platelet count in pregnant Nigerians.
Subjects and Method: A prospective study was carried out in a tertiary institution in Enugu State, Nigeria over the 7 months period. Two hundred pregnant women in the 2nd and 3rd trimester and 50 non pregnant female controls were recruited and PCA ratio (coagulometric assay), PT, APTT and platelets count were also determined.
Results: there was a significant difference using ANOVA ($P < 0.0001$) in the mean platelet count for female non pregnant control subjects and the pregnant subjects in the second trimester and third trimester which were $226.34 \pm 69.16 \times 10^9$/L, $186.38 \pm 42.81 \times 10^9$/L and $167.46 \pm 49.63 \times 10^9$/L respectively. The *post hoc* multiple comparison showed that this was as a result the differences between control subjects vs. the pregnant subjects in their 2nd trimester of pregnancy ($P < 0.0001$), the control subjects vs. pregnant subjects in their 3rd trimester ($P < 0.0001$), as well as the 2nd vs. 3rd trimester ($P = 0.004$).
There were also significant difference in The mean PT and APTT for female non pregnant control subjects and Pregnant subjects in the second trimester and third trimester which were for PT,13.17 ± 0.77 s, 12.19 ± 0.84 s and 13.10 ± 2.09 s respectively and APTT 40.09 ± 3.44 s, 36.83 ± 3.61 s and 39.51 ± 4.44 s respectively. However, the *post hoc* multiple comparison showed that this finding was mainly due to the differences in PT and APTT between the control subjects vs. the pregnant subjects in their 2nd trimester of pregnancy and between the 2nd vs. 3rd trimester ($P < 0.0001$), The differences noted between the control subjects vs. pregnant subjects in their 3rd trimester for both PT and APTT were not contributory ($P = 0.960$) and ($P = 0.642$) respectively.
The female non pregnant controls and pregnant subjects in their 2nd and 3rd trimester with PCA ratio ≥ 4 were 20%, 52%and 27% respectively while those with APC sensitivity ratio ≤ 4 were 8%, 38% and 61% respectively. There was a significant difference ($P = 0.000$) in the mean PCA ratio of the non-pregnant controls and the pregnant subjects which were 4.27 ± 0.44, 3.87 ± 0.50 and4.34 ± 0.43 respectively. These changes were also statistically significant ($P < 0.0001$). The *post hoc* multiple comparison showed that these were due to differences between the non-pregnant control subjects vs. pregnant subjects in their 3rd trimester and the pregnant subjects between the 2nd trimester vs.3rd trimester ($P < 0.0001$). However, the differences between non pregnant control subjects vs. the pregnant subjects in their 2nd trimester of pregnancy were not contributory ($P = 0.671$).
Conclusion: There are changes in protein C activity (PCA ratio), PT, APTT and platelet count in our pregnant women. This largely would imply the possibility of thromboembolic disorders in pregnancy and hence the appropriateness of heightened index of clinical suspicion among our obstetricians and care givers.

PO 217

Thrombocytopenia and pregnancy

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The thrombocytopenia is related to 4–8% of the pregnancies, the rate of platelets is generaly moderate 70 Giga/L and the thrombopenia is insulated occurring especially after the third quarter or sometimes with the first quarter without maternal haemorrhage. It is necessary to differentiate this thrombocytopenia ITP (immune thrombocytopenia Purpura) which is much rarer (1–5 cases out of 10,000 pregnancies) with an important hemorrhagic risk, a foetal or neonatal haemorrhage, since at the time of a ITP, the antibodies anti platelets cross the placenta. The principal objective of our study is to detect on one hand these thrombocytopenia in order to pose a diagnosis as soon as possible of it and to avoid the massive haemorrhage which perhaps fatal, and on the other hand, the search for possible thrombocytopenia could be really a good indicator of pregnancy complications.
We followed 216 pregnant women presenting no characteristic obstétrical, for each one of them a blood numeration'platelets was done, by knowing that they all presented an initial biological assessment (biochemical, hematologic and serologic). At the time of discovered of a thrombocytopenia, a blood smear is then carried out in order to eliminate on the one hand, false thrombocytopenia, and on the other hand, to study the other cellular lines (red and white cells) to check if the cytopenia is insulated or associated (see the quantitative aspect on NFS). At 20 of them, a 9.25% frequency, we especially find a thrombocytopenia in the third quarter, and for three pregnant women only more precociously at the end of the first. Anaemia is present among 182 women (84.25%) during the last quarter (Iron Anaemia deficiency or by mixed deficiency). No hemorrhagic sign was announced, the thrombocytopenia was not symptomatic. The rate of platelets varied between 65 and 110 G/L (Algerian Standards: 120–380 G/L) Among the etiologies, two cases of DIC (disseminated intravascular Coagulation) were diagnosed (Assessment of consumption carried out) three cases of TIP where the rate of platelets fell from 65 to 42 G/L at the end of the pregnancy with caused Caesarean, 1 HELLP syndroma, and 14 of them were purely related to the pregnancy, the rate of platelets returned to normal 10–20 days after the delivery. This preliminary study enabled us to quickly detect the three cases of TIP in order to avoid the hemorrhagic risk at the foetus or the

new-born baby more especially as the maternal rate of the platelets does not reflect that of the new-born baby at all, indeed, for the three cases the rate was lower than 30 G/L and the treatment was very quickly founded, the NFS must be systematic at the new-born baby and the premature. The frequency of the thrombocytopenia at the pregnancy being of 6.48%, the initial anamnesis and assessments must be essential to interpret allthe results obtained.

PO 218

Plasma thrombin generation and circulating nucleosomes in women with early abortions

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Background: Miscarriages are distressing complications of pregnancy affecting about 15% of pregnancies, and 2% are recurrent.
Chromosomal abnormalities are considered the leading cause of early abortions, other causes are represented by uterine abnormalities, endocrine disorders, infections, immunological disorders. However, about 50% of recurrent miscarriages is unexplained.
As thrombophilic conditions are associated with an increased risk of obstetric complications we performed a case control study aiming to evaluate the association between early abortions, thrombin generation and levels of circulating nucleosomes, a marker of tissue damage and inflammation with prothrombotic potential.
Materials and Methods: Twenty-six women who were enrolled at the time of early abortion and the same number of healthy women of comparable gestational age were included in the study. The levels of circulating nucleosomes and thrombin generation were measured in the two groups. Quantification of circulating total nucleosomes was determined using a commercially available ELISA kit and nucleosomes were quantified in arbitrary unit (AU). Thrombin generation was determined by calibrated automated thrombinography (CAT).
Results: Plasma thrombin generation was measured in the absence of triggers (recalcification) or in the presence of tissue factor (TF, 5 pM). The thrombin generation was significantly lower in cases with respect to controls under both conditions: in the presence of TF the thrombin peak in cases was 267.7 nM and in controls 321 nM ($P = 0.007$) and the endogenous thrombin potential (ETP) 1286 ± 327.2 nM/min and 1526 ± 270.5 nM/min, respectively ($P = 0.004$).
Circulating levels of nucleosomes were similar in cases (median value 56 ± 22 AU) and controls (median value 62.5 ± 26 AU).
Conclusions:These preliminary results provide evidence that lower levels of thrombin generation are associated with an increased risk of early miscarriages suggesting that a state of hyper-coagulability may favor the implantation of the embryo. Further studies are needed to better understand the role of circulating nucleosomes and thrombin generation in early miscarriages.

PO 219

The features platelet hemostasis in pregnant women with missed abortion

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The problem of miscarriage in their social value is one of the leaders in modern obstetrics. In the reproductive loss structure the rate of missed abortion (MA) is 10–20%. Last years much attention in studying of is MA genesis are attended to hemostatic disorders, including genetically determined forms of thrombophilia, hyperhomocysteinemia, antiphospholipid syndrome, etc. Therefore, a detailed studying of the platelet morphofunctional characteristics can improve the diagnosis and treatment of MA.
The features of the morphofunctional state of peripheral blood platelets were analyzed in the I trimester of gestation in 35 pregnant women with physiological, 40 – with MA, as well as in 20 somatically healthy women of reproductive age. Investigations were carried out in the real time using novel information biotechnology based on the apparatus-and-program complex (APC «Bioni», Westtrade LTD, Moscow, Russia) for cellular diagnostics using computed cytomorphometry (module computer laser phase-interference microscopy module «Bioni-CPM») and microelectrophoresis (microelectrophoresis module «Bioni-MEPh»). Dynamics of the morphofunctional parameters of the cellular membranes (electrokinetic parameters of the separate cells and cellular population as a whole, percent of immovable cells, an average fluctuation amplitude, cell distributions in amplitude (histograms), asymmetry, and excess) was estimated as well as the computer platelet images (three dimensions of whole cells and their parts, different cross-sections and histogram) and optic-geometrical parameters of each platelet and its distribution by sizes. At one time standard hemostasis studies of peripheral blood in patients were performed including the platelet number, ADP-induced aggregation. Analysis of coagulation hemostasis was performed by determining the activated partial thromboplastin time (aPTT), prothrombin time (TV), prothrombin index, number of fibrinogen data thromboelastography. Determined the presence of antiphospholipid antibodies (aPL), lupus anticoagulant (LA).
The circulating platelet multiplicity reflects cell distinctions in size, density, metabolic, functional, biochemical features and the level of megakaryocyte polymorphism. When MA revealed marked heterogeneity of platelet hemostasis: reduced percentage of resting platelets (49% vs. 56% in physiological pregnancy), increased content of activated cells (32% and 14% vs. 28% and 11%, respectively), the average in the population value of the diameter, perimeter, area and volume of platelets increased by 19.2%, 15.9%, 39.1% and 44.4%, respectively. Apparently, this is due to the processes of conversion of the cell hemostasis towards activation and implementation of hypercoagulability. The subpopulation of large platelets (with diameter > 5 microns) were isolated and characterized. Outside of pregnancy the macroform content does not exceed 2%, in physiological pregnancy (I term) – 4%, MA – increases to 7%. All patients with MA were followed up 7–10 days after emptying the uterus against the background of antiplatelet and anticoagulant therapy. The effectiveness of treatment was evaluated by the activation status of the restoration and normalization of morphometric parameters of circulating platelets.
There were significant correlation relationship between the platelet morphometric parameters, different morphological cell types, and the most important indicators agregatogramms. On the basis on these results, a system of objective and informative criteria for the timely MA diagnosis and the adequacy of pathogenetic therapy.

PO 220

The clinical outcome of 103 pediatric ITP patients in one center

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Background: Immune thrombocytopenic purpura (ITP) is a common disease in children that can be seen in two clinical forms as acute and chronic.
Aims: In our study, clinical course and treatment responses of patients with childhood ITP were evaluated in terms of determining causes of disease and the factors affect on chronicity.
Methods: The files of 103 patients followed as ITP and sufficient information can be accessed between march 2006 and February 2011 at Gaziantep University Faculty of Medicine, Department of Pediatric Hematology were investigated for history, clinical examination, laboratory findings, clinical course and treatment responses.

Results: Average age at diagnosis was 73.0 ± 50.7 months and female/male ratio was 1.1. Thirty-three percent of the patients had history of previous infection and 3.9% had history of vaccination. Bruising (75.7%) and epistaxis (35.9%) were most common compliants at admission. Most of the patients were admitted in spring (29.1%) and summer (27.2%). In our ITP patients 33.3% had previous viral infection, 20.6% had antinuclear antibody (ANA) positivity, 29% had antithyroglobulin antibody positivity. At the admittion 50.5% of the patients were diagnosed as acute ITP and 49.5% were diagnosed as chronic ITP. The age of diagnosis > 10 years, admission platelet count > 10,000/mm^3, and those with insidious onset, chronity was significantly higher than those without a history of previous infection ($P = 0.009$, $P = 0.001$, and $P = 0.003$ respectively). Asthe preferred initial treatment with intravenous immunoglobulin (IVIG) and steroid there was no difference in terms of the treatment response and relaps rate ($P = 0.097$, $P = 0.62$ respectively). Between follow up without treatment, IVIG, and steroid treatments there was no difference in terms of chronicity ($P = 0.33$).
Conclussion: In children, ITP can be seen after previous infection or vaccination and may be associated with underlying autoimmun disease. Although the prognose is variable, age at diagnosis, platelet count at admission, and previous history of infection are risk factors for chronicity.

PO 221

The serum TPO levels, not a good biomark in children immune thrombocytopenia

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Objective: To explore the relationship between serum TPO levels and the prognosis of children Immune Thrombocytopenia (ITP) during their onset period.
Method: The children (< 14 years) with new-diagnosis ITP were included in this study in Hematology Center ward of Beijing Children's Hospital from April 2012 to September 2012. The serum thrombopoietin (TPO) levels were tested in all patients. All of the children were followed in the 3rd month and were divided to two groups: Remission group (platelet count > 100×10^9/L) and Non-remission group platelet count < 100×10^9/L according to the platelet count. Several clinical data of the children with ITP were collected included: the first platelet count, megakaryocytes count of bone marrow for the ITP diagnosis, response to treatment measure, platelet count in the 3rd month followed period. And the relationship between these clinical data and serum TPO levels were analysed.
Result: There were 33 children with new-diagnosis ITP were included in this study. Age: from 2 months to 12 years old, median age is 3.0 years old. Six cases in non-remission group and other 27 cases in remission group. Correlation analysis showed no significant correlation between TPO levels and platelet count before therapy ($P = 0.775$,$r = -0.051$) and megakaryocyes count of bone marrow ($P = 0.773$,$r = -0.051$). similarly, no significant correlation were founded between TPO levels and response of treatment ($P = 0.907$, $r = -0.021$). The TPO levels were no significant difference between non-remission group and remission group ($P = 0.456$).
Conclusion: There is no significant correlation bewteen serum TPO levels and several clinical data of Children ITP. The TPO levels can't be used as a biomark for evaluating the prognosis of children ITP.

PO 222

Safety and efficacy of laparoscopic splenectomy in children with chronic immune thrombocytopenic purpura

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Background: Prevalence of Immune thrombocytopenic purpura (ITP) in Russian children range 5.5–6.5 in 100,000. Fifteen percent of them suffer from chronic ITP with excessive bleeding and poore quality of life. Treatment mode for this group is splenectomy.
Aims: To evaluate safety and efficacy of laparoscopic splenectomy (LS) in children with chronic ITP.
Methods: During the period 2000–2012 we observed 61 children with chronic ITP, who had excessive impending bleedings (35 – epistaxis, 20 – menorrhagia, 7 – kidney bleedings, 4 – intestinal bleedings, 6 – gum bleedings, 7 – eye bleedings, 2 – cerebrovascular accidents, 1 – hemothorax). Pre-operative conservative treatment in all patients included intravenous immunoglobulin (IVIG), steroids, interferon-alpha, anti-D-Rh immunoglobulin, danazol with no sufficient effect. The age of the patients varied from 2 to 17 years. The mean disease duration was 51.1 months. Laparoscopic splenectomy was performed in all patients. Three trocars – two 5 mm and 10 mm – were used in all cases; Ligasure instruments were employed for dissection and vessels ligation and transection (including the vascular pedicle).
Results: Mean platelet count during the surgery was 110.3 × 10 (9)/L (range 12–383 × 10(9)/L). Mean surgery time was 65 min (range 30–195 min). There were no indications for conversion. Five children required red cells transfusion in the early postoperative period, 29 – had reactive postoperative pancreatitis. No fatal complications occurred. There were no relaparoscopies or relaparotomies in our series of 61 patients. After LS 35 (57.4%) children have stable normal platelet count without bleedings; 26 (42.6%) children after LS need additional drug therapy- danazol, IVIG, steroids. After surgery and additional drug treatment nine children (14.7%) still have severe thrombocytopenia with episodes of bleeding.
Conclusion: LS in combination with conservative therapy was effective in 85.3% of actively bleeding ITP children after unsuccessful nonsurgical therapy.

PO 223

Comparison of length of stay for inpatients with immune thrombocytopenia treated with anti-Rh(D) vs. those treated with intravenous immunoglobulin or steroids

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Background: Anti-Rh(D) is an effective treatment for immune thrombocytopenia [ITP]. It has a more rapid onset of action (1 day) vis a vis glucocorticoids (3 days) and intravenous immunoglobulin [IVIG] (4 days). A direct comparison of length of stay [LOS] for adult inpatients receiving these therapies has hitherto not been performed.
Aims: To determine whether anti-Rh(D) therapy for ITP results in shorter mean LOS compared to treatment with glucocorticoids or IVIG.
Methods: With institutional review board approval, a retrospective chart review was conducted to assess length of stay in relation to treatments proffered for ITP. The defining diagnosis of ITP [coded 287.31] was rendered from the computerised record at St. Luke's-Roosevelt Hospital Center. In a period spanning 01SEP2005 through 29FEB2012, 303 admissions were identified, of which 78 were just for

active ITP, and in which ITP-related complications or other co-morbidities did not impact on LOS. Single agent therapy – glucocorticoids, anti-Rh(D), or IVIG – was administered in 50 of the 78 admissions; combination therapy was given in the remaining 28. LOS was extracted from the medical record. An average LOS was tabulated for each treatment group. Age and gender were also recorded. An apriori one-way ANOVA calculation was used to compare LOS among patients treated with prednisone, dexamethasone, or IVIG. The student's *t*-test was used to compare LOS between patients treated with anti-Rh(D) vs. the group of patients treated with prednisone, dexamethasone, or IVIG (http://www.physics.csbsju.edu/stats/).

Results: There were 45 adult and 5 pediatric admissions. The median age was 43 years, range 3–78 years. The male:female ratio was 1.1:1. Mean LOS (days) was similar for patients treated with prednisone ($n = 7$) 2.714 [95% CI 1.841–3.588], dexamethasone ($n = 7$) 3.000 [95% CI 2.127–3.873], or IVIG ($n = 10$) 2.500 [95% CI 1.769 thru 3.231], $P = 0.66$. Mean LOS was shorter for patients treated with anti-Rh(D) ($n = 26$) 1.888 [95% CI 1.383 thru 2.386] in comparison with the group of patients treated with prednisone, dexamethasone, or IVIG ($n = 24$) 2.71 [95% CI 2.186 thru 3.230], $P = 0.027$. There were no complications noted in the treatment arms.

Summary/Conclusions: LOS is 1 day shorter for ITP patients treated with anti-Rh(D) vs. those treated with glucocorticoids or IVIG. According to the findings of this study, anti-Rh(D) is a rapidly effective, well-tolerated therapy for ITP.

PO 224

Successful management of ITP during pregnancy: weekly IVIg is an option

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Background: Primary ITP is an autoimmune disorder characterized by isolated thrombocytopenia (peripheral blood platelet count $< 100 \times 10^9$/L) in the absence of other causes or disorders that may be associated with thrombocytopenia. Pregnancy does not increase the risk of developing ITP nor does it exacerbate preexisting ITP, although anecdotal reports of exacerbation of ITP during pregnancy with improvement after delivery. Treatment is largely based on the risk of maternal hemorrhage. Studies have shown that pregnancy in women with ITP can proceed safely with low hemorrhagic risk, with around 1/3 of patients requiring intervention. Throughout the first 2 trimesters a platelet count at 20 to 30×10^9/L or higher do not routinely require treatment, unless the patient is symptomatic or to produce an increase in platelet count to a level considered safe for procedures. They should be monitored more closely as delivery approaches. Corticosteroids are the first line of therapy in pregnant women; intravenous immune globulin (IVIg) is commonly used in steroid resistant patients. Other treatments such as intravenously administered anti-D (IV anti-D) and splenectomy during pregnancy have been reported. The mode of delivery is determined by obstetrical considerations.

Case report: A 20-year-old woman was admitted to the hospital during week 20 of her first pregnancy because of thrombocytopenia (3×10^9/L) without bleeding. She had no history of infection or drug intake. A subclinical hypothyroidism was diagnosed. Laboratory studies revealed a hemoglobin of 11.7 g/dL, WBC counts $12{,}300 \times 10^9$/L (normal differential), and normal coagulation and serological tests. She was started on oral doses of 60 mg of prednisone daily and IVIg (1 g/Kg/day for 2 days) with a complete response. Despite being maintained with steroids, there was a significant drop in platelet count after 14 days. At this time she presented with petechiae on the trunk and limbs, and she was noted to be hypertensive. IVIg was administered again and a single weekly IVIg (400 mg/Kg) infusion was given, which provided a normal platelet count. In the absence of any response and because of hypertension, steroid was tapered to 30 mg. Cesarean section was performed and she gave birth to a female infant with a normal platelet count.

Summary: Due to the paucity of RCTs, recommendations for the diagnosis and management of ITP in pregnancy are mainly based on clinical experience and expert consensus. First-line treatment of ITP in pregnancy include corticosteroids, IVIg, IV anti-D and splenectomy. There are no comparative trials of corticosteroids and IVIg in pregnant women; however, response rates are similar to those in non-pregnant patients. For the management of maternal ITP patients failing firstline treatment, azathioprine, cyclosporin A, splenectomy may be some options to be used before delivery. In our patient, a single weekly IVIg infusion was sufficient to prevent hemorrhage and provide an adequate platelet count for delivery. IGIV therapy offers a therapeutic modality in managing the difficult patients with ITP throughout their pregnancy.

PO 225

CXCR4 gene variation is associated with minimal platelet count of childhood primary immune thrombocytopenia

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Background/Aim: Primary immune thrombocytopenia (ITP) of childhood is an autoimmune disease characterized by abnormally increased destruction of platelets and decreased megakaryopoiesis. Chemokine receptor, CXCR4 is involved in the migration, homing, mobilization, proliferation and survival of hematopoietic stem cells. In addition, CXCR4 is shown to have an impact on the survival of platelets derived from ITP patients.

Methods: Two single nucleotide polymorphisms (SNPs) of the CXCR4 gene (rs2228014 and rs2471859) were assessed in 100 children with ITP and 126 healthy controls. The genotypes were analyzed by tetra ARMS polymerase chain reaction and confirmed by direct sequencing.

Results: Compared with controls, the rs2228014 and rs2471859 genotypes were not different in ITP patients ($P = 0.225$ and 0.759, respectively). Further analysis of the relationship between *CXR* polymorphisms and clinical features also showed that the rs2228014 and rs2471859 genotypes were not associated with protection from chronicity and steroid-dependence in ITP patients. However, the minimal platelet count were significantly decreased in patients with rs266085 genotype C/C ($P = 0.03$).

Conclusion: The findings of this study suggest that *CXR* gene variation may be associated with the minimal platelet count of childhood ITP.

PO 226

Decreased interlukin-7 in peripheral blood is due to the negative feedback of apoptosis-resistance and over-expression of pro-inflammatory cytokines of lymphocytes in primary immune thrombocytopenia

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Background: Primary immune thrombocytopenia (ITP) is an autoimmune disease with many immune dysfunctions, including over-proliferation and apoptosis resistance of auto-reactive lymphocytes. As a critical cytokine for development and survival of lymphocytes, whether interlukin-7(IL-7) is involved in ITP remains unclear.

Aims: This study aimed to determine the effects of IL-7 on the cytokine production and survival of peripheral blood mononuclear cells (PBMCs) and bone marrow mononuclear cells of (BMNCs) of ITP.

Methods: The levels of IL-7 in PB and BM serum and the production of IFN-γ, tumor necrosis factor (TNF)-α, and IL-10 were measured by ELISA. Besides, flow cytometric analysis of cell apoptosis was performed by staining with annexinV-FITC/Propidium Iodide (PI). The proliferation rate of PBMCs was examined by BrdU incorporation method.
Results: The result showed that the plasma IL-7 levels in peripheral blood (PB) from ITP patients were lower than that of the normal controls, and it had correlation with therapy and platelet counts, but the levels of IL-7 did not change in bone marrow (BM) of ITP patients compared with that of normal controls. The results also showed that IL-7 promoted the proliferation of lymphocytes both in PB and BM. Additionally, we also found that the exogenous IL-7 up-regulated the secretion of interferon-γ, tumor necrosis factor-α as well as IL-10.
Conclusion: These findings suggest that decreased IL-7 in PB is a consequence of the negative feedback of apoptosis-resistance and overexpression of pro-inflammatory cytokines of lymphocytes in ITP patients.
Hui Yuan Li and Dong Lei Zhang contributed equally to this study.

PO 227

Experience in treatment with alpha interferon in children with hemangioma in Srinagarind Hospital

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Background: Hemangioma, most common benign vascular tumor, has natural history of proliferation in the first year of life followed by spontaneous resolution. The treatment is not universal standard with indication as following: impaired organ function and Kasabach Merritt syndrome (KMS). Alpha-interferon inhibits angiogenesis, endothelial cell migration and proliferation.
Objective: To evaluate the outcome of alpha-interferon for the treatment of hemangioma.
Methods: We recruited 35 patients with hemangioma receiving alpha-interferon followed at hemangioma clinic between January 2001- January 2011.The patients' data including sex, age, type and location of hemangioma, indication, duration, outcome and complications of treatment were reviewed.
Design: Retrospective descriptive study.
Measurements: We evaluate of the outcomes including improvement of organ function, resolution of Kasabach Merritt syndrome (KMS) and decrease hemangioma size.
Results: the number of patients was 35 patients (female = 21, male = 14) with mean age 3.2 months (range 5 days-15 months), loss follow up in five patients. Hemangioma was divided into cavernous (9), capillary (10), mixed (7). Location of hemangioma includes head and neck (21), extremities (7), mediastinum (3), intraosseous (1), liver (2), larynx and epiglottis (2), and scrotum (1). Average age of treatment was 4.7 months (range 5 days-18 months). Duration of response in 29 patients was 4.7 months (range 1–6 months). Indications of treatment included KMS (1), KMS with impaired organ functions (2), impaired organ functions (30), infection (2), intracranial (1). Eleven patients were decrease in size of mass > 50%, 14 patients were decrease in size < 50% but 23 patients (82.1%) were improved organ function and resolution of KMS. Complications of treatment were elevation of liver enzymes (3), and fever (6).
Conclusions: The use of alpha-interferon was well response to improve organ function and decrease hemangioma size. Due to spastic diplegia, serious complication of alpha-interferon from previous case report studies, the consideration of this treatment should be discussed with parent in light of pro and cons.

PO 228

APC and PAR-antagonists' protective effects on inflammated mast cells and mast cells co-cultivated with rat neurons

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Backgrounds: Serine proteinases of blood coagulation system – thrombin and APC – are signal molecules that regulate cell functions via protease-activated receptors (PARs). PAR activation with thrombin results into transcription factors activation, increase of inflammatory mediators, expression of adhesive molecules on cells' surface [Angiolillo D.J., et al., 2010]. But APC through the same receptor with thrombin helps to stabilize cells and suppress inflammation [Griffin et al., 2007]. Mast cells are rich with PARs and so they contribute to connection between coagulation and inflammation. Activated mast cells secrete vasoactive and inflammatory factors, growth factors, and cytokines. It was suggested that mast cells can promote blood-brain barrier (BBB) damage. So stabilization of BBB after acute stroke via mast cells regulation may become a new pharmacological target [P.J. Lindsberg, et al., 2010]. It was shown previously that APC and iPAR 1-III can significantly decrease histamine, IL 1b and IL 6 release from peritoneal rat mast cells [Rusanova A. et al., 2009, Strukova S. et al. 2011]. But there are very few data APC and PAR-antagonists effects on TNF release from mast cells during acute inflammation and neuron survival at co-cultivation with inflammatory mast cells after APC and PAR-antagonists. Therefore it constitutes the aim of the present study.
Aims: To estimate secretory activity of mast cells in experimental peritonitis and APC/PAR-antagonists influence on it. To study interaction between mast cells and hippocampal neurons in co-culture.
Methods: Mast cells were isolated from rat peritoneal cavity and purified by centrifugation on Ficoll gradient. Histamine secretion was measured by spectrofluorescent methods, TNF secretion was measured by ELISA. Hippocampal neurons were isolated from newborn rat cubs and cultivated for 10 DIV (Gorbacheva et al., 2009). Mast cells were pre-incubated with APC or PAR-antagonists for 15 min and then co-cultivated for 1DIV with hippocampal neurons. To estimate the contents of co-cultures immunostaining with mast cells tryptase and synaptotagmin was performed. Neuron survival was estimated by morphological staining with SYTO13, Hoechst and Ethidium Bromide. Statistical analysis: non-parametric Mann-Whitney criterion. All the studies that involve experimental animals were performed according to ethic protocol approved by Lomonosov MSU.
Results: In short times of *in vivo* model (intraperitoneal injection of Thioglycolate broth) APC effect on TNFα release from mast cells resulted into 76% decrease of TNFα amount detected in mast cells supernatant. It was also shown that APC (1 mkM) pre-incubated with mast cells increased neuron survival by 12% compared to control group without mast cells and by 32% compared to co-cultures with untreated mast cells. The results also indicate that iPAR's influence on cell survival was more effective in tested concentrations of 2.5 mkM. Total cell survival was increased for 40% and level of apoptosis and necrosis was significantly suppressed.
Conclusions: The results show that APC and iPAR 1-III may be considered as effective cytoprotectors in our research model. If APC and iPAR can possibly modulate and stabilize mast cells that may be a background for investigation of mast cell-nerve interaction and cross-talk in different models.

PO 229

Splitomicin inhibits fMLP-induced superoxide anion production in human neutrophils by activate cAMP/PKA signaling inhibition of ERK pathway

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Splitomicin, is a cell-permeable lactone derived from naphthol and known to be a potent selective inhibitor of Sir2 (silent information regulator 2). Previous studies have demonstrated that naphtholic compounds pos- sess an inhibitory effect on neutrophils. Here, we present our investigation on the inhibitory effects of splitomicin in human neutrophils. The primary goal of our study was to locate a possible candidate on inflam- matory reactions and to hopefully develop a novel anti-inflammatory therapy. Neutrophils were prepared following standard procedures. Neutrophils induced by either fMLP (1 μM) or PMA (100 nM) were observed using a flow cytometer and the intracellular production of superoxide anions was investigated at different splitomicin concentrations. The cytosolic Ca++ influx concentration was measured using a fluorescence spec- trophotometer, and Mac-1 expression was detected with a flow cytometer. The MAP kinases were measured using western blotting. Our results showed that splitomicin inhibited superoxide anion production by fMLP (1 μM) and NaF (20 mM) in a concentration-dependent manner (37.5–450 μM). Splitomicin (300 and 450 μM) also suppressed fMLP-induced intracellular calcium ion mobilization and extracellular-signal regu- lated kinase (ERK) phosphorylation. Moreover, splitomicin could inhibit fMLP-induced Mac-1 expression and increase cAMP levels in human neutrophils. Our data demonstrated that splitomicin exhibits a noticeable inhibitory effect on superoxide anion production in human neutrophils. This negative effect was well- correlated with increased cAMP levels via PKA activity and the subsequent inhibition of ERK (p42/p44) phos- phorylation to decrease superoxide anion production.

PO 230

Prospective study of inflammatory biomarkers in patients undergoing peritoneal dialysis

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Background: Peritoneal dialysis (PD) is a kidney replacement therapy for patients in the end stage renal disease (ESRD). The PD has long-term complications, including inflammation, fibrosis and neoangiogenesis, which make this therapy ineffective for proper removal of solutes.
Aims: The aim of this study was to evaluate inflammation biomarkers in patients undergoing PD and to investigate the association with peritoneal solute transport rate (PSTR).
Methods: Forty-five patients who were under PD therapy for more than 6 months in the Nephrology Center of Hospital Sao Joao de Deus – Brazil, were eligible for this longitudinal study. IL-2, IL-4, IL-6, IL-10, IFN-γ, TNF-α plasma levels were measured, at baseline and after 6 months on PD, by flow cytometry (BD-FACSFortessa), using BD Cytometric Bead Array (CBA) Human Th1/Th2/Th17 Cytokine kit. PSTR of small solutes was evaluated using the dialysate-to-plasma ratio (D/P) of creatinine after a 4-h dwell. The Ethics Committee of the Hospital Sao Joao de Deus approved this study and written informed consent was obtained from all subjects. Statistical analysis was performed using SigmaStat 2.03 program and ANOVA test.
Results: At baseline 12 patients were classified as low-transporters, 25 as low-average transporter and 8 as high-average transporters. There was not difference between the respectively groups with regard to IL-2 (3.0 vs. 3.1 vs. 2.6 pg/mL), IL-4 (1.8 vs. 2.2 vs. 1.6 pg/mL), IL-6 (8.3 vs. 7.8 vs. 5.9 pg/mL), IL-10 (2.2 vs. 2.9 vs. 2.2 pg/mL), IL-17A (5.0 vs. 7.3 vs. 5.1 pg/mL), TNF-α (2.0 vs. 2.5 vs. 2.2 pg/mL) and INF-γ (3.2 vs. 3.5 vs. 2.8 pg/mL) plasma levels. Of the 45 patients, 12 (26.6%) were increased of the PSTR after 6 months follow-up. However, no significant changes were observed for cytokines levels during this time.
Summary/Conclusions: No association between inflammation markers and the PSTR in PD patients after 6 months follow-up was observed. Moreover, further studies with a larger number of patients need to be developed to better understand the pathogenesis of peritoneal dysfunction.
Supported by FAPEMIG, CNPq and UFSJ – BRAZIL.

PO 231

Role of protein S in murine model of allergic asthma

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Background: Protein S may exert an anticoagulant activity by enhancing the anticoagulant activity of activated protein C and/or by directly inhibiting the prothrombinase complex. Protein S itself may also directly regulate inflammatory responses and apoptosis. Recent studies have suggested that coagulation and anti-coagulation factors are associated with asthma, but the relationship between asthma and protein S is unclear.
Objective: To evaluate the effect of protein S in allergic bronchial asthma. On this purpose, we compared the development of bronchial asthma in wild type and human protein S transgenic mice.
Methods: Bronchial asthma was induced by sensitization and challenge with ovalbumin (OVA). Mice treated with saline were used as control. The effect of inhaled rhTM was assessedby administering it prior to OVA exposure. Airway inflammation was evaluated by measuring the number of inflammatory cells and the levels of cytokines in bronchoalveolar lavage fluid (BALF). Airway hyperresponsiveness was measured using a plethysmograph.
Results: Airway hyperresponsiveness was significantly decreased in protein S transgenic mice compared with their wild type counterparts. The number of eosinophils in BALF was decreased in protein S transgenic mice compared with their wild type counterparts.
Conclusions: These results suggest that protein S inhibits the development of bronchial asthma.

PO 232

Protective effect of thrombomodulin in murine asthma is dose dependent

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Background: Thrombomodulin (TM), the thrombin receptor on the endothelial cell surface, plays an important role in coagulation and inflammation by inactivating thrombin and activating protein C. Previously we reported that recombinant human TM (rhTM) is protective against murine asthma. However, the effect of different concentrations of rhTM on asthmatic inflammation remains unclear.

Objective: To evaluate the dose-dependent effect of inhaled rhTM on airway inflammation and hyperresponsiveness in a murine asthma model.
Methods: Bronchial asthma was induced by sensitization and challenge with ovalbumin (OVA). Mice treated with saline were used as control. The effect of inhaled low dose to high dose rhTM was assessedby administering it prior to OVA exposure. Airway inflammation was evaluated by measuring the number of inflammatory cells and the levels of cytokines in bronchoalveolar lavage fluid (BALF). Airway hyperresponsiveness was measured using a plethysmograph. Particle size distribution of each different rhTM was measured by Spraytec.
Results: The number of eosinophils in BALF and airway hyperresponsiveness was decreased by rhTM in a dose-dependent manner compared to saline treated mice. rhTM concentration of 3.75 μg/ml was associated with the lowest number of BALF eosinophils and airway hyperresponsiveness, and with the smallest particle size.
Conclusion: These results suggest that the effect of rhTM in murine asthma is dose- and particle size-dependent.

PO 233

The role of ETX in the pathogenesis of vascular complications of diabetes

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Background: Vascular complications of diabetes can be largely classified into macroangiopathy such as cardiovascular disease, stroke, and peripheral artery disease, and microangiopathy such as diabetic nephropathy. Preventing the complications of these vessels can significantly improve on QOL. However, the progression of vascular complications mechanism remains unclear.
Endotoxin (ETX) is a lipopolysaccharide (LPS) in the gram-negative bacteria of the outer membrane of the cell wall, and is recognized as a causative agent of lethal shock, high fever, DIC, etc. ETX contributes to acute and chronic inflammations, and triggers the innate immune response characterized by cytokine release and immune system activation. It was known that ETX induce reduce the dysfunction of the kidney.
Aim: The aim of this study was to clarify the role of ETX in vascular complications of diabetes.
Method: We obtained individual informed consent in the Traditional Outpatient Department for Diabetes, Internal Medicine, Shiga University of Medical Science from 2005 to 2011 upon obtaining approval from the ethics committee of the university, and measured the amounts of ETX in the blood (343 patients).The study protocol is in accordance with the Declaration of Helsinki.
Insulin resistance model mice by the ingestion of 70% glucose solution for 4 weeks were used in this study, insulin resistance in mice was confirmed by Oral Glucose Tolerance Test (OGTT). We measured the blood flow in the tail of mice by using the Doppler blood flow meter, and then. We also examined the amount of ETX in the blood and kidney.
Result: We found that ETX was detected in the blood of 148 patients with type 2 diabetes, and the patients who are detected ETX was also significantly increased the level of urine albumin compared with undetected patients. ETX was detected in the blood and kidney of insulin resistance model mice. We also found that the blood flow of the insulin resistance model mice was significantly reduced compared with controls.
Conclusion: We found that ETX was detected in blood of patients in type 2 diabetes, the renal function in the patients was significantly reduce. We also found that ETX was detected in blood and kidney of insulin resistance model mice, the function of the blood vessel was reduced. These data suggest that ETX may plays a pivotal role in the pathogenesis of vascular complications of diabetes.

PO 234

Venous thrombosis and the incidence of factor V Leiden and factor II A20210 mutation in the cardiac pacing patients

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Background: The pathogenesis of venous thrombosis (VT) is multi-factorial, involving the acquired and genetic factors. Additionally to the predisposing factors (e.g. pacemaker implantation), genetic predisposition due to molecular abnormalities of the coagulation pathway components have been encountered in the subjects with thromboembolic disease. In the patients of European ancestry, common mutations within the coagulation factor V (FV Leiden) gene and the factor II (G20210A prothrombin mutation) gene have been demonstrated to account for a large number of thromboembolism cases. There is a scarcity of studies focused on the contribution of genetic risk factors to VT (i.e. FV Leiden and G20210A prothrombin mutation) in the cardiac pacing patients.
Aim: To assess whether FV Leiden and G20210A prothrombin mutation are associated with venous thrombosis in the cardiac pacing patients.
Methods: The study group comprised 95 subjects (38 females, mean age 71 $\pm$ 7.58 years) with a cardiac pacemaker. Cardiac pacemakers were implanted due to SSS, AVB (II, III gr.), atrial fibrillation cum brady-arrhythmiae, respectively. All subjects were subsequently followed up for 18 months, whereupon in 10 subjects VT developed. Subjects were divided according to the non-occurrence (Group A; $n = 85$; mean age 71.2 $\pm$ 7.6 years) or occurrence (Group B; $n = 10$; mean age 72.6 $\pm$ 7.2 years) of VT in the post-implantation period, respectively. A transthoracic echocardiogram (TTE) and a venous ultrasound examination were performed.
Screening for FV Leiden and the G20210A prothrombin mutation were carried out by standard methods. Also the levels of D-dimers, fibrinogen, tissue factor (TF), factor VII, plasminogen activator inhibitor-1 (PAI-1), interleukin-6 (IL-6) and hsCRP) were determined before and at the 7th day, within 6 and 12 months after pacemaker implantation, respectively.
Results: The two groups did not differ with respect to the pacing mode. The symptoms of VT occurred in 10 subjects in Group B at a mean of 13.06 months (7–17 months), i.e. in 11.76% of the entire study population. The subclavian and brachiocephalic veins on the pacemaker side were occluded. Three subjects (20.0%) in Group A and six subjects (7.0%) in Group B were the carriers of FV Leiden ($P = 0.01$), whereas 3 (3.5%) subjects in Group A had the G20210A prothrombin mutation.
Initially, the plasma levels of prothrombotic (D-dimers, fibrinogen, TF, factor VII, PAI-1) and proinflammatory (IL-6, hsCRP) markers were appreciably higher in Group B than in Group A ($P < 0.001$). In all study subjects the values continued to rise until the 7th day after pacemaker implantation. Within 6 and 12 months after the procedure the trend towards increasing the plasma levels of these markers was observed in Group B only. In Group A the levels returned to normal.
Conclusion: It is highly recommendable that all cardiac pacing patients be screened for FV Leiden and G20210A prothrombin mutation. Coexistence of FV Leiden with the acquired VT risk factors increases the risk of thrombosis after pacemaker implantation.

PO 235

Impact of inherited thrombophilia on the risk of recurrence venous thromboembolism (VTE) in Georgian population

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Background: Factor V Leiden (1691G/A) and prothrombin gene mutations (20210G/A) are the most common genetic markers that predispose to a first episode of venous thromboembolism (VTE). However, whether the presence of these mutations confer an increased risk of recurrent venous thromboembolism is controversial.
Methods: A total of 70 patients with venous thromboembolism were genotyped by PCR analysis for the prothrombin gene (PTH) mutation and factor V Leiden (FVL) and were followed prospectively for recurrent VTE.
Aim: The aim of the present study is to estimate the impact of inherited thrombophilia on the risk of recurrence venous thromboembolism (VTE) onset in Georgian population.
Results: A total of 26 patients (37%) suffered recurrent VTE, while the rest of the patients 44 (63%) had the first episode of venous thromboembolism. In 20 patients out of 70 were found the genetic markers of inherited thrombophilia. In 10 patients (38%) out of 26 with recurrent venous thromboembolism were detected mutations that predispose to inherited thrombothilia, while in 44 patients with first episode of venous thromboembolism, mutations were identified in only 23% of cases. Thus, presence of mutations in patients with inherited thrombophilia were associated with increased risk of recurrent VTE. In these data, the recurrence rates were higher among those with the factor V Leiden (8 patients with reccurent VTE had factor V Leiden and 2 patients had both, factor V Leiden and prothrombin gene mutations. The results also showed the presence of double heterozygous of FVL and PTH mutations in the group of patients with recurrent VTE.
Conclusions: In a prospective evaluation of 70 patients, the presence of inherited thrombophilia was associated with a significantly increased risk of recurrent VTE, particularly among those with factor V Leiden mutation. Differently from other populations the role of mutations responsible for development of inherited thrombophilia in the pathogenesis of recurrent VTE has not been yet studied in Georgian population. The preliminary studies of mutations indicate that the investigated mutations significantly increase the risk of onset of recurrence venous thromboembolism (VTE) in Georgian population. The future investigations in this area would be especially important.

PO 236

Familial screening in the case of Budd-Chiari syndrome with multiple thrombi due to Arg42Ser mutation in the protein C gene

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Background: Protein C is the precursor of a vitamin K-dependent serine protease that plays an important role in the regulation of blood coagulation. Heterozygous protein C deficiency is inherited in an autosomal dominant fashion, and hereditary deficiency in this protein is associated with a high risk of thrombotic disease. Patients with protein C deficiency are at increased risk of recurrent venous thrombo-embolic events. A C-to-A substitution at nucleotide number 1387 within exon III of the protein C gene (*PROC*) is designated protein C Osaka 10. A c.125C>A change was identified in the proband and encodes an Arg42-Ser mutation. Arg42 is located at the cleavage site recognized by the enzyme that normally processes protein C to its active form. On the other hand, the frequency of heterozygous protein C deficiency may be as high as 1/200 to 1/500 in healthy adults; however, those affected do not exhibit thrombotic manifestations. Thus, the contribution of protein C deficiency to thrombogenicity remains to be determined.
Aim: We investigated the contribution of Arg42Ser mutation to thrombogenicity. We analyzed difference between the patients complicated thrombosis and no thrombosis with the same *PROC* mutation.
Methods: A 34-year-old Japanese female was admitted to our hospital for Budd-Chiari syndrome with multiple venous thrombi caused by an Arg42Ser mutation in *PROC*. We investigated genetic analysis of family members and analyzed their character.
Result: An Arg42Ser mutation in *PROC* was also identified in the patient's father and younger brother, whereas the sequence of her mother's DNA in this region was found to be wild-type. The levels of total cholesterol and LDL cholesterol in our patient were higher than those of her younger brother and father. Idiopathic high levels of hepatic enzymes were detected in our patient's younger brother as well as her father, both of whom exhibited hypertriglyceridaemia.
Summary/Conclusions: Our present study reveals that the patient's brother or father did not experience thrombotic events, even though they harbored the same Arg42Ser mutation in *PROC* with a concomittant decrease in protein C activity. We suggest two hypotheses to account for the absence of detectable thrombosis in our patient's family members, despite these individuals harboring the same mutation as our patient with a concomittant decrease in protein C activity. The first hypothesis maintains that elevated levels of total cholesterol may be a risk factor for venous thromboembolism (VTE). Moreover, in this case, we noted no recurrence of thrombosis after statins were administered to our patient for 18 months. Our second hypothesis maintains that levels of protein C activity were elevated by physiological increases in the levels of triglycerides and hepatic enzymes, findings consistent with a report that protein C activity is relatively high in patients with hypertriglyceridaemia.

PO 237

Protein C deficiency: a case study

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Background: The inherited thrombophilias are a heterogeneous group of genetic disorders associated with an elevated risk of venous thromboembolism (VTE). Causes of inherited thrombophilia include the factor V Leiden mutation, the protrombin gene mutation, dysfibrinogenemia, and deficiencies of protein C, protein S, and antithrombin III. The function of protein C is to inactivate factor Va and factor VIIIa.
Aims: Protein C deficiency by plasma level alone is found in 1 in 200 to 1 in 500 persons in the general population. However, many affected individuals remain asymptomatic throughout life. Protein C deficiency is present in approximately 2–5% of patients presenting with VTE. This case report has demonstrated an increased risk of multiple venous thromboembolic disease in patients with protein C deficiency.
A case study: A 55-year-old woman got pain in the left hypohondrium accompanied by fever. She was hospitalized and during her stay in the hospital she started complaining about pain in her left lower leg.
Results: A CT of the abdomen and thorax was performed and it showed the following findings: a) The scanned part of the chest shows a small pleural effusion on the left side, with small plate-like atelectasis; b) On the level of mediastinum trunkus pulmonalis with defects in filling the lobar and segmental parts which points to pulmonary thromboemboly; c) Abdominal aorta with defects in filling (possible thrombosis) in the length of 5.4–1.8 sm proximally from trunkus celia-

cus; d) Liver, gallbladder and pancreas with normal findings; e) Spleen with diffuse hypodense zones with different sizes which do not show colouring after application of i.v. contrast and point to infarct zones; f) The other organs in the small pelvis are KT regular. A colour Doppler sonographic screening of the circulation of the lower extremities showed DVT of the left lower leg. EKG and Ehocardiogpraphy of the heart with normal findings. Laboratory tests (coagulatory and biochemical) with the following findings: lightly increased cholesterol 5.45 (3.50–5.20), LDH 660 (208–378), D-Dimeri positive (3200 ng/ml), PT, aPTT and TT point to hypercoagulation condition. Diagnosis: DVT of the lower leg, Pulmonary thremboemboly, Thrombosis of the abdominal aorta, Infarct of the spleen. The therapy was as follows: LMWH (Low Molecular Weight Heparin) in therapeutic doses together with oral anticoagulant therapy for 10 days until achieving a therapeutic INR (target 2.5; range 2.0–3.0) as well as acetylsalicylic acid of 100 mg per day. The control tests performed after 1 month show slight regression in relation to the previous CT findings. Further tests for exclusion of neoplastic processes were carried out as well as genetic tests for hereditary thrombophilia. A heterozygous deficit of Protein C was determined.

Conclusion: The risk of VTE increases with age and among heterozygotes thrombosis is unusual before age 20 years. Treatment of a patient with protein C deficiency depends upon the individual patient's risk of thromboembolic disease. Patients that have had multiple thromboembolic episodes or are at high risk of further episodes may be considered for long-term oral anticoagulation therapy.

PO 239

Platelets function as enhancers of the dendritic cell-mediated allergic response

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Dendritic cells (DCs) are widely distributed in the body to enhance the innate immune response by cytokine production through antigen recognition and initiate acquired immunity by priming naïve $CD4^+$ T cells. Thus, DCs are the master cells in activating immune responses. Recently, it has been demonstrated that the interaction between thymic stromal lymphopoietin (TSLP) and human DCs plays an essential role in evoking inflammatory Th2 responses in allergy through expression of CCL17 by DCs, which recruits memory Th2 cells to the skin.

Meanwhile, platelets represent an important linkage between inflammation, thrombosis, and atherosclerosis. Platelets express many immune mediators such as cytokines and TNF superfamilies. However, few researchers have suggested that platelets have the ability to modulate DC-mediated immune responses.

We newly demonstrated that TRAP (thrombin receptor agonist peptide)-activated platelets expressed RANKL that is a molecule expressed on activated T lymphocytes working to maintain survival and maturation of DCs. The activated platelets promoted CD86 expression on DCs through the RANKL. We further clarified that the activated platelets enhanced the production of CCL17 induced by the DC stimulated with TSLP. Thus, platelets have the ability to enhance the DC-mediated Th2 response and may contribute to the allergic inflammation. In conclusion, our study may provide new insights of platelet function into the possible mechanism of allergic response that stems from DCs.

PO 240

Modern approach for the detection of pulmonary embolism and our results of it surgical cure

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Background: More than 6 500,000 people worldwide died from pulmonary embolism every year. According M.Verstaete 65% of Pulmonary Embolism were not diagnosed.

We propose to begin to think about the possibility of PE at the early stage as the patients is admitted to ICU of the Hospital and according with the ECG, and cardiac enzimes to do the PA blood pressure at once. If it increased to do. KT with contrasting.

The aim of our study was to develop indications to pulmonary embolectomy and to evaluate results of surgical treatment of PE.

Material and Methods: From January 2007 to September 2012, 239 patients with suspicion on massive PE were sent to our hospital. Diagnosis of PE was confirmed in 175 patients.

Intravenous infusion of thrombolytic agents under dynamic ultrasound control in combination with symptomatic intensive therapy was used in patients with moderately expressed signs of an overload or dysfunction of the right ventricle, pulmonary artery pressure below 50 mm Hg, and absence of intracardiac (thrombus) pathology. Indications to pulmonary embolectomy was: PE of high risk with the right ventricle dysfunction and PAP more than 50 mm Hg, the presence of absolute contraindications to thrombolytic therapy or its inefficiency, the presence of intra cardiac blood clot, and also the central or mixed localization of blood clots in pulmonary artery.

Methods: Seventy-seven patients with acute massive PE were operated. Disease duration from the moment of the first clinically significant episode of PE was in average 5.3 ± 1.7 days. Operation in all these cases was emergent. Hospital mortality rate was 5.19%. The condition of 73 patients was satisfactory, breathing rate at rest was 16–18 in a min, pressure in PA decreased to 23.8 ± 7.0 mm Hg. The choice of a method of an open embolectomy depends on localization of thromboemboli in the pulmonary artery tree and the right heart, and also on presence of accompanying intra cardiac pathology.

We consider justified embolectomy without cardiopulmonary bypass in general surgery hospitals in case of the isolated unilateral embolic defeat of large pulmonary arteries. We give preference to operation with artificial blood circulation in specialized departments of cardiovascular surgery in case of bilateral defeat. It should be noted that thrombolytic therapy before operation isn't contraindication to surgical treatment. Conclusion. Thrombolytic therapy demands constant monitoring of the patient condition, thus the echocardiography is highly informative, safe and available method of the research, allowing to define further tactics of maintaining patients with PE and to define indications to surgery. Surgical treatment of PE is 'a method of the second line', applied in case of impossibility or an inefficiency of thrombolytic therapy.

PO 241

Treatment of occluded stented segments in the venous system by ultrasound accelerated catheter directed thrombolysis

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Introduction: Stent placement in the venous system is an increasingly used treatment modality in chronic venous obstruction as well as additional treatment after thrombolytic therapy in iliofemoral DVT. Reocclusion of the recanalized venous tract in these patients is the most

important complication and manifests as a rethrombosis of the stented segments in up to 51% of cases. Reocclusion mostly occurs within the first 6 months after stent placement. We report on our experience in treating in-stent (re) thrombosis with ultra-sound accelerated catheter directed thrombolysis in patients referred to our tertiary center.
Methods: Retrospective analysis of patients treated for venous stent occlusion, after PTA and stent placement for either chronic venous occlusive disease or persistent vein compression in patients with acute DVT. Duration of the occlusion and suspected clot age were assessed using patient complaints and typical findings on duplex ultrasonography (DUS). DUS and venography were used to assess patency of the venous tract and to determine the cause of reocclusion. Acute treatment of occlusion consisted of ultrasound accelerated catheter directed thrombolysis. Additional procedures included: PTA, stent placement, and creation of an arteriovenous-fistula.
Results: We identified 12 patients from our database who were treated between January 2009 and October 2012. Mean age was 38 (16–54), and 50% were male. In four patients May-Thurner syndrome was found. Four presented with C1 disease, 1 with C2, 3 with C3, 2 with C4 and 1 with C5. Indications for stenting were; treatment of underlying obstruction after initial thrombolysis in acute DVT in five patients and treatment of chronic venous obstructive disease in seven patients. Technical success was achieved in 67% of patients. Complications during the intervention were; minor bleeding complications in two patients, positive blood cultures for staphylococcus aureus in one and a heparin-induced thrombocytopenia in one. Patency rate was 67%, as all successfully treated segments remained patent, during a mean follow-up of 10 months (1–24). Additional treatments were; restenting in four patients and creation of an arteriovenous fistula in three patients.
Conclusion: Treatment with catheter directed thrombolysis of thrombosed stent tracts is feasible and safe, in this population with complex secondary venous disease. Adequate treatment of these underlying causes of rethrombosis after stenting yields excellent patency rates.

PO 242

Polyphosphatehosphate is an haemostatic agent *in vivo*

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Background: Polyphosphate is an inorganic polymer of linear linked phosphate units that is abundantly found in nature and used in technical processes. Polyphosphate are procoagulant mediators in plasma.
Aims: Here, we analyzed synthetic polyphosphate of 70 phosphate units of as hemostatic agent in clinical relevant bleeding models in swine.
Methods and Results: Polyphosphate efficiently initiated real time thrombin formation in a factor XII-dependent manner in plasma. Topical applied polyphosphate terminated bleeding from liver and spleen wounds similarly to fibrin sealant or factor XII activator Kaolin and largely reduced blood loss, whereas tri-phosphate was inactive. Electron microscopy, histology and immunohistochemistry revealed that polyphosphate triggered fibrin meshwork formation and improved fibrin fiber structure at the wound site. Polyphosphate-triggered inflammatory leukocyte recruitment, edema and vessel activation was minor. Polyphosphate was degraded to inactive fragments with a half-life of 2 h by plasma-borne phosphatases.
Summary/Conclusion: The data identify polyphosphate as an inexpensive hemostatic agent with broad therapeutic implications.

PO 243

Hemorrhagic diathesis in patient with multiple myeloma – case report

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Background: Myeloma cells usually produce the monoclonal immunoglobulin (M-Ig; paraprotein) that interferres with platelets and causes thrombocytopathy. On other hand it acts in the coagulation cascade, prolongs the basic coagulation tests and clinically leads to the complex disorder of hemostasis associated with the very high risk of bleeding.
Case-report: Authors describe a case of 62-years old patient with mutiple myeloma and hemorrhagic diathesis. In the significant hyperproteinemia and paraproteinemia were found in the laboratory tests (the total serum proteins 122 g/L). Because of abnormal level of M-Ig that interferred with the coagulation factors prolonged standard coagulation tests were detected – slightly prolonged prothrombine time (PT; 70% INR; 1.17), more prolonged activated partial thromboplastine time (APTT; 40.1 s) and not measurable thrombine time (TT; more than 3 min). In full blood count severe thrombocytopenia was found. These laboratory findings were clinically manifested as an extensive hematomas in axillar and thoracic regions, with the size of 22 × 8 cm on the left, and 14.5 × 6 cm on the right. The patient underwent the standard treatment to stop bleeding (hemostyptics, transfusions of thrombocytes concentrates, fibrinogen), except transfusion of fresh frozen plasma which was contraindicated due to severe hyperproteinemia. In spite of this treatment and repeated transfusions of erythrocytes concentrates signs of circulatory instability became evident. Surgical hemostasis was repeatedly contraindicated by vascular surgeon due to severe thrombocytopenia and coagulopathy. Therefore the recombinant activated factor VII (rFVIIa) was used. Effect of this factor – shortening the standard coagulation times except of the thrombine time – was obvious after first intake of rFVIIa. Several doses of the rFVIIa were needed. In spite of normalized laboratory findings the control computer tomography of chest revealed progression of the bleeding. On other hand the clinical performance status of patient was improved and lower need of the transfusion treatment was detected. The treatment with rFVIIa continued till stabilization of patient (the total dose 45 mg of rFVIIa).
Conclusion: None of the standard coagulation tests may monitor the effect of the rFVIIa treatment and hemostasis status *in vivo*. The standard coagulation tests were not helpful in the management of the massive or life-threatening bleeding in patient with the multiple myeloma.

PO 244

The effect of adenosine/lidocaine/Mg2+ on correcting coagulopathy following traumatic hemorrhagic shock

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Background: Previous studies have shown that the use of Adenocaine® (adenosine and lidocaine) and Mg^{2+} can rescue and stabilize the heart during hypotensive resuscitation and provide a full correction of coagulopathy (reversal of aPTTand PT) in a severe hemorrhage rat model.
Aims: To shed light upon the potential mechanisms of action of adenocaine®/Mg^{2+} on the reversal of coagulopathy as observed in our hemorrhagic shock rat model, we have investigated if adenosine, lidocaine and magnesium has an effect on *in vitro* coagulation in human plasma.
Methods: APTT and PT clotting times in human pooled normal plasma were examined at doses, up to 5 mM each, of adenosine, lidocaine and magnesium and combinations thereof.
Results: APTT clotting times in human pooled normal plasma were significantly shortened upon increasing concentration of lidocaine 0–5 mM ($P = 0.029$) and $MgSO_4$ ($P < 0.0001$). Dose analysis of adenosine (0–5 mM) showed no difference in APTT. No significant

differences in PT clotting times were observed with adenosine, lidocaine or magnesium sulphate (0–5 mM) in human normal pooled plasma. The combined use of adenosine:lidocaine:magnesium (1:3:2.5 mM) decreased the APTT and PT clotting times by an average of 4 and 2 s, respectively. The doses tested here are approximately 30 times higher than what would be present in the circulation of our rat hemorrhagic shock model after bolus infusion of the Adenocaine®/Mg^{2+}, for which our previous studies indicated correction of APTT and PT clotting times. These differences observed between our present and previous studies may be attributable to variations between the *in vitro* and *ex vivo* analysis and between the two species.

Summary: Our previous investigations concluded that Adenocaine®/Mg^{2+} has coagulation restorative properties in addition to being able to rescue and stabilize the heart. The present findings suggest that the correction of APTT and PT clotting times observed in the rat are either not due to the effect of increasing adenosine, lidocaine or magnesium alone or in combination, relying other physiological factors operating during traumatic hemorrhagic shock, or are not necessarily transferrable to the human situation. The mechanisms of this resuscitation regime on coagulation parameters warrant further investigation.

PO 245

Fibrinogen therapy of severe bleeding based on whole blood rotational thromboelastometry

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Background: Fibrinogen is an under-recognized coagulation factor critical for producing effective clot in surgical patients. Moreover, fibrinogen plasma levels are reported to be predictive of perioperative bleeding. Although, the normal fibrinogen plasma levels are between 200 and 400 mg/dL, the target fibrinogen level in a bleeding patient is not known. Most transfusion algorithms recommend supportive therapy with fibrinogen when plasma levels are less than 100 mg/dL. In this situation, fibrinogen concentrates is a better option than fresh frozen plasma or cryoprecipitate to restore adequate fibrinogen plasma levels. Perioperative monitoring of blood coagulation is critical to better understand causes of hemorrhage, to guide hemostatic therapies, and to predict the risk of bleeding during the consecutive anesthetic or surgical procedures. Point-of-care coagulation monitoring devices assessing the viscoelastic properties of whole blood (i.e. rotation thromboelastometry), may overcome several limitations of routine coagulation tests in the perioperative setting.

Aim: The aim of this retrospective study was to evaluate in a cohort of adults with severe bleedings, treated with fibrinogen concentrates, the effects in terms of red blood cells (RBC), plasma, platelets and fibrinogen administration, 30 days mortality and recovery time in intensive care units, if the indication to treatment with fibrinogen was based on thromboelastometry or on the fibrinogen plasma level.

Methods: We retrospectively evaluated 81 consecutive patients with acute severe bleeding, which had necessitated supportive therapy with fibrinogen concentrate. In 40 patients (cases) fibrinogen was administered according to thromboelastometric profile (ROTEM®) and 42 (controls) the administration of fibrinogen was based on the determination of plasmatic fibrinogen according to the test of Clauss.

Methods: We observed a consumption of RBC (14 ± 8 vs. 12 ± 8 U), plasma (12 ± 8 vs. 9 ± 6 bags) and platelets (1 ± 2 vs. 1 ± 1 bags) similar (P = ns) in cases and in controls. The average amount of administered fibrinogen was significantly higher in patients monitored with ROTEM® compared to the control group (6 ± 3 vs. 4 ± 3 g, $P < 0.05$). In cases we observed a lower 30 day mortality than in the control group (28% vs. 37%) and a lower recovery time in intensive care units (12 ± 10 vs. 17 ± 15 days). These differences have not proved statistically significant.

Conclusions: The data of our study, although preliminary, indicate that the amount of fibrinogen administered according to the results of thromboelastometry was higher than that given by the value of the plasma fibrinogen obtained by the Clauss method. This difference could improve patient outcome in terms of 30 days mortality and recovery time in intensive care units. Prospective and larger studies are needed to confirm the results of our study.

PO 246

BDNF acts as an autocrine cell proliferation factor in TPO stimulated megakaryocytic cell line

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Background: While human platelets contain brain-derived neurotrophic factor (BDNF) and release it upon activation, a previous report on MEG-01, a megakaryocytic cell line, found no trace of BDNF production, and the pathophysiological function of platelet BDNF has remained elusive.

Aims: We sought to investigate whether MEG-01 can produce BDNF under various conditions. Furthermore, to examine the pathophysiological function of BDNF on megakaryocytic lineages, we evaluated the effect of BDNF on proliferation and polyploidization of MEG-01.

Methods: MEG-01 cultured with or without thrombopoietin (TPO) was subjected to western blot analysis to detect BDNF in MEG-01. Proliferation of MEG-01 stimulated with TPO, BDNF, or the combination of TPO and BDNF was accessed in the presence or absence of BDNF neutralizing antibody. Polyploidization assay was performed by measuring DNA contents of MEG-01 cultured with TPO and/or BDNF using flow cytometry with propidium iodide staining.

Results: Western blot analysis revealed no BDNF signal in MEG-01 in the absence of TPO, whereas BDNF production was clearly detected in MEG-01 stimulated with TPO. In proliferation assay, TPO added to the cell culture increased the cell number, and BDNF alone also accelerated MEG-01 proliferation. The combination of TPO and BDNF further accelerated MEG-01 proliferation. A BDNF-neutralizing antibody significantly suppressed cell proliferation of MEG-01 stimulated with TPO, suggesting that BDNF serves as an autocrine proliferation factor. On the other hands, BDNF did not positively affect the ploidy of MEG-01.

Conclusions: In this study, we demonstrate that MEG-01 produces BDNF in the presence of TPO and that it serves to potentiate cell proliferation without changing its ploidy. Our *in vitro* findings suggest that BDNF regulates MEG-01 proliferation in an autocrine manner, and that BDNF may be a physiological autocrine regulator of megakaryocytic progenitors, constituting a family of megakaryocyte-colony stimulating factors (MEG-CSF).

PO 247

Comparison of platelet microparticles quantification between patients with Alzheimer disease and individuals without cognitive impairment

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Introduction: Microparticles (MP) are small membrane fragments that are released during cell activation and apoptosis. Once MP are formed, they can move carrying membrane proteins from their cell of origin. The most common MP are derived from platelets. Increased number of platelets MP has been observed in patients who have an increased platelet activation as in Alzheimer disease (AD). Considering that senil plaques in the brain are formed by β amyloid and that

platelets are the main source of the amyloid precursor protein (APP) in the blood of humans, it can be assumed that platelet activation in patients with AD may contribute to the pathogenesis of this disease. This study describes the initial attempts made in characterizing the phenotype of PMP in AD.

Objective: To investigate whether levels of platelet microparticles (PMP) in the blood of patients with Alzheimer's disease (AD) are increased comparing to individuals without cognitive impairment (controls).

Methods: The study was approved by the ethics committee at the Federal University of Minas Gerais (UFMG), Brazil, and blood samples of the participants were collected after informed consent by themselves or relatives. PMP were determined in plasma from 118 subjects with similar age and socioeconomic status (AD, $n = 59$ and controls, $n = 59$), selected in the University Hospital at the UFMG, by flow cytometric technique using Annexin V (capable of recognizing phosphatidylserine present on the surface of PMP). Furthermore, it is also used PE fluorochrome for labeling of monoclonal antibodies against CD41a platelet surface. PMP isolated from plasma were then analised according to the size and granularity.

Results: No significant difference in the values of PMP (%) between the control group (27.6%) and DA (28.3%) was found. The lack of significant difference between the two groups might be partially attributed to the lifestyle changes of patients with Alzheimer disease, namely diet controlled by family members and caregivers. The use of medications that decrease platelet activation by these patients may also have a role. However, the lack of platelet profile data before installing the DA preclude any inference about the possible correlation between the plasma level of PMP and development of this devastating disease.

Conclusion: PMP levels in plasma samples were not different between control and Alzheimer disease groups, with considerable results overlapping in both groups.

Supported by CNPq, CAPES and FAPEMIG – BRAZIL.

PO 248

Identification and isolation methodologies for blood microparticles

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Background: Blood microparticles have been shown to be involved in a number of disease processes such as inflammation, vascular injury, angiogenesis and thrombosis. There have been extensive studies conducted into the quantification and characterization of microparticles and their association with certain disease processes. However, the methods used to study microparticles have lacked standardization, and the optimal protocols remain unclear, making it difficult to compare results across different studies.

Aim: To review published methods used for isolation and characterisation of microparticles in human blood.

Methods: The PubMed database was searched using the search terms: microparticles, endothelial, method, protocols, blood, platelet, isolation for the 5 year period of 2008–2012.

Results: Blood samples are typically collected in the atraumatic fashion with a large needle (19G or 21G), to reduce stress and endothelial reaction. In order to avoid contamination the first 3 ml is usually discarded, while the remainder is collected in a vacutainer containing citrate (0.105–0.129 M). Centrifugation parameters tend to have the most variation in the overall testing for microparticles. Strategies ranged from a two step centrifugation process to detecting microparticles directly from the platelet poor plasma, the rationale behind the different approaches included whether to run the risk of losing microparticles through centrifugation. Storage of the microparticles following centrifugation was achieved by a variety of freezing processes as this step was shown not to affect the microparticle count. The most frequently used are snap freezing in liquid nitrogen as well as freezing samples at -80 °C.

Detection of microparticles is accomplished by utilizing the expression of antigens on the surface of the various forms of microparticle, i.e. endothelial-derived (EMP), platelet-derived (PMP), leukocyte-derived (LMP) and exposing them to their complementary antibody. PMPs are detected mainly by CD31, CD41 and CD42 antibodies; LMPs are detected by the CD45 antibody; EMPs are detected by CD144, CD62E, CD146, CD54, CD31, CD105 and vWF antibodies. As PMPs and EMPs are both detected by CD31, this can be exploited to identify EMP in platelet free plasma by using CD31 +/CD41- marker.

Conclusion: There are multiple protocols utilized for microparticle isolation and characterisation. Standardisation is crucial in studying these important cellular elements, if we are going to be able to compare results of various studies.

PO 249

Prothrombotic microparticles concentration after percutaneous coronary intervention and stent implantation

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Background: Platelet activation accompanying numerous diseases as well as medical procedures can result in thrombotic complications. Microparticles (MP) are shedded upon platelet activation, and may be used to assess platelet function.

Aims: To measure MP concentration in the plasma of patients (pts) with coronary artery disease (CAD) taking aspirin and clopidogrel, before as well as after elective percutaneous coronary intervention (PCI) with or without stent implantation.

Methods: One hundred and thirty pts (76 men, 54 women) with CAD. During elective PCI stent implantation (bare-metal stent or drug-eluting stent) has been performed in 35 pts. MP concentration was measured using ZYMOPHEN MP-Activity test of HYPHEN BioMed Research Company before, 24 h and 1 month after PCI. In the control group there were 26 healthy blood donors not taking antiplatelet drugs.

Results: The influence of PCI and stent implantation on the generation of prothrombotic microparticles (MP) has been presented in tables 1 and 2. (M-men; W-women; MPc – microparticles concentration, VC-variation coefficient).

Table 1: MP concentration in plasma (nM) in patients subjected to percutaneous coronary intervention.

Table 2: Variation of MP concentration in plasma in patients subjected to percutaneous coronary intervention.

The mean microparticles concentration in the control group was 4.2 ± 2.3 nM.

Conclusions: Microparticle concentration in patients with coronary artery disease was higher than in the control group. In women it increased after coronary angiography, and in men after stent implantation. The average results did not exceed the values regarded as normal, 20% of results were > 10 nM, considered as the cut-off value for pathological results.

PO 250

Anticoagulant activity of sulfated polysaccharides from the green seaweed Cladophora falklandica

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Background: Some sulfated polysaccharides were found to have anticoagulant properties. Seaweeds from the Cladophorales synthesize sulfated xyloarabinogalactans. Aqueous extracts from *C. falklandica* showed as major constituents galactose and arabinose, and high percentage of sulfate. Taking into account the increasing interest in the

development of new antithrombotic agents, these polysaccharides deserve to be investigated as potential anticoagulants.
Aims: To evaluate the anticoagulant activity of the water extracts of *C. falklandica*.
Methods: The room-temperature water extracts (CX1-CX3) were evaluated. Determinations of prothrombin time (PT), activated partial thromboplastin time (APTT) and thrombin time (TT) were assayed according to established methods. Normal platelet depleted citrated plasma (900 μL) was mixed with 100 μL of each polysaccharide extracts, in different concentrations, and incubated for 1 min at 37 °C. Saline solution was used as control. TT-like assays were also performed with purified fibrinogen (3 mg/mL) instead of plasma. Results were expressed as ratios obtained by dividing the clotting time achieved with the extract by the time achieved with the control. In order to performed fibrinformation studies, clots were generated by adding thrombin (0.05 IU/mL) and calcium chloride (20 mM) to the preincubated plasma. The kinetics was evaluated measuring optical density (OD) at 405 nm every one min up to constant values. Assays were carried out in polystyrene strips, in the presence of different concentrations of CX2 (10–100 μg/mL) or saline solution as control. The sigmoid curves obtained (OD vs. time) were characterized by three parameters: lag phase, maximum velocity achieved and final network OD. All assays were performed in quadruplicate.
Results: The second room temperature water extract (CX2), comprises arabinose as the major component (60.6%), galactose (23.5%) and xylose (10.5%), with a high percentage of sulfate, with a molar ratio carbohydrate: SO_3 of 1:0.7. CX2 was the most active one in coagulation tests. This fraction significantly prolonged PT, APTT and TT, in concentration dependent manner; for CX2 solution (100 μg/mL) the ratios were 1.8, 5.2, and 4.1, respectively. For the TT-like assays the ratio was 2.7, for the same concentration of CX2. The kinetics analysis of fibrinformation with CX2 showed statistically significant differences vs. control in lag phase (0.5 ± 0.0 min vs. 6.0 ± 0.0 min, $P < 0.001$), Vmax (408.5 ± 30.4 min vs. 212 ± 23.8 min, $P < 0.001$) and in the final network OD (0.709 ± 0.002 vs. 0.801 ± 0.011, $P < 0.001$).
Conclusions: The second water extract of *C.falklandika* showed anticoagulant activity by global coagulation tests with normal plasma; therefore CX2 could potentiate thrombin inhibition by antithrombin and/or heparin cofactor II. Besides, TT-like assays with purified fibrinogen suggest that, at least one of the mechanisms involved would be direct thrombin inhibition. By other hand, a probable procoagulant effect was shown by fibrinformation kinetics. In other to understand these findings further studies are currently undertaken.

PO 251

Purification method thrombin-like enzyme and fibrinogenolytic enzyme from Agkistrodon blomhoffi ussuriensis snake venom

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Background: Snake venoms contain a variety of proteolytic enzymes affecting the host coagulation process. Snake venom fibrinogenolytic enzymes act on fibrinogen, leading to defibrinogenation of blood and a consequent decrease in blood viscosity. And thrombin-like enzymes are responsible for *in vitro* blood-clotting activity present in several snake venoms is a protease which resembles at least in part thrombin, a multifunctional protease that plays a key role in coagulation.
Aims: The purpose of this study was purification of fibrinogenolytic and thrombin-like enzymes (TLE) from snake venom.
Methods: The crude venom of *Agkistrodon blomhoffii ussuriensis* was used as a raw material for obtaining of fibrinogelytic enzyme and TLE. The venom separation was started with affinity chromatography on Blue Sepharose FF and followed by Phenyl Sepharose HP, Source 15S for fibrinogelytic and Sephadex G25 SF for TLE. The TLE activity was monitored by detection of the clot formation on coagulometer and fibrinogenolytic activity was determined by enzyme electrophoresis.
Result: The TLE was a single chain glycoprotein with 27 kDa molecular weight and fibrinogenolytic enzyme was a heterodimer protein that includes two polypeptides with 36 kDa molecular weight. Purified TLE cut from fibrinogen molecule only fibrinopeptide A and that allows make it to class α-specific TLE. Fibrinogenolytic enzyme was cleaved on β-chain quicker than α-chain and slower than on γ-chain.
Conclusion: *Agkistrodon blomhoffii ussuriensis* snake venom contains 36 kDa fibrinogenolytic enzyme that can be purified by affinity and 2 more ion exchange chromatography and 27 kDa TLE that can be purified by same affinity and size exclusion chromatography.

PO 252

Alterations in natural coagulation inhibitors in acute leukemias

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Background: Inherent inhibitors of coagulation carefully control the blood clotting system *in vivo* and ensure that blood propagation does not lead to occlusion of the vasculature. TM/protein C/protein S pathway is important in modulating coagulation. Acute leukemia development is accompanied by various abnormalities of hemostasis with nearly all patients demonstrating subclinical activation of coagulation. Our previous study revealed a low plasma concentrations of anticoagulation proteins at the moment of diagnosis of acute leukemia.
Aim: The aim of this study was to estimate the concentrations of selected endogenous anticoagulation proteins in patients with acute leukemia receiving chemotherapy.
Methods: Blood samples were obtained from 20 adult patients with acute leukemia (AML-13; ALL-7) at the following time intervals: before treatment (0) and on the 7th (1), 14th (2) and 21st (3) day of chemotherapy. The patients were followed-up in the Department of Hematology, University Hospital in Bialystok. They were not under the oral anticoagulant treatment and had normal liver function. Control samples were obtained from 20 healthy subjects, age- and sex-matched. Plasma concentrations of protein C (PC), total protein S (PS), soluble thrombomodulin (sTM) and soluble endothelial protein C receptor (sEPCR) were measured by ELISA method, using commercial tests. The patients with AML and ALL underwent the induction regimens according to generally applicable standards.
Results: The low concentrations of PC, found in ALL patients at diagnosis (76.1 ± 26.9%), were elevated on the 7th (109.9 ± 28.2%) and 14th (100.8 ± 31.3%) day of the treatment, then became reduced at 21st day to approximately normal value (89.1 ± 27.3%). In patients with AML the concentration of PC remained low during all the time of treatment (0–63.0 ± 20.8%; 1–62.1 ± 17.2%; 2–57.4 ± 14.3%; 3–50.3 ± 17.2%). There were no differences in the levels of total PS in both groups of leukemia during the treatment. The concentrations of sEPCR which were low at diagnosis of acute leukemia, were rapidly reduced on the 7th day and remained downregulated thereafter (AML: 0–82.8 ± 57.1; 1–46.0 ± 10.3; 2–44.9 ± 10.6; 3–36.5 ± 10.5 ng/ml; ALL: 0–79.7 ± 34.7; 1–44.3 ± 15.9; 2–47.3 ± 31.4; 3–44.9 ± 25.7 ng/ml). The study revealed that sTM concentrations, which were elevated in acute leukemia at the diagnosis (AML; 72.2 ± 101.7; ALL: 140.6 ± 199.3 ng/ml), were reduced at 7th and 14th (AML: 1–64.1 ± 32.5; 2–64.3 ± 38.0; ALL: 1–71.1 ± 25.2; 2–66.6 ± 19.8 ng/ml) day of treatment in order to reach very high values at the 21st day (AML: 124.5 ± 139.4; ALL: 120.7 ± 159.8 ng/ml).
Conclusions: Alterations in the concentrations of the physiological inhibitors of blood coagulation may result from different pathogenesis of acute leukemias as well as of different treatment of AML and ALL. This study indicates that basal coagulation system markers (PC, PS, sTM, sEPCR) may be helpful in monitoring patients presenting a disorder of this anticoagulant pathway, especially during treatment of acute leukemia. Nevertheless, some limitation of this study is a small number of enrolled patients.

PO 253

Venous thromboembolism prophylaxis with rivaroxaban subsequent to enoxaparin in major orthopaedic surgery

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Background: Venous tromboembolism is an important complication for which precautions should be taken after major orthopaedic surgery (elective hip and knee replacement, hip fracture).

Aim: Short and long period pharmacoprophylaxis are suggested for the prevention of venous thromboembolism in all guidelines following major orthopaedic surgery. In this study, we evaluated the efficiency of new oral anticoagulant prophylaxis following low molecular weight heparin.

Methods: Seventy-two major orthopaedic surgeries (32 total knee arthroplasty, 19 total hip replacement, 16 hip fracture) had been performed between January 2012 to October 2012 in Yeditepe University Hospital. Thirteen patients were male and 59 patients were female. The mean age of the patients was 69 (range; 43–96). Forty-one patients had risk factors related to venous thromboembolism. After 12 h from the surgery; 2 × 30 mg enoxaparin was given subcutaneously to all patients throughout following in hospital. After the discharge from the hospital; 1 × 10 mg rivaroxaban was prescribed to all of them. Short-term prophylaxis (for 10 days) was administered to the patients with knee prosthesis. Long-term prophylaxis (for 30 days) was administered to the patients with hip prosthesis and patients operated for hip fracture who had two or more risk factors.

Results: Venous doppler ultrasonography was performed to all patients after 6–12 weeks from the surgery. Venous thromboembolism was not detected in any of the patients.

Conclusions: Venous thromboembolism prophylaxis with enoxaparin following by rivaroxaban is an effective modality in patients who undergo major orthopaedic surgery.

PO 254

Start-register (Survey on anticoagulated patients register): the first year of activity

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Background: Oral anticoagulation treatment with vitamin K antagonist (VKA) has been shown to be effective in numerous randomized clinical trials, but despite the potential clinical benefits they are underused especially in patients at high risk of bleeding. In the last years many efforts are directed towards the development of novel orally anticoagulants (NOA) and many clinical trials were conducted to compare the efficacy and safety of the different therapy. However, in the setting of clinical trials, there is a very tight control therapy, resulting in lower rates of therapeutic failure. This makes difficult not only the assessment of the value of NOA but the evaluation of their health-economic benefits.

Aims: The purpose of START-Register is to provide information to help physicians on evaluation of treatment option, aiding treatment selection in their patients. In addition it is also aimed to improve our knowledge on the disease, (including epidemiologic, diagnostic, prophylactic and therapeutic information), on analysis of different possible treatments (monitoring, dosages, frequency of controls), and on therapy used (protective efficacy, complications during therapy or after suspension).

Methods: Participatings enroll all patients that meet predefined eligibility criteria, and data of each patient including indication to treatment, underlying conditions and type of anticoagulant were collected on the web-site of the register.

Results: From September 2011 to December 2012 active participating members are 68; 3016 patients (males 54%) were prospectively enrolled. The indication to treatment was: atrial fibrillation in 58% of patients, thromboembolic events in 30%, mechanical heart valve in 5%. Patients underwent elective cardioversion was 6%. VKA were used in all patients enrolled and bridging therapy with low-molecular-weight-heparin was used in 42% of patients.

Conclusion: START-Register, a multicenter observational prospective and non-interventional register, will allow us to enhance our understanding of the risk-benefits of the various anticoagulant drugs and therapy options.

PO 255

Dabigatran overdosis vs. disseminated intravascular coagulation

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Background: Dabigatran is a direct thrombin inhibitor obtained from dabigatran etexilate, an oral prodrug that is hydrolyzed in the liver. Dabigatran is 80% renally excreted with a half-life of approximately 13 h with a glomerular filtration rate (GFR) of > 80 ml/min, and 18 h with a GRF of 30–50 ml/min. Peak plasma concentration is in the range of 100–400 ng/ml. Trough concentrations are in the range of 20–150 ng/ml.

Clinicians and pathologists require knowledge how routine coagulation assays are affected not only with therapeutic doses but also with overdose levels. Although there is a dose-dependent effect of dabigatran on laboratory clotting assays, the results are dependent on the last dose of drug taken and the factors that influence pharmacokinetics such as GFR. There is also a marked variation of results concerning the reagents used, mainly in fibrinogen measurements. These way clotting assays (PT, aPTT, TT and fibrinogen) may be misinterpreted by clinicians.

Aims: Presentation of a clinical case with bleeding complications, and how the misinterpret of coagulation assays evolution can influence diagnosis.

Methods: Eighty-three years old female, with personal history of atrial fibrillation, asthma, type two diabetes, hypertension, osteoporosis and dyslipidemia, treated with dabigatran, amiodarone, metformin and multiple analgesia. She was admitted for analgesic therapy adjustment and decision on possible surgical intervention, following ancient osteoporotic fracture of L4. At admission, her laboratorial control revealed anemia, leukocytosis and thrombocytopenia, elevated RPC and abnormal renal function. No coagulation tests were done. On the second day of hospitalization, without apparent triggering factor, starts prostration, hematochezia, bruising at venipuncture sites and oral cavity bleeding during nasogastric intubation. New laboratorial control revealed not clottable PT, aPTT and fibrinogen Clauss, with a severely worsening of inflammatory/infection markers. Given these results, clinicians formulated two diagnostic hypotheses: severe DIC by sepsis vs. iatrogenic effects caused by dabigatran.

Pathologists were asked to interpret coagulation assays. Platelets and D-Dimmers were done and the diagnosis of severe DIC was concluded to be unlikely/excluded.

Therapy with dabigatran was discontinued. Multiple transfusion therapy was administered, on the third day the bleeding diathesis stopped and on the fifth day the coagulation assays normalized.

Conclusions: New oral anticoagulant, dabigatran, a direct inhibitor of thrombin, can induce big changes in coagulation assays: not clottable TP, aPTT and Clauss fibrinogen.

In the case presented, the results (prolonged aPTT and PT, falsely low fibrinogen) associated with severe hemorrhage were wrongly inter-

preted, suggesting DIC. In reality, the patient had no changes in fibrinogen (hypo or dysfibrinogenemia). By the other hand, D-Dimmer and platelets measurement could help to exclude DIC.
Despite disruption of dabigatran immediately after start of bleeding complaints, these persisted for two more days and coagulation assays took 5 days to become normal, although the half-life of dabigatran is shorter.
The use of these drugs in the general population and outside the context of clinical trials induces differences regarding the safety established. These differences may be particularly relevant in populations underrepresented in these trials, like the elderly and individuals with impaired renal function, as in this case.

PO 256

Efficacy and safety of thromboprophylaxis with use of LMWH and warfarin in the traumatology-orthopedic hospital

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In 2011 in our hospital were performed 20 584 orthopedic operations, 6 230 of them were associated with high venous thromboembolism (VTE) risk. Total fatality rate was 0.07% (15 cases). Lethality from pulmonary embolism (PE) compounded 0.01% – two cases.
We studied efficacy and safety of our local Protocol of Thromboprophylaxis, based on the 8th edition of ACCP Guidelines, 2008.
The investigation was retrospective. We analyzed medical cards of 732 patients after total hip or knee replacement (THR, TKR) in 2011. Most of surgery operations were performed with neuroaxial anesthesia. There were 68.7% women. The mean age of the patients was 56.7 ± 10.7. Primary arthroplasty was performed in 663 cases (87.9% – THR, 12.1% – TKR), revision surgery – in 55 cases (THR – 91%, TKR – 9%). The mean duration of primary joint replacement was 93.0 ± 27.8 min, revision arthroplasty – 129.7 ± 51.5 min. Inpatient treatment was 13.4 ± 0.7 days after surgery. A 99.87% of surveyed patients were supposed to have high risk of VTE. A 88.5% of all patients were older than 40 years, therefore, they appeared to have extra risk factor of VTE. A 6.3% of aged upwards 75 years composed a group of particularly high risk of VTE and hemorrhagic complications associated with anticoagulants. The VTE prophylaxis by low molecular weight heparins (LMWH) was started 12 h before surgery in 100% cases. The mean duration of a course LMWH was 10.3 ± 0.8 days (dalteparin sodium was received by 71.6% patients, nadroparin calcium – 17.6%, enoxapain sodium – 9.2%). In one case low dosage of dalteparin was used due to the improper risk stratification. Since the 7th-8th days all patients received warfarin. It recommended to 99.7% of patients after the release. Early mobilization (77.3% of patients) and graduated elastic compression stockings (37.9%) were used as unspecific thromboprophylaxis. We detected no hemorrhagic complications, which lead to cancellation of anticoagulants. Four VTE were observed (0.52%). One patient (woman, 63 years old) died due to pulmonary embolism at 9th day after THR. For other patient (woman, 83 years) PE was diagnosed at 10th day after surgery, at 29th day – sharp cardiovascular insufficiency and death were developed. Both cases of fatal PE after THR were associated with cancellation LMWH while target level of INR was not yet achieved by warfarin intake. Superficial vein thrombosis was detected in two patients (0.26%) by ultrasonography at 8–10 days after surgery.
The study showed that routine use of the Protocol was effective and safe. However, have been revealed insufficient use of mechanical VTE prevention methods and difficulties with selection of warfarin dosage. Thus, more frequent use of mechanical thromboprophylaxis and use of new oral anticoagulants, which doesn't require laboratory monitoring and selection of the dose, can raise the efficacy of the Protocol. Monitoring of Observance of the local Protocol and the analyses of all VTE cases are necessary to establish the causes of these complications and to find ways to improve it.

PO 257

Identification of brodifacoum exposure and subsequent monitoring of treatment and recovery using functional markers of vitamin K status

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Background: Superwarfarins are highly potent, freely available vitamin K antagonist type anticoagulants that are widely employed as rodenticides. They exert their effect by blocking the vitamin K epoxide reductase complex (VKOR) thereby limiting the recycling of vitamin K from the inactive, oxidised form (vitamin K 2,3-epoxide) back to the metabolic form (vitamin K hydroquinone). Vitamin K is an essential micronutrient utilised as a cofactor for vitamin K dependent carboxylase which is required for the post-translational activation of various vitamin K-dependent proteins, most notably in this case, coagulation factors II, VII, IX and X.
Aims: We describe a case of superwarfarin exposure in a 20 year old male who presented in April 2011 (approximately 6 weeks post superwarfarin exposure) with left calf pain and the subsequent use of functional vitamin K status markers to successfully monitor recovery and guide treatment. At presentation, ultrasound showed extensive haemorrhage. The patient's Hb was 6.2 g/dL, PT > 120 s and APTT > 240 s. He was immediately given 3500 units of 4-factor Prothrombin Complex Concentrate and 20 mg IV phytomenadione (vitamin K_1 [K_1]) (Konakion). The INR subsequently corrected, however it increased once more over the following days to 1.4. In response, the daily phytomenadione dose was increased to 40 mg. The INR corrected and remained normal with continued daily K_1.
Methods: A serum sample was collected and a superwarfarin screen performed by normal-phase HPLC with diode array detection. K_1 and vitamin K_1 2, 3-epoxide (K_1O) were measured by reversed-phase HPLC with in-line chemical reduction and fluorescence detection. Undercarboxylated prothrombin (PIVKA-II) was measured by ELISA.
Results: Brodifacoum, a long-acting superwarfarin with a clearance half-life of approximately 20–100 days was detected 100 μg/L. Superwarfarin action was confirmed by an elevation in the circulatory concentration of PIVKA-II to levels greater than those seen in anticoagulated patients (> 7 AU/mL) and by detection of K_1O at a highly elevated concentration relative to K_1. Monthly monitoring of brodifacoum and vitamin K markers was performed and is ongoing. Brodifacoum was not detectable in any subsequent samples (collected at intervals since April '11) presumably due to hepatic pooling; however its ongoing effects were evidenced by elevated PIVKA-II and K_1O. PIVKA-II remained elevated until 180 days post-presentation and then returned to normal (undetectable [< 0.2 AU/mL]), indicating complete correction of the coagulation defect by ongoing phytomenadione administration. K_1O remains highly elevated relative to K_1 (ratio = 1.2–3.7) 530 days post-presentation.
Conclusions: The use of novel laboratory markers conferred a number of benefits not seen with conventional coagulation assays: Confirmation of diagnosis by the direct measurement of brodifacoum; evidence that ongoing action was not a consequence of repeated-exposure; monitoring of treatment with vitamin K; biochemical evidence of continued vitamin K antagonism 18 months post-exposure. These results indicate this patient still requires treatment with phytomenadione at this time with further monitoring of vitamin K markers to determine when treatment should be discontinued.

PO 258

In vitro assessment, using thrombin generation, of the applicability of Prothrombin Complex Concentrate as an antidote for Rivaroxaban

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Background: Rivaroxaban has been approved as antithrombotic agent for prevention of venous thrombo-embolism for specific indications. At present no antidote is appointed and no guidelines have been formulated for the measurement of Rivaroxaban reversal.

Objectives: In this *in-vitro* study, we evaluated the influence of prothrombin complex concentrate (PCC: Cofact) on the anticoagulant effects of Rivaroxaban as measured by prothrombin time (PT) and various thrombin generation tests.

Methods: Plasma and whole blood samples from healthy volunteers were spiked with Rivaroxaban in concentrations up to 800 μg/L, PCC was added to these samples in concentration ranges as used clinically to reverse anticoagulant effects of vitamin K antagonists (VKA). PT, endogenous thrombin potential (ETP) and calibrated automated thrombography (CAT) assays were measured with varying tissue factor (TF) concentrations.

Results: Addition of PCC to Rivaroxaban spiked donor samples did not result in normalization of PT and thrombin generation lag time In contrast, normalization of ETP and CAT area under the curve did occur. However the response on PCC addition was strongly TF concentration dependent and in whole blood less PCC was required for Rivaroxaban reversal as compared to plasma.

Conclusions: PCC does not neutralize the lengthening effect on PT and thrombin generation lag time of Rivaroxaban anticoagulated blood *in vitro*, however total thrombin formation could be normalised. Performance of the different thrombin generation tests is assay condition dependent. Therefore, prospective studies are needed tot clarify which assay condition and parameter describes *in vivo* haemostasis best in Rivaroxaban patients that are treated with PCC.

PO 259

Defibrotide interactions with newer oral anticoagulants and antithrombotic agents

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Introduction: Clinical trials of defibrotide, a polydisperse mixture of porcine-derived single-stranded oligonucleotides demonstrate benefits for the treatment and prophylaxis of hepatic veno-occlusive disease (VOD) in hematopoietic stem cell therapy. This study investigated the effect of defibrotide on the anticoagulant and antiprotease actions of the newer oral anticoagulants.

Materials and Methods: Whole blood drawn from 25 healthy individuals was supplemented with 250 ng/mL of each of the individual oral anticoagulant drugs alone and with 100 μg/ml defibrotide. Celite activated clotting time (ACT) measurements were made. For the plasma based assays, a fixed concentration of defibrotide (100 μg/mL) was supplemented with defibrotide to pool plasma alone and with each of the individual oral anticoagulants (0–1000 ng/mL). To investigate the effect of defibrotide on agonist induced platelet aggregation PRP was prepared with varying amounts of defibrotide (0–100 μg/ml). To test the effect of defibrotide on warfarin, plasma samples from patients with INR range of 1.5–3.0 were supplemented with 100 μg/ml and the PT/INR was re-determined.

Results: While apixaban and rivaroxaban did not prolong the ACT, dabigatran produced a modest increase at 250 ng/mL; addition of newer oral anticoagulants to defibrotide supplemented plasma did not prolong the PT. Dabigatran showed a slight interaction in the aPTT assay. Defibrotide, did not impact agonist induced platelet aggregation. Defibrotide, alone and with newer oral anticoagulants, did not increase the PT/INR values of plasma samples collected from warfarin treated patients at concentrations of up to 100 μg/ml.

Conclusions: Based on the indications for the new oral anticoagulant drugs, their circulating levels and rapid clearance, it is unlikely that defibrotide will produce any interactions with these drugs.

PO 260

Dabigatran etexilat overdose and life-threatening gastrointestinal bleeding

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Background: New oral anticoagulants – dabigatran, rivaroxaban, apixaban and others – are small-molecule, selective inhibitors that bind to the selective coagulative site of vitamin K-dependent factors Xa and IIa. These drugs are attractive for patients due to the peroral drug form, a fixed dose administered once or twice daily to patients with normal body weight and renal function. There is no need for laboratory drug efficacy monitoring when the standard conditions are met.

One great disadvantage is the fact that elderly people can easily overdose on it and their renal function can change over time rapidly. A specific antidote has not yet been developed.

We would like to present our experience with life-threatening bleeding that was managed successfully. Our case report deals with a 83-year-old lady with numerous comorbidities.

(diabetes mellitus, ischemic heart disease, etc.)

She was admitted to hospital for weakness and pre-shock status, when she had been treated with the standard dose of dabigatran (Pradaxa®, Boehringer Ingelheim) 150 mg twice daily for atrial fibrillation. Melaena wad occurred shortly before admission, verified in the hospital, and so the urgent gastrointestinal tract examination was indicated. The laboratory results of both the blood count and haemocoagulative parameters were severe even critical (Hb 76 g/L, APTT- R 3.16, TT more than 120 s, fibrinogen 0.33 g/L, D-dimers 0.81 mg/FEU). dTT was 500 ng/ml ten h after the last dose of dabigatran.

Volume resuscitation and complete haemodynamic support was self-evident.

The lower dose of FEIBA (approximately 25 IU per kg b.w.) was administered to the patient as a bolus immediately before gastroendoscopy. A bleeding stomach ulcer was proved and successful surgical haemostasis followed. The persisting pathological haemocoagulative parameters were normalized by fresh frozen plasma infusions during following 2 days, so APTT – R became lower to 1.8, PT- R 1.66, while pathological thrombin time more than 120 s persisted.

In spite of the fact that the woman was resuscitated because of malignant bradycardia, now she is again being treated with dabigatran etexilate for persistent atrial fibrillation.

Conclusion: This is our first experience with FEIBA used for dabigatran overdosing treatment. We were afraid to administer higher dose of FEIBA due to the hypercoagulative woman′s state. rFVIIa would have been the further step to stop bleeding.

PO 261

Incidence of thromboembolic and bleeding events according to the timing of prophylaxis after orthopedic major surgery

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Background: Venous thromboemolism, including deep vein thrombosis and pulmonary embolism represents major concern after orthopedic major surgery. But concern about bleeding complication after surgery

may make orthopedic surgeon hesitate to start anticoagulation with low-molecular weight heparin (LMWH) for more than 6 days in real practice even though it is recommended to retart 6–8 h after wound closure.

Aims: This study is designed to provide the information regarding the incidence of venous thromboembolism or bleeding according to the timing of anticoagulation with LMWH after major orthopedic surgery.

Methods: Patient who underwent major orthopedic surgery with femur fracture from July 2013 to December 2013, were retrospectively identified and reviewed with chart. Orthopedic surgery included bipolar hemiarthroplasty and closed reduction with interal fixation. Patients who received prophylaxis with LMWH within 5 days of surgery were classified as group 1, and after 6 days as group 2. Patients who need to have briding therapy due to anticoagulation therapy with warfarin were excluded. Whether there are events of venous thromboembolism or bleeding complication were followed for 30 days of procedure. Major bleeding was defined as drop of hemoglobin level of 20 g/L or more, or leading to transfusion of 2 or more units of whole blood or red cells or bleeding in critical organ.

Results: Sixteen patients were included. There were 5 men and 11 women, ranging in age form 67 to 90 years with a mean age 78.8. Site of femur fracture was right in 11 patients. Preoperative LMWH injection was done in 14 patients (87.5%). Mean number of injection after operation was 9.4. Interval between surgery and injection postoperative was 4.72 $\pm$ 1.98 (range, 0.5 to 8 days) overall, 3.55 $\pm$ 1.52 (range 0.5 to 5 days) in Group 1, and 6.67 $\pm$ 0.75 (Range 6–8) in Group 2. Pulmonary embolism occurred in one patient 2 days after procedure in Group 2. Major bleeding occurred in one paitent 3 days after procedure in Group 2.

Summary/Conclusion: Limitation is small number of data, but no one showed bleeding complication in Group 1. It is important to follow the recommendation that prophylaxis with LMWH be started 6–8 h after wound closure to prevent venous thromboembolism.

PO 262

Profiling anticoagulants from hematophagous animals: an on-line post-column bioactivity assay with parallel mass spectrometric identification

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Background: Cardiovascular and cerebrovascular diseases are among the most dreaded killers in most developed countries. There have been numerous anticoagulants that have been developed to circumvent deaths and debilitation due to these diseases. Over the years, natural sources such as plant and animal extracts have proven to contain a wide array of pharmacologically important compounds. Since the salivary gland extracts of hematophagous animals like ticks and mosquitoes are rich in anticoagulants, these exogenous proteins can be used as therapeutic agents based on their highly specific targeting. These pharmacologically active anticoagulants inhibit central enzymes of the blood clotting cascade. As these inhibitors are present in min amounts, they are difficult to identify using traditional analytical scale chromatographic approaches.

Aim: We propose to identify novel potent inhibitors of the key enzymes involved in the blood coagulation cascade.

Methods: We propose to identify novel potent inhibitors through the development of a miniaturized workflow for on-line post-column profiling of the crude salivary gland extracts. In this miniaturized workflow, purification is first performed via nano liquid chromatography. Thereafter, the eluent is split into two lines, one being fed into an on-line post-column bioassay where its anticoagulant properties are tested. The other is fed into a mass spectrometer for compound identification. As the bioassay and MS-guided identification are carried out in parallel, we will be able to match the proteins with its corresponding activity. In this manner, profiling of the salivary gland extracts is achieved through a single tandem workflow. The development of this technique, counters the constraints posed by the limited amounts of exogenous compounds available from these hematophagous animals. This miniaturized workflow will be used to profile the salivary extracts of ticks and mosquitoes.

Conclusion: With the identity of the anticoagulants from the hematophagous animals being elucidated, they can be recombinantly expressed and purified from suitable expression systems. This will allow the study of the mechanisms of these inhibitors so that their therapeutic potentials can be explored further.

PO 263

Are the complications of anticoagulant treatment with vitamin K antagonists in atrial fibrillation gender dependent?

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***Background*:** It is well known that patients with atrial fibrillation are at great risk for thromboembolic complications. Long-term anticoagulant treatment is recommended in that case. Atrial fibrillation affects males more than females. Several studies have shown that females are at higher risk of thromboembolic complications. Still there are insufficient evidence about gender-related complications.

Aims: The aim of our study was to compare the incidence of thromboembolic and haemorrhagic complications between genders. We also assessed the risk of these complications in relation to the CHA_2DS_2-VASc and HAS-BLED score and time spent in therapeutic range.

Methods: The study included 224 patients treated with vitamin K antagonists, followed for 851 patient-years in the Departmant of Haematology, Haemostasis and Prevention of Thrombosis, Clinical Centre of Vojvodina. Our group consisted of 119 (53%) male and 105 (47%) female patients. International Normalized Ratio measurements have been done periodically and maintained at recommended range of 2.0–3.0. Thromboembolic and haemorrhagic complications were registered during regular controls by clinical examination, diagnostic procedures such as nuclear magnetic resonance, computing tomography scan, endoscopic procedures, then laboratory analyses (examination of urine sediment) and patient history. Risk of ischaemic stroke was estimated using CHA_2DS_2-VASc score, while HAS-BLED score was used to assess the risk of haemorrhagic complications.

Results: All patients were classified into three categories by CHA_2DS_2-VASc score- at low risk 4 (1.8%), moderate risk 16 (7.1%) and high risk 204 (91.1%). The most numerous were patients over 65 years- 184 (82%). The most frequent comorbidities were hypertension (70%), vascular diseases (38%) and diabetes mellitus (23%). Risk of thromboembolic complications was higher in male patients ($P = 0.002$). Using HAS-BLED score 84 (38%) patients were at low risk and 140 (62%) were at high risk of haemorrhagic complicationts, but there was no significant difference in the incidence of complicationts between genders ($P = 0.915$). Both genders were at the same risk of haemorrhagic complications ($P = 0.358$). One hundred and thirty-nine (62%) patiens with stable INR had the same incidence of thromboembolic and haemorrhagic complications compared to those with labile INR- 85 (38%).

Summary/Conclusions: Small sample study showed that all patients were at great risk of thromboembolic complications, male more than female. Complications of anticoagulant treatment were not gender dependent.

PO 264

Laboratory coagulation assays and ROTEM thromboelastography in monitoring the reversal of dabigatran with FEIBA and haemofiltration

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Background: Dabigatran, a direct thrombin inhibitor, is increasingly prescribed in the management of atrial fibrillation. There remains no effective reversal agent for patients presenting with bleeding or requiring emergency surgery. In the case of life threatening bleeding, the only effective treatment recommended by the manufacturers is haemodialysis. *FEIBA* (factor VIII inhibitor bypassing agent) has been purported as a potential reversal agent for dabigatran. However, there is limited data on the clinical efficacy in humans. Clinical management is further complicated by routine coagulation laboratory assays, which can only provide limited information in these emergency situations. *ROTEM* thromboelastography represents a whole blood haemostasis assay capable of providing rapid results in the perioperative and emergency setting. We present a clinical case of dabigatran reversal in a 74-year-old patient who presented with septic shock and acute renal injury secondary to duodenal ulcer perforation.
Aims: To present our clinical experience using *FEIBA* in the perioperative setting for the reversal of dabigatran. To discuss the potential role for *ROTEM* thormboelastography in monitoring dabigatran reversal. Finally, to highlight the limited efficacy of CVVH (continuous venovenous haemofiltration) in plasma clearance of dabigatran.
Methods: *ROTEM* thromboelastography (INTEM, EXTEM, FIBTEM), dabigatran assays (BIOPHEN DTI Kit HYPHEN BioMed), APTT assay reagent (SynthASil IL) and PT assay reagent (recombiplastin-IL).
Results: This 74-year-old patient was admitted with a 3 day history of abdominal pain and septic shock. Radiological investigations confirmed a perforated duodenal ulcer requiring emergency surgery. On admission, APTT>210s and PT>140s compatible with dabigatran accumulation secondary to acute kidney injury (eGFR 20 [baseline > 60]) corresponding to dabigatran levels 1.68 μg/ml. *ROTEM* Thromboelastography performed at baseline: INTEM CT 1660s, MCF 5 mm, while α-angle and CFT were immeasurable. The patient was taken to theatre. Pre-operatively, Vitamin K 10 mg IV and *FEIBA* 2500 IU (50 IU/kg) were administered.
Immediately post *FEIBA*, *ROTEM* thromboelastography parameters show an improvement: INTEM CT 1336s, MCF 23 mm, CFT 1651 and α-angle 13°. The patient underwent an omental oversew of the duodenal ulcer with no significant bleeding complications. There was minimal bleeding noted by the surgical team perioperatively. At two h post *FEIBA*, INTEM parameters suggest decreasing haemostatic efficacy (CT 2036s, MCF 5 mm and the α-angle and CFT were immeasurable). Dabigatran levels were 1.71 μg/ml, APTT and PT were prolonged again with no end point. The patient was transferred to the intensive care unit where CVVH (Xenium XPH 130 filter) was started for management of acute kidney injury. CVVH was noted to have little effect on dabigatran levels (pre = 1.71 μg/ml, post = 1.56 μg/ml). Post-operatively no clinical signs of bleeding were detected and no further doses of *FEIBA* were administered. The patient subsequently died 2 days post surgery with complications related to bowel ischaemia.
Summary: We highlight an effective clinical response to *FEIBA* for dabigatran reversal in the perioperative setting. We demonstrate the potential role for *ROTEM* thromboelastography in monitoring dabigatran reversal. Additionally, as demonstrated by dabigatran assay monitoring, CVVH does not achieve plasma clearance of dabigatran and therefore cannot be considered as a therapeutic option.

PO 265

Are point-of-care devices useful for monitoring bleeding risk under rivaroxaban treatment?

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Objective: Monitoring of rivaroxaban may be required in emergency situations when acute lifethreatening bleeding or thrombosis occurs or when operations need to be planned. The aim of this study was to investigate *ex vivo* the time- and concentration-dependent effects of rivaroxaban on point-of-care devices (POCT).
Methods: POCT was performed in blood samples of 27 consecutive patients who received 10, 15 or 20 mg/od of rivaroxaban; the samples were collected prior to and two h after drug intake at steady state. Twenty-five patients receiving phenprocoumon and 20 healthy individuals were also enrolled in this study. The whole blood prothrombin time (PT), activated partial thromboplastin time (aPTT) and activated clotting time (ACT) were determined using the GEM PCL Plus coagulation system. Additionally, PT was measured on the CoaguCheck XS. In citrated plasma the PT, aPTT and drug concentration were determined.
Results: Two h after the drug administration, significant, concentration-dependent prolongations of PT, aPTT and ACT were observed to different extents. The PT measured using GEM PCL Plus (range 15.1–116.5 s) was most sensitive to the rivaroxaban plasma concentrations (range 0.0–430.1 ng/ml). The PT ranges determined using CoaguCheck XS (11.2–26.4 s) or Neoplastin Plus® (12.0–37.6 sec in plasma) resulted in decreased good differentiation between the maximum concentration and through levels of the rivaroxaban administration.
Conclusion: Whole blood PT values that are assessed with POCT systems, such as the GEM PCL Plus, enabling dose-dependent determination may be helpful to monitor the target specific oral anticoagulants as rivaroxaban in critical situations.

PO 266

Topical use of antithrombotics

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Background: Antithrombotics are usually administered intravenously, subcutaneously or orally. However, topical application of anticoagulants is reported.
Aims: To review the available literature regarding clinical conditions, topical anticoagulant treatment and resulting positive or adverse effects and subsequently attempt to ascertain their safety and efficacy.
Methods: Interrogation of MEDLINE/Pubmed databases for topical anticoagulant treatments between 1/1/1990 and 11/12/2012 using the search terms 'topical' and: heparin; warfarin; low molecular weight heparin; tissue plasminogen activator; urokinase; streptokinase; activated protein C. Review articles were used for cross-referencing and corroboration and excluded from further analyses.
Results: There were 42 studies reported in 11 different clinical conditions. Most studies were randomized, placebo controlled trials, prospective cohort studies or case reports. The clinical conditions in which topical anticoagulants were used included microangiopathy, acute haemorrhoids, periodontitis, dermatitis, phlebitis, burns, ocular conditions and surgery, blunt force impact, scars, clinical conditions associated with superficial venous thrombosis (SVT), and other.
The most commonly used topical anticoagulant is heparin, used in 83% of the studies analyzed. Other treatments utilized were activated protein C (APC), tissue plasminogen activator (tPA), streptokinase and low molecular weight heparin (LMWH). APC, tPA and LMWH were administered as liquids, whereas heparin and streptokinase were

applied via more than 1 method, such as gels, pastes, liquids and sprays.
Dosage of different anticoagulants, if they were stated, varied depending on clinical conditions.
Most studies reported mean improvements or resolution of symptoms/condition in patients. A 8.6% of studies reporting heparin treatment had no resolution or improvement of outcome and 1 study related to streptokinase treatment reported no outcome. A further 19.0% of studies reported adverse effects, such as allergic reactions, mild to moderate pain, mild contact urticaria and pruritus. A 16.7% of which involved heparin and the remaining 2.3% APC. However, it was not made clear in 9.5% of studies if the negative effects were a direct result of the anticoagulant treatment investigated.
Summary/Conclusion: Topical anticoagulant treatment is used in differing ways in different clinical situations and gives rise to variable outcomes. Further research has to be conducted to match clinical conditions with their safest and most effective topical treatment.

PO 267

Improving vitamin K antagonists management through daily low dose vitamin K supplementation

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Background: VKA therapy is a lifesaving treatment, used in the primary and secondary prevention of both arterial and venous thrombosis. It is characterized by a high intra/inter-individual variability due to drug interactions, genetic factors, intercurrent diseases and dietary vitamin K intake. As shown in literature, instability of anticoagulation may be associated with a low dietary intake of vitamin K. As a consequence of INR variability an increase of bleeding and thrombotic complications is observed.
Case report: A 79-year-old man, without significant previous diseases, was admitted to a cardiological division, because of the onset of atrial fibrillation at high frequency. Warfarin was immediately started in association with low molecular weight heparin at therapeutic dose. Five days after discharge the patient was re-admitted because of epistaxis and over-anticoagulation, showing a PT INR = 12, immediately normalized with concentrated prothrombin complex and vitamin K.
One month after restarting anticoagulation patient condition was as follow: very low quality treatment (time in the therapeutic range = 5%), high frequency of INR monitoring (2–3 times/week), very low mean warfarin posology (1.75 mg/week). Within possible non genetic causes of high variability, only the concomitant treatment with amiodarone could have only partially explained the high sensitivity to AVK. CYP2C9 and VKORK1 genotypes were measured and showed aplotype A/A VKORK1 that is associated to a major sensitivity to warfarin. To control the high INR variability, we decided to treat the patient with a supplementation of low dose of vitamin K (50 mcg/die). Rapidly we observed a stabilization of INR levels, with an improvement of quality treatment: time in the therapeutic range reached the 65%; frequency of INR monitoring was reduced to 1 control each 2–3/weeks, warfarin posology was stabilized at 3.75–5 mg/week.
Discussion: This case report shows us that therapeutic instability of warfarin treatment is strongly associated to the high sensitivity and low dose requirement. As known, genotyping both CYP2C9 and VKORK1, helps to identify patients that show an early response to VKA therapy with a consequent risk of overdosage and haemorragic complications.
Besides variations in dietary intake of vitamin K can have a significant effect on anticoagulant response to oral anticoagulants. In this case we observed that supplementation with a low dose of oral vitamin K contributes to improve anticoagulant stability, with a consequent reduction of both hemorrhagic and thrombotic risks. For this reason a healthy dietary regimen containing enough fruits and vegetables should be recommended to each patient on vitamin K antagonists despite previous recommendations. Probably direct oral anticoagulants may improve and simplify anticoagulation management of very AVK unstable patients.

PO 268

A personalized system to enhance INR monitoring

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Background: Warfarin is the most commonly used oral anticoagulant drug and dose requirements are influenced by genetic and non-genetic factors. Due to the difficulty in determining an individual's proper warfarin dose, therapy is typically initiated with a standard dose followed by INR monitoring. A Personalized Medicine Interface Tool (PerMIT) is a software utility that supplies critical guidance to warfarin dosing decisions by modeling the dose requirements and response characteristics of individual patients based on their genotypic and physical characteristics.
Aims: The central objectives of this study were to 1) test the hypothesis that in comparison to patients who demonstrate above average time in therapeutic range, patients who experience a low time in therapeutic range demonstrate a greater proportion of dosage adjustments which are inconsistent with status of achieving pharmacokinetic steady state, and 2) to determine the proportion of above range INR's which are preceded by trends in the plasma concentration time profile that would have predicted the out of range INR event.
Methods: We conducted a retrospective analysis of fifty patient treated with warfarin. A database including patient characteristics, CYP2C9 and VKOR C*1* genotypes and the first thirty days of warfarin dosages and INR measurements was created to estimate maintenance dose and graphical display of the calculated S-warfarin plasma concentrations ([Cp]) and INR measurements. From the graphical display of the patient's measured INR values and calculated plasma concentration time profiles, the following data were extracted:
A Time in therapeutic range (TTR, 2.0–3.0) was determined based on linear interpolation
B Average daily dose administered when the INR was within range
C C.Number of INR measurements of > 3.0 that were predicted by the concentration time profile
D percentage of dosage decisions that were consistent with interpretation of the plasma concentration time profile
Methods: The time maintained within the target therapeutic range (TTR) was significantly greater ($70 \pm 0.15\%$ vs. 31 ± 0.15, $P < 0.0001$) for those patients where 60% or more of the dosage decisions were consistent with the plasma concentration time profile. Overall 9 of 28 (32%) INR measurements that exceeded 3.0 were predicted by a preceding increase in plasma concentration. Within the group with the greater average TTR, above range INR's were more common (18 vs. 10) and were less often predicted by the plasma concentration time profile (22% vs. 50%). This appears to be associated with fluctuations in the INR which are independent of changes in plasma S-warfarin concentration. Among the patients with the lower TTR, a greater proportion of INR's were below range (54.7% vs. 26.6%). In each of the groups, the average difference between the PerMIT estimated maintenance dose vs. the average daily dose for the TTR period was approximately 0.95 mg/d.
Conclusions: These data support the hypothesis, that a patient's INR response can be maintained within the therapeutic interval for a greater proportion of time when dosing decisions are guided by the plasma concentration time profile.

PO 269

The effect of application of fresh frozen plasma on prothrombin time in the patients overdosed with oral anticoagulant medicaments

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Background: Risk factors in patients who receive oral anticoagulant therapy (OAT) are age, severe kidney disease, liver disease and cardiovascular diseases, as well as unstable anticoagulant effect. According to current recommendation, in case of bleeding, when INR is larger that 9, fresh frozen plasma (FFP) is administered, in a dose of 10–20 ml/kg or concentrate of prothrombin complex 50 U/kg and 1 mg of the vitamin K intra venous.
Aims: The aim of the paper was to determine to what extent the application of FFP improve protrombin time (PT) in the bleeding OAT overdosed patients.
Material and Methods: To determine coagulation status, blood samples were taken by standard procedure. PT was done using recombinant rabbit thromboplastin (HemosIL) with ISI 1.1 on ACL 7000 apparatus. Control PT was done 12 h after the administration of FFP. Retrospective counting was done from the protocol for hemostasis and delivery of blood and blood products.
Results: From 1 January 2011 to 31 December 2011, 40 patients were treated in our institution due to bleeding. Most frequent forms of bleeding were gum bleeding (7%), nasal bleeding (35%) and rectorragia (58%). In 14 patients, INR was immeasurable, while in remaining 26 it ranged from 7.83 to 14.4. After FFP was administrated in the dose of 15 ml/kg, 10 patients returned to therapeutic range (INR 2–4), out of whom only one belonged to the group with immeasurable INR.
Conclusion: In overdosed OAT patients, the administration of FFP only does not reduce INR to therapeutic range, which is also found in the paper by other authors.

PO 270

Use of protrombin complex concentrates for urgent reversal of dabigatran in the emergency department: a pilot study

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Background: Hypothetically prothrombin complex concentrates (PCC) could overcome the anticoagulant effect induced by thrombin and factor Xa inhibitors because PCC contains coagulation factors II, VII, IX, and X in a high concentration and they are known to enhance thrombin generation. While in an animal model infusion with PCC reversed the biological effect of rivaroxaban, data for dabigatran are not as clear-cut.
Objectives: The aim of this study was to describe our experience from the emergency department with the urgent reversal of anticoagulation with dabigatran using a prothrombin complex concentrate.
Methods: An observational study of hemorrhagic complications was carried out in patients treated with dabigatran over a period of 12 months (July 2011 to June 2012).
Results and Conclusions: We report a pioneer experience in five patients on the suitability of PCC used for the reversion of the anticoagulant effect of a direct thrombin inhibitor. PCC was used successfully in our emergency department and was able to control major bleeding events. Based on the small amount of data, we suggest further evaluation of the use of PCC in reversing massive hemorrhage caused by dabigatran.

PO 271

Comparative study of medication compliance to two anticoagulants in the prevention of venous thromboembolism in orthopaedic surgery

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Background: Rivaroxaban (Riv) is a selective, direct Factor Xa inhibitor indicated in the prevention of venous thromboembolism in adult patients undergoing elective hip or knee replacement surgery (HKRS). It was introduced in the pharmacotherapeutic formulary of the Hospital Centre of Cova da Beira (CHCB) on February/2011. It is administered orally, which is a potential advantage in terms of compliance when compared to enoxaparin (Eno).
Aims: The aim of this study was to compare adherence to Eno vs. Riv in adult patients undergoing elective HKRS. The occurrence of adverse drug reactions (ADRs) was also compared in both groups.
Methods: Cross-sectional study of outpatient compliance to Eno or Riv, in patients undergoing KHRS in CHCB, from February/2011 to April/2012. The evaluation of medication adherence was carried out using a validated questionnaire and the occurrence of ADRs was evaluated in a structured interview.
Results: The study included a total of 84 patients, who underwent elective knee (45 patients) or hip (39 patients) surgery; 42 patients were subjected to therapy with Eno (18 knee + 24 hip) and 42 with Riv (27 knee + 15 hip). In all, 90.5% patients were considered adherent to medication, but it was not observed a significant difference ($P = 0.71$) between patients anticoagulated with Eno (92.9% adherent) or Riv (88.1% adherent). Similarly, there was no significant difference ($P = 1$) in medication adherence between patients undergoing knee or hip surgery. However, there was a significantly higher occurrence of ADRs ($P = 0.002$) in patients treated with Eno (38.0% patients reported ADRs attributable to this drug, mainly hematoma in the site of injection) when compared to patients treated with Riv (9.5% patients reported ADRs attributable to this drug, mainly gastrointestinal and skin disorders).
Conclusions: Although it was not observed a significant difference in adherence to subcutaneous Eno vs. oral Riv, which may be potentially attributed to the short-term anticoagulation therapy (2–5 weeks), the occurrence of ADRs was significantly lower in patients treated with the oral anticoagulant. This difference in drug-related adverse events differs from other studies that detected similar adverse-event profiles. From a methodological point of view, this is a small cross-sectional study and our results must be considered exploratory in nature.

PO 272

The course of D-Dimer levels in patients getting Rivaroxaban in therapeutical doses

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Background: D-Dimer levels are associated with thromboembolic risk. Patients with atrial fibrillation or after thromboembolic diseases with high D-Dimer levels have a higher risk of thromboembolic diseases.
Usually D-Dimer levels fall to normal range during an effective anticoagulation.
Aims: To view the course of D-Dimer levels in patients receiving Rivaroxaban.
Patients and Methods: We investigated the course of D-Dimer levels before and 4 weeks after starting oral anticoagulation with Rivaroxaban in 56 patients in during the year 2012.
The Rivaroxaban dose was 15 or 20 mg per day according to renal function. The patients had atrial fibrillation or venous thromboembolic disease and had an indication for oral anticoagulation (e.g. CHADS2-Score > 2, recurrent thromboembolic disease, severe throm-

bophilia). There was no patient with acute thromboembolic disease, so that no patient had an indication for 2 × 15 mg/day.
Results: Of these 56 patients: 26 patients (46%) had normal D-Dimer levels before and after intake of Rivaroxaban17 patients (31%) had D-Dimer levels above normal range before therapy with Rivaroxaban an normalized after 4 weeks of Rivroxaban therapy nine patients (16%) had D-Dimer levels above normal range before starting rivaroxaban and D-Dimer levels fell, but stayed above the normal range four patients (7%) had normal D-Dimer during therapy with vitamin-K-antagonist and D-Dimer levels rose above normal range after switching to RivaroxabanSummary:
In summary about 23% of our patients had D-Dimer levels above the normal range despite anticoagulation with Rivaroxaban.
No thromboembolic event occurred during the investigation in these 56 patients regardless of the D-Dimer level. However, the follow up was too short because the investigation time was only up to 12 month.
Conclusion/Discussion: It is not known, what it means clinically that a subgroup of patients have D-Dimer levels above normal range despite oral anticoagulation with Rivaroxaban.
Does it mean, these patients have a higher thromboembolic risk than patients with normal D-Dimer levels during anticoagulation?Do these patients need higher Rivaroxaban doses for effective anticoagulation, for example 15 mg twice daily?A long term follow up of these patients is needed to get more clinical data and to see, it the elevation of D-Dimer during anticoagulation is clinical relevant.

PO 273

Safety of enoxaparin sodium (subcutaneous injection kit) used in clinical settings in patients undergoing abdominal surgery (general surgery and gynecology) – specified drug-use survey

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Background/Aims: A specified drug-use survey in Japanese patients who underwent abdominal surgery (general surgery and gynecology) was conducted from March 2009 to June 2012 to evaluate the safety of CLEXANE® Subcutaneous Injection Kit 2000 IU used in clinical settings.
Patients and Methods: The survey included patients who started treatment with CLEXANE® Subcutaneous Injection Kit 2000 IU for cesarean section and for whom preoperative creatinine clearance could be calculated using the Cockcroft formula. In this survey, patient characteristics at enrollment, adverse drug reactions (ADRs), bleeding ADRs, and incidence rate of pulmonary thromboembolism were investigated.
Results: In this survey, 769 cases were collected by continuous registration in participating facilities from 131 facilities in Japan and 9 cases were excluded from the safety analysis. Of the remaining 760 patients (528 general surgery cases and 232 gynecology cases), two patients were excluded from the efficacy analysis, resulting in 758 patients (526 general surgery cases and 232 gynecology cases) evaluated for efficacy. The mean age of patients in the safety analysis was 61.9 years. BMI indicated 33.9% were obese (≥25 kg/m^2) and 66.1% were non-obese (< 25 kg/m^2); malignant tumor was the most frequently observed underlying disease. The mean duration of study treatment was 5.9 days.
There were 100 adverse effects (81 patients) observed, with an incidence rate of 10.7%. Among these, 35 adverse effects (30 patients) were hemorrhagic adverse effects with an incidence rate of 3.9%. The common adverse effects were abnormal hepatic function, posttreatment hematoma and increase in alanine aminotransferase, all of which are known adverse events of the drug. Seventeen severe adverse effects (11 patients) were observed with an incidence rate of 1.4%. Furthermore, pulmonary thromboembolism and deep vein thrombosis were observed in 4 (0.5%) and 1 (0.1%) patients, respectively, but none of these had a fatal outcome (death).
Conclusions: The specified drug-use survey on CLEXANE® Subcutaneous Injection Kit 2000 IU used in clinical settings in patients undergoing abdominal surgery (general surgery and gynecology) did not reveal any potential new safety signals.

PO 274

Availability of laboratory monitoring of new oral anticoagulant (rivaroxaban)

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Background: Bleeding is still a frequent complication for patients on treatment with newly developed oral anticoagulants. One of the main reasons for this is the rejection of the need of laboratory monitoring. Rivaroxaban (R) is a direct factor Xa inhibitors and can be taken orally in fixed doses once or twice a day. R doesn't have readily available assay to measure its anticoagulant effect (AE) so far.
The aim of this study was to evaluate the possibility of measuring AE of R by one of well-known routine methods – blood recalcification technique (RT).
Methods: From 2012 to 2013, we have studied 309 patients (pts) (M/F 158/151; mean age 59 ± 8 years, range 39–68 years) with acute, symptomatic, objectively confirmed (bilateral ultrasound) proximal deep-vein thrombosis (DVT), without symptomatic pulmonary embolism. Pts initially received therapeutic doses of LMWH (enoxaparin): 1 mg/kg twice daily. In 49 pts there was no any net clinical or ultrasound benefit after 5–7 days of initial anticoagulant treatment, therefore we decided to replace LMWH on R to all of them (after preliminary investigation of RT). We started therapy with recommended doses of R: 15 mg twice daily for 3 weeks and then 20 mg per day. Method of RT in short: the basic idea of RT is the recovery of ionized calcium initial concentration in citrated blood (balanced RT). In our method we use lower concentration of calcium than in balanced RT. Empirically we have found that this alteration increases sensitivity of the test to detection of blood hypercoagulation (BHC). RT was determined by thromboelastography (TEG 5000, the USA): time of reaction 'r' – normal range = 660–960 s. Blood analysis was taken after 3–4 h of LMWH injection or swallowing R.
Methods: Forty-nine pts had BHC (range = 375–428 s) after 5–7 days of initial treatment with LMWH assessed by TEG. Replacement by R increased 'r' to normal or hypocoagulation values (range = 685–3450 s). Twenty-eight percent pts had significant blood hypocoagulation after 3 days of therapy R (range = 1220–3450 s). All the changes of R doses (to 20–15–10 mg daily) were induced by TEG values (aimed value – normocoagulation of blood). Net clinical and objective benefits of R therapy began to be detected on the 3rd to the 12th day. There was no case of bleeding in our pts.
Conclusions: Laboratory monitoring is possible and desirable in some cases for the selection of doses of rivaroxaban. TEG is a simple and available method for dose adjustment of new oral anticoagulant (R) therapy. This assay allows individualizing the therapy making it more effective and safe.

PO 275

New treatments and old effects: Dabigatran-induced leukocytoclastic vasculitis

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Background: Oral anticoagulant therapy is increasingly used for the prevention and treatment of thromboembolic complications of vascular disease. Until recently, we have depend on the vitamin K antagonist class drugs, now the new oral anticoagulants (direct thrombin inhibitors and factor Xa inhibitors) are available. Bleeding is the most serious complication of the use of this drugs. All trials (RE-LY, ROCKET AF, ARISTOTLE) showed non-inferiority or superiority for the efficacy outcome vs. warfarina and rates of major bleeding complications were similar or even reduced. We report a case with a unpublished complication in a patient who starts treatment with dabigatran.

Case report: A 70 year old man with medical history of hypertension and smoking years ago. Diagnosed in February 2005 of atrial fibrillation, then started anticoagulation with acenocumarin which was stopped in June 2005 after successful electrical cardioversion.

In July 2007 was diagnosed with persistent atrial fibrillation CHA_2DS_2-VASc 2, HAS-BLED 2 and starts again acenocumarin therapy with INR (International Normalized Ratio) target between two and three.

From the begining of treatment had regular levels of INR with regular dosis of acenocumarin (16 mg weekly) until October 2011 when was admitted with ischemic stroke of cardioembolic origin. This occurred 24 h after cardioversion. The INR on admission was 1.83. And it was complicated with hemorrhagic transformation. The hospitalization lasted 4 months. At the time of discharge the patient was independent for almost all basic activities of daily living.

In November 2012 acenocumarol treatment was suspended and initiate treatment with dabigatran 150 mg twice daily. The rest of the usual treatment remained unchanged for months.

Just 10 days after the change the patient developed lower limb palpable purpuric lesions, some with vesicles of hematic content. We reduced dabigatran dose to 110 mg twice daily. But the skin lesions continued to evolve with pelvic extension and appearance of superficial necrosis (pictures). Blood count was normal, INR 1.44, TTPAr 2.15 and creatinine 1.34 mg/dl. Detailed coagulation study was normal.

Skin biopsy disclosed leukocytoclastic vasculitis.

Dabigatran was suspended and began low molecular weight heparin at prophylactic doses, oral prednisone and topic treatment.

Actually the lesions improved (pictures) and the patient has resumed acenocoumarin therapy without further incident. Next days, the results to rule out another possible etiology of vasculitis will be ready.

Discussion: In addition to unexpected bleeding, skin reactions like photosensitivity, maculopapular vesicular urticarial eruptions, purple toes syndrome, skin tissue necrosis, and vasculitis are one of the uncommon adverse effects of the vitaminK antagonists therapy.

We report a leukocytoclastic vasculitis with skin tissue necrosis case. The cause of this was seemingly dabigatran because of the close temporal relationship between exposure to the drug and the onset of the symptoms, and the resolution of the lesions after dabigatran was discontinued. Illustrates this observation a new rare association between vasculitis and dabigatran? Clinicians should be aware of this potential adverse effect and recommend interrupting the drug intake when temporal relation is evocative.

Like all new drugs, we need more experience in daily clinical practice, outside clinical trials.

PO 276

Laboratory testing in new oral anticoagulants

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New oral anticoagulants (NOAC) showed clinical benefits and 'non inferiority' in compare with traditional and were approved in many countries. They don't need in laboratory control and are used in fix dozes. But the problem of interpretation of testing results in laboratory haemostasis for these patients in routine practice is unsolved.

The Aim to estimate the routine haemostatic parameters in patients received NOAC and compare it with thrombotic and haemorrhagic events.

Methods and objectives: Thirty-one patients with non valvular atrial fibrillation were included – 24 received direct thrombin inhibitor dabigatran (42–81 years old, 11 male, 13 female) in dose 110 mg bid (11) and 15 mg bid (13) and seven patients received direct anti-Xa inhibitor rivarixaban 20 mg od (64–76 years old, 3 male, 4 female). Quik test, APTT, thrombin time (TT), FVIII activity, vWF antigen, D-dimers (D-d) were measured (STA Compact, Stago reagents). Observational time – from 3 up to 6 months. The results are presented as median, 25 and 75 percentiles (Me [25%; 75%]); statistic Mann-Witney U-criteria was used. In all cases renal function was estimate before treatment and glomerular filtration rate (GFR) were calculated: it fluctuated from 43 ml/min to 98.8 ml/min.

Methods: We found significant prolongation of APTT and TT in dabigatran group: 43.7 [30.0;51.3] s and 148.6 [51.2;218.1], respectively. The mean values in rivaroxaban group were 28.0 [26.0;30.0] s and 17.1 [16.4;17.7], $P < 0.01$. Markers of activation (FVIII, vWF, D-d) varied widely in both groups and didn't decrease during observational time. There were no significant differences between two dabigatran dosing groups or any correlation with GFR, probably in the result of small groups.

We had no thromboembolic complications, but 3 hemorrhagic events: macrohematuria and sclera hemorrhage (dabigatran), vaginal hemorrhage (rivarixaban).

Conclusion: There is significant prolongation of APTT and TT on dabigatran in distinction of rivaroxaban, without correlation with adverse events. It was surprised the absence of activation markers redaction under NOAC therapy. We need further investigation with higher number of patients.

PO 277

Influence pharmacogenetics analysis on adequacy and safety of therapy by indirect anticoagulants

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In clinical practice pharmacogenetics analysis and pharmacogenetics algorithm dosing of indirect anticoagulants are introduced to optimize the therapy these drug.

The aim to estimate influence pharmacogenetics analysis for adequacy and safety of therapy indirect anticoagulants at inhabitants of Northwest region of the Russia.

Materials and methods. Prospective not randomized cohort research. The pharmacogenetics analysis (PGA) is performed to 100 patients receiving therapy of coumarin. Supervision term over patients is 6 months. PGA was performed with polymerase chain reaction in real time. All patients were divided into four groups according to received prothrombin test results. Data processing was held with use of Microsoft Excel 2003 and SPSS 17.0 software.

Methods: Therapeutic hypocoagulation has been reached at 58% patients for average 6 ± 4.2 days (criterion efficiency of therapy is level international normalised ratio 1.8–3.0). The normal genotype of

PGA (CYP2C9, VKORC1-1639 G>A) has been revealed at 25% patients, 70% patients had the episodes of bleedings, genotype defined propensity to superfluous hypocoagulation has been revealed in 20% cases and 44% patients had bleeding. The supporting dose calculated on Gage et al. model receved of the all patients made 5.3 ± 0.7 mg.
Conclusions: The pharmacogenetics analysis will not replace adequate laboratory monitoring with level INR which reflecting safety therapy of indirect anticoagulants.

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Intensity of bleeding following tooth extraction is moderated by personality traits independently of anticoagulant therapy

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Background: Anticoagulation with dose adjusted vitamin-K antagonists (VKA, therapeutic range of the international normalized ratio (INR) of 2–3) and low dose aspirin (65–330 mg daily) enhance bleeding following tooth extraction. However, intensity of bleeding varies substantially between patients. Guidelines disagree regarding interruption or continuation of anticoagulation for tooth extraction.
Aim: We hypothesized that personality traits may moderate bleeding intensity following tooth extraction independent of anticoagulant therapy with VKA or aspirin.
Methods: A total of 180 patients (77 female, 103 male, 49.9 + 15.3 years [mean, standard deviation]) underwent tooth extraction without interruption of anticoagulation. The study was accepted by the local university ethics committee and patients gave written informed consent prior participation. Sixty three patients did not take any anticoagulant (group 1), 60 patients were on aspirin (group 2), and 57 patients on VKA (INR 2–3, group 3). Patients completed a validated state-trait anxiety inventory (STAI), and a personality inventory with 12 dimensions (Freiburger personality inventory [FPI]). Based on results of a focus group with patients before tooth extraction, and a self-developed questionnaire (ZAI) was set up on general attitudes regarding general feeling and anxiety before tooth extraction. The 43 items of the ZAI were reduced to six factors by multifactorial regression analysis. Dentists (JF, PH) rated bleeding intensity with a score from 0 to 9 according a standardized protocol during and following the intervention. The moderating effects of the STAI and FPI dimensions on the bleeding index were calculated by correlation matrices and for the factors of the ZAI by multinodal logistic regression analysis.
Results: Intensity of bleeding after tooth extraction was higher in group 3 (score < 6 in 63%, score > 6 in 37% of patients) compared to group 1 (92% and 8%) and group 2 (78% and 22%) and higher in group 2 compared to group 1 (all $P < 0.005$, chi-square test). Personality traits influenced the bleeding intensity independently of anticoagulation: higher anxiety values of STAI questionnaire and of the FPI dimensions self consciousness, somatic distress, and emotionality correlated positively ($P < 0.0001$) and lower values for satisfaction ($P < 0.02$) and extraversion ($P < 0.003$) correlated negatively with increased values of the bleeding index. Moderating factors for a higher bleeding index of the ZAI were fear for dentist visits, treatment, complications and risks of intervention (all $P < 0.001$).
Conclusion: The intensity of bleeding following tooth extraction is influence by anticoagulant therapy with VKA and aspirin and independently of the anticoagulants by certain personality traits and previous experiences of patients with tooth treatments. Personality traits and specific experiences of patients with previous and fears for other surgical interventions may also influence bleeding intensity of other surgical interventions independently of anticoagulation. The effect of new oral anticoagulants in this context remains to be determined.

PO 279

Deep vein thrombosis of the penis: an unusual but severe complication of prostatic abscess: case-report and review of the literature

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Venous thrombosis of the penis has most often been related to the superficial venous system, an affection commonly referred to as Mondor's penile disease. Thrombosis of penis deep venous system (PDVT) has been rarely reported and must be clearly distinguished because it may cause serious clinical complications.
In this report, we describe the case of a 66-year-old patient, hospitalized for prostatic abscesses and septic shock, presenting with a PDVT. Drainage of prostatic abscesses and treatment of septic shock (antimicrobial with piperacillin/tazobactam and support treatments) were immediately undertaken. Patient was also treated with anticoagulant therapy (low-molecular-weight heparin and vitamin K antagonists. Duplex ultrasound showed vein recanalization after 6 months of anticoagulant treatment. Local clinical evolution was favorable despite severe initial manifestation presenting with a high probability of major necrotic sequel of penis.
This is the first reported observation of PDVT associated with infection. At our knowledge, only five cases of PDVT have been reported in the literature. Outcome was negative in 3 cases resulting in penis necrosis or persistent erectile dysfunction. PDVT has been associated with Behçet's disease and heterozygous factor V mutation, Henoch-Schonlein purpura and heterozygous factor II mutation, hyperhomocysteinemia, activated protein C resistance and heterozygous factor V mutation, trauma and elevated factor VIII levels. Duplex ultrasound can be used to easily distinguish between superficial and deep venous penile systems. Additionally, it allows analyses of arterial blood flow as well as penile structures. Use of other imaging techniques, such as MRI or CT-scan, provide additional and different information that may be helpful in better targeting a differential diagnosis, in evaluating an associated neoplasia, or in detailing proximal extension of thrombosis.
Anticoagulant treatment should avoid potentially serious complications in penile structures drained by the deep venous system, thus hindering thrombosis extension to still patent segments. Additional therapeutic options (i.e. thrombolysis or thrombectomy) may be considered depending on the severity of the clinical picture and according to availability of resources and skills personnel. Identification and treatment of an underlying cause provoking PDVT should always be looked for. Searching for other prothrombotic conditions must also be undertaken to optimize anticoagulant treatment duration.

PO 280

Over-representation of thoracic outlet obstruction in patients with unprovoked upper extremity deep vein thrombosis

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Background: Upper extremity deep vein thrombosis (UEDVT) is a rare condition. Compared with lower extremity DVT, UEDVT has lower risk of pulmonary embolism but it may cause considerable morbidity due to post-thrombotic syndrome. The optimal duration of treatment was 3 months according to the ACCP guidelines. Primary or unprovoked UEDVTs are a subset of UEDVTs that occur in the absence of established risk factors such as catheter-induced injury or malignancy. A potential cause of primary UEDVTs has been postulated to be venous thoracic outlet obstruction. Paget-Schroetter Syndrome (PSS), effort-induced axillary or subclavian vein thrombosis resulting from vigorous and sustained upper extremity movements have been described before. However, the prevalence and role of thoracic outlet

obstruction, in particular intermittent venous outflow obstruction as a significant risk factor for UEDVTs remain unclear.

Aims: To evaluate the correlation between positional venous thoracic outlet obstruction and UEDVT.

Methods: Nine consecutive cases of unprovoked UEDVT referred over a 2 year period (2010–2012) to a tertiary care center in Hamilton, Ontario, Canada were reviewed. All patients were screened for thrombophilia. Contrast-enhanced 3D magnetic resonance angiography (cMRA), combined with provocative manoeuvres with the arms in abducted and resting positions, was performed for all patients to look for thoracic outlet obstruction. Additional T2-weighed images were performed with the affected arm in resting position to identify soft tissue structures including brachial plexus. Detailed chart reviews were conducted to look for additional risk factors for thromboses.

Results: Three of 9 (33.3%) patients had a history of sustained arm movements. Of these patients, all had evidences of thoracic outlet obstruction causing venous compression on MRA. The other 6 (66.6%) patients did not have a history of vigorous or sustained arm movements. Four of these six patients (66.7%) had evidence of thoracic outlet obstruction causing venous compression on MRA. None of the nine patients had clinical symptoms consistent with positional venous thoracic outlet syndrome. Overall, seven of nine patients (77.8%) had evidence of venous thoracic outlet obstruction on MRA when the affected arm was in abduction position, with one patient having obstruction with arms in both abduction and resting positions.

Summary/Conclusions: Positional thoracic outlet obstruction causing intermittent venous blockade is over-represented in patients with unprovoked UEDVT. If available, cMRA combined with provocative manoeuvres should be performed in this patient population to define the underlying pathology associated with the venous obstruction. Whereas thoracic outlet obstruction presents as an ongoing factor associated with UEDVT, the need for extended therapy beyond 3 months in this patient population should be readdressed. Further recurrence may also be prevented by therapeutic strategies to avoid positional venous obstruction.

PO 281

Venous thrombosis and plasma homocysteine levels in the cardiac pacing patients

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Background: Despite major advances in the techniques of pacemaker implantation, these procedures are still associated with many complications, occurring both early and late during the follow-up. Patients who undergo the implantation of a pacemaker, or a defibrillator, are at risk of thrombosis associated with transvenous leads. Increased homocysteine levels are related to the occurrence of venous thrombosis (VT), but whether this relation is actually causative still remains unclear, as does the association between this amino acid and the complications in the cardiac pacing patients.

Aim: To assess whether the occurrence of venous thrombosis and plasma homocysteine levels are associated with the actual mode of cardiac pacing.

Methods: The study group comprised 92 subjects (38 females, mean age 71 ± 7.58 years) with a cardiac pacemaker. Subjects were divided according to the actual mode of cardiac pacing into Group A (64 subjects; mean age 69.96 ± 7.86 years) with physiological (DDD) pacing and Group B (28 subjects; mean age 73.27 ± 6.79 years) with non-physiological (VVI) pacing, respectively. Cardiac pacemakers were implanted due to SSS, AVB (II, III gr.), atrial fibrillation cum brady-arrhythmiae, respectively. Transthoracic echocardiography was performed prior to and after pacemaker implantation. Screening for C677T polymorphism of MTHFR was carried out by the standard method. The plasma homocysteine levels before and after 7 days, within 1, 3, 6 and 12 months of pacemaker implantation were measured. The levels of D-dimers, fibrinogen, tissue factor (TF), factor VII, plasminogen activator inhibitor-1 (PAI-1), interleukin-6 (IL-6) and hsCRP) were also determined.

Results: The two groups differed with respect to the pacing mode. During the 18-month follow-up period 10 subjects developed VT, i.e. 11.76% of the entire study population (5 in Group A and 5 in Group B; $P = 0.12$). The symptoms of VT occurred in 10 subjects within the post-implantation period (mean of 13.06 months; 7–17 months). The carrier frequency for 677T allele was similar in both study groups (0.296 vs. 0.327; p = ns). There were 5 (7.81%) and 3 (11.54%) homozygous for allele 677T of the MTHFR gene (Group A and Group B, respectively, $P = 0.61$). At baseline, plasma homocysteine levels were higher in Group B subjects (14.98 ± 3.47 μM *vs.* 13.07 ± 3.48 μM; $P = 0.02$). Also after pacemaker implantation, mean plasma homocysteine levels were significantly higher in Group B subjects ($P = 0.04$). Also, in the Group B subjects only, after pacemaker implantation, plasma homocysteine levels remained increased non-significantly for up to 3 months (14.98 ± 3.47 μM *vs.* 16.75 ± 6.69 μM *vs.* 15.40 ± 50 μM *vs.* 15.54 ± 3.05 μM; for baseline, 7 days, 1, and 3 months, respecively), and then spontaneously reverted to baseline values.

Conclusions: The MTHFR 677TT genotype and plasma homocysteine levels were not related to the risk for VT. The elevated homocysteine levels following pacemaker implantation may well be attributable to the actual mode of cardiac pacing.

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Ageing of the venous valves as a new risk factor for venous thrombosis in the elderly – the BATAVIA pilot study

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Background: Increasing age is a strong risk factor for venous thrombosis (VT). Previously, we have shown that ageing leads to damaged venous valves, i.e. thicker and less functional valves. Ageing of the venous valves has, as yet, not been investigated *in vivo* in relation to the risk of venous thrombosis.

Aims: The aim of this study is to assess the effect of valve thickness and function on the risk of venous thrombosis.

Methods: Analyses were performed in the Biology of Ageing and Thrombosis: Appraisal of Valve thickness and function, and *in vivo* Assessment – the BATAVIA study. We will invite 100 patients aged 70 years and older with a first deep venous thrombosis of the leg between 2008 and 2011 and 100 controls subjects without thrombosis. We will perform an extensive ultrasound examination of the venous valves in the popliteal vein in the non-affected leg, as the non-affected leg represents the stage of the ageing process of the venous valves in the affected leg prior to the thrombotic event.

We excluded all patients with a recurrent VT in the contralateral leg and patients with a history of deep venous surgery in the lower extremities.

The valves of the popliteal vein were imaged with a 9 MHz linear probe using B-mode ultrasonography. Valve closure time (VCT) was assessed as an indicator for valve function using an automatic inflatable cuff. After the examination, a short interview was conducted to obtain information on post-thrombotic syndrome and varicose veins.

This study is still ongoing. Currently we present the results from the pilot study ($n = 20$). At the time of the ISTH conference, complete results of this study will be available.

When complete data for cases and control subjects are available, relative risks adjusted for age and sex will be estimated by calculating odds ratios (OR) and their 95% confidence intervals (95% CI). This study was approved by the Medical Ethics Committee of theLeidenUniversityMedicalCenterand all participants gave written informed consent.

Results: Currently, measurements are available for the first 20 patients. Mean age of the participants was 77.8 (range 74–90). In 18 of 20 participants valve parameters could be measured. Mean thickness of the valves was 0.43 mm (range 0.31–0.53 mm). Mean VCT was 0.35 s (range 0.2–1.2 s), which shows that venous reflux was present in some patients.
Conclusion: This pilot study shows that *in vivo* visualization of venous valves and measurement of valve thickness by ultrasound is feasible. Complete results will show whether ageing of the venous valves, i.e. an increase in valve thickness and a decrease in valve function, is associated with an increased risk of venous thrombosis in the elderly.

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Management and treatment outcomes of venous thromboembolism (VT)

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Background: Deep vein thrombosis (DVT) and pulmonary embolism (PE) collectively known as VT are acute medical emergency affecting 1/1000 inhabitant. VT is often predisposed by risk factor. The symptoms of VT are non-specific and the diagnosis requires objective tests. Anticoagulation is the mainstay of VT treatment. Clinical outcomes after VT include recurrent thrombosis, cancer and mortality.
Aim: The aim of this study was to explore risk factors, clinical presentation, treatment modalities and mortality after VT.
Methods: This study represents cases of VT registered in the Thrombosis Registry of Oestfold the 'The Troll registry' during the period 2005–2011. This quality control registry includes base-line and follow-up data on all patients managed for VT in Oestfold county of Norway. First registration is considered as index event. The register is approved by the Norwegian Social Science Data Service.
Results: In total 1308 objectively verified VT events were registered in 1231 patients 660 (54%) males. These were classified as DVT in 683 (52%), PE in 565 (43%) and both in 60 (5%). Median age at diagnosis was 64 years (IRQ = 50–76). At time of data analysis 322 patients were dead, including 131 (20%) of DVT patients, 182 (34%) of PE and 11(18%) of those with both diagnosis. There was significantly higher mortality among PE compared to DVT ($P < 000.1$). Median time to death in DVT and PE were 0.9 years (Range = 0–6) and 0.5 years (0–7), respectively. Thirty-five percent of those having PE and 21% of DVT died within first 90 days following diagnosis.
A risk factor was identified in 798 (61%) of the events; these were: previous VT 321 (25%); family history of VT 192 (15%); immobilization 220 (17%), including 101 (8%) orthopedic surgery, other general surgery 50 (4%), trauma 57 (4%), medical disease 12 (1%); active cancer 262 (20%); long-haul flights 120 (9%); contraceptive pills/HRT 107 (8%); sedentary life style 82 (6%); known thrombophilia 60 (5%); pregnancy 21 (2%).
Pain, swelling and/or tenderness were presenting features of DVT in 482 (75%); 539 (83%); 351 (55%), while dyspnea and/or chest pain were found in 353 (66%) and 271 (50%) of PE, respectively. Seventy-one (13%) of PE had received antibiotic by general practitioner (GP) last 4 weeks before diagnosis. A 524 (77%) of DVT and 11 (2%) of received ambulant treatment. Median duration of hospital stay in DVT/PE was 4 and 5 days. Twenty (4%) of PE and 12 (2%) of DVT received thrombolysis.
Summary/Conclusions: This study describes unselected population of VT. A recognizable risk factor of VT was found in 61%. Despite wide use of thromboprophylaxis 17% of VT were predisposed by a surgical or medical condition requiring immobilization. These should have been prevented if adequate thromboprofylaxis was given. Thirteen percent of PE were misdiagnosed by GP as respiratory tract infection indicating the need for greater awareness for this condition. There was significantly higher mortality among PE patients than DVT.
Such quality control registries are required to monitor the quality of management and unravel eventual weaknesses.

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Non-inherited causes of newly diagnosed venous thromboembolism in Croatia in 2011: Croatian Cooperative Group for Hematologic Diseases (CROHEM) study

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Background: Venous thromboembolism (VTE) is a major medical health problem associated with significant morbidity and mortality. VTE is a multifactorial disease, resulting from a complex interaction of genetic and acquired factors. The incidence and characteristics of VTE in Croatia has not been well described.
Aims: To investigate non inherited causes of VTE among newly diagnosed patients with secondary VTE in Croatia.
Methods: Croatian Cooperative Group for Hematologic Diseases (CROHEM) conducted observational non interventional epidemiological study from January 1st until December 31st 2011 analyzing medical records of newly diagnosed patients with VTE from 5 major general hospitals (Sibenik, Knin, Koprivnica, Slavonski Brod, and Varazdin) in 4 different Croatian counties (Sibenin-Knin County, Koprivnica-Krizevci County, Brod-Posavina County, and Varazdin County). Study was approved by a medical ethics committee of each hospital.
Methods: There were 642 new cases of VTE in 2011; 389 (60.59%) of them with deep vein thrombosis (DVT), 218 (33.96%) with pulmonary embolism (PE), and 35 (5.45%) cases of both conditions. There were 280 (43.6%) males (median age 65 years) and 362 (56.4%) females (median age 74 years, $P < 0.001$ compared to male). Among them, 371 (57.8%) were secondary and 271 (42.2%) were idiopathic VTE. Recidivism of VTE was diagnosed in 78 patients (12.15%). There was no difference in recidivism between secondary and idiopathic VTE. Although patient population with VTE was in general old (median age 71 years), patients with secondary VTE were younger than those with idiopathic VTE (median age 69 vs. 74 years, $P < 0.001$). There was no gender difference between them. The most frequent causes of secondary VTE were cancer (40.4% of secondary VTE) and trauma, surgery and immobilization (37.5% of secondary VTE), while other causes of secondary VTE were less frequent (other diseases (e.g. neurological diseases with paralysis, autoimmune diseases, inflammatory bowel diseases) (17.9%), sepsis (9.7%), central venous catheter (3.25%), pregnancy [2.16% of secondary VTE]). More than one non inherited risk factor for VTE had 43.40% of patients with secondary VTE. Among all 642 VTE patients, 69 (10.75%) died, 35 (50.73%) of them having VTE as a main cause of death. More patients died among secondary VTE (55 deaths of 371 secondary VTE, 14.8%) than among idiopathic VTE patients (14 deaths of 271 idiopathic VTE, 5.2%) ($P < 0.001$).
Summary/Conclusion: Many cases of secondary VTE had more than one underlying non inherited condition for development of VTE. The most common risk factors for VTE were malignancy, trauma, surgery, and immobilization. Secondary VTE had higher mortality than idiopathic VTE. As non inherited risk factors for VTE, such as surgery and cancer, are applicable to more people, improved thromboprophylaxis in these settings might substantially lower the incidence of VTE.

PO 285

Audit of completion of compulsory documented risk assessment for venous thrombosis

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Background: Venous thromboembolism (VTE) is historically a poorly managed risk in hospitalised patients and it is responsible for the deaths of an estimated 60,000 people in the UK each year. In order to reduce the risk of VTE, the Belfast Health and Social Care Trust introduced a thromboprophylaxis policy in December 2011 which gave detailed guidance on appropriate VTE risk assessment for all hospitalised patients. This policy is in conjunction with a standard printed VTE risk assessment which has been part of the hospital drug kardex since 2010. In March 2012, we initiated auditing of the completion of the trust VTE risk assessment and have continued auditing on a monthly basis since then. We started our audit in one hospital base within the trust and have since progressed the audit to involve all four hospitals within the Belfast Trust, encompassing 2111 inpatient beds.

Aims: The overall aim of our audit is to increase the completion rate of VTE risk assessment within the Belfast Trust. We have set our target completion at 95%.

Methods: Three auditors collected data on a monthly basis. Every inpatient drug kardex was reviewed for completion of the VTE risk assessment. Data was collated and written feedback was given to each ward within the hospital regarding their current performance.

Results: At the start of the audit, the completion of VTE risk assessment at site one was 53.9%. After 9 months of auditing and feedback, the completion has improved to 72%. At site two, initial completion was 48.5%. This has only improved to 51.3% following 4 months of auditing and feedback. The completion rate for site three, which is the regional elective orthopaedic unit, was initially 58.2% and this has dramatically improved to 91.8% after 4 months of auditing. At site four, which is in the early stages of audit, the initial completion of 40.6% has improved to 43.6% after 2 months of audit.

Conclusion: Since the introduction of the VTE risk assessment and Trust Policy, the completion target of 90% has only been achieved at one hospital site within the Belfast Trust. The completion rate at the remaining three sites is improving, but at a much slower rate. Our audit is on-going on a monthly basis at all sites and we hope to see continued improvement in all hospitals within the trust through feedback to wards and heightened awareness of the need for VTE risk assessment.

PO 286

Is MTHFR 677 genotyping a link that can be missed from the chain of assessment of the venous thrombosis risk?

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Background: Venous thrombosis is common cause of morbidity and mortality in most developed countries. It may develop as a consequence of numerous inherited and acquired risk factors, and a sinergy of these factors further increases the risk of this disease. Efficient recognition and management of risk factors for venous thrombosis are very important in reliable risk stratification, and recommendations about analyses available for detecting risk factors that would respect cost-benefit aspects are necessary for routine clinical practice with individuals already affected by the thrombotic process and for prevention of this disease.

Aim: The aim was to clarify whether determination of homocysteine or methylenetetrahydrofolate reductase (MTHFR) 677 genotyping has a greater clinical significance in the daily practice of preventing venous thrombosis and its complications.

Methods: A total of 110 subjects were enrolled. Among them 55 venous thrombotic patients (case group, mean age 41.24 ± 12.13 years) and 55 healthy controls with no history of any thrombotic event (control group, mean age 42.47 ± 10.92). Patients and controls were age and sex matched. Plasma homocysteine concentration was determined using a fluorescence polarization immunoassay method. Hyperhomocysteinemia was defined as plasma homocysteine levels above 12 μM. Detection of the MTHFR 677 C or MTHFR 677 T allele was performed through real-time PCR on ABI PRISM 7000 Sequence Detection System instrument, using the assay for allelic discrimination. Statistical analyses were performed using the Statistic 10 software. Informed consent was taken from the subjects. An institutional committee had approved the study protocol.

Results: Statistically significantly higher mean homocysteine concentration was found in cases compared to in controls (12.94 ± 5.08 μM vs. 9.72 ± 3.82 μM; $P < 0.001$), and there was significantly higher incidence of hyperhomocysteinemia in cases (63.6% vs. 18.2%; $P < 0.001$). Comparison of subjects with idiopathic venous thrombosis and those with secondary venous thrombosis did not show a significant difference in relation to homocysteine levels (13.47 ± 6.85 μM vs. 12.46 ± 2.73 μM; $P = 0.980$). On the basis of the results of genetic testing for mutations in the MTHFR 677 gene, cases and controls were classified into homozygous carriers of the mutation (677 T/T), heterozygous carriers (677 T/C) and non-carriers (677 C/C). There were no significant differences in homocysteine concentrations between the three case subgroups (13.02 ± 2.09 μM vs. 12.55 ± 5.98 μM vs. 13.84 ± 6.50 μM; $P = 0.169$) as well as between the three control subgroups (9.21 ± 2.37 μM vs. 10.07 ± 4.90 μM vs. 10.13 ± 2.58 μM; $P = 0.706$). Also, there were no significant differences in incidence of three genotypes between cases and controls ($P = 0.650$). However, comparison of the levels of homocysteine between the subgroups of patients and subgroups of healthy controls showed that there is no significant difference in homocysteine levels in the 677 T/T carriers in case group compared to 677 T/T carriers in control group (13.84 ± 6.50 μM vs. 10.13 ± 2.58 μM; $P = 0.114$), while there is significant difference between 677 T/C carriers in case group and 677 T/C carriers in control group (12.55 ± 5.98 μM vs. 10.07 ± 4.90 μM; $P = 0.014$), and between non-carriers among patients and non-carriers among controls (13.02 ± 2.09 μM vs. 9.21 ± 2.37 μM; $P < 0.001$).

Conclusion: Our results suggest that determination of plasma homocysteine concentration has a much more significant clinical role in the prevention of venous thrombosis compared to MTHFR 677 genotyping.

PO 287

Factor VII levels and genetic polymorphisms in severe preeclampsia

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Background: Preeclampsia (PE) is a syndrome characterized by the onset of hypertension and proteinuria in normotensive pregnant women, after the twentieth week of pregnancy. The PE is associated with a hypercoagulable state even more evident that the physiological condition of pregnancy. The actual model accepted for the blood coagulation process detaches the tissue factor (TF) as the essential factor to trigger the coagulation cascade pathway, which leads to fibrin blood clot formation. When vascular damage occurs, blood is exposed to TF that binds to factor VII (FVII), activating it (FVIIa). The TF/FVIIa complex activates directly factors X and IX, resulting in thrombin generation and consequently in fibrinogen cleavage into fibrin monomers. Previous studies suggest a relation between FVII gene polymorphisms, -402GA and -401GT, and its plasma levels.

Aim: The aim of this study was to evaluate FVII plasma levels, the FVII gene polymorphisms and the occurrence of severe PE.

Methods and Results: A group of 94 pregnant from Brazil with severe PE were evaluated. Plasma levels of FVII were not significantly different when compared the genotypes AA X AG X GG regarding the -402GA polymorphism ($P = 0.078$) and the genotypes GG X GT X TT in -401GT polymorphism (0.082). The correlation between the polymorphisms -402GA and -401GT and FVII plasma levels was also not significant ($P = 0.144$ and 0.053, respectively).
Summary/Conclusion: The results demonstrated that there isn't an association between reduced levels of FVII and the presence of alleles -402G and -401T in pregnant with severe PE.

PO 288

Investigation on expression pattern of VKORC1 in mouse brain

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Background: The role of vitamin K is well-known in the coagulation cascade. Additionally, vitamin K plays also a role in bone metabolism and cell cycle regulation. Vitamin K dependent (VKD) proteins are classified according to their structure and homology: proteins belonging to coagulation (factor II, VII, IX and X, protein C), proteins important for cell cycle (growth arrest protein 6 (Gas6) and protein S), calcium-binding proteins (osteocalcin and matrix gla protein, MGP) and proteins with unknown function (proline-rich gla proteins 1 and 2 and transmembrane gla proteins 3 and 4). Biological activation of VKD proteins depends on posttranslational γ-carboxylation by γ-glutamyl carboxylase (GGCX). Vitamin K is reduced to hydroquinone by the enzyme VKORC1 (Vitamin K epoxide reductase complex, subunit 1). Hydroquinone then serves as a cofactor for GGCX, rendering VKD proteins biological active. Thereby, hydroquinone is oxidized to epoxide which is reduced by VKORC1 back to the hydroquinone form of vitamin K (known as vitamin K cycle).
Aims: The function of vitamin K in blood coagulation is well established. However, the role beside coagulation is less understood. The analysis of mRNA expression of VKORC1 in brain could clarify the overall knowledge of vitamin K.
Methods: A probe with a length of 558 bp from murine VKORC1 cDNA was cloned into the pBluescript SK II + vector comprising T3 and T7 RNA polymerase promoters. By *in vitro* transcription DIG-labeled RNA probes were generated. *In situ* hybridization (ISH) was performed using paraffin mouse brain sections.
Results: We examined the expression pattern of VKORC1 in brain of 11 days (P11) old mice by ISH. VKORC1 mRNA was present in the Purkinje cells of the cerebellum and in the pyramidal neurons of the hippocampus.
Conclusion: Based on our results we suggest that VKORC1 is expressed in mouse brain and an additional function of vitamin K beside coagulation should be taken into account. Therefore, further studies are needed to study the detailed role of VKORC1 in the context of brain development and function.

PO 289

Establishment of a reference interval for canine FVIIa level in healthy client owned dogs

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Background: Administration of recombinant human activated FVII (rhFVIIa) is efficacious and safe for treatment of FVII deficiency as well as inhibitor complicated haemophilia A and B. Dogs are phenotypically appropriate models for human FVII deficiency and haemophilia research, and administration of rhFVIIa has proven effective in dogs. Still, little is known about normal canine FVII and FVIIa levels in healthy dogs.
Aims: Establish a reference interval for activated canine FVII clotting activity (cFVIIa:Clot) based on samples from a population of healthy normal dogs.
Methods: Stabylite plasma from 41 healthy dogs of different breeds, ages and sexual status, were analysed in a species specific FVIIa:Clot assay, with all canine reagents. A standard curve was generated with recombinant activated canine FVII (rcFVIIa) and used to convert clotting times to FVIIa concentrations. The reference interval (RI) was calculated using The Reference Value Advisor.
Results: The 95% RI for cFVIIa:Clot was 1.4–29 ng/ml, mean 5.3 ng/ml, similar to what has previously been reported for human FVIIa:Clot at 4 ng/ml (0.5–8.4 ng/ml).
Conclusions: The results demonstrate that dogs have a similar FVIIa level to humans, which further supports the dog as a valuable animal model for FVIIa research.
This study was approved by the Companion Animal Ethics and Administrative Committee at the DepT. of Veterinary Clinical and Animal Sciences, University of Copenhagen, and by the Novo Nordisk Ethical Review Committee. For all dogs recruited the owners provided a signed consent form.

PO 290

Congenital factor VII deficiency and surgery in a single center experience

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Background: Congenital factor VII (FVII) deficiency is a rare hemorrhagic disorder that can cause excessive bleeding during and after surgery in affected patients. The recombinant form of activated factor VII (rFVIIa, NovoSeven^{R} from Novo Nordisk, Bagsvaerd, Denmark), which was developed as a second-generation bypassing agent, has recently been used in the management of bleeding for patients with congenital FVII deficiency.
Aims: We review the results of surgical procedures in patients with congenital FVII deficiency at our center and describe the safety and effectiveness of surgery with rFVIIa or with an antifibrinolytic agent without rFVIIa.
Methods: We reviewed the results of nine surgical procedures in six patients with congenital FVII deficiency at the Kyung Hee University Hospital at Gangdong, Seoul, Korea, between January 2008 and December 2012. We administrated rFVIIa preoperatively in seven patients and postoperatively in six patients. We administrated rFVIIa pre-and postoperatively. FVIIa was administrated every 4 h with the first dose being administered 0.5 h preoperatively at least 48 h with dose 15–30 /kg. After 48 h, the frequency was reduced according to the patient's clinical status. FVII levels were measured before testing and then again after the intravenous administration of routine dose FVIIa.
Results: Between January 2008 and December 2012 at our center, nine operations were performed successfully and no complications were observed in the six patients with congenital FVII deficiency. The median age of the patients was 10 years (range, 2–37 years). The median level of FVII activity was 2% (range, 0.6–7%). Four major orthopedic procedures, 1 tonsillectomy, 1 hydrocelectomy and 3 dental extractions were performed. The median duration of hospitalization was 6 days (range, 0–15 days). rFVIIa was administered at all procedures, except the dental extraction that was performed using only antifibrinolytic agents without any replacement. FVII levels were very various

depending on each individual. No bleeding or thrombogenic complications were observed in any case.

Conclusion: Patients with congenital FVII deficiency who require surgery can be treated efficiently and safely with rFVIIa or antifibrinolytic agents. rFVIIa was well tolerated and maintained effective hemostasis and showed good clinical outcome after the major surgery. Knowledge of the patients' previous bleeding history and their response to rFVIIa should be helpful in managing bleeding during surgery in patients with factor VII deficiency.

PO 291

Pharmacodynamic effects of escalating dosages of a new recombinant human Factor VIIa (LR769) in congenital hemophilia A or B patients

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Background: Congenital Hemophilia A and B treatment may be complicated by the development of inhibitors to the deficient factor. In such cases factor replacement becomes ineffective, and use of a bypassing agent is needed to treat bleedings or in the peri-operative period. A recombinant form of activated Factor VII has been available for this purpose. In this study, the pharmacodynamic effects of a new recombinant human Factor VIIa (LR769) produced by LFB/rEVO Biologics are tested.

Aims: The objectives of the study are to assess the pharmacodynamic effects over time of LR769 after a single dose of 25, 75, and 225 μg/kg in hemophilia A or B patients with or without inhibitors.

Methods: Two sites in the USA and 1 in the Netherlands are participating. The study was approved by the IRB/EC of the institutions and performed consistent with the Declaration of Helsinki. Informed consent was obtained prior to any study activities. It is an open label, randomized, cross-over study. Fifteen adult hemophilia A or B patients with or without inhibitors will be treated in three dosing cohorts. Each patient is treated in two cohorts on two separate occasions, according to one of three sequences: 25 and 75 μg/kg, 25 and 225 μg/kg, or 75 and 225 μg/kg. Each dose cohort consists of 10 administrations.

Serial sampling to obtain plasma for performing several pharmacodynamic parameters are taken at baseline and up to and including 24–36 h after administration. Assays performed include a) Thrombin generation assay output (at variable assay conditions and made sensitive to variations at high doses), including lag time, time to peak, peak, and endogenous thrombin potential; b) activated partial thromboplastin time; c) prothrombin time; d) Prothrombin fragments 1 + 2; e) D-dimer; f) thrombin-antithrombin complex; g) thromboelastometry using RoTEM, including evaluation of lysis.

Results: The first cohort has been successfully treated without any safety issues, and dosing is continuing. At the time of the conference the evolution over time of the pharmacodynamic parameters will be presented.

Summary: In order to provide hemophilia patients with inhibitors additional therapeutic options, a new recombinant human FVIIa (LR769) is being developed. This study provides important information in the target population on the pharmacodynamic effects of LR769 in order to determine the appropriate dose and frequency of administration for further clinical efficacy studies.

PO 292

Pharmacodynamic effects of two recombinant FVIIa products in anticoagulated healthy volunteers

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Background: For many years, recombinant human activated coagulation factor VII (rhFVIIa) has been used for the treatment of haemophilia A or B patients with inhibitors to factor VIII or IX. The effectiveness of treatment is determined by assessment of bleeding cessation, however, a lack of a clear correlation between pharmacodynamic (PD) and pharmacokinetic (PK) parameters hinders dose optimisation and development of new rhFVIIa products. Therefore PK and PD properties of two rhFVIIa products (LR724 and eptacog alfa) were assessed in healthy volunteers, who were pre-treated with fondaparinux to induce an anticoagulated state. LR724 is a novel rhFVIIa product under development.

Methods: This double-blind clinical study was approved by an independent medical ethics committee and informed consent was obtained from all participants. Thirty-six healthy male subjects were dosed in a randomised fashion to placebo ($n = 6$), 15, 45 or 90 μg/kg rhFVIIa (6/0:6/6:6/6, LR724/eptacog alfa). Subjects received 5 mg fondaparinux subcutaneously 2 h prior to intravenous bolus administration of study drug. FVIIa activity and antigen, thrombin generation (TGT), coagulation times, and markers of coagulation activation were assessed regularly up to 24 h. Based on the observed effect profiles, a PK-PD model for PT and TGT lag time was developed with NONMEM.

Results: Both products induced a dose-dependent increase in FVIIa activity; up to 1000-fold baseline at 90 μg/kg. Apparent half-life ranged from 1.9 to 2.8 h. Reversal of the fondaparinux-induced anticoagulant state was observed: significant reductions for TGT lag time (−15.9% and −20.9% for 45 and 90 μg/kg) and related parameters (time to peak, velocity index) and increase of AUC and peak value, PT (−21.8% and −26.0% for 45 and 90 μg/kg) and APTT (−5.6% at 90 μg/kg). Prothrombin fragment 1 + 2 (+96.0%) and TAT complex level (+83.9%) were only significantly increased at the highest dose level of rhFVIIa. D-dimer and fibrinogen levels did not change significantly.

The FVII activity time-course followed 2-compartment kinetics, and was modelled, including endogenous FVII activity as a cosine function of clock time, fitted to data from the placebo group. This PK model was incorporated into a maximum effect PK-PD model to describe the effects on PT and TGT lag time. For PT a flat baseline time-profile was used and for TGT lag time a fondaparinux (anti-Xa) concentration-dependent component. Adding a 1-compartment PK model for anti-Xa data from the placebo group resulted in a robust model for the interaction between fondaparinux and the effects of the two products on TGT lag time.

Conclusion: By applying a fondaparinux challenge, pharmacodynamic effects of LR724 and eptacog alfa could successfully be evaluated and modelled in healthy volunteers. This clinical study demonstrated that PT and TGT lag time are good markers to accurately assess the relation between rhFVIIa dose level and procoagulant effect. Simulations with this model may guide future drug development, and demonstrated that repeated administration of both 45 and 90 μg/kg rhFVIIa at a 2- or 3-h interval would result in a maximal pharmacodynamic effect.

PO 293

The association of FXIII val34leu polymorphism with cerebrovascular thrombosis in Iranian patients

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Background: A G to T substitution in exon 2 of the FXIIIA gene results in the replacement of Valine with Leucine in amino acid 34. It has been suggested that this substitution (FXIII Val34Leu polymorphism) would be a predisposing factor for intracerebral hemorrhage or a protective factor against venous thrombosis. Furthermore, FXIII Val34Leu polymorphism has a significant heterogeneity among different population.

Aim: An investigation into the role of FXIII Val34Leu polymorphism in cerebrovascular thrombosis in Iranian patients using a case-control study.

Method: Thirty-three patients with cerebrovascular thrombosis who were admitted to Iranian Blood Transfusion Organization (IBTO) thrombosis and hemostasis laboratory were selected as case group. The control group were consisted of 100 age and sex matched healthy subjects. FXIII Val34Leu polymorphism were genotyped by using polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) method. Cfo1 was used as the restriction enzyme.

Results: No significant differences was observed between the prevalence of Val/Val (wild type) genotype in case (75.8%) and control groups (64.0%). On the other hand, the prevalence of FXIII Val34Leu polymorphism was not significantly different in cases (24.02%) and controls (36%) with a Pv of 0.151 and odds ratio of 0.569 (95% confidence interval: 0.233–1.392).

Summary/Conclusion: According to the similar results in both groups, we concluded that the presence of FXIII Val34Leu polymorphism is not associated with cerebrovascular thrombosis in Iranian population.

PO 294

The evaluation of cases with Factor X deficiency in Southern East of Turkey

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Backgrounds and Aim: Factor X (FX) is the component of both extrinsic and intrinsic coagulation cascade and is the first enzyme of the common pathway which results in thrombus. Congenital FX deficiency (FXD), an autosomal recessive inherited disease, is an extremely rare coagulation defect with only 50 cases of congenital FXD been reported in the world literature so far, with the incidence of 1:500,000–1,000,000 in the general population. In this study we aimed to investigate the clinical and laboratory data of the patients diagnosed with FXD applied to our clinic with bleeding diatesis.

Methods: The files of the 15 patients (7 female, 8 male) diagnosed and followed-up for FXD within the last 4 years were evaluated retrospectively. The patients' age, gender, presenting complaints, physical examination findings, factor levels, the clinical follow-up results and treatments were recorded.

Results: The mean age of the patients was 29 months (min-max:1–144 months). The most presenting complaints were easy bruisability (n = 8; 53%) and epistaxis (n = 8; 53%). The other presenting complaints were gum bleeding (n = 5; 33%), hemarthtosis (n = 5; 33%), subcutanous hematoma (n = 4; 26%), oral mucousal bleeding (n = 3; 20%), hematuria (n = 1; 6.6%), gastrointestinal bleeding (n = 1; 6.6%), respectively. FX leveles were < 1% in 6 patients, 1–5% in 4 patients, > 5% in 5 patients. Heparin added-PCC was used for prophlaxy (n = 11; 73%). Any treatment related complication was not observed.

Conclusions: The clinical features in patients with FXD is independent from factor levels. There is not any single prophylactic scheme for all patients. Heparin added-PCC can be used safely for effective prophlaxy. We suggest that family history is important when considering prophlaxy and in patients with life-threatining bleeding or with FXD sibling the prophlaxy should be introduced in the early course.

PO 295

Safety and pharmacokinetics of three doses of a new recombinant human Factor VIIa (LR769) in congenital hemophilia A or B patients

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Background: Congenital Hemophilia A and B treatment may be complicated by the development of inhibitors to the deficient factor. In such cases factor replacement becomes ineffective, and use of a bypassing agent is needed to treat bleedings or in the peri-operative period. A recombinant form of activated Factor VII has been available for this purpose. In this study, a new recombinant human Factor VIIa (LR769) produced by LFB/rEVO Biologics is tested. The goal is to provide patients with Hemophilia A and B who develop inhibitors a cost-effective alternative treatment option.

Aims: The objectives of the study are to assess the safety and pharmacokinetic (PK) properties of LR769 after a single dose of 25, 75, and 225 μg/kg in hemophilia A or B patients.

Methods: Two sites in the USA and 1 in the Netherlands are participating. The study was approved by the IRB/EC of the institutions and performed consistent with the Declaration of Helsinki. Informed consent was obtained prior to any study activities. It is an open label, randomized, cross-over study. Fifteen adult hemophilia A or B patients with or without inhibitors will be treated in 3 dosing cohorts. Each patient is treated in 2 cohorts on 2 separate occasions, according to one of three sequences: 25 and 75 μg/kg, 25 and 225 μg/kg, or 75 and 225 μg/kg. Each dose cohort consists of 10 administrations. Safety assessments include physical examinations, hematology and biochemistry labs, adverse events, anti-rhFVIIa antibodies and antibodies to host related impurities. PK samples to determine FVIIa activity (with a modified clotting method insensitive to TFPI) are taken at baseline and up to and including 24–36 h after administration.

Results: The first cohort has been successfully treated without any safety issues, and dosing is continuing. At the time of the conference full safety (on repeat exposure) and PK data will be available and presented.

Summary: In order to provide hemophilia patients with inhibitors additional therapeutic options, a new recombinant human FVIIa (LR769) is being developed. Safety and PK data that will be presented will provide the basis for further clinical efficacy studies.

PO 296

Extracorporeal shockwave lithotripsy (ESWL) in a patient with congenital fator VII deficiency and von Willebrand disease

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Background: Congenital factor VII deficiency is a rare autosomal recessive bleeding disorder, which prevalence is estimated in 1:500,000. Von Willebrand disease is the most common inherited coagulopathy,

with an incidence of 1%, and is one of the most frequent causes of bleeding in women with menorrhagia. An important complication of ESWL is perirenal/intrarenal hemorrhage, described in up to 20%-25% of the cases. We report the management of the ESWL procedure in a patient with association of these two coagulopathies.
Case report: A 30-year-old woman with von Willebrand disease and congenital FVII deficiency had symptomatic nephrolithiasis. Her complaints were: oral bleeding, menarche with excessive menses and necessity of blood transfusion, vaginal hemorrhage after the second delivery, and other episodes of metrorrhagia related to uterine myomas, controlled with hormonal contraceptives. Laboratory data showed prolonged PT (19%) and APTT (R = 1.38), FVII:C = 3%, FvW:Ag = 38%, FvW:RCo = 20%, RIPA = normal, FVIII: C = 41%. DDAVP test: adequate responsiveness to desmopressin. Ultrasonography of urinary tract: image compatible with stone in the right renal pelvis of 1.2 cm. The patient received one dose of rFVIIa (20 mcg/kg) and DDAVP (0.3 mcg/kg) before ESWL. rFVIIa was maintained at the same dose, 4/4 h for 24 h and 6/6 h in the second and third days; daily dose of DDAVP (0.3 mcg/kg) was continued for 2 days. rFVIIa was guided by testing FVII, and was 135% in the third day after ESWL. The patient developed mild hematuria for 4 days, as expected in these procedures, and low back pain requiring oral analgesia for 3 days. Ultrasonography after 1 month from the procedure showed no stones and the patient was asymptomatic.
Summary: There are few studies regarding ESWL in patients with coagulopathy in the medical literature, since this treatment option is contraindicated in the presence of uncorrected bleeding disorders. These studies address individuals with hemophilia and vonWillebrand's disease, but none of them reported patients with congenital factor VII isolated or in association with other coagulopathy. In this case, the best treatment option for nephrolithiasis was ESWL, whose stone-free rate described for calculi diameter of 2 cm or less is about 66% to 99%. Moreover, the percutaneous nephrolithotomy adds greater risk of complications. It is known that the level of factor VII is not a good predictor of bleeding and prior hemorrhagic history should be considered. Despite the history of bleeding of the patient reported, which could generate some doubts, low levels of factor VII and the procedure with great potential for bleeding justify the prophylactic use of rFVIIa. As the best of our knowledge, this is the first case report of a patient with factor VII deficiency and von Willebrand disease undergoing to ESWL whose management with DDAVP and rFVIIa resulted in good clinical course, with no abnormal bleeding.

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PO 297

Risk factors most predictive of venous thromboembolism among hospitalized children

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Background: Venous thromboembolism (VTE–deep vein thrombosis and pulmonary embolism) is rare in children and associated with hospitalization. VTE causes an increase in morbidity, mortality, hospital length of stay, and cost. Several risk factors for VTE in children have been described, yet those most predictive of VTE are ill-defined. A better understanding of which risk factors are most predictive of VTE may allow for identification of high risk patients, and application of selective thromboprophylaxis among those patients at greatest risk.
Aims: (i) Ascertain which among 96 putative VTE risk factors in hospitalized children ages 0–18 are most predictive of clinically overt 90 day VTE. (ii) Describe the predictive ability of these retained risk factors in the same cohort retaining only those risk factors meaningfully additive in enhancing risk prediction.
Methods: All hospitalizations to a pediatric hospital between 1 January 2005 and 31 December 2010 were identified. The electronic medical record was searched for ICD-9 codes and procedures demonstrative of clinically overt VTE and putative VTE risk factors. Logistic regression with applied weighted retention modeling placing value on retaining the fewest risk factors most predictive of VTE was employed. Bootstrap analysis provided an unbiased estimate of predictive performance.
Results: Among 93,742 admissions, 783 VTE events (0.84%) occurred. Those risk factors retained as predictive of 90-day VTE varied by age group, and are ordered from most to least predictive. Among ages 0–1 year old (214/25,483; 0.84%) ICU stay, prior VTE, hypercoagulability, peripherally inserted central venous catheter (PICC), congenital heart disease, elevated Charlson Comorbidity Index (CCI) and chronic respiratory failure (CRF) were predictive with an area under to receiver operating characteristic curve (AUC) of 0.88. In ages 1–14 (387/55,085; 0.7%) CCI, ICU stay, prior VTE, hypercoagulability, PICC, central venous catheter, thrombocytosis, and obesity were retained with an AUC = 0.85. In ages 14–18 (182/13,174; 1.38%) risk factors of elevated CCI, IUC stay, prior VTE, height, cancer congestive heart failure, sepsis, hypercoagulability, L-aspiraginase therapy and PICC were associated with an AUC = 0.85. Among all children ages 0–18 (783/93,742; 0.84%) ICU stay, CCI, prior VTE, PICC increasing weight, hypercoagulability, and cancer were most predictive with an AUC = 0.85.
Summary: VTE among children is rare and most prevalent in the youngest and teenagers. Identifying the risk factors most predictive of VTE can aid in the identification of high risk patients. Next steps might include prospective validation of VTE risk assessment models in children, and investigating the benefit of selective thromboprophylaxis among children at highest risk for VTE.

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Non-catheter deep venous thrombosis in pediatric patients: data from a single hospital

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Background: Although the incidence of deep venous thrombosis has increased over the last decade, non catheter-related (NC-DVT) is uncommonly seen in childhood and adolescence.
Aim: To determine the demographic, clinical and treatment of NC-DVT in children in a single centre.
Methods: Clinical data were retrospectively reviewed of all DVT admitted to our service from 2000 through 2012 and analysed NC-DVT.
Results: One hundred and eighteen pts were admited in our service (91 pts with catheter-related DVT and 27 with non catheter related thrombosis – the mean age was 12 year [range 1–15 years] Site of thrombosis were: femoral 17, jugular 4, popliteal 3, inf cava 2, subclavia 1). Diagnosis was made using venous compression ultrasonography Doppler and TC SCAN. The most frequent underlying clinical condition were: infections 14 pts (sepsis 5, abscess 3, otomastoiditis 2, osteomyelitis 1, supurative adenitis 1, varicella 2. Staphylococcus aureus was the predominant pathogen 6 pts (43%). Other risk factors associated were malignancy 3 pts (ALL 1, NBL 1) autoimmune disease 4 pts, nephrotic syndrome 2 pts, burn 1 pts. Only 3 pts (all adolescences) had spontaneous deep thrombosis: 1 Homozygous V Leiden factor, 2 negative thrombophilia screen: Prothrombin 21210, MTHF, factor V Leiden, PAI, lupus anticoagulant, protein C, protein S. antithrombin, antiphospholipid antibodies. Only 2 pts had positive familiar history of venous thrombosis. Anticoagulation began with unfractionated heparin 2 pts, LMWH 25 pts followed by anticoagulation with acenocumarol. LMWH anti Xa level was obtain in 12 pts and adjust level of 0.5–1 U/ml. Post- thrombotic syndrome was seen in pts 26% of children.
Conclusions: Non catheter deep venous thrombosis in children is usually multifactorial, in associations with underlying disease. The strongest risk factors in our patients was infections. In our population spontaneus deep thrombosis was rare 11% of the NC-DVT and 2.5% of all patints included with deep venous thrombosis. The mainly group risk was present always on adolescents.

PO 300

Successful bivalirudin use in a child post liver transplant

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Background: Thrombosis of the hepatic and portal arterial/venous system is a severe complication associated with high morbidity following liver transplant in children < 10 kg. To prevent or treat thrombosis unfractionated heparin (UFH) is the anticoagulant used due to the short half life (1–2 h). The Stollery Anticoagulation guideline post liver transplant suggest 10 u/kg/h UFH, and in the absence of bleeding, increasing to age appropriate dosing. Effect is monitored using the aPTT and anti-Xa level. When UFH resistance occurs, and measured heparin effect is minimal, despite doses exceeding 35 u/kg/h, there are few available alternatives in children. Bivalirudin, a direct thrombin inhibitor, has advantages, as demonstrated in adults, including short half life (25 min), no reliance on hepatic metabolism or antithrombin (AT) levels, monitoring using the aPTT, and renal clearance. Unfortunately, there is little safety and efficacy data in children, with a few case reports and one dosing study published and there is lack of reversibility. However, the use of bivalirudin in children who have challenges with UFH anticoagulation, including heparin resistance and low levels of antithrombin, may provide more consistent anticoagulation.
Aim: To describe the first use of bivalirudin as thromboprophylaxis in a child post liver transplant.
Methods: A 14 month old male child with maple syrup urine disease (branched chain ketoaciduria) who underwent a cadaveric donor liver transplant.. Pre transplant thrombophilic testing including antiphospholipid antibody, lupus anticoagulant, Factor V Leiden, prothrombin gene 20210, Protein C, S, and AT was normal. Post-transplant he was started on UFH 10 u/kg/h post transplant and increased to 20 u/kg/h. Eight days post transplant, the child developed HAT, AT and anti-Xa levels were 0.5 u/ml and 0.1 u/ml, respectively. One month later, the child was retransplanted with a cadaveric pediatric graft. In the first 48 h, two episodes of HAT requiring intraoperative tPA to clear the thrombus occurred. Following these events, UFH dosing was maximized (up to 36 u/kg/h) with AT concentrate administered when the level was < 0.4 u/ml. Despite adequate AT levels, the UFH doses were as high as 30 u/kg/h and unable to achieve an anti Xa of 0.35 u/ml. Consequently, 3 weeks post 2nd transplant, a bivalirudin infusion of 0.6 mg/kg/h was started 2 h after UFH was discontinued aiming to maintain the PTT 60–80 s. Informed consent and ethics approval were obtained.
Results: A maximum of 6 mg/kg/h (avg 3–4 mg/kg/h) achieved the desired PTT. After 3 weeks of this regimen, the child was stable, free of thrombosis and was transitioned to age appropriate enoxaparin for 1 year.
Summary/Conclusions: Bivalirudin is an alternative to UFH in children following liver transplant who are heparin resistant, have low AT levels and do not have severe renal failure. Further studies to determine safety and efficacy are urgently required.

PO 301

A case of extensive recalcitrant IVC thrombosis in a teenager with Behçet disease

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Background: Behçet is a rare immune-mediated relapsing multisystem vasculitis most commonly presenting during the 3rd–4th decade of life. Prevalence ranges depending on geography (1/15,000–500,000). Behçet's is characterized by a triad of uveitis, recurrent oral and genital ulcers and is diagnosed mainly by clinical criteria. Vascular involvement may occur in 40% with a potential dismal outcome. Vasculitis with associated thrombosis is more common in the venous system of the lower extremities followed by the vena cava. Although Behçet's has variable manifestations, venous thrombosis is not common in the pediatric population.
Case Report: Seventeen year-old African-American male presented with 1½ months of worsening fatigue and subsequent shaking chills, night sweats and weight loss. He was admitted to a local hospital with extensive evaluation during a 2 week hospitalization revealing elevated inflammatory markers (ESR > 140 mm/h; CRP > 80 mg/L), nodular pulmonary infiltrates, complete thrombosis of common iliac veins, inferior vena cava (IVC) with thickened walls, and renal veins. After transfer to a tertiary center on warfarin, MRV confirmed extensive venous thrombosis with potential vasculitis, and extensive collaterals. Based on pustulosis at IV sites, acneiform rash, scrotal ulcer, history of recurrent oral ulcers, he was diagnosed with Behçet's and began high dose methylprednisolone with resolution of systemic symptoms within a few days. He underwent successful catheter directed thrombolysis (EkoSonic Device and tPA) with stent insertion in both iliac veins. Shortly thereafter he experienced acute IVC obstruction with significant LE edema that resolved after stent insertion at the level of a narrowed possibly hypoplastic hepatic IVC. Due to a Heparin dependent antibody ELISA (1.068 OD), Argatroban was utilized. Post thrombolysis, an Argatroban dose up to12 mcg/kg/min was required; dose prior to thrombolysis was 1.5 mcg/kg/min. Serotonin release assay was negative and he was discharged on enoxaparin with targeted

levels at the high therapeutic range. With steroid therapy inflammatory markers normalized. Five months later, with rising inflammatory markers (ESR 86 mm/h, CRP 46 mg/L), he complained of left LE symptoms and was found to have recurrent iliac vein and IVC thromboses. Repeated thrombolysis with stent insertion was performed due to severe residual stenosis. A few weeks later he reported worsening symptoms requiring repeated thrombolysis of left iliac veins. IVC imaging revealed narrowing without occluding thrombus. Post thrombolysis, Argatroban was utilized again requiring high doses; thereafter he was maintained on Enoxaparin and 81 mg aspirin daily. One week later the left iliac veins were re-thrombosesd without clinical symptoms; no intervention was performed. Currently he is maintained on Enoxaparin and 162 mg aspirin daily. Behçet's is managed with Infliximab, Methotrexate and steroids.

Summary:

1 Extensive thrombosis in pediatric patients with systemic symptoms should prompt consideration of vasculitis.
2 Control of vasculitis is critical to VTE outcome in Behçet's.
3 Aggressive anticoagulation post-thrombolysis is required with concurrent active vasculitis.
4 Observed IVC stenosis could be either congenital or resulting from vasculitis.
5 Post-thrombolysis, Agatroban dosing may significantly increase, perhaps due to higher accessible thrombin loads.
6 A false positive HIT ELISA may be observed during acute inflammation.

PO 302

Splenic infarction in a teenager associated with oral contraceptives, elevated lipoprotein a, and median arcuate ligament syndrome

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Background: Etiologies of splenic infarcts in adults are diverse, and include hematologic disorders (benign/malignant), hypercoaguable states (including oral contraceptives and lupus anticoagulant), embolic disorders (including paradoxical emboli), vascular disorders, collagen/autoimmune vascular disease, trauma and operative. The majority in adults are related to hematologic malignancies/myeloproliferative diseases or thromboembolic conditions. However, in pediatric patients splenic infarct in the absence of a hemoglobinopathy is rare, and etiologies differ compared to the adult population.

Case Report: A 14-year-old female Caucasian athletic teenager experienced sudden middle-left upper abdominal pain which resolved spontaneously, but recurred approximately three times the following week. Due to worsening consistent pain, she was evaluated and found to have elevated WBC (18.8 K/mcL) prompting CT scan that demonstrated a large splenic infarct. Ultrasound Doppler revealed a wedge shape infarct with patent splenic artery/vein. The patient utilized combined oral contraceptive for menometrorrhagia for 1½ years. Hematology evaluation 2 weeks after initial symptoms revealed an asymptomatic female with ESR 45 mm/h, CRP 2.5 mg/dL, D-Dimer 2127 ng/mL, platelet count 879 K/mcL, factor VIII (FVIII) activity 192%. Further evaluation revealed normal/negative; echocardiography, hemoglobin electrophoresis, ANA, rheumatoid factor, JAK 2, PNH, amylase and IBD markers. Thrombophilia work-up revealed elevated fasting lipoprotein a (182 nM) and compound heterozygous MTHFR mutation with normal homocysteine level. FVL, P20210, protein C, protein S, antiphospholipid antibody and ATIII were normal/negative. Approximately 1 month from initial symptoms, acute abdominal pain prompted repeat imaging for recurrence and complications including pseudocyst, abscess or rupture. CTA for vasculitis/vascular anatomy demonstrated abnormal inferior hooked shaped narrowed celiac artery with mild post stenotic dilatation. Ultrasound Doppler revealed high velocity in celiac axis and low resistance wave form at hepatic/splenic arteries. The patient had a very soft low-pitched epigastric bruit. The patient was treated with 81 mg aspirin daily.

Summary:

1 Thromboembolism in pediatric patients may result from combined risk factors. This rare case demonstrates splenic infarct with combined oral contraceptives, an elevated LPa and abnormal vasculature. This report emphasizes a variety of critical factors:
2 Combined oral contraceptives represent well-documented thrombotic risk factor; this risk decreases with increasing length of therapy but is not negligible.
3 LPa has gained increased awareness as a thrombotic risk factor in pediatric patients, but treatment remains without consensus. Niacin and/or aspirin have been reported in specific cases, yet concerns regarding niacin and stroke exist.
4 Although celiac artery thrombosis was not detected, the combination of median arcuate ligament syndrome and splenic infarct should raise the possibility of thromboembolism.
5 We suggest that splenic infarct evaluation should include early vascular anatomic imaging to assist in diagnosis and consideration of thromboembolism.
6 Compound heterozygous MTHFR mutation with a normal homocysteine did not contribute to this patient's risk.
7 This report provides additional important information to the few published articles addressing splenic infarction and thromboembolism, celiac artery thrombosis and oral contraceptives use, and median arcuate ligament syndromes in pediatrics.

PO 303

Inherited antithrombin deficiency: description of a pediatric series in a single center

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Inherited antithrombin deficiency (IAD) is associated with increased risk of arterial and venous thrombosis in children and adults. Antithrombin (AT) deficiency is classified depending on the phenotype, defined by plasma AT activity and antigen levels, as types: I (quantitative) or II (qualitative): IIa (reactive center defects), type IIb (heparin binding domain defects), and type IIc (pleiotropic defects). Very few clinical and laboratory data on IAD in neonates and children have been reported.

Objectives: To describe the clinical manifestations, phenotype and genotype analysis, treatment and outcome in a series of neonates and children with IAD evaluated in a single center. From March 1992 to March 2012, 613 patients (pts.) younger than 18 years old with objectively confirmed arterial and/or venous thrombosis were studied for inherited and acquired thrombophilia. Asymptomatic children with a family history of IAD were also evaluated. Patients with low AT activity in plasma were classified according to AT antigen levels. AT activity levels were measured by chromogenic amidolytic methods, using bovine factor Xa and thrombin-based assays. Antigen concentrations were tested by Laurell rocket electrophoresis and immunoturbidimetric test. Progressive anti-factor-Xa activity assay was utilized to identify type IIb IAD. Age-related local reference values were used. Molecular analysis of AT gene (SERPINC1) was performed at the Mayo Clinic College of Medicine (Rochester, USA).

Seven children from 6 unrelated families were diagnosed with IAD. Six children had a family history of thrombosis. Four patients (2 girls/2boys, age range 21 days-11.6 years) had thrombosis in one or more sites: intracardiac thrombosis, 1pt.; renal veins, 2 pts.; inferior vena cava, iliac and femoral veins, 2pts.; pulmonary embolism, 1 pt.; and arterial ischemic stroke (AIS), 1pt.. Infection was the only risk factor detected in 2pts. No pt. had catheter related thrombosis.

Type I IAD was detected in 2pts: 1pt. was heterozygous for SERPINC1 R129X, FV Leiden and PT20210 mutations while no AT

mutations were identified in a 6-year-old girl with AIS. Type IIb IAD, AT Budapest 3, was detected in 2pts. from Gypsy families, one of them was heterozygous and had S.*pneumoniae* sepsis before the first thrombotic event, and the other was homozygous and presented perinatal renal vein thrombosis with extension into inferior vena cava. In the three asymtomatic patients, type I IAD heterozygous for a new mutation (p.Lys71LysfsX113) was found in a 16.8 year-old adolescent and type IIa IAD heterozygous AT Cambridge I was detected in two 3.8 year-old twin girls. The therapy used was: AT concentrate, heparin and indefinite vitamin K antagonists (VKAs), 3pts; tissue plasminogen activator, 1pt; aortic thrombectomy, 1pt and aspirin, 1pt. All the pts. are alive. One pt. presented severe post-thrombotic syndrome, 1pt. developed unilateral renal atrophy, and 1pt. with renal impairment required kidney transplant and had recurrent thrombosis while receiving VKAs.
This is one of the largest pediatric series of IAD. The clinical manifestations and evolution were highly heterogeneous. A novel mutation was detected. The long-term follow-up of these patients with IAD variants, along with phenotypic and genotypic characterization, would allow for evaluation of their thrombotic risk.

PO 304

The assessment of risk factors for rhrombosis in children

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Background: Thrombosis occurs much less frequently in children than in adults.
Aims: We tried to estimate the risk factors for thrombosis in pediatric population.
Methods: We analyzed 70 cases (32 boys, 38 girls) in children referred to the Department in 2007–2012 due to thrombosis. All patients were assessed: age at onset, location and cause of the clot. All analyzed patients underwent screening for congenital thrombophilia.
Results: Age of thrombosis was from the first week of life to 17 years, median 6.5 years. Arterial thrombosis occurred in 11 (16%), venous in 59 (84%) patients. Thrombi were localized mostly in the deep veins of the extremities (n = 27, 38%) and venous sinuses central nervous system (n = 15, 21%). The most common causes of thrombosis were: congenital thrombophilia (n = 27, 39%%), infection (n = 15, 21%), surgery (n = 14, 20%), thrombocythemia was found in 2 (3%) patients. Two patients had hyperhomocysteinemia. Six (8%) patients had no underlying cause thrombosis. The patients with congenital thrombophilia (n = 27, 39%) had the following defects: protein S deficiency in 7 (26%), protein C deficiency in 4 (15%), antithrombin deficiency in 7 (26%), prothrombin gene mutation in three patients (11%), and mutation of the factor V Leiden in 6 (22%) patients. Eight patients with thrombophilia performed an additional risk factor for thrombosis.
Conclusions: The most common location of thrombosis in children are deep veins of the limbs. The most common causes of thrombosis in this age group are: inherited thrombophilia, infection and surgery. In all paediatric patients with thrombosis diagnostic tests in the direction of congenital thrombophilia are indicated.

PO 305

Clinical utility of age specific natural anticoagulant levels in children with thrombosis

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Background: Normal healthy children have lower levels of natural anticoagulants such as antithrombin (AT), protein C (PC), and protein S (PS) when compared to adults. Further decline in levels of natural anticoagulants is commonly seen in pediatric patients with thrombosis. It is therefore important to know the role of decreasing levels of natural anticoagulants in predicting thrombosis in children. The use of age appropriate, highly specific cut-off levels for AT, PC, and PS may assist the physicians simplify clinical decisions about additional confirmatory tests.
Aims: Our aims were to define highly specific cut-off values for natural anticoagulants in two groups of children aged < 10 years and 10–18 years (group 1 and group 2, respectively), and to assess the predictive ability of these cut-off levels to identify patients with thrombosis.
Methods: Retrospective review of charts from 2004 through 2010 was performed after institutional review board approval for patients on whom physicians requested a hypercoagulation panel in our pediatric tertiary care children's hospital. Three hundred and sixty-five patients were identified, and their AT, PC, and PS levels were extracted from the laboratory database. Receiver operating characteristic (ROC) analysis was done to define specific cut-off values for AT, PC, and PS levels with a high specificity of 90%. Positive likelihood ratios (LR+) were calculated to quantitate the probability of thrombosis in pediatric patients.
Results: Among 365 patients (age range: 3 days to 17 years), 182 (48.2%) patients had confirmed thrombosis. There were 174 patients (46.6%) in group 1 and 191 patients (53.4%) in group 2. The medians and interquartile ranges of the anticoagulant levels in the two groups were as follows: AT [99% (76–118) vs. 101% (89–112), P = 0.48], PC [78% (52–101) vs. 104% (85–133), P < 0.001], and PS [75% (59–89) vs. 80% (63–104), P = 0.03]. In group 1, cut-off levels for AT, PC, and PS were 56%, 34%, and 35%, respectively at 90% specificity. In group 2, it was found to be 86%, 73%, and 49% for the same specificity. The LR+ for group 1 were 1.3 for AT, 1.8 for PC, and 1.4 for PS that translated into an increase in the post-test probability of the presence of thrombosis by about 10%. For group 2, the LR+ were 9.1 for all the three laboratory variables that translated into an increase in its probability by 45%.
Conclusions: In children aged less than 10 years, a significant decrease of the natural anticoagulant levels from the age specific normal levels increase the probability of the presence of thrombosis by only about 10%. However, in children aged 10–18 years, a minimal decrease in their levels increases the probability by about 45%. Therefore, a fall in natural anticoagulant levels strongly predicts thrombosis in older children between 10 and 18 years of age. Physicians may consider additional testing to confirm thrombosis for this subset of pediatric patients in a hospital setting.

PO 306

Antiphospholipid antibodies in neonates and babies with clinically significant thrombosis

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Background: We note increase frequency of thrombotic events among newborns and infants. There are some condition in structure of prothrombotic factors in this group, including such common factors as premature, infections, blood vessel catheterisation, low level of antithrombin, protein S and protein C. However sometimes there is prognostically significant factor – rise of level of antiphospholipid antibody (APA). Children with high level of APA may need in long-term treatment with anticoagulants.
Aims: To estimate frequency of increasing APA in children, who had thrombotic events under 1 years old. To monitor APA titer during next few years.

Methods: We observed 42 children suffer from thrombotic events during first year of life. All episodes were confirmed with ray-path methods (ultrasound, CT, MR). Primary diagnostic furthermore includes determination of antithrombin, protein C, protein S level, lupus anticoagulant (LA). Concentrations of antibody to cardiolipin, phospholipids, betta-2-glycoprotein-1 were measured twice, with 3–4 month intertrial interval. During catamnesis all rates measured 1–4 times a year.
Results: Our group consist of 19 female and 23 mail. Six of them were premature newborns. First event of thrombosis until the age 1 month was in 12, between 1 and 3 month – 10, between 4 and 6 month – 4, and from 6 to 12 month – 16. Twenty children had venous thrombosis, 3 – arterial thrombosis, 15 – ischemic stroke, 2 – intracardiac, 1 – intracranial venous sinus. Level protein C less 30% had 2, protein S less 30% – 1, antithrombin III less 30% – 1. Relationship between catheter and thrombosis was in 19, infection as underlying condition was found in 20. Eight children had high level of APA in first detection. High level of APA revealed in 5 (11.9%) children at the second detection. Ten and more time increasing level of APA (antibody to betta-2-glycoprotein-1) fined in 2; 2–10 time increasing observed in 2; one patient had 1,5 time increasing of APA. LA was positive in 2. In 1 year of observation, level of APA had impaired in all patients. It become normal in one, and stays significantly higher in other.
Summary/Conclusions: Amount of rise level of APA in children, suffer from thrombotic events during first years of life is not so rare condition. These parameters must be checked in such patients.

PO 307

Neonatal cerebral sinus venous thrombosis associated with congenital chylothorax and congenital nephrosis: report of two unusual cases

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Background: In neonates, cerebral sinus venous thrombosis (CSVT) represents a serious thrombotic condition which is often multifactorial. Early diagnosis and timely management is increasingly considered to influence outcome as anticoagulant therapy prevents propagation of thrombosis.
Aim: We report CSVT presenting in association with two rare neonatal disorders: congenital bilateral chylothorax and congenital nephrosis.
Methods/Results: The first case is a full-term male infant who was found to have congenital bilateral chylothorax which required chest tube drainage. A routine head ultrasound (HUS) showed thrombosis of the superior sagittal sinus prompting CT venography (CTV) revealing extensive deep and superficial venous system thrombus formation (Images A & B). The second case is a male infant born at 34 weeks of gestation who was found to have dysmorphism and intra-uterine growth restriction. He subsequently suffered acute cardio-respiratory decompensation and was found to have congenital nephrosis as well as grade 4 bilateral intraventricular hemorrhage (IVH) on HUS. A subsequent head MRV and CTV demonstrated left transverse sinus thrombosis (Images C & D).
Discussion: Although the risk of systemic thrombophilia has previously described in both these conditions, there have not been any reports of neonate CSVT in association with congenital chylothorox to date and only one published case available in the context of congenital nephrotic syndrome. The exact mechanism of thrombophilia is poorly understood but it is plausible an acquired hypercoagulable state resulting from protein loss and likely volume depletion were responsible for the development of CSVT in both cases. The clinical threshold for suspecting CSVT should be low in any neonate presenting with seizures with a medical condition that results in loss of natural physiological anticoagulant factors and fibrinolytic factors.

PO 308

Analysis of different mechanisms through hydrogen sulphide (H2S) would inhibit platelet aggregation

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Background: Hydrogen sulphide (H_2S) is the most recent of the gasotransmitters to be identified and investigated. H_2S is an important endogenous modulator, which exhibits beneficial effects on the cardiovascular system. However, the mechanisms involved in the different effects on platelets pathways are still unclear.
Objective: In order to investigate H_2S inhibitory pathways on platelet function we performed several tests: the opening of KATP-channels, the cGMP and cAMP accumulation, fibrinogen binding (B-Fg) and Ca^{2+} influx.
Methods: Tests were performed using platelet rich plasma (PRP) (300×10^9 plat/L). Sodium hydrogen sulfide (NaHS) was used as H_2S donor and 100 μM glibenclamide (Glib) as a selective K + -channel blocker. Samples were preincubated with buffer, 100 mM glib (during 30 min) at 37 °C, then with or without NaHS (1, 5 and 10 mM) for 2 min before adding 2 μg/mL collagen as agonist. The expression of fibrinogen binding (B-Fg) and Ca^{2+} influx were assessed by flow cytometry using mean fluorescence intensities. cGMP and cAMP accumulation by RIA were measured as pmol/10^6plat. Student's *t*-test control vs. NaHS.: * = $P < 0.05$, ** = $P < 0.01$.
Results: H_2S significantly inhibited platelet aggregation ($P < 0.01$) induced by 2 μg/ml Col. Glib failed to block the inhibition induced by NaHS. The accumulation of cGMP and cAMP in presence of NaHS have no statistical difference 10 ± 2 vs. 9 ± 1 and 5 ± 1 vs. 4 ± 2 respectively. Levels of B-Fg (126 ± 12 vs. 15 ± 5)** were inhibited in the presence of H_2S, and Ca^{2+} influx was unaffected.
Conclusion: Platelet aggregation induced by collagen was significantly inhibited in a concentration-dependent by NaHS. The test with Glib ruled out the role of KATP channel in H_2S-induced antiaggregation. The accumulation of cGMP and cAMP confirmed that these are not the mediators of the inhibitory effect of NaHS.
This result, together with a significant blocked of B-Fg levels, suggest the inhibition on platelet function would be through another signaling pathway, such as CalDAG-GEFI/Rap1.Thus, H_2S may constitute a novel target for antithrombotic drug development.

PO 309

Platelet activation and abnormal Ca2+ homeostasis in women with gestational diabetes mellitus

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More than 50% women diagnosed with gestational diabetes mellitus (GDM) will develop type 2 diabetes mellitus in their future life. Platelets from patients with type 2 diabetes show abnormalities in intracellular Ca^{2+} homeostasis that are involved in platelet hyperaggregability and the development of thrombotic complications. The aggregational activity of platelets can be changed due to various factors, including reactive oxygen species (ROS). The aim of this study was to investigate the effect of H_2O_2 on aggregation and Ca^{2+} mobilization in human platelets from pregnant women with GDM and Type 1 diabetes mellitus (DM) *in vitro*.
We compared 36 pregnant women with GDM (24.68 ± 5.12 years), 14 pregnant women with Type 1 DM (24.31 ± 3.68 years), 24 healthy pregnant women (HPW) (23.96 ± 5.18 years) and 15 healthy non-pregnant women (HNPW) (23.82 ± 4.58 years). There were no significant differences in fasting glycaemia (5.38 ± 0.41) mM for GDM (6.27 ± 0.21) mmol/for pregnant women with Type 1 DM

(4.61 ± 0.17) mM for HPW and (4.82 ± 0.14) mM for HNPW (or in glycated hemoglobin levels (6.78 ± 0.21%, 6.94 ± 0.13%, 5.96 ± 0.56% and 5.83 ± 0.61% accordingly). Platelet aggregation and disaggregation was determined by light transmission using computerized analyzer of platelet aggregation. Intracellular Ca^{2+} concentration in the platelets was determined using the fura 2-AM.

The platelet aggregates produced under the influence of H_2O_2 are unstable, destroyed spontaneously, and the aggregation is replaced by disaggregation. In this study where gestational diabetic and Type 1 DM pregnancies compared to normal pregnancies, the disaggregation at the pregnant women with GDM and Type 1 DM was lower 1.2 and 1.3 times than in the control group, respectively. The resting platelet cytosolic Ca^{2+} concentration in group of pregnant women with GDM did not vary in compared with HPW. When platelets were treated with 1 μM thapsigargin (TG), a specific inhibitor of SERCA, plus ionomicin (50 nM), there was a transient increase in cytosolic Ca^{2+} concentration due to release followed by Ca^{2+} extrusion. Since the peak response was not significantly different in all groups of subjects, the amount of Ca^{2+} accumulated in the stores must be similar. However, the rate of decay cytosolic Ca^{2+} concentration to resting levels was significantly slower in platelets from pregnant women with GSD. Immediately after the addition to the platelets of H_2O_2, the intracellular concentration of Ca^{2+} did not change (the lag-period), but subsequently its concentration increased and then decreased to the basal level. The release of intracellular Ca^{2+} upon H_2O_2 stimulation was at the same level in all groups of women, but after the addition of 2 mM $CaCl_2$ to H_2O_2 stimulated cells the increase of intracellular Ca^{2+} concentration were greater in the pregnant women with GSD with respect to the healthy pregnant women.

We conclude that H_2O_2 was likely involved in the enhancement of intracellular Ca^{2+} concentration observed in platelets from pregnant women with GDM and Type 1 DM, which might lead to platelet dysfunctions. H_2O_2-dependent enhanced Ca^{2+} entry in diabetic donors might be explained by an increased amount of H_2O_2 or a greater sensitivity to oxidation by this ROS in diabetic platelets.

PO 310

Detection of high platelet reactivity induced by release reaction by light transmission aggregatometry and the ways to prevent it

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Background: High platelet aggregation plays a key role in thrombosis. The reveal of high platelets activity can help to prevent thrombotic complications by prophylactic therapy. Broad spectrum of methods proposed to solve this problem. We suggest the simple use of light transmission aggregation (LTA) for detection of high level of platelets aggregation induced by release reaction.

Aims: The aim of our study was to evaluate platelets features in atherosclerosis patients that can lead to additional risk of thrombotic complications and to fin the ways of its diminution.

Methods: Platelets aggregation in 724 patients (pts) with stable ischemic heart disease due to CAD and 36 healthy subjects (HS) was studied by simultaneous analysis of the mean aggregate size changes and light transmission (LTA) using an aggregation analyzer BIOLA (Russia). ADP at doses 1.0 and 5.0 μM was taken as an agonist. Platelet microaggregates were assessed by scanning electron microscopy. All patients did not receive any antiplatelet therapy before platelets investigation for at least 2 week.

Results: In 29 from 724 CAD pts platelets 1.0 μM ADP induced aggregation was significantly elevated and biphasic (48 ± 14.2% vs. 5.2 ± 2.8% in HS $P < 0.01$) during first 5 min of registration and didn't decrease in time. Second wave started after 2 min and quickly reaches a maximum as a result of intraplatelet agonists secretion. In most of CAD pts ($n = 695$) 1.0 μM ADP aggregation was 9.7 ± 5.4% without second wave and generally with disaggregation. 5.0 μM induced ADP aggregation was 53.2 ± 14.1% in this group vs. 56.8 ± 15.4% in other 29 pts (normal range 25–68%, $P = 0.3$) without second wave and disaggregation. In 29 pts spontaneous aggregation was recorded by LTA method and its level was the same as if it was ADP induced but started at 9th-14th min (45.7 ± 12.3%). In other CAD pts and HS spontaneous aggregation was not defined by LTA. In whole blood of all 29 pts were detected microaggragates contained from less than 10 platelets. In blood of the rest pts and HS microaggragates were absent. In 23 from 29 pts were neurological symptoms (12-dizziness, 4-depression, 8-cephalalgia, 1-TIA). Aspirin therapy decreased platelet aggregation to normal ranges in 25 pts and was ineffective in 4 pts in whom clopidogrel was medication of choice. In pts who were taking antiaggregant therapy microaggregates disappeared and the symptoms reduced.

Summary/Conclusions: high platelet aggregation is closely associated with risk of unfavorable thrombotic events at CAD pts. The appearing of microaggregateds in whole blood worsens microcirculation and lead to neurological symptoms. One of possible reasons is revealed by us release reaction, which induce not only the growth of agonist induced aggregation but also to spontaneous aggregation phenomenon. In most such cases aspirin normalized platelets status and clopidogrel can be a good substitution in cases of aspirin inefficiency. LTA is a good sensitive method in such situation, but the time of registration must be prolonged up to 20 min.

PO 311

Impedance aggregation comparing three different anticoagulant matrices performed on whole blood samples from healthy subjects

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Background: Whole blood platelet aggregation is measured on the Multiplate® analyzer (Roche Diagnostics) using impedance aggregation. The Multiplate® uses a test cell containing two pairs of electrodes made of highly conductive silver-coated copper. Activated platelets adhere and aggregate on the metal sensor wires causing increased electrical resistance. The increase of impedance is transformed to arbitrary aggregation units (AU) and plotted against time. The overall platelet activity is expressed by the parameter area under the aggregation curve (AUC), which is affected by the total height of the aggregation curve as well as by its slope.

Aim: To determine the effect of three different anticoagulants, which can be in use in different laboratories, for whole blood platelet aggregationusing seven different agonists to determine ranges appropriate for each anticoagulant.

Methods: Adult subjects ($n = 67$) were determined to be healthy and free from any anti-platelet medication for at least 10 days via a questionnaire assessment. Blood from the same healthy subjects was prospectively collected under informed consent in three different anticoagulant matrices: NaCitrate (sodium citrate, 3.2%), LiHeparin (lithium heparin, 75 USP units) and hirudin, 15 μg/ml). Platelet count was verified to be $> 100 \times 10^9$/L. Agonists were randomized and whole blood platelet aggregation was performed on the Multiplate analyzer within three h of sample collection. Agonists tested were ADP adenosine-5′-diphosphate (ADPtest), arachidonic acid (ASPItest), collagen (COLtest), thrombin receptor activating peptide (TRAP), ristocetin high dose (RISTO Hi), ristocetin low dose (RISTO Lo), and prostaglandin E1 reagent and adenosine-5′-diphosphate (ADP HS).

Results: The mean and ± 2 standard deviation (SD) AUC range was calculated for each agonist and anticoagulant combination. The results

are expressed below as 'mean U (range U)' for each combination. ADPtest (final concentration of 6.5 μM) with hirudin yielded 93.4 (55.4–131.2), LiHeparin yielded 102.6 (65.0–140.2), and NaCitrate yielded 80.5 (43.9–117.1). ASPItest (final concentration of arachidonic acid 0.5 mM) in hirudin resulted in values of 108.5 (68.6–148.3), with LiHeparin 112.7 (71.5–153.9), and with NaCitrate 75.8 (46.6–105). For Coltest (final concentration 3.2 μg/ml), hirudin yielded 86.8 (53.6–120), LiHeparin yielded 94.3 (58.3–130.3), and NaCitrate yielded 64.5 (39.5–89.5). TRAP (final concentration 32 μM) with hirudin resulted in values of 129.9 (83.7–176.1), with LiHeparin 129.4 (87.2–171.6), and with NaCitrate 104.5 (68.7–140.3). Ristocetin was tested in high and low concentrations (0.77 mg/ml and 0.2 mg/ml). Results for each concentration, high and low respectively, were as follows: with hirudin 117.1 (53.3–180.9), 13.1 (0–37); with LiHeparin 113.1 (63.5–162.7), 25.0 (5.4–44.6); with NaCitrate 92.6 (36.4–148.8), 11.2 (2.4–20.0). Only hirudin and LiHeparin were used in the ADP HS measurement (final concentration of 9.4 nM), with hirudin yielding 79.9 (35.1–124.7) and LiHeparin yielding 96.4 (49.8–143).

Summary and Conclusions: The mean results from identical samples treated with each agonist using different anticoagulants resulted in different ranges. For all agonists, 3.2% NaCitrate consistently yielded lower values, while LiHeparin yielded higher values. It is important for laboratories to utilize ranges that reflect the anticoagulant used in testing. Having improper ranges can result in possible misdiagnosis of patients.

PO 312

Changes in platelet aggregation after impact with vipoxin and its componends isolated from Vipera ammodytes venom

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Background: The neurotoxin vipoxin, isolated from the venom of *Vipera ammodytes meridionalis*, consists of basic, highly toxic phospholipase A_2 (PLA_2) and an acidic and non-toxic component acting as an inhibitor of PLA_2.

Aim: Objective of our study is to track the response of platelet aggregation in the presence of vipoxin and phospholipase A_2 in different concentrations, initiated by inducers, adenosine diphosphate (ADP), collagen and arachidonic acid (AA).

Methods: Vipoxin and its components were isolated from Bulgarian *Vipera ammodytes meridionalis*.

The turbidimetric method of Born (1962) was followed using a dual channel Lumi-aggregometer 700, Chronolog Corp, USA. Human platelet-rich plasma (0.45 ml) maintained at 37 °C was preincubated with vipoxin and PLA_2 for 2 min in a siliconized glass cuvette with constant stirring. The aggregation was initiated by adding ADP 10 μM, collagen 2 μg/ml and AA 0.5 mM and was traced out within 6 min.

The concetrations of vipoxin were 30, 50, 70, 90 μg/mL and a concentrations of PLA_2 were 15, 25, 35, 45 μg/mL.

Results: Different mechanisms of inhibition of platelet aggregation by PLA_2 toxin could be involved and tested in the process depending on the inductor used.

We found the inhibitory effect of phospholipase A_2 on platelet aggregation initiated by ADP, collagen and AA. The use of collagen and AA, inducing inhibition of platelet aggregation reaches 100% at the highest concentration of PLA_2 (45 μg/mL). Platelet aggregation in the presence of vipoxin is not affected and utilizing a three inducers.

Conclusion: The PLA_2 inhibits agonists-induced platelet aggregation in a dose-dependent mode. The results showed that the enzyme renders different effect according to inducers applied.

PO 313

New approach to determination of the intravascular platelets activation in patients with ischemic heart disease

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Background: Atherothrombotic processes and tromboishemic complications which dominate among the cardiovascular diseases are the leading cause of the patients with ischemic heart disease (IHD) death. However, the atherothrombosis diagnostics is often carried out not in the early stages, but in the late stages of its development, when we have the apparent clinically tromboishemic complications and acute cardiovascular events.

The goal of our study was to identify the diagnostic criteria for patient's stratification on the groups with thrombogenic risk on the basis of the overall evaluation new and previously known markers of atherothrombosis. This will improve the quality of early diagnostics of atherothrombosis, especially in patients with asymptomatic IHD.

Methods: Two hundred and fifteen people were studied: 144 patients with unstable angina, 49 individuals with stable angina and 22 healthy volunteers. The study included: electrocardiographic monitoring, echocardiography, 6-min walking test and coronary angiography. Laboratory studies included: blood test, measurement of cardio specific enzymes level, biochemical blood analysis with the definition of inflammation markers (C-reactive protein), levels of homocysteine, myeloperoxidase and endothelin-1. Platelet aggregation properties were studied by turbidimetric method on platelet aggregation analyzer with adenosine diphosphate disodium salt acid at the concentration of 0.5 and 2.5 μM. Hemostasis and coagulation system of natural anticoagulants were tested on hem coagulator. Morphometric characteristics of platelets – average platelets volume (APV), platelet width by volume distribution (PWD) were determined by the automatic cytoflowing hemoanalyzer. To verify the morphometric characteristics of platelets, which play an important role in atherothrombosis and in the development of tromboishemic complications in patients with IHD, we carried out a qualitative and quantitative evaluation using the atomic force microscopy (AFM). Specially developed computer algorithms were used for the estimation of the platelets state. All investigations were carried out initially and from 3, 6 and 12 months.

Methods: Using AFM were determined that the average height of platelets, the average height of clusters (aggregates) and the percentage ratio of pathologically active cells ($P = 0.04$) in patients with unstable angina is significantly greater than in patients with stable angina and in healthy person. This is indicates that there is an intravascular platelet activation. In patients with unstable angina the correlation between APV and the increase (%) abnormal platelet activity ($r = 0.71$), between APV and an average height of plate cells ($r = 0.52$) were significantly positive. The correlation of morphometric characteristics of platelets (APV, PWD) defined in the general blood analysis with morphometric parameters, verified by AFM, as well as the initial level of myeloperoxidase, homocysteine, antithrombin III, D-dimer, fibrinogen and von Willebrand factor was revealed. This indicates that AFM data may be used as diagnostic criteria of thrombogenic risk and repeated coronary events in patients with IHD.

Conclusions: The most informative diagnostic criteria are the platelet morphofunctional parameters such as APV, PWD the degree of adrenaline-induced platelet aggregation, the average height and the number of abnormal platelet activity identified by AFM, as well as markers of endothelial dysfunction, which is an independent predictor of unfavorable outcomes in patients with unstable angina.

PO 314

Inhibition of Platelet aggregation in Dengue infection does'nt correlate with anti DV-NS1 and anti PDI

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Background: Dengue infection is one of the major public health problems in Indonesia. The molecular mimicry between Non Structural 1 (NS-1) Dengue Virus to auto antigen on the platelets surface is currently the most evaluated pathomechanism of platelet function disorder.
Aims: In this study, we observed the correlation between anti NS-1 and anti PDI antibodies against platelets function disorder on secondary Dengue infection.
Methods: Fifty patients with secondary DV infection according to WHO criteria were observed by a cross sectional study. Patient's blood was collected on day 3, 5 and 7 after fever onset. Platelets aggregation test was done to prove the possibility of platelets dysfunction. Anti NS-1 and anti PDI antibodies were determined by solid phase ELISA.
Results: The inhibition of platelets aggregation was increased among day of observation. Means value of inhibition on day 3 is 46.6%, day 5 is 52.5% and day 7 is 56%. There is a significant difference ($P < 0.05$) of inhibition of platelet aggregation value between days of observation. The antibodies against NS-1 DV and PDI were detected in all 50 sera with the positive rate of 90% develop NS-1 antibodies and 72% of PDI antibodies, on day 3 of symptoms. The highest OD of NS-1 antibodies is detected on the day 3 and decreased on day 7. The OD of PDI antibodies was increased on day 3 and still increasing on day 7. There is weak correlation between anti NS-1 antibodies and the inhibition of platelets aggregation ($r = 0.160$; $P = 0.267$). There is no correlation between anti PDI antibodies and the inhibition of platelets aggregation ($r = -0.118$; $P = 0.578$). There is a significant correlation between anti NS-1 and PDI antibodies ($r = 0.386$–0.490), while the differences of OD between observation days are not significant ($P > 0.05$).
Conclusion: This study shows the kinetics profile of NS1 and PDI antibodies responses, which were detected by the third day of symptoms. Dengue patients' sera also inhibited platelets aggregation. NS-1 antibodies and PDI antibodies might have a role on the platelets aggregation dysfunction; however statistically there is no correlation between them. It is possible that other mechanism involve in the inhibition of platelets aggregation.
Keywords: anti NS-1 antibodies, anti PDI antibodies, inhibition platelets aggregation, secondary Dengue Infection.

PO 315

Impedance aggregometric analysis of thrombocyte function in pooled platelet concentrates in relation to storage time

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Background: To treat hereditary or acquired thrombocytopenia/-pathy, transfusion of platelet concentrates is a common procedure in Anaesthesiology and Intensive Care Medicine. Concerning quality controls of platelet concentrates, testing of volume, platelet count and remaining leukocyte content after preparation is required by German Medical Association or NHS guidelines. Before transfusion a visual check (colour change, swirling) must be performed. At the end of storage life pH values as well as sterility have to be monitored. A routine analysis of thrombocyte function in platelet concentrates is not prescribed. Modern point-of-care devices allow a fast and reliable monitoring of platelet function and are widely used in clinical routine. Impedance aggregometry (multiple electrode aggregometry, MEA, Multiplate®, Roche AG, Grenzach, Germany) was developed for time-saving whole blood analysis and is increasingly distributed.
Aims: The aim of the present study was the implementation of impedance aggregometry for thrombocyte function in pooled platelet concentrates and to correlate MEA results with storage time.
Methods: A) After collection from pooled platelet concentrates from 2 to 4 days of age, samples were diluted by additive solution (Composol PS®, Fresenius Kabi AG, Bad Homburg, Germany). Calcium chloride was added to obtain the target calcium concentration. MEA was performed according to the manufacturer's specifications. ASPItest (0.5 mM), ADPtest (6.5 μM) and TRAPtest (32 μM) reagents were used as specific platelet activators.
B) Platelet concentrates of 5 to 12 days of age were frequently analysed by impedance aggregometry in the same manner. Furthermore pH values, pO_2, pCO_2 and glucose concentrations were evaluated at the point of Multiplate® measurement and correlated with impedance aggregometric results.
Methods: A series of dilution yielded steady impedance aggregometric results for a platelet count of 400 per nl and a calcium concentration of 5 mM. With respect to specific thrombocyte activators ASPItest and TRAPtest showed reliable results for aggregation.
A) For platelet concentrates from 2 to 4 days of age the area under the curve (AUC) was in the lower clinical reference range; however results did not differ significantly between time points. For TRAPtest a significant difference was detected between day 3 and 4 ($P = 0.035$, One Way ANOVA).
B) Analysis of platelet function from day 5 to 12 after preparation resulted in a significant decline of the AUC in ASPI- and TRAPtest on day 12 ($P < 0.001$, ANOVA on Ranks). Linear regression analysis showed weak correlation between AUC in Multiplate® and pH values or glucose concentrations respectively ($R_{pH/ASPI} = 0.351$, $R_{pH/TRAP} = 0.347$, $R_{glucose/ASPI} = 0.398$ and $R_{glucose/TRAP} = 0.405$).
Conclusion: The results of our study suggest that multiple electrode aggregometry is not only suited as a point-of-care device for testing platelet function in whole blood, but might also be a useful tool for monitoring thrombocyte function in pooled platelet concentrates.

PO 316

Increased platelet activity associates with clinical severity of ST-elevation acute myocardial infarction

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Background: Hemangioma, most common benign vascular tumor, has natural history of proliferation in the first year of life followed by spontaneous resolution. The treatment is not universal standard with indication as following: impaired organ function and Kasabach Merritt syndrome (KMS). Alpha-interferon inhibits angiogenesis, endothelial cell migration and proliferation.
Objective: To evaluate the outcome of alpha-interferon for the treatment of hemangioma.
Methods: We recruited 35 patients with hemangioma receiving alpha-interferon followed at hemangioma clinic between January 2001- January 2011.The patients' data including sex, age, type and location of hemangioma, indication, duration, outcome and complications of treatment were reviewed.
Design: Retrospective descriptive study.
Measurements: we evaluate of the outcomes including improvement of organ function, resolution of Kasabach Merritt syndrome (KMS) and decrease hemangioma size.
Results: the number of patients was 35 patients (female = 21, male = 14) with mean age 3.2 months (range 5 days-15 months), loss follow up in 5 patients. Hemangioma was divided into cavernous (9), capillary (10), mixed (7). Location of hemangioma includes head and neck (21), extremities (7), mediastinum (3), intraosseous (1), liver (2), larynx and epiglottis (2), and scrotum (1). Average age of treatment was 4.7 months (range 5 days-18 months). Duration of response in 29 patients was 4.7 months (range 1–6 months). Indications of treatment

included KMS (1), KMS with impaired organ functions (2), impaired organ functions (30), infection (2), intracranial (1). Eleven patients were decrease in size of mass > 50%, 14 patients were decrease in size < 50% but 23 patients (82.1%) were improved organ function and resolution of KMS. Complications of treatment were elevation of liver enzymes (3), and fever (6).

Conclusions: The use of alpha-interferon was well response to improve organ function and decrease hemangioma size. Due to spastic diplegia, serious complication of alpha-interferon from previous case report studies, the consideration of this treatment should be discussed with parent in light of pro and cons.

PO 317

Mechanisms for Ang-(1-7) on the expression of talin1 induced by Ang? in human umbilical vein endothelial cells

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Background: Renin-angiotensin system (RAS) is one of the most important regulation systems, which has played an important role on regulating physiological function, especially at cardiovascular and cerebrovascular diseases development. Integrin is a major receptor family present in the cell surface, which can induce the conglutination between cells and extracellular matrix, activated integrin $\alpha II_b\beta_3$ is mainly involved in thrombus formation through inducing platelets adhesion and aggregation. Talin 1, as the essential factor in the process of activating $\alpha II_b\beta_3$, is recently specially concerned. Previous work have proved that angiotensin-(1-7) [Ang-(1-7)] could significantly inhibit the experimental thrombosis induced by angiotensin II(AngII). However, whether Ang-(1-7) and AngII can affect thrombosis formation by means of regulating talin 1 has not been reported. In this study, we will explore the possible mechanism for the influence of Ang -(1-7) on the expression of talin 1 induced by AngII in endothelial cells.

Aims: Previous data demonstrated that Ang-(1-7) had an inhibititory effect on the talin1 expression induced by AngIIin human umbilical vein endothelium derived cell line (HUVECs). However, its mechanisms were not fully understood. The purpose of this study was to explore the inhibitory mechanisms.

Methods: HUVECs were cultured in DMEM. Talin1 antigen was measured by ELISA Kit. Talin1 mRNA was examined by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR). Samples were devided into groups at random as follows: control, AngII,Ang (1-7),PDTC,AngII+ Ang (1-7) and AngII+ Ang (1-7) +PDTC.

Results: Compared with control, PDTC, which is the inhibitor of NF-κB pathway, had no marked effects on talin1 antigen when used alone, but PDTC could significantly inhibited the effects of Ang-(1-7) on talin1 expression induced by AngII($P < 0.05$).

Conclusion NF-κB pathway participates in the inhibitory process that Ang-(1-7) inhibited the expression of talin 1 induced by AngII.

PO 318

Differential platelet activation: granule release without aIIbβ3 activation indicates platelet functions independent of aggregation

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Background: Platelets are appreciated by their crucial role in maintaining adequate haemostasis but they are also involved in wound healing, inflammation, angiogenesis, and atherogenesis via the release of cytokines, chemokines, and growth factors from their α-granules.

Aim: To investigate the regulation of α-granule release and opening of glycoprotein IIb:IIIa ($\alpha_{IIb}\beta_3$) on the platelet surface after stimulation of their thrombin receptors.

Methods: Activation of $\alpha_{IIb}\beta_3$ or P-selectin expression on platelets stimulated with increasing concentrations of PAR-1 agonist or PAR-4 agonist, in the presence or absence of $P2Y_{12}$ antagonists (clopidogrel, AR-C69931MX or apyrase), or in the presence or absence of the prostacyclin analogue iloprost was measured with flow-cytometry using antibodies against P-selectin and activated $\alpha_{IIb}\beta_3$. Platelet-leukocyte complexes were measured with flow-cytometry, release of soluble α-granule markers PF-4, β-TG, RANTES/CCL5 and PDGF-AB was measured with ELISA.

Results: PAR-1 or PAR-4 activation in the presence of $P2Y_{12}$ antagonism (AR-C69931MX) resulted in 84% P-selectin expression compared to stimulation in the absence of $P2Y_{12}$ antagonist, whereas $\alpha_{IIb}\beta_3$ activation was reduced to 12% ($P < 0.05$). P-selectin expression was also only slightly reduced when platelets were activated with PAR-1 or PAR-4 in the presence of apyrase or iloprost (with iloprost 79% vs. without iloprost 100%; $P < 0.05$) while under the same conditions $\alpha_{IIb}\beta_3$activation was almost completely prevented (with iloprost 7% vs. without iloprost 100%; $P < 0.05$). When blood was collected from patients on clopidogrel, P-selectin expression on their platelets was 90% of platelets of untreated individuals. However, their $\alpha_{IIb}\beta_3$activation was reduced to 29% ($n = 4$). PAR-1 or PAR-4 stimulation of whole blood in presence of AR-C69931MX resulted in the formation of platelet-monocyte complexes (115% compared to stimulation in the absence of AR-C69931MX), while these complexes only express 27% of the $\alpha_{IIb}\beta_3$activation observed after stimulation in the absence of AR-C69931MX ($P < 0.05$). Similar results were found for platelet-granulocyte complexes. No differences were found in the supernatant of PAR-1 activated platelets in the presence or absence of $P2Y_{12}$ antagonist for levels of PF-4, β-TG, RANTES/CCL5, or PDGF-AB. When platelets were stimulated with ADP in the presence of AR-C69931MX, α-granule release was blocked.

Conclusions: When the $P2Y_{12}$ pathway is blocked, or when platelets are inhibited by a prostacyclin analogue, PAR-1 or PAR-4 stimulation still induced α-granule release while their $\alpha_{IIb}\beta_3$ activation is blocked. We propose that this differential activation is physiologically important because it enables the platelets to fulfil their role in inflammation, angiogenesis and atherogenesis.

PO 319

Evaluation of activation states of small GTPases in patient platelets

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Background: Small GTPases are molecular switches of cellular processes that plays important roles in activated platelets. Rap1 is implicated in aggregation. Rab27 and Ral is in granule secretion. Rho is in shape change and Rac is in aggregation and granule secretion. Activation states of platelets are monitored by P-selectin and activated GPIIb/IIIa levels on platelet surface. However, it is possible that some of these GTPases are activated in platelets.

Aim: First, to establish the method for measuring activation states of small GTPases. Second, to elucidate the activation states of each small GTPases in various diseases.

Method and Results: First we developed the method for isolating platelets without activation by a gelfiltration method. Little activation was cinfirmed by FACS analysis for P-selectin expression levels. Then, we generated and purified proteins containing binding region of GTP-bound active GTPases from E coli and established so called 'pull-down assays' that allowed us to measure the activation states of Ras, Rap1B, RalA, RalB, RhoA, Rac and Rab27. After the study plan was approval by the University ethics committee, we started to measure the activation states of these GTPases in platelets of patients with and without pulmonary hypertension. In spite of small number of patients

analyzed, Ral could be highly activated among GTPases analyzed in patients with pulmonary hypertension.
Summary: We established the method to evaluate the activation states of sevelral GTPase in a real clinical setting. We could present clearer data by analyzing more patients.

PO 320

Necrotic cell material from human renal tubular cells dose dependently stimulate platelet activation, aggregation and platelet-leukocyte complex formation

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Introduction: Acute kidney injury (AKI) is often the result of ischemia reperfusion injury (I/R), which leads to a complex interplay between coagulation-, inflammation and cell death processes, typically resulting in renal tubular epithelial cell necrosis.
Platelets play a central role in coagulation and inflammatory processes and therefore might play an important part in the pathology of AKI. However, the role of platelets in AKI is not known to date. Necrotic cell material can function as Danger-Associated Molecular Patterns (DAMP's) and activate innate immune cells, leading to an excessive immune response and tissue damage.
Objectives: We aimed to investigate the effect of necrotic human tubular epithelial cells on platelets.
Method: Human platelets in platelet rich plasma (PRP) or whole blood were stimulated with necrotic cell material derived from a human proximal tubular epithelial cell line (HK2 cells) which were repeatedly freeze-thawed in liquid nitrogen. Platelet activation and platelet-leukocyte formation were measured by FACS analysis.
Platelet aggregation was determined using multiple electrode aggregometry (Multiplate).
Results: Necrotic cell material from human TECs dose dependently stimulate human platelets activation and aggregation in PRP. Furthermore stimulation of whole blood with necrotic TECs increases platelet-leukocyte complex formation.
Conclusions: Our data show that necrotic cell material from human tubular epithelial cells (TEC), dose dependently stimulate human platelet activation and aggregation. Furthermore necrotic TEC material enhances platelet-leukocyte complex formation. This indicates that locally released renal necrotic TEC material during renal I/R could activate platelets, leading to enhanced coagulation and inflammatory processes. This could result in further loss of renal cells and ultimately renal function during AKI.

PO 321

Comparison of different brands of agonists for light transmission aggregometry using a multichannel analyser

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Background: Light Transmission Aggregometry (LTA) is considered as the golden standard technique for evaluation of platelet function. The results of a worldwide survey on the assessment of platelet function by LTA showed the high variability in LTA practices (Cattaneo et al., JTH 2009). The SSC Platelet Physiology published a draft guideline after the SSC meeting in Cairo (2010) in order to standardize LTA. Furthermore, the CLSI guideline is also a useful tool in standardizing this method. The society of hematological laboratories in The Netherlands (VHL) has set a goal for 2013 and 2014 to reduce unnecessary variances in LTA results by investigating the present differences between LTA methods and by stimulating all hospital laboratories to uniform there pre-analytical and analytical variables.
Aims: One part of the Dutch LTA standardization procedure was to investigate the three most used companies in The Netherlands, who provide agonists for LTA for which the source and nature is often unknown to the users of the technique. *E.g.* different types of collagen are available of which only the fibrillary type extracted from tendons is supposed to be functional as platelet activator.
Methods: In this study different brands of collagen (Biodata, Chronolog, Horm: 2 and 5 μg/mL), ADP (Biodata, Chronolog, Sigma: 2 and 5 uM), arachidonic acid (Biodata, Hart, Sigma: 1 mM) epinephrin (Biodata, ABP, Chronolog: 5 uM) and ristocetin (Biodata, ABP, Chronolog: 0.6 and 1.2 mg/mL) were compared as agonists for LTA in healthy volunteers. All tests were performed on a PAP8e (Biodata) multichannel LTA analyzer. Six healthy volunteers refrained from smoking, eating and drinking coffee or tea for 2 h. Blood was drawn into 105 mM citrated and buffered saline using a needle of 21 gauge. Each agonist was used in relevant concentrations, conform the draft SSC guideline (Cairo, 2010) and used for analysis of thrombocyte function in platelet rich plasma (PRP) of six healthy volunteers. Platelet count of PRP was standardized to 200/nL and PRP was prepared by centrifuging blood samples at 200 g for 10 min at ambient temperature without using a brake. ANOVA with Bonferroni correction was used for statistical evaluation.
Results: The variation over the eight channels of the PAP8e was 9% for arachidonic acid (0.5 mM), 6% for collagen (5 mg/L) and 5% for ADP (1.5 μM).
The maximum amplitude of the aggregation response after agonist activation was comparable between the studied brands in case of epinephrine, arachidonic acid and ristocetin. ADP showed a non-significantly lower response for Chronolog as compared to BioData and Sigma at a concentration of 2 μM. At a concentration of 5 μM no difference was observed. In case of collagen, BioData showed a significantly ($P < 0.05$) lower response as compared to Chronolog and Horm at concentration levels of 2 as well as 5 mg/L.
Conclusions: The used formulation of collagen and ADP (only low concentration) should be considered when implementing LTA procedures and comparing LTA-results between laboratories.

PO 322

Technical validation of light transmission aggregometry using Chrono-Log, PAR8 and PAP8e

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Background: Light transmission aggregometry (LTA) is a common method used to assess platelet function. The method has never been standardized and a report (Cattaneo et al., 2009) from the platelet physiology subcommittee of the SSC of the ISTH showed a large diversity in LTA practices and advised a worldwide standardisation programme.
Aims: We compared three devices for light transmission aggregometry using fixed agonist concentrations recommended by the SSC-ISTH (Cairo, 2010). Several variables and parameters were studied: reagents from different providers, reproducibility (channels), reference values, difference between adjusted and non-adjusted platelet rich plasma (PRP), amount of patient plasma needed for testing as well as agonist volume, user-friendliness, software possibilities and financial aspects (costs of exploitation and device).
Material and Methods: We tested three LTA devices Chrono-Log 490-4D, PAR8 (Hart Biological) and PAP8e (Biodata) using 4 device-specific agonists (except for TRAP14): ADP (5 μM) (Chrono-Log,

HART, Biodata), ristocetin (1.2 μg/ml) (Chrono-Log, ABP, Biodata), collagen (2 μg/ml) (Chrono-Log for all) and TRAP 14 (10 μM) (Bachem for all three devices). Citrated blood (3.2%) was collected from 30 healthy donors not taking any medication that affects platelet function, 18 females (21–58 years) and 12 males (20–54 years) with comparable mean platelet counts 263 ± 59 × 10^9/L and 250 ± 40 × 10^9/L. LTA was tested in adjusted (250 × 10^9/L) and non-adjusted PRP (recommendations ISTH-SSC 2010). Since the majority of maximal aggregations of the agonists was not normally distributed, results were expressed as medians and 25% and 75% quartiles (inter-quartile range. IQR).

Results: Chrono-Log needed 400 μL PRP whereas PAR8 and PAP8e used only 200 and 225 μL PRP. The differences between the channels on each device is < 3.3%. Reference values for maximal aggregation with four agonists were comparable for Chrono-Log and PAR8. PAR8 reference values of non adjusted PRP were (expressed as median [25–75% IQC]): 78 (75–86) for 5 μM ADP, 88 (86–90) for 2 μg/ml collagen, 92 (90–94) for 1.2 mg/ml ristocetin and 87 (87–89) for 10 μM TRAP 14. PAP8e results were significantly lower (e.g. 20% lower for 2 μg/ml collagen). Adjustment of PRP had no significant effect on results of healthy volunteers except for TRAP. PAR8 software was less user-friendly than the PAP8e software. PAP8e was slightly less expensive (both device and exploitation costs) than PAR8. Chronolog is the most expensive.

Summary and Conclusions: Reference values for 30 healthy volunteers are very comparable for Chrono-Log and PAR8 and IQR intervals appear to be very narrow. PAP8e showed significantly lower maximal aggregation levels. For all three devices adjusted PRP was comparable to non-adjusted PRP. Variation between channels was low. Modern LTA devices used less patient sample and were cheaper in hardware and reagents.

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PO 323

Fractal and euclidian geometrical descriptors of platelet shape

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Background: Platelet shape is traditionally classified into morphological categories (e.g. discoid, round, dendritic). Fractal Hausdorff-Besicovitch dimension (FD) and circularity (C) are mathematical descriptors for the surface roughness and roundness of irregular shapes. They can be applied to the outlines of platelets automatically detected on microscopic images.

Aims: In order to describe platelet outline structure in a mathematical accurate way suitable for statistical analysis a pilot study was performed to analyze roughness, roundness, area, peri- and diameter of platelet outlines as well as number, length and width of filopodia per platelet.

Methods: Nine hundred and twenty-eight microscopic images from six PRP-samples were acquired right after preparation. Fractal dimension (FD), circularity (C), area (A) and perimeter (P) were determined for each of the 6629 platelet-outlines (PLO). Corresponding original images of every fifteenth PLO were randomly assigned for computer assisted manual measurement of platelet-diameter and number, length and basal and apical width of filopodia (1). Automated outline detection and statistical analysis was performed using Matlab R2012b (MathWorks Inc., Natwick, USA).

Results: Mean FD of all PLO was 1.305 ± 0.078 and mean C was 0.741 ± 0.025. Only scatterplot of fractal dimension (FD) vs. perimeter (P) revealed the existence of three clearly disjoint groups of PLO (G1, G2, G3). G1-PLO had a significantly higher FD (1.337 ± 0.0075, $P < 0.001$) than G2- (1.265 ± 0.061) and G3-PLO (1.248 ± 0.056). As well the roundness of G1-PLO was higher than for G2- and G3-PLO (C_{G1} = 0.819 ± 0.188, C_{G2} = 0.665 ± 0.224, C_{G3} = 0.459 ± 0.186, $P < 0.001$). G1-PLO had lower diameter, area and perimeter and they showed less, shorter and smaller filopodia than G2- ($P < 0.001$) and G3-PLO ($P < 0.001$). Hence, PLO were automatically categorized into morphological characteristic groups with significant structural differences of platelet shape. According to our observations the smaller and rounder G1-PLO with less, shorter and smaller filopodia were mostly associated with floating, not yet adherent platelets, while the larger G2-PLO with more, longer and wider filopodia mostly represented adherent platelets. G3-PLO mainly consisted of aggregates of several platelets with large area, dia- and perimeter and numerous long and wide filopodia.

While FD values were not significantly correlated with number, length or width of filopodia, there was a significant correlation between circularity and cumulated length of filopodia (r^2 = -0.571, $P < 0.001$). Circularity clearly distinguished platelets with no (C = 0.918 ± 0.009, $P < 0.001$), few (< 3, C = 0.714 ± 0.008, $P < 0.001$) or many (> 3, C = 0.484 ± 0.019, $P < 0.001$) filopodia.

Summary/Conclusion: Fractal dimension and perimeter of platelet outline define three clearly disjoint groups that exhibit significant differences in platelets roundness and size as well as number, length and width of filopodia. The group of presumably floating platelets (G1) has significantly higher FD values than adherent (G2) and aggregated platelets (G3). Circularity is significantly different for platelets with different number and length of filopodia.

Our method can be applied to any sufficiently contrast rich image of platelets and allows the fully automated continuous numerical assessment and statistical analysis of platelet shape and its change. This will be of particular interest with regard to conditions accompanied by an increased activity of platelets (e.g. inflammatory diseases, contact activation, drug-testing).

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PO 324

Absence of clinically relevant interaction between sugammadex and aspirin on platelet aggregation and coagulation

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Background: The selective muscle relaxant binding agent sugammadex (Bridion®) is associated with a limited and transient prolongation of activated partial thromboplastin time (APTT) and prothrombin time (PT) in healthy male volunteers. Although *in vitro* spiking experiments showed no effect of sugammadex on platelet aggregation, it was considered necessary to assess in a clinical study the potential interaction between sugammadex and aspirin because of the widespread prophylactic use of aspirin.

Methods: A randomized, double-blind, placebo-controlled, 4-period cross-over design was performed in 26 healthy male volunteers (aged 25.7 ± 7.4 years, BMI of 22.8 ± 2.9 kg/m^2, normal coagulation). Each subject was assigned to receive a single intravenous dose of 4 mg/kg sugammadex or placebo at t = 0 in the absence (period 1 and 2) or presence of aspirin (period 3 and 4). Aspirin (75 mg, oral) was

administered daily for at least 7 days prior to period 3, until and including sugammadex or placebo administration in period 4. Collagen-induced whole blood aggregation, APTT, bleeding time, sugammadex plasma concentrations, tolerability and safety were assessed. Evaluation of clinically meaningful interaction was based on true geometric mean ratios (GMRs) and corresponding 2-sided 90% confidence intervals (CI) for the different $AUEC_{3-30\ min}$ treatment comparisons (APTT and platelet aggregation) or treatment comparisons at 5 min (bleeding time). The pre-defined clinically meaningful margin was 0.75 for platelet aggregation and 1.50 for APTT and bleeding time.

Results: Aspirin treatment inhibited baseline platelet aggregation before sugammadex/placebo administration by approximately 25% (from approximately 17 Ohm to 13 Ohm in both treatment groups). Platelet aggregation did not differ between sugammadex and placebo, neither in the treatment arm with nor in the treatment arm without aspirin. The GMR for sugammadex co-administered with aspirin vs. aspirin alone was 1.01 and corresponding lower limit of CI was 0.91, which excluded the pre-defined clinically meaningful effect margin of 0.75. APTT was on average 32–33 s in the placebo treatment arm for all time points and was not affected by aspirin therapy. Sugammadex dosing induced an immediate prolongation of approximately 3.5–4 s, which disappeared within 1 h post-dose. The GMR of statistical interaction between sugammadex and aspirin on APTT was 1.01 and corresponding 90% CI upper limit was 1.04, which was below the pre-defined non-inferiority margin 1.50. In addition, the GMR for sugammadex alone vs. placebo alone was 1.06 and corresponding 90% CI upper limit was 1.07, which did not exceed the pre-specified non-inferiority margin of 1.50. Bleeding time was variable and prolonged by aspirin treatment and no additional effect of sugammadex was observed. The GMR of bleeding time for sugammadex with aspirin vs. aspirin alone at 5 min was 1.20 and corresponding 90% CI upper limit was 1.45, which was below the pre-defined non-inferiority margin 1.50. The plasma concentration profiles of sugammadex were comparable for sugammadex alone and sugammadex plus aspirin. Sugammadex, either alone or in combination with aspirin, was generally well tolerated by all treated subjects.

Conclusion: This study showed no clinically relevant interaction between sugammadex and aspirin on platelet aggregation, APTT and bleeding time.

PO 325

A standardized flow chamber model for real-time visualization of whole blood platelet aggregation

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Background: Currently available standard laboratory models for the assessment of the platelet function are all performed in static conditions and may overlook important mechanisms of platelet function in the flowing blood. Standardized flow chamber models are needed for flow based platelet assessment.

Methods: This study aimed to develop a standardized flow chamber model for assessment of platelet adhesion and aggregation using commercially available hardware and disposable assay biochips. The models sensitivity towards shear forces, platelet receptor defects, platelet inhibitors and coating reagent was assessed and the reproducibility of the model was validated.

Results: The model is sensitive to: (i) high vs. low shear stress dependent platelet adhesion and aggregation ($P < 0.0001$), (ii) coating reagent as well as (iii) blockage of platelet receptors GP-IIb/IIIa, GP-Ib-α, GP-VI and inhibition induced by aspirin. Reproducibility: The average%CV declined over time from a mean of 28.7% at 0.5 min to a mean of 12.9% at 7 min.

Conclusion: This flow chamber model produces reproducible, physiologically and pathologically relevant findings, assessing whole blood platelet adhesion and aggregation. It fulfils all recent recommendations of flow chamber development and standardization from the ISTH scientific subcommittee on biorheology.

PO 326

Automation of light transmission platelet aggregation

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Background: The investigation of platelet function disorders by light transmission aggregometry has changed little over the last 50 years, with the use of manually operated instrumentation making the process time consuming and labour intensive. In the current study we have used samples from normal healthy donors to develop the potential of using a high throughput coagulation analyser (CS-2000i. Sysmex Corporation, Kobe, Japan) to automate platelet aggregation.

Methods: We assessed the ability of a CS-2000i with prototype software to perform platelet aggregation, examining the effect of varying reaction cuvette stirrer speed and the platelet count in platelet rich plasma (PRP) using: ADP (0.5–10 μM) and collagen (0.5–10 μg/mL); dose response with ADP (0.5–10 μM), Epinephrine (0.5–10 μM), Collagen (0.5–10 mg/μL), Ristocetin (0.75–1.25 mg/mL), Arachidonic Acid (0.12–1.0 mM); imprecision of response to ADP (2 μM and 5 μM). All platelet agonists were from Hyphen Biomed (Neuville sur Oise, France), and an AggRAM (Helena Biosciences Europe, Tyne and Wear, UK) aggregometer was used as the reference instrument.

Results: CS-2000i reaction cuvette stirrer speed was found to influence reaction sensitivity and was optimised to 800 rpm. There were no clinically significant changes in aggregation response when the PRP platelet count was 150–480 $\times$ 10^9/L, but below this there were changes in the maximum amplitude (MA) and slope (rate). For further experiments a standardised PRP count of approximately 250 $\times$ 10^9/L was then used. Dose response with ADP, Epinephrine, Collagen, Ristocetin and Arachidonic Acid were comparable between CS-2000i and AggRAM. Aggregation imprecision was similar on both systems (CS-2000i: MA for ADP 2 μM cv 5%, 5 μM, cv 12% slope 2 μM cv 6%, μM cv 10%. AggRAM: MA for 2 μM cv 9%, ADP 5 μM cv 6%, slope 2 μM cv 7%, 5 μM cv 3%).

Conclusions: Our preliminary studies with platelets from normal healthy donors indicated that optimal sensitivity using the CS-2000i was obtained with a reaction cuvette stirrer speed of 800 rpm, and that the PRP should be adjusted with autologous PPP to a count of 200–300 $\times$ 10^9/L. Aggregation with a PRP platelet count of less than 150 $\times$ 10^9/L showed poor sensitivity. Aggregation imprecision was comparable between the CS-2000i and AggRAM, with similar aggregation dose response profile from the commonly used platelet agonists. In addition to the advantage of its walk-away technology the CS-2000i also required a smaller PRP sample volume than the AggRAM (140 μL vs. 250 μL respectively). These data are encouraging but further studies are underway using clinical samples from patients with platelet disorders and subjects receiving various anti-platelet drugs.

PO 327

Measuring platelet aggregation integrals to predict thromboembolic complications in patients undergoing pipeline embolization for treatment of cerebral aneurysms

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Background: Thromboembolic complications remain a significant cause of morbidity and mortality in patients undergoing treatment for

cerebral aneurysms with flow diverting stents such as the pipeline embolization device (PED). While the prevalence of antiplatelet therapy non-responsiveness has been associated with adverse cardiac events in patients with acute coronary syndrome, the role of platelet non-responsiveness in thromboembolic complications after PED has not been fully examined. We recently found that the prevalence of clopidogrel and aspirin resistance in patients treated with PED was 27.6% and 30.3%, respectively, which was similar to that reported in ACS patients, but did not reduce the likelihood of aneurysm occlusion at 6 months. The maximum amplitude of platelet aggregation by light transmittance platelet aggregometry (LTA) has been the gold standard identifier to characterize antiplatelet therapy (clopidogrel and aspirin) non-responsiveness; however, in our experience, discrepancies exist between the maximum amplitude and degree of platelet disaggregation, which also reflects platelet responsiveness.

Aims: Here we sought to examine a role for LTA integrals (area under the curve, AUC) in predicting acute thromboembolic complications in patients receiving antiplatelet therapy prior to PED intervention.

Methods: All patients received clopidogrel and aspirin prior to LTA using 4 agonists (ADP 20 and 5 uM, arachidonic acid (ACA), and U46619). Calculation of the integral of each aggregometric reading was used to determine the AUC, which is a function of both the maximum amplitude and the degree of platelet disaggregation. One-way ANOVA was used to assess statistical significance for any association between AUC and thromboembolic complications. Complications were defined clinically by interventional neuroradiology after the procedure.

Results: Of the 79 patients treated with PED for cerebral aneurysms 15 experienced a major or minor complication. Of those 15 complications 5 were classified as thromboembolic and 10 were classified as non-thromboembolic (3 were hemorrhagic, the remaining were due to other etiologies including device malfunction and vascular stenosis). Although the average AUC generated from ADP 20 μM response curves was increased in patients with thromboembolic complications (17,978AUC ± 8545), as compared to the average AUC generated from the response curves of the patients with non-thromboembolic complications (11,641AUC ± 6567) or those without complications (11,833 ± 7876), the data did not achieve statistical significance ($P > 0.05$). There was no significant difference in the average AUC between any of the groups to the remaining three agonists (ADP 5 uM, ACA, and U46619).

Summary/Conclusion: Developing parameters that more completely assess platelet responsiveness is important for determining the optimal antiplatelet therapy prior to cerebral aneurysm treatment with flow diversion devices. While our study failed to reach statistical significance, we did see a trend showing increased ADP 20 uM aggregation response integrals in patients that went on to have thromboembolic complications, as compared to those patients without complications. We conclude that further investigation with increased sample size is needed to determine whether the AUC from aggregometric readings might be a helpful indicator of thromboembolic complication risk in the setting of flow-diverting stent intervention for cerebral aneurysms.

PO 328

Platelet activation might facilitate the immunogenicity of stressed biotherapeutics

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Background: The primary role attributed to platelets has been the support of hemostasis and the initiation of wound healing. Recently, it has become increasingly clear that platelets play an important role in the regulation of the intravascular immune system. Platelets express the IgG receptor FcgammaRIIa, MHC class I molecules, the immune costimulatory proteins CD40 and CD40 ligand as well as a variety of toll-like receptors. Furthermore, platelets can produce important immune modulators such as IL-1 beta, soluble CD40 ligand, serotonin and histamine. Although platelets are unable to transcribe new RNA, they contain a wide range of precursor mRNAs which they can use as templates to synthesize proteins de novo. Activated platelets can also bind to neutrophils and monocytes in circulation and modulate their functional activity.

Aim: We asked if protein aggregates as contained in stressed biotherapeutics might influence the activation status of platelets.

Methods: Flow-cytometric assays were established to analyze platelet activation and the formation of leukocyte-platelet aggregates (LPA) in citrated human whole blood obtained from healthy blood donors. Human monoclonal IgG1 and human polyclonal IgG were either used untreated or subjected to temperature or mechanical stress. Upregulation of CD62P, PAC-1, CD63 and CD154 on platelets and formation of LPAs were analyzed after 15 min incubation of human whole blood at 37 °C with the stressed and unstressed IgG preparations, respectively.

Results: Incubation of human whole blood with human IgG preparations subjected to either mechanical stress or temperature stress induced a marked increase in platelet activation associated with an upregulation of platelet activation markers when compared to the unstressed human IgG controls. Furthermore, stressed human IgG induced a significant increase in leukocyte-platelet aggregates when compared to unstressed preparations.

Conclusion: Based on our results, we believe that the potential role of platelet activation in the induction of unwanted immune responses against biotherapeutics that are given intravenously deserves further attention. Currently, we investigate if the activation of platelets by protein aggregates is restricted to IgG preparations or is a more general phenomenon that is also seen with non-IgG therapeutics.

PO 329

A novel flow cytometry-based platelet aggregation assay

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Background: The main function of platelets is to maintain normal homeostasis. Inefficient platelet production and/or defective platelet function results in bleeding disorders which summon a wide range of genetic traits and acquired pathologies. Several platelet function tests have been developed to date to be used in the clinic and in experimental animal models. In particular, platelet aggregation is routinely measured in an aggregometer, which requires normal platelet counts and significant blood sample volumes, making it difficult to analyze platelet function in thrombocytopenic patients, infants or small rodents.

Aim: To develop a novel flow cytometry-based platelet aggregation assay, in which 10–25-fold lower platelet counts can be used. To optimize the test to be used on either platelet-rich plasma (PRP) or whole blood from human subjects or mice.

Methods: Fluorescent labeling of platelets with 2 different dyes or fluorochrome-conjugated antibodies, and subsequent measurement of double-colored events on a flow cytometer.

Results: With this novel aggregation assay we are able to measure the early onset of aggregate formation which allows us to distinguish between aggregation mediated by αIIbß3 and α2ß1 integrins. This fea-

ture allows us to discriminate between aggregopathy accompanying Glanzmann thrombasthenia andLAD-III.
Further, this assay can be used for mouse platelets, both whole blood, prp and fetal platelets. A great advantage is the small volume which is necessary to perform an assay. This gives us the opportunity to follow platelet function in time in one mouse and embryonic mouse blood can be measured. In a Bernard-Soulier-phenocopying mouse model, we were able to distinguish the specific failure of vWF andCLEC-2 receptor mediated signaling required for proper platelet aggregation.
Summary/Conclusions: The presented principle stands as a promising user-friendly tool, which allows analysis of platelet aggregation in thrombocytopenic patients or infants, and facilitates studies in platelets obtained from experimental animal models without the need of special devises but a flow cytometer.

PO 330

Ex vivo and *in vivo* effect of aspirin on different platelet activity pathways

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Background: While aspirin inactivates the cyclooxygenase-1 (COX-1) enzyme responsible for the conversion of arachidonic acid (AA) in prostaglandin H_2, the *in vivo* and *ex vivo* antiplatelet effects of aspirin in different pathways may not be equal in all subjects.
Aims: We sought to investigate platelet aggregation in response to AA, epinephrine, collage and ADP at different concentrations in healthy controls before and after aspirin, and in subjects with stable coronary artery disease (CAD) on aspirin.
Methods: Fourteen healthy controls had blood collected during 3 consecutive weeks (visits 1, 2 and 3) and after a week of treatment with aspirin 81 mg daily (visit 4), and from 26 CAD patients taking aspirin 81 mg/day. Platelet aggregation from light transmission aggregometry (LTA) was measured in response to arachidonic acid (150 and 1500 μM), adenosine diphosphate (ADP; 1 and 2 μM), and epinephrine (0.4 and 2 μM). LTA was performed with and without *ex vivo* addition of aspirin (final concentration 30 μM) to platelet rich plasma (PRP). Paired samples were compared using paired *t*-test and unpaired samples were compared using standard *t*-test.
Results: Prior to aspirin ingestion in visits 1–3, addition of aspirin *ex vivo* to PRP decreased platelet aggregation in response to AA, ADP, collagen and epinephrine. Following aspirin use for 1-week, a significant decrease in platelet aggregation was observed for each agonist. The response to aspirin *in vivo* vs. aspirin *ex vivo* was similar for platelet aggregation in response to AA (150 μM: 2.5 ± 2.1 vs. 2.8 ± 1.8, $P = 0.8515$; 1500 μM: 7.0 ± 4.8 vs. 4.1 ± 2.4, $P = 0.949$) and ADP (1 μM: 10.2 ± 6.3 vs. 9.2 ± 5.5, $P = 0.243$; 2 μM: 33.7 ± 12.7 vs. 27.1 ± 13.6, $P = 0.065$). However, epinephrine induced platelet aggregation in response to aspirin *in vivo* was higher than aspirin *ex vivo* (0.4 μM: 22.6 ± 9.8 vs. 15.2 ± 6.5, $P = 0.0018$; 2 μM: 41.2 ± 20.1 vs. 25.9 ± 11.4, $P = 0.0019$) suggesting that responsiveness to epinephrine may be dose dependent. A similar pattern was observed in patients with CAD. There was no significant difference between AA- (150 μM: 4.7 ± 3.8 vs. 4.4 ± 4.5, $P = 0.659$; 1500 μM: 19.4 ± 18.6 vs. 15.5 ± 10.7, $P = 0.306$) and ADP- (1 μM: 27.2 ± 21.4 vs. 29.5 ± 20.7, $P = 0.141$; 2 μM: 50.0 ± 25.2 vs. 52.3 ± 21.8, $P = 0.334$) induced platelet aggregation in response to aspirin *in vivo* and *ex vivo*, while epinephrine induced platelet aggregation in response to aspirin *in vivo* was higher than aspirin *ex vivo* (0.4 μM: 26.1 ± 12.2 vs. 20.5 ± 12.4, $P = 0.001$; 2 μM: 41.9 ± 16.1 vs. 34.6 ± 16.6, $P = 0.005$). Not surprisingly, CAD patients had increased platelet aggregation in response to *in vivo* and *ex vivo* aspirin for each agonist compared with healthy controls.
Conclusions: *In vivo* and *ex vivo* aspirin decreases platelet aggregation in response to ADP, epinephrine, and AA. While the response to ADP and AA activation pathways is inhibited to a similar degree with *in vivo* and *ex vivo* aspirin, epinephrine induced platelet aggregation is higher following *in vivo* (approximately 5 μM) than *ex vivo* (30 μM) aspirin. These data suggest that aspirin affects multiple activation pathways and may affect different pathways in a dose dependent manner.

PO 331

Clinical features and bleeding patterns in children with Glanzmann thrombasthenia: single center experience

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Background: Glanzmann thrombasthenia is a rare autosomal recessive bleeding disorder characterized by a defect in platelet aggregation. The incidence is high in communities where consanguinity is prevalent.
Aim: To report the clinical features and bleeding patterns of children with Glazmann thrombasthenia at our center.
Material and Method: Patient files were retrospectively reviewed for demographic and clinical data.
Results: Twenty-four patients (12 girls and 12 boys) were diagnosed as Glazmann thrombasthenia at our center. Two patients were excluded because of insufficient clinical data in patient files. Positive family history of bleeding was noted in 8 (36%) patients, no history of bleeding in 4 (18%) and unknown in 10 (45%). Sixteen patients' parents were consanguineous. The median age of onset of bleeding symptoms was 2 months (1 week–7 years). All patients presented with easy bruising, 4 presented with hematoma after vaccination, 2 with gastrointestinal bleeding and 1 with bleeding after circumcision. On follow-up, the most common type of bleedings were; easy bruising (100%), epistaxis (54%), gum bleeding (42%), gastrointestinal bleeding (25%), oral mucosa bleeding (21%), bleeding from tongue (21%) and menorrhagia (4%). The most common indication for hospitalization was epistaxis (n:5). Fourteen patients were treated with thrombocyte transfusion, tranexamic acid, recombinant active factor VII and fibrin glue as a single or combined therapy; none of them had a major bleeding complication. Oral contraceptives were used in two patients with menorrhagia. Four patients had a surgical procedure on follow-up, 3 were circumcised and 1 had a dental operation. Circumcisions were performed with fibrin-glue and transamin, dental extraction was performed with transamin. No major bleeding or complication was noted after the procedures. No patient was lost due to bleeding on follow-up.
Conclusion: Glanzmann thrombasthenia is a se−vere hemorrhagic disease with clinical variability: epistaxis, gum bleeding and easy bruising are the most frequent bleeding types. Patients usually present early in life, 73% of our patients were ≤ 1 year when they first had a bleeding symptom. Although the bleeding problem is lifelong, the disease is associated with a low morbidity and mortality with good supportive care.

PO 332

Influence of angiotensin-(1-7) on the expression of talin 1 induced by angiotensin ?in endothelial cells

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Background: Renin-angiotensin system (RAS) is one of the most important regulation systems, which has played an important role on regulating physiological function, especially at cardiovascular and cerebrovascular diseases development. Integrin is a major receptor family present in the cell surface, which can induce the conglutination between cells and extracellular matrix, activated integrin $\alpha II_b\beta_3$ is mainly involved in thrombus formation through inducing platelets adhesion and aggregation. Talin 1, as the essential factor in the process of activating $\alpha II_b\beta_3$, is recently specially concerned. Previous work

have proved that angiotensin-(1-7) [Ang-(1-7)] could significantly inhibit the experimental thrombosis induced by angiotensin II(AngII). However, whether Ang-(1-7) and AngII can affect thrombosis formation by means of regulating talin 1 has not been reported. In this study, we will observe the influence of Ang -(1-7) on the expression of talin 1 induced by AngII in endothelial cells.

Aims: To study the effects of Ang-(1-7) on the expression of talin 1 induced by AngII in human umbilical vein endothelium derived cell line (HUVECs).

Methods: HUVECs were cultured in DMEM. Talin 1 antigen was measured by ELISA Kit.Talin 1 mRNA was examined by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR).

Results: (1) Compared with control, a gradual increase in talin 1 antigen ($r = 0.965, P < 0.05$) and talin 1 mRNA ($P < 0.05$), were due to the increasing concentration of AngII(10^{-10}–10^{-6} M) treated in HUVECs, and peak appeared in the 10^{-7} M. (2) When pretreated with Ang-(1-7) (10^{-9}–10^{-6} M), then treated by AngII, it inhibited the expression of talin 1 at antigen and mRNA level in the dose-dependent manner ($r = -0.987$, $P < 0.05$), and 10^{-6} M was the strongest concentration.

Conclusion: The present data suggested that AngII could increase the expression of talin1 in vascular endothelial cells, and Ang-(1-7) could inhibit this effect at mRNA level.

PO 333

Molecular study on the interactions between extracellular fragments of platelet thrombin receptor PAR4 and thrombin exosites and functional effects on platelet activation

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Introduction: Thrombin is a potent activator of platelets by the cleavage of the *N*-terminal of its receptors PAR 1 and PAR4 and it is well established its roll in haemostasis, thrombosis and vascular biology, but the detailed receptor mechanisms needs to be further elucidated to improve treatments for cardiovascular diseases. The focus of this project is centered in the details of thrombin interactions with its low affinity receptor PAR4 and how this would affect normal haemostasis or become the basis for new drug design. In this interaction with the PAR4 it is not known in detail the contributions of the different extracellular loops (ECL) of PAR4 or the exosites of thrombin: exosite I is related to PAR1 activation by its hirudin-like binding site, but it has not been experimentally discarded its contribution to PAR4 binding (1), on the other hand, exosite II is the natural binding site for heparin, a highly cationic patch (2) similar to exosite I but in the opposite side of the protein. Its binding to PAR4 would depend upon its electro-negativity and hydrophobic interaction with α-helix chains.

Methods: We propose possible ECL and PAR4 *N*-terminal region as candidates to interact with thrombin at exosite II. The most plausible sequence are: *N*-terminal anionic cluster (A54 to S66), and two amino-acidic sequence in the ECLII (both from S221 to S240).

Our first approach is performed with peptides that mimic the aforementioned PAR4 fragments in order to detect some binding and/or effect on thrombin. To test these peptides we use activation of platelets by thrombin measured, in flow cytometry (P-selectin exposure) and we evaluate an expected quenching of this activation by the presence of the peptides, and additional data from the use of exosite I and II blockers. The hypothetical bindings that result in these mentioned effects are also investigated by Surface Plasmon Ressonance (SPR). The next approach is the evaluation of the PAR4 cleavage by thrombin using Western Blot with monoclonal antibodies against PAR4, and again a parallel quenching effect of these peptides.

Results: Our data seems to confirm a hypothesized contribution of exosites I and II with different strength in the PAR4 activation with exosite II being the most important; moreover the use of PAR1 inhibitors and blockers of the exosite I and II in combination with the designed peptides give us differential results indicating contributions of both thrombin exosites. The results for gamma-thrombin, lacking exosite I, drive us to the conclusion that there is an interchangeable interaction with different extracellular parts of PAR4. The experimental data were confirmed by different methods such as the check of the PAR4 cleavage by Western Blot. These results give us a starting point for the development of further assays in living systems to study these interactions in detail.

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PO 334

The influence of neurotropic drugs on platelet aggregation *in vitro*

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Background: Neurotropic drugs of different groups are widely used nowadays. Besides the central action they influence other systems-immunity and hemostasis. Although there exist some discrepant data to this.

Aim: the research of influence of some neurothropic drugs on aggregation ability of trombocytes.

Methods: the aggregation of thrombocytes was examined by analyzer 'Biola' (Moscow) with turbidimetric method (Born's method 1962). Adrenalin, ADP (10 mcg/mL, collagen was used as inducers.

For influence of neurotropic drugs examination the plasm, rich of thrombocytes was incubating with diazepam, propranolol, haloperidol, flumazenil, dopamine at a temperature of 37 °C during 2 min. Measuring of drugs in the process of incubation was- for diazepam - 10 mcg/mL, propranolol- 10 and 15 mcg/mL, haloperidol- 10 mcg/mL, flumazenil- 10 flumazenil.

Statistical processing of results of research was conducted by using nonparametric method W-Wilcoxon, Mann Whitney.

Results: It was established that incubation of healthy men's plasm, which is rich of platelets with agonist of benzodiazepine receptors (diazepam 10 mcg/mL) refused the aggregation effect, in spite of adding inducers of aggregation (adrenalin [$P < 0.01$], collagen [$P < 0.01$], ADP [$P < 0.01$]). Adding flumazenil didn't influence on ADP, collagen, adrenalin-induced aggregation of blood platelets ($P > 0.05$) and didn't refuse diazepam effect ($P < 0.01$). Adding to the healthy man's plasm, rich of thrombocytes the blocking agent D2- of dopamine receptors of haloperidol also restrained the aggregation in the setting of inducers of different nature: adrenalin ($P < 0.01$), ADP ($P < 0.01$), collagen ($P < 0.01$).

The blocking agent of ß- adrenoreceptors propranolol also reduced aggregation after incubation with thrombocytes on traditional inducers – adrenalin ($P < 0.01$), ADP ($P < 0.01$) and collagen ($P < 0.01$). It should be mentioned, that propranolol had a dose-dependent effect: with increasing drug dose, the amplitude of curve of aggregation was decreasing.

Conclusion: Inhibiting influence of diazepam is caused by covalent bonding of drug with glycoprotein receptors GP IIb/IIIa, which have a common RGD- consequence for bonding with fibrinogen. Another probable mechanism is connected with metabolism of arachidonic acid. In this case diazepam changes the conformation of thrombocyte membrane, which influence the activity of phospholipase C, phosphoinositid's level, formation of thromboxane A2, brake action of mobilization Ca2+ and phosphorylation of protein P-47. The propranolol's effect can be explained only by it's prevention of dissociation of a to βg- subunit of G-protein, which leads not only to brakeage of response of β adrenoreceptors, but also others, including α- adrenoreceptors. Just only dissociation of subunits of G-protein can be the necessary condition of receptors' activation. As approval of this statement can be the fact of inhibiting of aggregation ability in case of adding not

only adrenalin, but adding ADP and collagen, biological effects of which are connected with other receptors and occur in case of dissociation of a to βg- subunit of G-protein. If this statement is true, we amplified signalling mechanisms in blood platelet in case of agonists adrenoreceptors actions. Similar mechanism is probable for blocking agent D2- of dopamine receptors- haloperidol.

PO 335

Antidepressant suppresses collagen-induced platelet activation

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Background and Aims: Selective serotonin reuptake inhibitors (SSRIs) are a class of most frequently prescribed antidepressants in the treatment of a variety of neuropsychiatric disorders as well as depression. Recent epidemiological studies have shown that SSRIs may increase the risk of abnormal gastrointestinal bleeding and may prevent ischemic heart disease events. Additionaly, some *ex-vivo* studies have shown that platelet functions of patients medicated with SSRIs may be impaired. To understanding these findings, it is suggested that SSRIs inhibit serotonin uptake into platelet, as well as into presynaptic neuron, to reduce platelet serotonin content in patients chronically medicated with SSRI. However, probably it is not the sole mechanism of platelet dysfunction. The aim of our study is to elucidate the mechanisms of SSRIs' effects on human platelets.

Materials and Methods: We purchased pharmaceutical ingredients of S-citalopram, paroxetine, fluvoxamine, sertraline, which are marketed SSRIs for clinical use, and those of R-citalopram (the optical isomer of S-citalopram) and metabolites of both citalopram isomers. Each material was resolved in platelet rich plasma (PRP) taken from healthy volunteers and incubated for 90 min at 37 degree Celcius.

(1) Platelet aggregation induced by ADP, collagen, arachidonic acid, epinephrine or STA_2, was measured with a light transmission aggregometer.

(2) Collagen-induced cytosolic calcium increase with/without S-citalopram, R-citalopram or the metabolites, was measured with fluorescent indicator fura-2 and a cytosolic ion analyzing device.

(3) Expression of CD62Pand phosphorylation of spleen tyrosine kinase (Syk) induced by collagen with/without S-citalopram were measured with a flow cytometer.

Results:

(1) All SSRIs and the related materials suppressed platelet aggregation induced by collagen significantly, but did not suppress platelet aggregation by the other agonists.
(2) The both citalopram isomers and the metabolites suppressed collagen-induced cytosolic calcium increase.
(3) S-citalopram suppressed collagen-induced CD62P expression and Syk phosphorylation.

Summary: Our data show that, after only 90-min incubation, SSRIs suppress collagen-induced platelet aggregation specifically, and that the non-SSRI related materials, which do not inhibit serotonin uptake, also suppress similarly. These facts indicate that there are other mechanisms of SSRIs' impairing effects on platelet functions than decrease of platelet serotonin content, above mentioned.

Glycoprotein VI (GPVI) is a major collagen receptor on platelet and forms a complex with an Fc receptor, which has intracellular docking sites for Syk. Following activation of GPVI and Syk phosphorylation, calcium is released from dense tubular system. Our data show that S-citalopram, a typical SSRI, inhibits both of Syk phosphorylation and cytosolic calcium increase induced by collagen. These facts indicate that SSRIs may suppress collagen-induced platelet activation by inhibiting some steps before Syk activation in GPVI pathway.

Conclusion: SSRIs may affect in early steps of GPVI signaling pathway.

PO 336

Platelet aggregation pattern amongst multiracial healthy blood donors performed in National Blood Centre (NBC), Malaysia

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Background: Platelet aggregation test using light transmission aggregometry is considered as gold standard for assessment of platelet function. Laboratory performing the test is highly encouraged to establish its own reference range, as different population/ethnic background may have different aggregation pattern. Malaysia is a multiracial country comprises of three major ethic groups i.e Malay, Chinese and Indian. National Blood Centre (NBC) is the referral centre for the nation to conduct platelet function study using platelet aggregation test for inherited platelet disorders.

Aims: This study aims to determine the platelet aggregation pattern and subsequently estimate the reference range for platelet aggregation test performed in three major ethnic in Malaysia i.e Malays, Chineses and Indians at Hemostasis Laboratory, National Blood Centre, Kuala Lumpur.

Methods: This study was conducted from September 2012 to January 2013. There were 60 informed consented healthy blood donors of different ethnic group participated in the study. Blood were drawn into 3.2% sodium citrate tube. Subsequently, platelet rich plasma (PRP) was obtained and platelet aggregation test were done using PAP-8E, BioData. Five panel agonists were used i.e. ADP (10 μM), ristocetin (1.5 mg/ml), arachidonic acid (1.0 mM), epinephrine (10 μM) and collagen (5 μg/ml and 0.19 mg/ml) as per mentioned in the guideline. Reference ranges were taken as mean ± 2SD of final aggregation in percentage.

Results: Analysis showed that mean ± 2SD for ADP (10 μM) is 82–92%, ristocetin (1.5 mg/ml) is 98–103%, arachidonic acid (1.0 mM) is 90–97% and collagen (0.19 mg/ml) is 88–95%. About 48% of respondent had 0% aggregation in response to collagen (5 μg/ml) (Median = 3%). We also noticed that 30% of respondent had < 20% aggregation in response to epinephrine (10 μM) which might due to variability in the sensitivity of its receptor among normal person (Median = 86%). Therefore, the reference range for collagen (5 μg/ml) and epinephrine (10 μM) could not be estimated.

There were no differences observed in pattern/percentages of platelet aggregation amongst the three major ethnic groups or amongst different gender for all agonists. However, the percentages of platelet aggregation to all agonists were significantly higher as compared to Caucasian population. These findings could indicate variability in platelet receptors sensitivity amongst different population.

Conclusion: Results obtained from this study could provide an insight for the dedicated laboratory at National Blood Centre when performing platelet aggregation test among the three main races. This estimated reference range can substitute the reference range provided by manufacturer which is based on Caucasian population. Future study may look into variability in sensitivity of receptors seen amongst Malaysians population in particular for epinephrine and collagen agonist.

PO 337

Prasugrel shows less intraday variability in its antiplatelet effect compared to ticagrelor in cynomolgus monkeys

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Background: Prasugrel is the 3rd generation thienopyridine prodrug while ticagrelor is a direct acting non-competitive $P2Y_{12}$ antagonist. In

phase three studies, both agents reduced the ischemic event rates compared to clopidogrel. A new ELISA-based vasodilator-stimulated phosphoprotein (VASP) phosphorylation assay in whole blood provides a more specific test of $P2Y_{12}$ inhibition than other platelet function tests.

Aims: The objectives of this study included a comparison of the antiplatelet effects of single and 7-day multiple dosing of prasugrel and ticagrelor in cynomolgus monkeys using the human VASP phosphorylation assay and light transmittance aggregometry (LTA).

Methods: Platelet VASP phosphorylation was determined in whole blood by ELISA and expressed as the platelet reactivity index (PRI). ADP-induced platelet aggregation in platelet-rich plasma was determined by LTA. The relationship between PRI and platelet aggregation was evaluated.

Results: Single oral dosing of prasugrel (0.3 and 1 mg/kg, $N = 6$) resulted in potent and significant antiplatelet effects, which were sustained up to 24 h. Each mean PRI was 84, 21 and 16% at 0, 4 and 24 h, and each mean platelet aggregation induced by 20 μM ADP was 57, 24 and 27%, respectively, in 0.3 mg/kg prasugrel group. Ticagrelor (3 and 10 mg/kg, $N = 6$) also showed significant effects with maximum decreases similar to prasugrel, but its effects were diminished at 24 h. Each mean PRI was 82, 13 and 69% at 0, 4 and 24 h, and each mean platelet aggregation induced by 20 μM ADP was 58, 20 and 54%, respectively, in 3 mg/kg ticagrelor group. In this single-dose study, strong correlations between the PRI and platelet aggregation induced by ADP (5 and 20 μM) were observed (correlation coefficient of 0.93 and 0.96, respectively). Multiple dosing of prasugrel (1.8 mg/kg loading dose, 0.3 mg/kg once daily maintenance dose, $N = 10$) showed more rapid antiplatelet effects, that were sustained over 24 h. Each mean PRI was 93, −2, 0, 1, 1 and 3% at 0, 1, 4, 12, 16 and 24 h on Day 1, respectively. Similar low PRI values were observed on Day 7 with mean PRI of 7, 1 and 6% at 0, 4 and 12 h, respectively. Twice a day multiple dosing of ticagrelor (10 mg/kg maintenance dose following a single 20 mg/kg loading dose, $N = 10$) also showed significant antiplatelet effects during the day, but intraday changes in antiplatelet effects were observed. Each mean PRI was 93, 29, 4, 22, 9 and 12% at 0, 1, 4, 12 (12 h = predose), 16 and 24 h on Day 1, respectively. Similar intraday changes in the PRI were observed on Day 7 with mean PRI of 7, 5 and 24% at 0, 4 and 12 h, respectively. The multiple-dose study also showed strong correlation between the PRI and platelet aggregation.

Summary/Conclusion: Prasugrel showed potent antiplatelet effects with longer duration of action throughout the entire day. Ticagrelor also showed potent antiplatelet effects but more intraday variability in platelet inhibition compared to prasugrel was observed. The present study also showed the human ELISA VASP assay can be utilized for monkey studies.

PO 338

Methods for detection ASA resistance *in vitro*

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Background and Aim: Acute myocardial infarction (AMI) is frequently also associated with platelet gene polymorphism as platelet enzyme cyclooxygenase 1 (COX-1). Dual antiplatelet therapy is part of the secondary prophylaxis in the treatment includes regularly acetylsalicylic acid (ASA). Polymorphism of COX-1 gene is not the only cause of ASA resistance. By use of the impedance aggregometry after *in vitro* administration of ASA i tis possible to distinguish exogenous (noncompliance or patient problems of absorption of the active substance) from endogenous causes in failure of antiplatelet therapy.

Material and Methods: We examined a group of 90 patients with AMI who were receiving antiplatelet therapy such as aspirin by impedance aggregometry on Multiplate analyzer (MEA). We used as agonist arachidonic acid (1 mM). Patients without adequate response to treatment were subjected to *in vitro* incubation with ASA. The dose was equivalent to a daily dose of 100–500 mg/day. Patients who were not respond on antiplatelet therapy were also selected for *in vitro* measurements. Samples of blood were incubated with an appropriate amount of ASA (18.2 mg/ml, diluted 1:2, 1:4, 1:8 and 1:16 NaCl) for 5 min. Subsequently, the detected change in the aggregation ability after the addition of arachidonic acid by MEA. The DNA samples were examined receptor polymorphism COX-1 (position 842 A/G; rs10306114).

Results: Patients, in the effective treatment, are with ASA inhibition of aggregation under 220 AUC*min. The resulting data demonstrate that the number of patiens (2/3–66%) *in vitro* testing is responding to treatment. Absolute data will be presented in this communication.

Conclusion: With this method, we were able to eliminate the deficiency of platelet receptor, and thereby reveal irregular drug intake or malabsorption. The majority of patients were detected insufficient response to the presence of ASA. We can conclude, that in the above-mentioned causes of failure of antiplatelet therapy it is necessary to change treatment. An ineffective preventive use of ASA may be also caused by aspirin resistance. On the other hand, the cause of aspirin resistance may be due to a number of factors such as low absorption, inadequate dosage of ASA, genetic factors or interactions with other medications in the given cases.

Supported by the Grant LF-2013-004.

PO 339

The influence of different types of TRAP peptides on platelet aggregation, using different light transmission aggregometry devices

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Background: Light transmission aggregometry (LTA) is the recommended method to assess platelet function. Recommendations for standardizing LTA are advised by the platelet physiology subcommittee of the SSC of the ISTH. One of the recommendations is the use of Thrombin Receptor Activation Peptide (TRAP) as an agonist. Although the concentration (10 μM) is standardized, there is no recommendation for the type of TRAP to use.

Aims: Comparison of platelet aggregation in healthy volunteers with two types of TRAP agonist on three different LTA devices. TRAP aggregations were measured both in adjusted (A) and non adjusted (NA) platelet rich plasma (PRP).

Material and Methods: Citrated blood (3.2%) was collected from 30 healthy volunteers, 18 females (21–58 years) and 12 males (20–54 years) with comparable mean platelet counts $263 \pm 59 \times 10^9$/L and $250 \pm 40 \times 10^9$/L. In part one of this study, 13 healthy donors (7 females and 6 males) were tested using the inhouse method with TRAP14 (10 μM) (Bachem) and the CH490-D (Chronolog). Also tested was TRAP6 (10 μM) (Hart Biologicals) using PAR8 (Hart Biological) and PAP8e (Biodata).

In the second part, 17 healthy donors (11 females and 6 males) were analysed with only one TRAP reagent (TRAP14, 10 μM, Bachem) on all three LTA devices. TRAP aggregation was tested in both adjusted (250×10^9/L) and non-adjusted PRP. The healthy donors didn't use any medication that is known to inhibit platelet function for at least 10 days before blood collection. A normal response is defined as a single monophasic wave with a minimal maximal aggregation of 70%.

Results- The first study ($n = 13$) showed a normal response to TRAP14 in NA and A PRP with the CH490-D in all subjects. In NA PRP, 85% (11) showed a normal response to TRAP6 with the PAR8 and only 60% (7) with the PAP8e. In A PRP, only 55% (7) showed a normal response to TRAP6 with the PAR8 and only 30% (4) with the PAP8E.

In the second study ($n = 17$) TRAP14 was compared on all three devices. In NA PRP, 100% (17) showed a normal response to

TRAP14 using CH490-D and PAR8. Only 65% (11) showed a normal response to TRAP14 using the PAP8e (NA PRP). In A PRP, 94% (16) showed a normal response to TRAP14 on the CH490-D, 70% (11) with the PAR8 and only 40% (7) with the PAP8e.
Conclusions: Although the recommended concentration of 10 μM was used for both types of TRAP, TRAP6 shows more abnormal responses in healthy volunteers as compared with TRAP14 on the PAR8 and PAP8e device. Therefore recommendations should be made on the type of TRAP that is used for platelet function testing. Although there are more healthy volunteers with a normal response on TRAP14 compared to TRAP6 with the PAP8E, there is still a markedly abnormal response when compared with CH490-D and PAR8. Laboratories should therefore standardize and validate there method for TRAP aggregation. Because of the high number of abnormal responses found when using A PRP, it is recommended to perform TRAP aggregation in NA PRP.

PO 340

Platelet aggregation in patients with chronic cerebral ischemia, carried the AGT-174T/M, AGT-235M/T, AGTR1-1166F/C, ACEI/D polymorphisms

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Background: Cognitive dysfunction syndrome is a companion set of brain disorders. The major share of cognitive deficits based on the neurodegeneration of Alzheimer's type, and cerebrovascular disorders.
Aim was to study the platelet aggregation and genetic polymorphism of some proteins of the renin-angiotensin-aldosterone system (AGT-174T/M, AGT-235M/T, AGTR1-1166A/C, ACEI/D) in patients with chronic cerebral ischemia, complicated by cognitive dysfunction.
Methods: Clinical group consisted of 207 (135 women and 72 men) patients aged 66 ± 10 years, with cognitive deficits with chronic cerebral ischemia without signs of decompensation. The criteria for inclusion, as well as the objective of establishing the degree of cognitive decline rating scales were used: MMSE, tracking test, clock drawing test, Khachinsky's scale, Hamilton's test. The vascular nature of the lesion was confirmed by neyrovizulizatsion and scale ischemia NINDS-AIREN. The PCR method in real time to identify of genetic polymorphism was applied. ADP-, collagen- and adrenalin-induced platelet aggregation was measured.
Methods: Among all patients, cognitive deficits bore signs of mild dementia (MMSE (M ± δ) – 20.2 ± 2.7 points), without of depression (Hamilton (M ± δ) – 7.99 ± 2.4 points) against chronic cerebral ischemia (Khachin (M ± δ) – 6.27 ± 0.9 points, neuroimaging data). The study found all the desired mutations in the homo-or heterozygous. To assess the effect of the mutant allele carriers on platelet aggregation, all patients were divided into 5 groups: 1) homo-and heterozygous (AGT-174M | | AGTR1-1166C | | ACE-Del, $n = 12$); 2) homo-and heterozygous (AGT-174M | | ACE-Del, $n = 15$); 3) homo-and heterozygous (AGTR1-1166C | | ACE-Del, $n = 68$); 4) ACE-Del, $n = 68$); 5) patients carried of the 'wild' alleles studied polymorphisms in the homozygous state ($n = 32$). It was established that in all patients the slight platelets hyperaggregation on low doses of ADP. Spontaneous platelet aggregation ranged from 1.22 ± 0.39 to 1.56 ± 0.35 optical units and did not differ between groups of observation. When comparing the average size of the aggregates formed in making inductors (ADP, adrenalin), marked the lowest platelet aggregation in patients – carriers of the three 'unfavorable' alleles. Severe induced hyperaggregation observed in patients carriers of wild-type alleles for all studied polymorphisms (*U*-test, $P < 0.05$) with a maximum radius of aggregates on ADP- induction. When comparing the average size of the aggregates formed in making inductors marked the lowest platelet aggregation in patients – carriers of the three 'unfavorable' alleles.
Summary. In patients with chronic cerebral ischemia, the wild-type allele carriers studied genetic polymorphisms of angiotensin-aldosterone system registered a high platelet aggregation with the same radius of aggregates (resistance) with a decrease in the ADP concentration. The patients – carriers of the mutant allele aggregation properties of blood platelets relatively pronounced.

PO 341

Reduced platelet reactivity in critical limb ischemia patients

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Objective: Despite aspirin therapy a substantial amount of patients will experience cardiovascular events (CVE). In the current study, we tested baseline platelet activation and platelet reactivity in critical limb ischemia (CLI) patients, a patient population with a particular high cardiovascular risk, hypothesizing that platelets from CLI patients display increased baseline activation and are hyperreactive. Furthermore, we investigated the effect of aspirin therapy and the presence of high-on-aspirin platelet reactivity (HAPR) in patients with CLI with a novel platelet reactivity assay.
Methods: Baseline platelet activation and platelet reactivity in response to five major platelet agonists were determined, in 23 CLI patients (11 on aspirin, 9 on oral anticoagulant and 3 on aspirin + clopidogrel) included in the Juventas-trial (clinicaltrials.gov: NCT00371371) and in 17 healthy controls. Platelet activation was quantified with flow cytometric measurement of platelet P-selectin expression and fibrinogen binding. Aspirin effectiveness was measured by the additional stimulatory effect of arachidonic acid (AA) stimulation to convulxin (CVX) stimulation (AA-effect).
Results: CLI patients not on aspirin compared to healthy controls, showed higher baseline platelet activation: P-selectin expression was 59.4 ± 4.8 vs. 40.3 ± 8.6 ($P < 0.001$). Platelet responsiveness to the tested agonists was not different between cases and controls, except for responsiveness of fibrinogen binding to CVX with or without AA, which was lower in cases than in controls ($P = 0.009$ and $P = 0.037$, respectively). Presence of cardiovascular risk factors was inversely correlated with *in-vitro* reactivity of circulating platelets of CLI patients. Use of aspirin did not substantially modulate platelet responsiveness in CLI patients, except from the effect of AA, while a profound effect of clopidogrel was observed. Furthermore, the AA-effect seemed to properly identify the effect of aspirin and HAPR in CLI patients.
Conclusions: Baseline platelet activation in CLI patients is increased compared to healthy controls, while the *in-vitro* response to stimulatory agents was either not different or decreased.. Additionally, several cardiovascular risk factors were inversely correlated with *in-vitro* reactivity of circulating platelets. HAPR was observed in a substantial proportion of the CLI patients.

PO 342

The use of a rapid, whole blood platelet factor 4 clotting assay for the early detection of hypercoagulability and guide to heparin therapy in equines

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Background: Hemostasis research using animal models has significantly influenced clinical, diagnostic and pharmaceutical advance-

ments in human coagulation, thrombosis and drug therapy. Platelet Factor 4 (PF4), a protein primarily secreted from within the platelet, is a strong contributor to the coagulation status of the patient and is a specific heparin antagonist. It is released from the alpha granules of platelets when the latter are activated by trace thrombin insufficient to clot plasma fibrinogen. As a result, PF4 provides a means for determining the degree of a hypercoagulable state, defined here as a state in which activated products and intermediates normally absent from circulating blood are detected intra-vascularly or are released from tissues.

Aims: This study describes the utility of PF4 testing in the treatment of equine patients admitted to veterinary clinics with clinical diagnosis of acute laminitis, an equine disease that is caused by inflammation of the sensitive soft-tissue, or laminae, of the foot, and is a major cause of lameness and death if not medically treated immediately. Methods.

Patients were diagnosed with acute laminitis as evidenced by abnormal sensitivity, pain, heat and significantly decreased circulatory status in the hoof capsule by radiograph and venogram. Subjects were tested using a rapid PF4 assay using citrated whole blood with a < 3-min turn-around time to determine hypercoagulability. A 500 μL of blood was added to the PF4 reagent vial, containing co-lyophilized calcium chloride and unfractionated heparin, and clotting was measured in a semi-automatic coagulometer. Based on the results of the PF4 assay, where it is known the clotting time is inversely related to the PF4 level, heparin therapy was modified in subjects where a hypercoagulable state was determined.

Results: Over 30 horses suffering with laminitis secondary to colic, endotoxemia, sepsis, EHV-1 and colitis were successfully treated with simultaneous conventional therapy and heparin. These animals, destined for euthanasia, were subsequently spared and recovered fully. Conclusions.

The use of heparin as an adjunct to traditional therapies results in better therapeutic outcomes than traditional therapies alone. Heparin dosing will depend on the PF4 level and clinical condition of the patient. The availability of an easy, whole blood functional PF4 assay is a significant step toward the rapid detection of a patient's hypercoagulable state. Current commercial PF4 assays are either radioimmuno or ELISA methods that are labor intensive and require 45 min to h to perform. It is the intent that studies using a rapid PF4 assay will transition to human applications, providing timely and clinically useful information that has never before been possible.

PO 343

Evaluatyion study of a new, direct whole blood platelet function assay and its application

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Background: Platelet function assay is not only helpful in the diagnosis of certain congenital and acquired platelet defects which leads to bleeding disorders, but also plays a key role in the study of thrombotic disease pathological machanism, clinical diagnosis, antithrombotic therapy, monitoring the responseness of anti-platelet drug, etc..

Aims: The purpose of this study is to develop a method using a continuous automatic platelet counting assay based on the Coulter principle to test platelet aggregation function by PL-11 platelet function analyzer (Nanjing Sinnowa Medical Science & Technology Company, Nanjing, China) and also to evaluate its performance in clinical application.

Methods: Whole blood sample anticoagulanted with sodium citrate were taken from 247 healthy volunteers (125 men, 122 women, between the ages of 18–82 years), from June to December 2012 at the First Affiliated Hospital of Soochow University, China. The study conformed to the ethical guidelines of 2004 Declaration of Helsinki and was approved by the institutional Ethics Committee at the First Affiliated Hospital of Soochow University, China. The informed consent was obtained from all healthy donors. Platelet aggregation in 0.5 ml of whole blood was induced with ADP or collagen by PL-11 using a continuous automatic platelet counting assay based on the Coulter principle. The intra-assay and inter-assay coefficient of variation (CV), carry-over rate, accuracy, linearity of PL-11 were also evaluated, respectively. The correlation of platelet aggregation was detected between the PL-11 and MPG-3E photoelectric turbidimetry.

Results: All the parameters were conformed to the standard of Clinical Laboratory Improvement Amendment 88. Both of the intra-assay and inter-assay CV values were less than 5%; carry-over rate was less than 1%; the accuracy and the linearity was excellent ($r > 0.99$). The correlation between the PL-11 and MPG-3E was $R^2 = 0.9439$. The maxium of platelet aggregation induced by ADP or collagen using PL-11 was 17.7–54.3%, or 19.8–61.1%, respectively.

Conclusion: Analytical results of the PL-11 platelet analyzer are accurate and reliable. It has a potential in clinical application.

PO 344

Evaluation of 195 referral cases with suspected hereditary platelet function disorders using platelet aggregometry and ATP release

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Background: Although hereditary platelet function disorders (PFDs) are common in the general population but in our country except for severe cases, they are not well recognized due to limited diagnostic tools for these problems.

As a reference coagulation laboratory in Iran, after being equipped with lumi-aggregometer during recent 2 years, we have started to check our referral patients suspected to PFDs for light transmission platelet aggregometry (LTA) using different dilutions of agonists (instead of one dilution which was used in the past) and check ATP release in highly suspicious cases for platelet secretion abnormalities.

Aims: To evaluate our cases suspected to PFDs with new panel of platelet function assays especially for finding the prevalence of platelet secretion abnormalities which has never been diagnosed or classified in our hemophilia centers.

Methods: During a 7-month period from November 2011 until May 2012, we have investigated 195 patients with bleeding problems who referred for platelet aggregation test to our special coagulation laboratory of Iranian Blood Transfusion Organization. Twelve normal healthy controls were also evaluated for determination of reference ranges of dilutions of agonists for LTA. Platelet secretion and LTA were measured in platelet rich plasma using Crono-log Lumi-aggregometer (model 700X). We have applied four platelet commercial agonists including ADP (5.10 and 20 μM), arachidonic acid (0.5 mM), collagen (2 and 5 μg/ml) and ristocetin (1.5 mg/ml) for the aggregation test. Due to high expenses of Luciferin-Luciferase reagent, ATP release was investigated only in patients with suspicious secretion abnormality in LTA. Results were analysed by SPSS version 16, Polar Engineering.

Results: Among 195 referred patients (66 male and 129 female), 91 patients were abnormal (quantitative and/or qualitative platelet defects) and 104 cases were quiet normal in platelet count and function. Abnormal cases laid on the category of Bernard Solier Syndrome (BSS) (4 cases) (2% of 195 cases), Glanzman thrombastenia (GT) (9 cases, 4.6%), platelet secretion abnormality (13 cases, 6.7%), low platelet count with normal function (14 cases, 7.7%), unclassified PFDs (46 cases, 23.6%), drug-affecting abnormalities (3 cases) and PFD due to afibrinogenemia (one case). Six cases of GT (5 new and 1 known case) and 3 cases of BSS (2 new and 2 known cases) were confirmed by flowcytometric analysis. Bleeding score (Tosetto et.al) was ≤ 2 in 62% of 195 cases, 3–5 in 28.4% and ≥ 6 in 9.6%. Interestingly

two cases that previously diagnosed as GT and BSS several years ago, laid on the platelet secretion defects category.

Conclusion: It seems that the two rare and severe platelet function abnormalities (GT and BSS) have a relatively higher incidence in Iranian patients. About 8% of patients were classified as platelet secretion abnormality. We guess if we did platelet secretion test in all patients with abnormal platelet aggregation pattern, it's prevalence was higher than 8%. Since this study is the first evaluation of ATP release in Iran, reevaluation of registered patients with PFDs and comprehensive studies should be done to find patients with platelet secretion disorders.

PO 345

The efficacy of anti-Rh D, and vincristine sulphate in immune thrombocytopenic purpura

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Background: Immune thrombocytopenic purpura (ITP) is a benign disease prevalent in infancy, and pediatric age. The critically important complication is related mortality secondary to intracranial bleeding. The therapeutic objective is to decrease its treatment-related side effects, while maintaining platelet counts at a safe level.

Aims: In this study we wanted to investigate the effects of anti-Rh D immunoglobulin in acute, and vincristine sulphate therapy in chronic ITP. We intended to determine the time to the onset of treatment effect.

Methods: Anti-Rh D immunoglobulin (Win Rho SDF®, 300 mcg, 1500 IU, Cangene Corporation, Winnipeg, MB, Canada) was administered to the patients with diagnoses of acute (n = 26), and chronic (n = 14) ITP, while 6 cases diagnosed as acute ITP received vincristine sulphate therapy. Anti-Rh D immunoglobulin was administered at a dose of 50 μg/kg given as a 5-min IV infusion. Chronic cases with ITP who were not responsive to any treatment modality as corticosteroids, intravenous immunoglobulin (IVIG) and Anti-Rh D immunoglobulin received a vinca alkaloid, i.e. vincristine sulphate at weekly IV doses of 0.02 mg/kg (max. 2 mg/dose) for 4 weeks. WBC measurements were performed at the end of 24, and 48 h, and on 5., 7., and 14. days, and at 1. months of the treatment to determine platelet counts. Side effects of the drug therapy were closely monitored. Informed consent forms were obtained from patients and/or their parents. Statistical analyses were performed using 11.5 Statistical Package Program (SPSS v. 11.5). For intergroup comparisons Student-t test, and for comparisons between genders *chi*-square *test* were used. $P < 0.05$ was considered as statistically significant.

Methods: Anti-Rh D administration increased platelet counts within the first 24 h of therapy in cases with acute ITP, and in those with chronic ITP paltelet counts increased only after 48 h of therapy. Even though Anti-Rh D immunoglobulin is not recommended in cases with emergency, in our cases diagnosed as acute ITP, it was found to be effective within the first 24 h of therapy. In our cases we didn't observe any potential adverse effects of Anti-Rh D immunoglobulin (which exerts its effects via blockade of Fc receptors of the reticuloendothelial cells) when given to Rh-positive patients.

Our cases with a diagnosis of chronic ITP responded to vincristine sulphate at a lower extent within the first week of therapy when compared with anti-Rh D immunoglobulin treatment. However especially at 2. weeks, a marked increase in platelet counts was observed. None of our cases experienced any treatment-related adverse effect.

Summary/Conclusions: Anti-Rh D immunoglobulin raised platelet counts to higher levels in acute ITP, rather than chronic ITP. Anti-Rh D immunoglobulin is an efficient, cost-effective, and safe drug to be used in the treatment of acute ITP.

In chronic ITP, vincristine sulphate induced an increase in platelet counts in the long term i.e. 2 weeks. Vincristine sulphate should be used as a last resort in cases of need for its long term effect, and only with a short-acting drug like anti-Rh D immunoglobulin.

PO 346

Thrombotic complications in patients with adult chronic immune thromobocytopenic purpura

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Background: Immune thrombocytopenic purpura (ITP) is autoimmune disease characterized by platelet destruction and decreased platelet production. Occasionally in ITP a life-threatening hypercoagulable state may develop. There are reports of thrombotic events after splenectomy or management of ITP with intravenous immunoglobulin (IVIg). There are also rare reports of intracranial thromboses and ischaemic heart strokes. We present 5 adult chronic ITP patients with different thrombotic events during the long course of ITP hystory.

Material and Methods: We present five patients (4 female and 1 male), with median age 56 (range 43–60), suffering from ITP for median 10 years (range 3–32), four of them were splenectomized, but in all of them with transient response to splenectomy. In further disease course in four patients different treatment modalities were used at the tine of low number of platelets and in one patient the prednisone was the only therapeutic modality. In a-53-year-old woman after splenectomy the number of platelets normalized but on 21st day after splenectomy a portal vein thrombosis developed. She was on LMW heparin and recovered completely. In the second patient after viral infection when platelet count dropped to 1×10^9/L IVIg 0.400 mg/kg/day for 5 consecutive days was given. She responded to the therapy with normalization of platelet count but after 5 days she developed acute anterolateral myocardial infarction with creatin kinase of 2260, troponin 27,8. She was treated with IV heparin and she completely recovered. The other three patients (aged 56, 60 and 56 years) developed cerebral vascular insult (CVI) confirmed by CT in two and MRI in one patient. Two patients were without treatment and one patient was with corticosteroids treatement. At the time of CVI the number of platelets was 21×10^9/L, 18×10^9/L, and 22×10^9/L respectively. All three patients recovered, one, without sequel and two patients with neurologic sequel.

Conclusion: In the first patient with thrombotic event after splenectomy we advise cautiousness after splenectomy for possible post-splenectomy thrombotic complications in the subset of ITP patients who may have persisting platelet activation. In second patient IVIg have promoted thrombosis by increasing bloodviscosity, activating platelets, or causing vasospasm. In patients with CVI anti-platelet antibodies, which are present in most cases of chronic ITP complement mediated fragmentation of platelets and the release of platelet microparticles was a factor contributing to microthromboses.

Kex words: hypercoagulability, immune thrombocytopenic purpura, ischaemic stroke.

PO 347

Successful use of Recombinant Factor VIIa for menorrhagia control in a patient with Glanzmann's thrombasthenia

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Glanzmann's thrombasthenia is a congenital bleeding disorder caused by defective platelet glycoprotein IIb/IIIa, which is essential for the binding process between platelets and fibrinogen. The standard treatment for bleeding is platelet transfusion but repeated transfusion may result in the development of anti-platelet antibodies rendering future platelet transfusion ineffective.

Menorrhagia is common problem in patients with Glanzmann's thrombasthenia, particularly during first menstruations and may sometimes require blood transfusion.
A 14-year-old girl with Glanzmann's thrombasthenia suffered from menorrhagia monthly. Her menstrual cycle was 28 days and menstrual duration was over 15 days. To control menorrhagia patient need repeated platelet transfusion. Over time platelet transfusions had often had a suboptimal result and were complicated by allergic reactions. Alternative effective agents were needed. Hormonal therapy (oral contraceptives), hemostatic drugs were not helpful and patient needed iron supplement for iron deficiency anemia due to massive monthly blood loss. She could not go to school during menstrual period, her activity was disrupted and normal life stood paralyzed.
We report on a patient with Glanzmann's thrombasthenia with menorrhagia, despite documented suboptimal effectiveness of platelet transfusions and complicated allergic reactions, menorrhagia controlled successfully with monthly use of recombinant factor VIIa.

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Evaluation of laboratory desmopressin testing and clinical effects in patients with hereditary and acquired thrombocytopathies: results of a retrospective multicenter study

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Background: The necessity of desmopressin testing (DT) in patients with thrombocytopathies (TP) prior to the first therapeutic use is a matter of controversy and the test itself is not standardized. Aim of study was to develop recommendations on a standardized test in patients with TP.
Patients and Methods: Data were collected from patients tested between January 2000 and September 2010 in 13 centres of the 'Competence Network Hemorrhagic Diatheses East' : age, gender, diagnosis, bleeding history, medication, results of coagulation testing, way of desmopressin administration, dose, side effects, time points of blood sampling, coagulation parameters (von Willebrand factor antigen – VWF:Ag, collagen binding activity – VWF:CB, ristocetin cofactor activity – VWF:RCo, factor VIII activity – FVIII:C, activated partial thromboplastin time – aPTT, PFA-100® closure times – CT with collagen/epinephrine and collagen/adenosine diphosphate cartridge – coll/epi and coll/ADP) and clinical applications. Criteria for desmopressin response were defined as follows: increase of basal values of VWF:Ag and VWF:CB or VWF:RCo of at least 1.5-fold and shortening of CT values reaching the reference range (coll/epi: 85–165 s, coll/ADP: 71–118 s) within 120 min after desmopressin administration.
Results: Seventy-five patients (42 children, age range: 3–17 years; 33 adults, 20–83 years) with hereditary TP (aspirin-like defect: $n = 34$; 25 non-classified TP; 9 storage pool defect; 4 MYH9-associated macrothrombocytopenia; 3 receptor defect) and 15 adults with acquired TP (14 medication-induced, 1 essential thrombocythaemia) were included. Desmopressin was administered i.v. in 56 patients (42 children/14 adults), intranasally (i.n.) in 32 (1/31) and s.c. in 2 adults. The i.v. dose ranged from 0.07 to 0.4 μg/kg (median: 0.3 μg/kg); the absolute i.n. dose from 300 to 600 μg. After desmopressin administration blood was collected mainly at 60, 120 or 240 min measuring CT, VWF:Ag, VWF:CB and FVIII:C. In 6 patients (7%) mild side effects such as facial flush were reported. Of 84 patients evaluated for response, 57 (68%) showed a response and 27 (32%) a non-response. Responders were significantly younger than non-responders and the response was dependent on the way of administration (i.v.: 43 responders/11 non-responders; i.n.: 13/15). After desmopressin administration a significant shortening of CT values was observed reaching the reference ranges. The desmopressin-induced increase of mean values of VWF:Ag, VWF:CB, VWF:RCo and of FVIII:C ranged from 1.2 to 2.7fold.
Gender and blood group did not influence the time course of any of the measured parameters. The maximal desmopressin effects were observed between 30 and 120 min after desmopressin application. For 20 patients (7 children, 13 adults), 23 clinical applications were described including mainly dental/oral surgical interventions ($n = 6$), herniotomies ($n = 3$) and menorrhagia ($n = 3$). Three patients who were classified as responders in the DT suffered from bleeding despite desmopressin application.
Conclusion: An i.v. dose of 0.3 μg/kg and the determination of PFA 100® CT coll/epi and VWF:Ag measured before and 60 min after desmopressin application is recommended as test standard for patients with TP. A prospective study is needed for the validation of these recommendations and for the evaluation of clinical relevance of test results.

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Congenital amegakaryocytic thrombocytopenia – the first case diagnosed in the Czech Republic and successfully treated by means of hematopoietic stem cell transplantation from matched unrelated donor

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Background: Congenital amegakaryocytic thrombocytopenia (CAMT) is a rare recessively inherited disorder characterized by severe thrombocytopenia with a reduction or absence of megakaryocytes in the bone marrow and the development of progressive bone marrow failure. It manifests mostly in the neonatal and infant period. Level of endogenous thrombopoietin (TPO) is high, but platelets or progenitor cells do not respond to it due to mutations of thrombopoetin receptor – c-mpl. In some patients additional anomalies (heart defects, CNS anomalies and orthopedic anomalies) have been reported. The only causative cure for CAMT is hematopoietic stem cell transplantation (HSCT).
Methods: A case of a female baby born in 33 week of gestation is described. The child presented at birth with hematomas and petechiae on the body. Severe thrombocytopenia (14×10^9/L) was recognized. No serious structural congenital anomalies were detected. Intravenous immunoglobulin application as the first treatment attempt had no effect on the level of thrombocytes. Platelet concentrates had to be repeatedly administered in the span of 7 to 8 days once the number of thrombocytes fell below 20×10^9/L. Because of recurrent drop of hemoglobin level red blood cells had to be transfused every 3 weeks. Immunological cause of thrombocytopenia was excluded by serological testing and genotyping of human platelet antigens. Trephine biopsy revealed hypocellular haematopoiesis. A few dysplastic megakaryocytes were found, no other significant features of hematopoiesis dysplasia were observed. TPO level was significantly increased. Other causes of congenital thrombocytopenia (Fanconi anemia, Shwachmann -Diamond syndrome and Dyskeratosis congenita) were systematically examined and ruled out. Molecular genetic testing was performed, mutation in the c-mpl gene was not however revealed. At the age of 6 months the child underwent successful allogeneic bone marrow transplantation from matched unrelated donor. Peri and post-transplant period was without major complications or signs of severe graft vs. host disease (GVHD). Patient has been nowadays surviving 4 years after transplantation without immunosuppression in a good quality of life. Platelet count does not fall beyond normal ranges.

Summary: CAMT is a rare cause of congenital bone marrow failure syndrome. Even though the diagnostic testing (c-mpl gene mutation) is known, cases with not proven mutation were observed. In those affected, bone marrow failure ensues usually within the first 5 years.
The excellent results of the allogeneic HSCT can be achieved only if highly specialized laboratory techniques are at disposition, matched donor is available and transplantation is performed by experienced transplant unit team at early stage of disease.

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Haemostatic management of pregnant women with platelet function disorders: utility of PFA-100

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Background: Defects in platelet function are a common cause of mucocutaneous bleeding, however characterisation of this heterogeneous group of disorders is hampered by the limitations of currently available tests. Consequently, there is little available guidance on monitoring of women with platelet function disorders during pregnancy and labour. Diagnostic uncertainty leads to variable management of these patients between centres and frequently leads to infusion of pooled platelet concentrates. The PFA-100™ is a rapid, global test of shear-dependent platelet function that may be sensitive to quantitative and qualitative defects in platelets and VWF and may provide clinically useful information.
Aims: To evaluate the use of PFA-100™ in informing haemostatic management of delivery in a cohort of pregnant women with platelet function disorders.
Methods: The course, management and outcome of pregnancy and delivery in a tertiary obstetric unit were evaluated retrospectively alongside haemostatic parameters including PFA-100™ analyses.
Results: Nine pregnancies in eight women were evaluated. Prolongation of both collagen/epinephrine and collagen/ADP closure times were demonstrated by PFA-100 analysis outwith pregnancy in all cases, in association with abnormalities of platelet light aggregometry, normal platelet counts, VWF levels and function. All were uncomplicated, singleton pregnancies and delivered at term. Antifibrinolytic therapy (tranexamic acid) was used routinely prior to and/or following delivery. Serial PFA-100 analyses were performed during pregnancy; correction to normal values was demonstrated in four cases, while remaining abnormal throughout pregnancy in five cases. Five of the nine deliveries were vaginal, with two requiring episiotomy and forceps delivery; there were two cases of emergency Caesarian section (C-section) and two planned C-section. Of the five cases with persistently prolonged closure times, three received treatment with desmopressin during labour, two of whom went on to receive combined spinal and epidural anaesthesia (CSE) and C-section uneventfully with no additional haemostatic measures. Further doses of desmopressin were administered as necessary, including prior to epidural catheter removal. The third, in whom desmopressin did not result in correction of PFA parameters, had an uncomplicated vaginal delivery. The remaining two cases with prolonged PFA closure times had vaginal deliveries with no bleeding complications, using alternative forms of analgesia. Of the four cases with normalised PFA-100 closure times, two were vaginal deliveries without CSE, one of which was forceps with episiotomy. One patient required emergency C-section with CSE anaesthesia in her first pregnancy and CSE and elective C-section in her second, which was complicated by uterine atony and significant haemorrhage. No patients in our cohort received prophylactic pooled platelet concentrates.
Conclusions: Our data suggest that PFA-100 analysis may be useful in monitoring selected patients with platelet function disorders during pregnancy: PFA-100 analysis was shown to directly influence haemostatic management of delivery in at least six of the nine cases studied. We propose that women with previously demonstrated abnormalities in PFA-100 that fully correct during pregnancy (or following DDAVP) be considered for regional anaesthesia and delivery without routine use of platelet concentrates. Therefore, PFA-100 may be useful in tailoring haemostatic support and avoiding unnecessary use of platelets in these patients.

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Spontaneous duodenal hematoma in a patient with Glanzmann's thrombasthenia

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Background: Glanzmann's thrombashtenia (GT) is a rare congenital disorder of platelet function associated with a prolonged bleeding time, a normal platelet count, abnormal clot retraction and defective platelet aggregation. Mucocutaneous bleedings including gastrointestinal bleeding are common in GT patients but spontaneous intramural duodenal hematoma is extremely rare (1–2).
Aim: We report a child who had GT presented with signs and symptoms of duodenal obstruction. We aimed to pay attention a rare presentation of GT patients.
Case and Methods: A 5-year-old boy with a known GT was brought to the pediatric emergency room by his parents. The patient had been complainting of the acute onset of periumblical and right upper quadrant pain and vomiting. There was no melena and hematemesis in admission. No history of trauma was present. The abdomen was flat with tenderness in the midepigastrium and right upper quadrant without evidence of peritoneal irritation. Rectal examination reveled black stool.
Results: Abdominal ultrasonography revealed appearent intramural duodenal hematoma and ileoileal invagination. Invagination was resolved via rectal barium application. Intramural hematoma was resolve recurrent administration of thrombocyte suspension. Erythrocyte suspension was given because of deep anemia. The patient did not need further surgical intervention. Intramural hematoma completely regressed on day 10.
Discussion: Intramural duodenal hematoma is generally seen after abdominal trauma. It has been reported spontaneous intramural duodenal hematoma in coagulation disorders such as Von Willebrand disease, immune thrombocytopenic purpura, polyartheritis nodosa, hemophilia A (1–3). Only one report about GT and intramural duodenal hematoma was found in the literature and GT (1). The diagnosis of duodenal hematoma should be considered in any patient who has abdominal pain and a coagulation disorder. İntramural hematoma may be abundant even requiring blood transfusion. Nonoperative management of an obstructing duodenal hematoma was successful and potentially life-threatening surgery was avoided.

PO 352

Use of recombinant factor VIIa in the management of 3 bleeding episodes in a patient with Bernard-Soulier syndrome

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Introduction: Bernard- Soulier syndrome (BSS) is a rare congenital platelet autosomal recessive disorder characterized by defective platelet adhesion and manifested by spontaneous and often profuse bleeding.
Objective: To report the efficacy of recombinant factor VIIa (rFVIIa) in the management of bleeding episodes in a patient with BBS refractory to platelet transfusions.

Report: We reported the case of a 20 year-old woman with BBS. Born from a first degree consanguineous marriage. Family history: 10 siblings, five of them suffering from BBS and 3 died in early infancy by severe haemorrhage. The patient was admitted twice for severe melena and once for menorrhagia scored 150 (Higham et all (1990) British Journal of Obstetrics and Gynecology). Despite oral contraception and Platelet transfusion, bleeding persisted with severe anaemia. She was administered Intravenous rFVIIa (NovoSeven®) (90, 56 μg/Kg, 3doses at 2 h). She was administred parenteral iron supplement which corrected anaemia.
Conclusion: Considering the refractory to platelet transfusion and platelet alloimmunization risk in our patient, we administered rFVIIa which permitted to stop the haemorrhage.This case highlights the potential benefits of rFVIIa in helping to control severe bleeding episodes in patients with BBS.

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Availability of a microchip flow chamber system as a screening test of platelet storage pool disease

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Background/Aims: Platelet storage pool disease (SPD) is platelet disorders characterized by a reduction in the number or content of α-granules, δ-granules or both. SPD is diagnosed with electronic microscopy and quantitative measurement of platelet ADP/ATP, which need specialists. Although SPD presents prolonged bleeding time, a standard screening test, its accuracy depends on individual technique and sometimes it shows false-negative. We hypothesized that an automated microchip flow-chamber system, Thrombus-formation Analysis System (T-TAS, Fujimori Kogyo), which has been reported to be useful for evaluations of antiplatelet therapy (Hosokawa, Thrombosis and Haemostasis 2013), might be available as a screening test for platelet functional disorders. We evaluated a family-case of α/δ SPD with T-TAS.
Case: A 6-year-old boy presented with intracranial bleeding after a head injury, and postoperative ruptured suture. His mother and the elder brother also repeated subcutaneous hemorrhage and a nasal bleeding since childhood. They had no clinical symptom except for bleeding.
Methods: In T-TAS, hirudin-added blood was injected to a microchip which surface was coated by collagen, and platelet thrombus formation was monitored with a pressure sensor. The blood flew through the duct in microchip at the shear rate of 1000 s. Flow pressure curve was visualized for 10 min and the time when the pressure in the microchip reaches 10 kPa (T_{10}) was evaluated. Platelet aggregation was further evaluated with standard assays using platelet aggregometer, PRP313M (IMI) and Multiplate (Verum Diagnostica).
Results: Platelet count and size was normal in all cases. His bleeding time was 15 min and his mother's was 6 min, while other common coagulation tests were normal. In PRP313M using platelet rich plasma, their platelet response to 5 μg collagen and 1.7 μg ristocetin were 48.6%-58.3% and 27.3%-55% compared to normal control, respectively. In Multiplate using whole blood added hirudin, response to ristocetin, ADP and collagen were within normal range. In T-TAS, however, thrombus formation was not observed during measurement time (T_{10} > 10 min, reference 2.4–6.6 min) in all cases. Farther investigations for definite diagnosis were performed. Electronic microscopy revealed few α-granules. In quantitative analysis of ADP/ATP released from washed platelets stimulated by convulxin or PAR1P, ADP was 0–1.09 μM at 1.0×10^6/μL platelets (reference 11.7–13.7 μM) or 0–1.55 μM (10.5–12.0 μM), respectively, and ATP was 0 μM (14.0–16.3 μM) or 0 μM (13.7–14.8 μM), respectively.
Conclusion: In this α/δ SPD family case, standard platelet function tests were nearly normal except for prolonged bleeding time in the propositus, but T-TAS could show abnormal platelet aggregation in all cases. Therefore, T-TAS might have high sensitivity to platelet disorders and be a candidate as a screening test for platelet functional disorders including SPD.

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Features of patients with immune trombocytopenic purpura south-east of Turkey

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Background and Aim: Acute immune (idiopathic) thrombocytopenic purpura (ITP) is characterized by thrombocytopenia, spontaneous formation of purpura, petechiae, ecchymoses and mucosal bleedings and it is also a disease which can be diagnosed with exclusion of other thrombocytopenia symptoms. However, the disease is important in aspect of severe organ bleedings and risk of becoming chronic. In this paper, clinical and sociodemographical features of cases with ITP evaluated in South-East of Turkey.
Materials and Methods: Total 151 patients, who were diagnosed with ITP, were evaluated. The patients' male/female ratio was 1/1.06. The average age was between 5.1 ± 3.4. Seventy-five percent of the patients were diagnosed as acute ITP and 25% of the patients were diagnosed as chronic ITP.
Result: Tendency to chronicity was highest in the 2–10 years age group ($P = 0.01$). The most frequent presenting symptom was skin findings in both the acute and the chronic group. A 64.6% of the cases with insidious onset had become chronic. It was determined that season and gender had no effect on chronicity of ITP (< 0.05). A 68.4% of the acute cases had history of infection and seropositivity was demonstrated in 1%. Bone marrow aspiration findings did not alter the diagnosis in any patient. In the first treatment, the patients responded partially positive against steroid; the ratio of becoming chronic and, in the acute ITP group the ratio of positive response against steroid was higher and this difference was found significant ($P = 0.001$ and $P < 0.05$).
Conclusion: The preference of economical and effective treatment methods for the ITP patients will prevent morbidity and mortality deaths relating to ITP.

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Successful IVF and pregnancy in Glanzmann's thrombasthenia under cover of recombinant factor VIIa

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Case Report: A 35 year-old woman with glanzmann's thrombasthenia (GT) with no further conservative treatment options for primary infertility underwent *in vitro* fertilization (IVF). Ultrasound guided follicle aspiration was performed under cover of recombinant factor VIIa (rFVIIa). The patient became pregnant during the first treatment cycle.
Pregnancy itself was uneventful. During pregnancy, the frequency of epistaxis increased significantly and could be treated successfully by local application of tranexamic acid and bipolar coagulation during one episode.
In 38 + 1 weeks of gestation, primary caesarian section was performed due to the elevated risk of peripartal bleeding again under cover of

rFVIIa. Four HLA compatible platelet concentrates had been prepared as stand by therapy. An apparently healthy child was delivered. Despite no evidence for increased intraoperative bleeding, postoperative hemoglobin concentration decreased to 6.7 g/dl, necessitating transfusion of two erythrocyte concentrates. Recombinant FVIIa was reduced successively and discontinued on day 10 after delivery. Under this regimen including tranexamic acid 1000 mg tid p.o.), no further relevant bleeding complications occurred and lochia were even below average.
The decision for rFVIIa over platelet transfusions was guided by the idea to prevent alloantibody formation against the GPIIb/IIIa complex.
This case report demonstrates feasibility and safety of IVF in women with GT under specific treatment.

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Course of pregnancy, labour and perioperative management of a patient with Bernard-Soulier Syndrome – case report and literature review

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Background: Bernard-Soulier syndrome (BSS) is a rare hereditary thrombocytopathy characterized by large platelets, thrombocytopenia of variable degree, and bleeding symptoms. The disease is caused by dysfunctional GPIb/IX/V heterocomplex or its absence.
We present the course of pregnancy, delivery and postpartum period of a patient with a grave Bernard-Soulier syndrome complicated by a history of VTD and epilepsy.
Case Report: A 39-year-old patient was admitted to the Clinic in 15th gestational week of her second pregnancy (one miscarriage) due to grave anemia and thrombocytopenia (Plt = 3G/L). She had a history of chronic ITP treated with corticosteroids since infancy, splenectomy performed at the age of 25 and was first diagnosed with Evans' syndrome 6 years ago. The diagnosis was then discarded following negative Coombs' test. After skull injury in early childhood she developed grand mal epilepsy at the age of 14. Since the age of 25, the patient experienced numerous mucosal bleeding episodes from GI tract, epistaxis, easy bruising and gingivial bleeds. She also suffered from viral hepatitis C and had a history of deep venous thrombosis. During pregnancy she was administered several platelet, FFP and RBC transfusions, she was treated with corticosteroids and immunoglobulins as well as argon coagulation procedures. Immunological tests confirmed BSS diagnosis (decreased expression of GP Ib/IX by 58% and GP Ib – by 63.5%).
The course of pregnancy was uneventful except for severe thrombocytopenia. The patient was admitted to the II Ob/Gyn Dept in 37th completed week of gestation. Cesarean section was performed and the male fetus from the breech position was born weighting 2780 g, 53 cm, 1st min Apgar score – 10. The postoperative period was complicated by wound and mucosal bleeds in spite of extended treatment. Perioperative and anesthetic management included platelet, FFP, RBC transfusions, corticosteroids and a selection of appropriate drugs and techniques.
Conclusion: The case of BSS pregnant woman highlights the need for establishing both hematological and obstetrical care standards for management of this rare disease.

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RFVIIa inhalation for life threatening pulmonary hemorrhage in a case with refractoriness to platelet transfusion

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Background: Refractoriness to platelet transfusion is a common complication of repeated platelet transfusion in relapsed acute leukemia, stem cell transplantation, aplastic anemia, and thrombasthenia. It is associated with high mortality rate due to massive intracranial, intrapulmonary or internal bleeding, despite aggressive treatment with platelet and red cell transfusion, high dose steroids, immunoglobulin, anti-fibrinolytic agents and intravenous recombinant factor VIIa (i.v. rFVIIa).The aim of this presentation is to indicate that localintra-pulmonary administration of rFVIIa may be more effective than the intravenous route. We present a 51 years old male with resistant acute lymphoblastic leukemia who who failed to respond first line and second line induction chemotherapy and became refractory to platelet transfusion. On day 17 after receiving a third line induction chemotherapy he developed rapidly progressive cough, shortness of breath and hemoptysis. He was febrile, severely neutropenic with WBC 0.5×10 ⑩/L, Hb 85 g/L, platelets 7×10 ⑨/L despite repeated platelet transfusion. Coagulation profile and chemistry tests were normal. He was transferred to the intensive care unit, intubated and given single donor platelet transfusion along with rFVIIa 90 mcg/kg i.v. every three h in addition to red cell transfusion, fresh frozen plasma, methyl prednisolone, tranexamic acid and immunoglobulin. Pulmonary hemorrhage continued despite a rise in platelet count to 28×10⑩/L and consumption of four doses of single donor platelets and rFVIIa. At this point, same dose of rFVIIa was put in 5 ml normal saline and given intrapulmonarily by nebulizer.
Results: Pulmonary hemorrhage was stopped almost immediately and platelet count continued to rise. He was extubated 5 days later and transferred back to the ward on day 10 with WBC 5×10 ⑨/L, Hb 107 g/L, and platelets $117 \times 10$⑨/L.
Conclusion: rFVIIa inhalation may be more effective in cases with pulmonary hemorrhage.

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Time dependent effect of aspirin intake on circadian rhythm of platelet reactivity: a pilot study

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Background: Hemangioma, most common benign vascular tumor, has natural history of proliferation in the first year of life followed by spontaneous resolution. The treatment is not universal standard with indication as following: impaired organ function and Kasabach Merritt syndrome (KMS). Alpha-interferon inhibits angiogenesis, endothelial cell migration and proliferation.
Objective: To evaluate the outcome of alpha-interferon for the treatment of hemangioma.
Methods: We recruited 35 patients with hemangioma receiving alpha-interferon followed at hemangioma clinic between January 2001- January 2011.The patients' data including sex, age, type and location of hemangioma, indication, duration, outcome and complications of treatment were reviewed.
Design: Retrospective descriptive study.
Measurements: We evaluate of the outcomes including improvement of organ function, resolution of Kasabach Merritt syndrome (KMS) and decrease hemangioma size.
Results: the number of patients was 35 patients (female = 21, male = 14) with mean age 3.2 months (range 5 days-15 months), loss follow up in five patients. Hemangioma was divided into cavernous

(9), capillary (10), mixed (7). Location of hemangioma includes head and neck (21), extremities (7), mediastinum (3), intraosseous (1), liver (2), larynx and epiglottis (2), and scrotum (1).Average age of treatment was 4.7 months (range 5 days-18 months). Duration of response in 29 patients was 4.7 months (range 1–6 months). Indications of treatment included KMS (1), KMS with impaired organ functions (2), impaired organ functions (30), infection (2), intracranial (1). Eleven patients were decrease in size of mass > 50%, 14 patients were decrease in size < 50% but 23 patients (82.1%) were improved organ function and resolution of KMS. Complications of treatment were elevation of liver enzymes (3), and fever (6).

Conclusions: The use of alpha-interferon was well response to improve organ function and decrease hemangioma size. Due to spastic diplegia, serious complication of alpha-interferon from previous case report studies, the consideration of this treatment should be discussed with parent in light of pro and cons.

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Can abnormal high mean platelet volume be used as a screening factor for detection of metabolic syndrome and coronary heart disease?

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Background: Platelets or thrombocytes are the smallest cells in the blood, heterogeneous with respect to size, density, age and metabolic function, initiating the process of coagulation when a blood vessel is broken.

Mean platelet volume (MPV) is a measurement that describes the average size of platelet cells in the blood. The medical literature abounds with studies of various diseases showing a higher MPV in the affected group than in the control group. This is a confirmation of platelet activation in these disorders. Nevertheless, in these studies, the 'abnormal high' MPV is still within the normal range (7–11 fL) even if it is higher than the MPV control group (also within the normal range).

In fact, in day by day practice, in patients with platelet activation, we cannot guess that the MPV is 'abnormal' (compared to control group) although within normal range.

Aims: A. To select consecutive patients visiting the emergency room with MPV above the upper limit of the normal range, and without any disease supposed to cause an abnormal MPV. B. To perform tests on these patients that may reveal pre-diabetes (PDM)/diabetes mellitus (DM), metabolic syndrome (MS) or coronary artery disease (CAD). C. To decide if a naive high MPV can or cannot be used as a screening test for these diseases.

Methods: During 6 months 792 consecutive patients with MPV higher than 11.5 fL (normal: 7.0–11.0 fL) were checked for every disease known to cause high MPV. One hundred and seventy-seven naive patients (those with high MPV but without a clear etiology) have been selected for our study.

Out of 792 patients, 177 patients were found to be naïve and only 30 were interested in undertaking supplementary tests in order to explore the possibility of DM, MS or CAD.

Results: The MPV was 12.3 ± 0.74 (range: 11.5–15.2 fL).

Study population: 47% were males and 53% females; the age was 41 ± 8 (range: 28 – 58 year); Ashkenazi Jews 10%, Sepharadi 43%, Arab (Muslim, Christian and Druse) 43%.

All the selected patients were tested for fasting glucose, HBA1c, CRP and high sensitivity CRP (HS-CRP), triglycerides (TG), total cholesterol (TC), LDL-cholesterol (LDL-C) and HDL-cholesterol (HDL-C).

Blood pressure and waist circumference were measured. TTE and treadmill tests were performed. BMI was calculated for every patient.

MS was found in 33.3% of patients (in Israel's general population, prevalence is 15–17%) and *P* value was 0.022 – statistically significant.

P-DM was found in 30% of patients (in Israel's in general population ages 35–45, prevalence is 15%), the *P* value was 0.028 – statistically significant.

Treadmill test was negative in all patients and TTE demonstrated diastolic dysfunction ± calcified valve in only 16.6%.

Conclusions: A high MPV in a naive patient can be used as a screening factor for P-DM/DM and MS. It does not predict the presence of CAD.

Limitation of this study: the cohort of patients is low; this study will be continued to make possible a better statistical analysis.

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Metabolomics response of human platelets to collagen interaction

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Background: Metabolomics is the study of total metabolites in a biological system, these studies rely predominantly on NMR and Mass Spectrometry techniques. This approach is yet to be applied comprehensively to platelets, specifically their response to collagen interaction.

Aims: To identify changes in the platelet metabolome in response to collagen receptor engagement. A focus will be placed on the lipidome, and the use of highly specific ligands for different collagen receptors should allow identification of lipid metabolism pathways which are involved in the different receptor axes.

Methods: Three different methods for total lipid extraction are tested, the most effective extraction method for the lipid species of interest is then utilised. Lipid extracts are analysed on a variety of Mass Spectrometry instruments including a Q Exactive Thermo Scientific spectrometer and a LTQ Orbitrap Velos spectrometer. Data is subjected to relevant multivariate statistical analysis such as PLS and PCA. This form of analysis can also be applied to the study of lipid rafts as some platelet collagen receptors have been shown to localise to rafts such as GPVI.

Results: The most efficient lipid extraction method was selected for the broadest range of lipid species of interest possible. This method has been utilised for successful lipid extraction from platelets stimulated by different ligands.

Conclusions: Metabolomics provides a novel method of studying the platelet metabolome and highlighting responses to collagen interaction. The identification of specific lipid species involved in the platelet response to collagen interaction will shed light on uncharacterised receptor axes.

References:

1 Ruggeri, ZM. Nature Medicine 2002 Paglia et al., *J Chromat* 2012.

PO 361

Whole blood aggregometry and platelet derived microparticles enumeration in acute leukemia patients

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Background: Hemostasis is a complex and efficient system maintaining a delicate balance between a prohemorrhagic and a prothrombotic state. Platelets and platelet derived microparticles (PMPs) resulted from platelet membrane remodeling in response to activation or apoptosis play important roles not only in physiological hemostasis, but also in pathological conditions.

In hematological malignancies, the hemostasis impairment can occur both in terms of bleeding, as well as thrombosis. The prediction of

these complications is difficult because of the lack of laboratory markers.
Aims: This study aimed to assess the platelet function and the circulating PMPs levels in patients with acute leukemia and their possible role in hemorrhagic and thrombotic complication prediction.
Methods: Platelet function testing was achieved through whole blood aggregometry using adenosine diphosphate (ADP), colagen and thrombin receptor activating peptide (TRAP) as agonists.
Circulating PMPs levels were evaluated by flow cytometric method.
We investigated 24 patients with acute leukemia at the moment of diagnosis, prior specific treatment initiation and a control group of 16 healthy subjects.
The study was approved by the Ethics Committee of Coltea Clinical Hospital and informed consent was obtained from every participant of the study in accordance with the Declaration of Helsinki.
Results: The results showed that compared with the control group, patients with acute leukemia displayed reduced platelet aggregation response in 19 cases (79%), increased response in 3 cases (13%) and normal response in 2 cases (8%). The reduced platelet aggregation response was associated with hemorrhagic complications development in 8 patients (42%). The increased response to agonists was associated with major thrombotic events (myocardial infarction and stroke) occurrence in all the 3 cases (100%).
The level of PMPs in 21 out of the 24 patients (88%) was not significantly different from the control group, but in 3 patients the circulating PMPs number was much higher, two out from these three patients (67%) developing major thrombotic events during disease progression.
Conclusions: These findings indicate that platelet hypoaggregability precedes bleeding complications, while platelet hyperreactivity could predict major thrombotic events in patients with acute leukemia. Notwithstanding our study group was too small, this hypothesis requiring further investigations.
Our data suggests that PMPs enumeration doesn't offer significant information on acute leukemia patients hemostasis status, except rare isolated cases.

PO 362
A systems biology study of platelet GPVI signalling

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Background: Platelet activation occurs in response to a diverse range of signals from the environment and is critical for haemostasis. The complexity of platelet activation *in vivo,* which results in the engagement of multiple cell surface receptors and intracellular signalling pathways, renders this process difficult to study. Although many of the proteins that participate in these pathways are known, their interactions and stoichiometry are still poorly understood. The interdisciplinary techniques of systems biology can enhance our understanding of such complex biological systems that operate over many space and time scales.
Aims: Through close collaboration between mathematicians and biologists we hope to enhance our knowledge surrounding platelet activation and aggregation. To aid this we build stand-alone mathematical models representing individual signalling pathways that can be closely linked to experimental data. Mathematical models are often difficult to utilise in a laboratory setting so we aim to provide interactive tools that enable experimentalists to quickly generate and test hypotheses automatically comparing numerical simulations to experimental data. Analysis of mathematical models should provide us with an enhanced quantitative understanding of the interactions involved, extending the utility of data sets beyond that solely available through intuition alone.
Methods: We present an initial mathematical model of the early subcellular events following the GPVI receptor complex activation. In order to deveop such a model current biological knowledge of the key components involved needs to be captured. Estimations of protein concentrations, binding affinities and rate constants for the key reactions are essential for an accurate model. Such quantitative data requires different experimental approaches and is currently poorly represented in the platelet literature. We compare numerical simulations of our mathematical model to this experimental data focussing on the changes in the phosphorylation states of specific tyrosine residues or key components within the early stages of the GPVI signalling pathway. Through parameter sensitivity and estimation techniques we determine key aspects of the signalling network that effect its dynamics.
Results: A mathematical model is presented that quantitatively captures the time course of kinase activation that follows ligands binding to the GPVI receptor. Our early experiences and difficulties in trying to determine the precise time and location rich data required to parameterise this model are discussed. To enable use of our model in a laboratory setting we are developing a user interface that allows biologists to compare numerical simulations to experimental data. Mathematical analysis of our model highlighted the importance of platelet shape, structure and protein copy numbers of specific proteins to the regulation of these early events.
Summary/Conclusion: By concentrating on detailed models of subsets of individual signalling pathways we are able to validate current knowledge of individual components, their interactions and stoichiometry before we compile them into a complete mathematical model of platelet signalling following activation via combinations of many agonists.

PO 363
Immature platelet fraction and mean platelet volume in diabetes mellitus

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Background: Dysregulated platelet-endothelial interactions have been recognized as an important pathogenic mechanism in the development of atherosclerosis. Accelerated atherosclerosis is the main underlying factor contributing to the high risk of vascular events. In patients with diabetes mellitus (DM) in particular, platelet abnormalities play a pivotal role in the lead up to atherothrombotic events.
Aims: Immature platelet fraction (IPF), absolute IPF and mean platelet volume (MPV) was evaluated to investigate the platelet status in DM subjects.
Methods: We investigated peripheral blood IPF, absolute IPF and MPV of 366 patients with DM, 30 with metabolic syndrome (MetS), and 54 controls. The MPV, IPF, absolute IPF and other blood cell indices were measured using Sysmex XE-2100 (Sysmex, Kobe, Japan) and compared between each groups. That of the DM patients were analyzed according to the presence of diabetic complications and glycemic control ($\leq$ 6.5% HbA1c vs. 6.6–7.9% HbA1c vs. $\geq$ 8% HbA1c).
Results: The DM group had a significantly higher MPV (10.35 vs. 10.00 fL, $P = 0.0118$), IPF (%) (2.20 vs. 1.70%, $P = 0.0201$) and absolute IPF (4.80 vs. 4.60 $x10^9$/L, $P = 0.0429$) than the control group. The MetS patients also had higher those markers than the control, however statistically significant differences were not found. There was significant differences in IPF (%) between DM patients with and without cardiovascular disease ($P = 0.0384$). Among DM patients, IPF(%) and absolute IPF showed significant difference based on the HbA1c concentration ($P = 0.0142$ and 0.0034).
Conclusion: IPF is higher in the patients with diabetes or MetS than the control, moreover, associated with cardiovascular disease and poor glycemic control.

PO 364

Responses of different platelet activation pathways in health volumteers with Aspirin/Clopidogrel administration

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Aim: To investigate the inhibition profiles of aspirin and clopidogrel on COX-1 and P2Y12 receptor, and potential interaction between them.
Method: Twenty healthy male volunteers were admitted and divide into two groups, which take clopidogrel (75-mg loading dose and maintenance) or aspirin (100-mg loading dose and maintenance) in consecutive 7 days. Platelet inhibition (%), CEPI-CT/CADP-CT, and CD62p were observed on the 1st, 3rd, 5th and 7th day after medicine intake and withdraw.
Results: Both CEPI-CT and CADP-CT changed significantly ($F = 27.2$ and 25.3, $P < 0.05$) after clopidogrel use, only CEPI-CT changed statistically after aspirin administration ($F = 36.7$, $P < 0.05$). Inhibition on COX-1 induced by arachidonic acid (inhibitionAA) increased statistically ($F = 35.1$, $P < 0.05$) after aspirin intake. In clopidogrel group, it was P2Y12 inhibition increased statistically ($F = 24.8$, $P < 0.05$), but not inhibitionAA ($F = 1.85$, $P > 0.05$). CD62p expression decreased in both groups (clopidogrel: $F = 28.7$, $P < 0.05$; aspirin: $F = 20.7$, $P < 0.05$) after intake.
Conclusions: COX-1 activation may interact with P2Y12 receptors in clinical settings and POCT in platelet function helps to monitor in dual-therapy informatively.

PO 365

ELISA-VASP assay: preanalytical stability of whole blood samples

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Background: Phosphorylation of VASP (Vasodilator Stimulated Phosphoprotein) is the most relevant biomarker mirroring the P2Y12 activation by ADP at a molecular level. P2Y12 antagonists play a major role in cardiology. While the flow cytometry-based assay (PLT VASP/P2Y12) is widely used, a new ELISA-based assay (CY-QUANT VASP/P2Y12) was recently developed at BioCytex.
Aims: To determine the stability of whole blood (WB) samples stored at room temperature (RT) before analysis by ELISA-VASP. Stability of WB samples from healthy donors as well as treated patients was investigated over up to 4 days after blood sampling.
Methods: WB samples from healthy subjects and patients undergoing antiplatelet treatment were collected and stored in the dark at RT. Aliquots were taken out using a syringe and needle from the unopened tubes each day from D0 to D4 and readily analyzed by ELISA-VASP. Evolution of the Platelet Reactivity Index (PRI) values was examined over time. Five independent series of samples ($n \geq 30$) from presumed healthy donors were respectively analyzed by ELISA-VASP at t = 0 (D0) and after 1 (D1), 2 (D2), 3 (D3) and 4 days (D4) of storage at RT.
Methods: Platelet Reactivity Index (PRI) values at each time were compared using the non parametric ANOVA on ranks test (Kruskal-Wallis). Although a statistical discrepancy between t = 0 and the following days was detected, the difference was minor and did not interfere with the interpretation of test results. Results from patients will be discussed as well.
Conclusion: The whole blood stability of samples stored at room temperature prior to analysis using the CY-QUANT VASP/P2Y12 is acceptable for at least 48 h or even longer for samples from healthy subjects.

PO 369

The participation of gamma/delta and alfa/beta T- lymphocytes in coagregates forming with platelets in patients with coronary artery disease

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Background: Recent studies accumulate evidence that platelets, beside their haemostatic activity, participate in inflammation, immunity and atherosclerosis. In a previous study we demonstrated that CD4 + lymphocytes interacted with platelets and formed heterotypic aggregates. We also found that platelets support CD4+ lymphocyte adhesion to the subendothelial extracellular matrix under flow conditions by formation of heterotypic clusters that are dependent on platelet adhesion and aggregation and mediated by CD40L, PSGL-1 and $_1$-dependent integrins.
Aim: Aim was to investigate the participation of gamma/delta and alfa/beta T- lymphocytes in coagregates forming with platelets in healthy individuals and patients with coronary artery disease.
Methods: In this study, we used the blood of twenty healthy men and thirty-two patients with coronary artery disease (as anticoagulant sodium citrate 3.8% were used). Blood samples were pre-incubated with specific antibodies for platelets, T-cell and gamma/delta and alfa/beta T- lymphocytes detection (Beckman Coulter). The monoclonal antibodies anti-CD3-FITC (Beckman Coulter, Brea, CA, USA), anti-TCR PAN alfa/beta -PE (Beckman Coulter, Brea, CA, USA), anti-TCR PAN gamma/delta -PC5 (Beckman Coulter, Brea, CA, USA), anti-CD41-PC7 (Beckman Coulter, Brea, CA, USA), anti-CD45-ECD (Beckman Coulter, Brea, CA, USA), and their negative controls were used to label gamma/delta, alfa/beta T- lymphocytes and platelets, respectively. Counting of cell aggregates and the definition of lymphocytes phenotype in the composition of these aggregates was performed using flow cytometry FC 500 (Beckman Coulter, Brea, CA, USA). A minimum of 5000 lymphocytes was counted per test.
Methods: It was observed that in the normal whole blood the number of lymphocyte-platelet aggregates were $3.37 \pm 0.8\%$ of the total number of leukocytes. The percentage of T-cell-platelet coagregates were $72.15 \pm 5.7\%$ of the total number of lymphocyte-platelet aggregates. In total T cell-platelet aggregates T gamma/delta were $6.27 \pm 2.4\%$ and T alfa/beta $85 \pm 6.4\%$. Patients with coronary artery disease and progressive atherosclerosis have increased number of lymphocyte-platelet aggregates (LPA) compared to healthy donors ($P < 0.001$). Administration of aspirin (cyclooxigenase-1 inhibitor) corrected the number of LPA to the values in healthy individuals. Conclusions. So in whole blood of healthy donors T gamma/delta – cells interact with platelets, but in T cell-platelet adhesion alfa/beta T-lymphocytes take a greater role. Perhaps it is important for T-cell migration and participation of platelets in the immune response and atherogenesis. Our data point on lymphocyte-platelet interaction in atherosclerosis with coronary artery disease. This process depends on cyclooxigenase-1 related platelet function.

PO 370

Guideline for diagnosis of inherited diseases of platelet function: interdisciplinary S2K guideline of the GTH (e..V.); AWMF-Registry Nr. 086-003

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Background: Congenital disorders of platelet function are a heterogeneous group of disorders which are often not detected until bleeding

occurs. In clinical settings only a few methods have proven to be useful for identification and classification.

Aims: The THROMKID quality project was aimed to obtain information on the means of investigating platelet function employed by clinical centres in German-speaking countries.

Methods: A patterns-of-practice survey was conducted from 2005 to 2007. Consensus conferences were held 2009 and 2011. Results were summarized in the guideline AWMF-Registry Nr. 086-003 (http://www.awmf.org/) 2012.

Results: For a rational diagnostic approach, a stepwise algorithm is recommended. Patient history and clinical investigation is mandatory. Von Willebrand disease and other coagulation disorders should always be ruled out prior to specific platelet testing. Platelet count, size, volume (MPV) and morphology may guide further investigations. The PFA-100® CT is suited for screening for severe platelet defects. Platelet aggregometry allows assessment of multiple aspects of platelet function. Flow cytometry enables diagnosis of thrombasthenia Glanzmann, Bernard-Soulier syndrome and storage pool defects. Molecular genetics may confirm a putative diagnosis or pave the way for the identification of new defects.

Conclusion: Consensus guidelines that reliably guide clinicians may improve diagnostic and therapeutic approach to affected patients.

PO 371

Lymphocyte-Platelet Adhesion in patients with chronic viral hepatitis C

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Background: In the works of many researchers shows the significant role of the immune system in the pathogenesis of chronic viral hepatitis C. It is now widely studied intercellular interactions, especially between hemostasis and immunity. It is established, that the platelets are relevant to inflammatory reactions: adhesive interaction between platelets and leukocytes are important links in the mechanisms for migration of leukocytes to zone of damage, and, consequently, the inflammation and the development of immune and reparative reactions (Vitkovsky Y.A. et al., 1999–2011). At the same time, many aspects of the immune response with hepatitis C remains unknown, although this is important both in the theoretical aspect to understanding immunopathogenesis, and in practical terms, to forecast the course of the disease.

Aim: The Aim was to study the performance adhesion of lymphocytes with platelets in patients with chronic viral hepatitis C.

Methods: Thirty-two persons with chronic viral hepatitis C were examined. Group included 10 women (31.3%), 22 men (68.7%), in age 37.68 ± 2.0 years old. Duration of the illness up to 5 years was revealed at 59.1%, over 5 years – at 40.9%. The first degree of the biochemical activity was determined at 59.1% of patients with chronic viral hepatitis C, the second – at 40.9%. LTA was studied by Vitkovsky's method (1999). Indicator of LTA expressed number of units of lymphocytes with platelets on 100 cells (norm – 13–15%). Degree of adhesion (LTD) defined as number of platelets, formed contact on lymphocyte surface (norm – 3.0 ± 0.3).

Methods: It is established that at patients with chronic viral hepatitis C the LTA-index decreased to 10.0 ± 1.32%. The average LTD reduced to 2.46 ± 0.29. Thus at the persons had illness HCV less than 5 years ago, LTA consisted 10.23 ± 1.81%, LTD – 2.35 ± 0.24; at the patients who had diseases more than 5 years ago – 9.67 ± 2.01% and 2.58 ± 0.61 respectively. At patients with the first degree of biochemical activity of process had minimal LTA-index – 8.62 ± 2.48%; LTD – 2.48 ± 0.40. Patients with the second degree of HCV activity showed 12.0 ± 1.68% of LTA-indicies; an 2.39 ± 0.39 of LTD.

Summary: Thus, in the case of chronic viral hepatitis C the adhesion of lymphocytes with platelets decreased. This decrease was more in persons with a longer duration of the disease.

PO 372

Comparison of TRAP-stimulated platelet releasate between children and adults using two dimensional-Differential-In-Gel Electrophoresis (DIGE)

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Background: Platelets play important roles in hemostasis and thrombosis. In response to physiologic stimuli such as thrombin, platelets release their granular contents that mediate both thrombosis and vascular repair. Recent studies of the platelet proteome demonstrated that secretion of platelet proteins is increased in patients with cardiovascular disease and that these secretory proteins play potential roles in the inflammatory process. Hence the study of platelet releasate or secretory proteins has become important.

Aims: This study aimed to characterise the differences in platelet releasate from healthy children and adults upon activation with thrombin receptor activating peptide (TRAP).

Methods: Blood samples were collected from adults (n = 4, age 22–41 years old) and children (n = 4, age 2–6 years old). All participants were healthy, with no family history of haematological disorders and had not taken any antiplatelet medication 10 days prior to sample draw.

Washed platelet suspensions were prepared and activated with 0.5 U/L TRAP. Protein released from the activated platelets was isolated and stored at −80 °C until analysis by 2 dimensional Differential-In-Gel Electropheresis (DIGE). Releasate protein from each individual was randomized for labeling with either Cy 3 or Cy 5 cyanine dyes. Equal amount of protein from all the samples were pooled and labeled with Cy2 acting as internal standards. The internal standard was included in all gels to minimize variation between gels. Labeled proteins were separated on 7 cm-immobilized pH gradient (IPG) strips (pH 4–7) as first dimension isoelectric focusing. The strips were then electrophoresed on 4–12% Bis-Tris zoom gels.

Gel images were scanned by Typhoon Trio Imager and analyzed for differentially expressed proteins using DeCyder software v7.0. The biological variation analysis module was used for matching multiple gels and calculating the relative abundance of protein spots comparing children to adults. The filtering parameters were set to select protein spots that had a P-value of < 0.05 and a 1.5 fold difference in relative abundance.

The differentially expressed protein spots were excised for future identification of differentially expressed proteins by mass spectrometry.

Results: On average, 245 protein spots were detected on each gel. The DeCyder analysis identified twenty protein spots that showed at least 1.5 fold differential expression, of which seven protein spots were significantly different (P value < 0.05). Quantitative changes in protein abundance were mainly observed in proteins related to platelet activation and signaling pathway.

Summary/Conclusion: This study demonstrates that there are some differences in the platelet releasate of children and adults following activation with TRAP. Future studies are required to further characterise the age-specific differences in platelet releasate across the spectrum of age. Such studies will contribute to the understanding of the normal platelet physiology across age.

PO 373

Platelet-derived growth factor (PDGF-BB) and platelets in myeloproliferative neoplasms

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Background: Platelet-derived growth factor, first discovered 30 years ago, is a small molecule belonging to the heparin-binding family of polypeptide growth factors. There are four isoforms of PDGF: PDGF-A, PDGF-B, PDGF-C, and PDGF-D, which in active forms are present as dimers. PDGF-BB is released from activated platelets, however, numerous studies have shown that it is also produced by endothelial cells, fibroblasts, smooth muscle cells of blood vessels, astrocytes, neurons, monocytes, cytotrophoblast cells and tumor cells. Platelet-derived growth factor is involved in angiogenesis, embryogenesis, as well as cancer development and progression. The studies in a variety of pathological conditions indicate that tumor cells by secreting cancer procoagulant, induce platelet activation and PDGF release from the platelets alpha-granules.
Aims: The aim of this study was to evaluate the concentration of platelet-derived growth factor (PDGF-BB) and platelet count in patients with myeloproliferative neoplasms.
Methods: The study involved 69 patients with myeloproliferative neoplasms (mean age 62.42), hospitalized and diagnosed at the Clinical Ward of Hematology of the Dr. J. Biziel University Hospital No. 2 in Bydgoszcz, Poland. These patients were enrolled in the study at the time of the diagnosis of MPNs and prior to the implementation of appropriate treatment. The study group included 40 patients with essential thrombocythaemia (ET), 18 with polycythemia vera (PV), 5 with chronic myeloid leukemia (CML) and 6 with the primary myelofibrosis (PMF). The control group consisted of 31 healthy volunteers to correspond to the age (the mean age of 58,22) and gender to the study group. In blood samples was determined PDGF-BB concentration using ELISA test (R&D Systems, USA). Platelet counts were measured using an automated hematology analyzer. The study was approved by the Bioethics Committee of Collegium Medicum in Bydgoszcz, the Nicolaus Copernicus University in Torun, Poland.
Results: In the current study, there was a significantly higher concentration of PDGF-BB in all patients with MPNs, as compared to the control group (Me = 253,84 pg/ml vs. 118,78 pg/ml, $P = 0.001097$). There was also significantly higher number of platelets (PLT) in patients with myeloproliferative neoplasms compared to the controls (Me = 713,42 $\times 10^9$/L vs. Me = 250,39 $\times 10^9$/L, $P < 0.000001$). Furthermore, a detailed analysis of the results in four subgroups revealed that the concentration of PDGF-BB was significantly higher only in ET (Me = 244,53 pg/ml) and PV (Me = 322,94 pg/ml) patients, compared to the control group (Me = 118,78 pg/ml). Moreover, platelet counts were significanlty higher in ET (X = 955,49 $\times 10^9$/L), PV (X = 397,29 $\times 10^9$/L) and CML (X = 598,20 $\times 10^9$/L) patients in comparison with controls (X = 250,39 $\times 10^9$/L). Analysis of correlations revealed statistically significant, positive correlations between PDGF-BB and PLT count ($R = 0.602941$, $P = 0.010407$), PDGF-BB and red blood cell count (RBC) ($R = 0.632353$, $P = 0.006454$), and PDGF-BB and hematocrite (HCT) ($R = 0.534314$, $P = 0.027140$) in PV patients.
Conclusions: Coexistence of high PDGF concentrations and an increased platelet count in patients with MPNs indicates a platelet-derived source of PDGF. Significant correlations between PDGF and platelet count as well as the number of red blood cells may suggest an increased cells proliferation dependent on angiogenesis in PV patients.

PO 374

Biomarkers for post thrombotic syndrome: a systematic review and meta-analysis

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Background: Post thrombotic syndrome (PTS) is a serious condition that occurs in 20–50% of patients following deep vein thrombosis (DVT). Currently, no curative treatment options are available. Biomarkers can be of use in both exploring PTS etiology and developing risk stratification tools for PTS. Potentially, this could increase the options for preventive or therapeutic measures according to the individual risk profile.
Aims: This review gives an overview of the current knowledge on biomarkers in relation to PTS.
Methods: A systematic search was executed in Pubmed, Embase/Medline, and Cochrane Central Register of Controlled trials to identify all publications on biomarkers in relation to PTS. Where possible, results of studies were pooled and a meta-analysis was performed using Review Manager 5.1 (The Cochrane Collaboration).
Results: A total of 24 papers were included in this review. In patients after DVT, increased D-dimer appeared to be associated with the development of PTS, according to meta-analysis of three studies [OR 2.04 (95%CI 1.02–4.08)] $P = 0.04$. Neither thrombophilia nor increased Factor VIII (FVIII) was associated with PTS in patients after DVT [OR 0.93 (95%CI 0.72–1.18)] $P = 0.54$, [OR 1.78 (95%CI 0.88–3.57)] $P = 0.11$ Factor V Leiden (FVL) was associated with post thrombotic ulceration [OR 11.42 (95%CI 6.37–20.48)] $P < 0.00001$. Due to extensive heterogeneity, meta-analysis could not be performed for markers of inflammation and tissue remodelling in relation to PTS.
Conclusion: Based on the findings of this review, we pose that D-dimer as well as FVL are associated with an increased risk for PTS and post thrombotic ulceration respectively, most likely explained by either prolonged clotting, or impaired clot lysis. Hypercoagulability as such appears not to be associated with PTS. The role of inflammation still has to be elucidated. More standardized research methods are needed, to increase comparability of future studies.

PO 375

Diagnosis and prevention of post-thrombotic syndrome (PTS)

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Background: Up to 50% of the patients develops PTS after deep venous thrombosis (DVT). PTS has negative impact on thequality of life and DVT-related costs. The Villalta and CEAP clinical scores are most frequently used tools for the diagnosis of PTS. To prevent PTS, it is recommended to wear compression elastic stocking (CES) for 2 years.
Aims: To determine the: 1- rate of PTS after DVT, 2- agreement between Villalta and CEAP scores, 3- association between PTS and duration of use of CES.
Methods: This cross-sectional study included patients treated for DVT in Oestfold Hospital-Norway 3–9 years prior to recruitment. Villalta and CEAP scores were assessed by the investigators. PTS was classified into four categories by Villalta score: severe (> 14), moderate (10–14), mild (5–9) and absent (< 5), and into eight categories by CEAP scale. The duration of use of CES was retrospectively registered. The study was approved by the Regional Ethics Committee and written informed consent was obtained from all patients.
Results: Median duration of observation after first DVT in 128 patients [median age 61 years; males 89 (67%)] was 5.5 years (range 3.5–8.7 years). Twenty-five patients had recurrence in ipsilateral

extremity and 13 in contralateral extremities after the first episode. In the Ipsilateral extremity 72 patients (56%) had no PTS, 38 (30%) had mild 12 (9%) had moderate and 6 (5%) had severe PTS assessed by the Villalta score.
The distribution of CEAP classes in ipsilateral extremities were C0 (38%), C1 (17%), C2 (15%), C3 (10%), C4a (16%), C4b (2%), C5 (2%) and C6 (0%).
There was a weak correlation between continuous Villata score and CEAP classes ($r = 0.27$, $P < 0.000$). Of 126 patients with both scores only 67 had concordant results regarding the presence or absence of PTS (kappa 0.09, $P = 0.2$).
CES was worn for 3, 6, 12 or 24 months by 31%, 34%, 29% and 6% respectively. Median durations of using CES in patients with no, mild, and moderate PTS (Villalta) were 6, 6 and 12 months and for C0-1, C2-3, C4a-b were 6, 6, 6 months respectively.
Summary/Conclusions: The proportion of patients having PTS according to Villalta in the ipsilateral extremity (44%) was comparable to other studies. Higher proportion of patients was deemed to have PTS in according to CEAP (62%). There was no very week correlation and absolutely no agreement between the two scores. Although, it is unknown which of two tests more accurate diagnose PTS, however, the International Society of Haemostasis and Thrombosis recommend Villalta score as a standard for diagnosing PTS. Interestingly, the grade of PTS does not seem to be affected by the duration of using CES. Further studies are needed to determine the optimal duration for using CES.

PO 376

Spontaneous acute serebral hematoma in a child with F XIII deficiency

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Background: Factor XIII deficiency is a very rare bleeding disorder with an estimated incidence of one in 3–5 million individuals. The clinical manifestation of factor XIII deficiency are neonatal haemorrhage and lifelong bleeding diathesis. The incidence of intracranial haemorrhage (ICH) is higher in factor XIII deficiency than any other congenital bleeding disorders.
Aim: We are reporting here the clinical outcome of a young child with intracranial bleed due to factor XIII deficiency.
Results: Five years old boy was admitted to emergency room with vomiting, headache and poor feeding. His symptoms had started 3 days prior to the admission. There was no history of trauma and bleeding. The patient had been followed up by haematology department with diagnosis of factorXIII deficiency since 10 days old (his factor XIII levels was% 2). On physical examination, he was lethargic and disorientated, and plantar reflexes was extensor bilaterally. Also there was no motor dysfunction, facial asymmetry, fever and neck stiffness. Computed tomography showed a right periventricular haematoma and ventriculer dilatation due to foramen monro obstruction. The patient was managed successfully with fresh frozen plasma transfusion and supportive treatments.
Conclusion: Early diagnosis is crucial due to risks associated with ICH, commonly experienced during childhood.

PO 377

Major surgery in emergency in a patient with deep factor X homozygous deficiency: use of the thrombin generation test for management

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Background: Factor X (F.X) deficiency is a rare, severe bleeding disorder, recessively inherited. Although haemorrhages are frequent and often life-threatening there are few reports on its handling in emergency conditions.
Aim: In surgical procedures, since there is no specific F.X concentrate available, PCC concentrates containing F.X but also the other vitamin K dependent factors are administered with a theoretical risk of thromboembolic complications. We assessed in emergency condition whether monitoring of thrombin generation concomitantly to plasma F.X was helpful for adjusting the dosages.
Patient and methods: Our patient, a 30 years old female presented with pelvic and abdominal violent pain due to intraperitoneal rupture of a large ovarian cyst with intracystic haemorrhages. The deficiency in F.X (at 2%) was diagnosed at 6 years because of frequent ecchymoses post mild traumas. She also presented previous post tonsillectomy haemorrhages and abundant menorrhages. At basal level, during surgery and post -surgery period, plasma F.X level was monitored chronometry, Stago, Asnières, France) concomitantly to Endogenous Thrombin generation Potential (ETP) with a calibrated automated thrombography (CAT) (Stago, Asnières, France) allowing also to measure the Lag time (LT), the Peak Height (PH), the Time to Peak (TTP) and the Velocity index (VI); fibrin monomers (FM) (Stago, Asnières, France) were measured too in order to detect any coagulation activation.
Results and discussion: At basal level F.X was at 2%, but ETP was in the normal range: 1771 nM/min vs. 1419–2060 nM/min in controls, PH and VI were decreased at 188 nM and 52 nM/min vs. 282–388 nM and 114–196 nM/min in controls respectively, LT and TTP were prolonged at 7.2 min and 10.8 min vs. 1.43–2.13 min and 3.33–4.73 min in controls respectively. Before surgery (cystectomy with cure of hemoperitoneum), the patient received 20 IU/kg of F.X (present in CONFIDEX ®, CSL Behring SA, France) in order to obtain a F.X level at 50%. Then she received 10 IU/kg/day during 5 days followed by 7.5 IU/kg/day/2 days of F.X to obtain a residual plasma F.X level at about 30–35%.With this procedure, ETP was only slightly increased (1.5 fold vs. the basal level), PH and VI remained in the normal range, LT and TTP were improved. There was no coagulation activation since FM remained undetectable during 6 days. Neither haemorrhages nor thrombotic complications were observed.
Conclusion: This observation illustrates that toraise F.X plasma level at 50% in pre-surgery followed by a residual plasma level at 30–35%, in agreement with the normalisation of the generation of thrombin parameters data, allowed a surgery with high haemorrhagic risk. Such conclusion must be confirmed by future cases reports.

PO 378

The rare coagulation disorders in the west Algeria: diagnosis and screening

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Background: The rare coagulation disorders present significant difficulties in diagnosis and management and her frequency in the general population is low.
Aims: Diagnosis and screening of rare coagulation disorders in west Algeria.
Method: This study was conducted from 2008 to 2012 in west Algeria on All the subjects with bleeding tendency without any acquired causes, were selected for further investigations.
Screening tests including platelet counts, prothrombin time (PT), activated partial thromboplastin time (aPTT), and fibrinogen assay.
Patients with prolonged aPTT were tested for factor VIII, IX, XI and von Willebrand factor (antigen and Ristocetin cofactor activity).
Patient with prolonged PT and aPTT were tested for factor II, V, VII, and X.
Patient with low factor V level were tested for factor VIII.
Result: Out of 127 patients diagnosed with inherited bleeding disorders, 47 (37%) subjects had rare bleeding disorders. Among them,26

were male and 21 patients were female. Median age of all the patients was 14 years, (range 6 months to 65 years).
The rare bleeding disorders that were found in our population included deficiency of factor II:{n = 04 (3.14%)}, factor V:{n = 07 (5.51%)}, factor VII: {n = 16 (12.60%), factor XI: {n = 5 (3.94%)}, Combined deficiency of factor V and VIII: {n = 12 (9.45%)}, fibrinogene dificiency {n = 3 (2.36%)}.
In 47 patients withe rare coagulation disorders, the mean bleeding score (Tosetto,2006) was 2.3 (range 0–13).
Conclusion: The diagnosis of rare bleeding disorder is dificult in our contries (lack of reagent). this results dont reflect the real frequency of rare coagulation disorders in west Algeria. The perspective is to organise and develop the diagnosis and management of rare coagulation disorders.

PO 379

Prenatal diagnosis for rare bleeding disorders

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Background: Inherited deficiencies of blood coagulation factors are usually associated with lifelong bleeding tendency. Rare bleeding disorders (RBDs) represent 3–5% of all inherited coagulation deficiencies, and are usually transmitted as autosomal recessive traits. Congenital factor VII deficiency is one of rare bleeding disorders with prevalence of around one individual per 500,000. Severe bleeding manifestations tend to occur mainly in homozygous or compound heterozygous individual. It is an autosomal recessive disease.
Aim: To avoid patient was born, prenatal diagnosis is a very good method for the disease history positive family. A women asked for prenatal diagnosis for her second conception. She have a congential factor VII deficiency baby-boy, who was diagnosed at his 2-age.
Methods: Coagulation activity was assessed by one-stage clotting method detected on IL coagulation analyser. The gene of facotor VII, including all exons and intron boundary, was amplified by PCR and seqenced.
Results: Biological assessment for this proband showed significant prolonged prothrombin time and the activity and antigen of factor VII were 2.8% and 3.62% respectively. Other factors of haemostasis (V, II and X) were in the normal range for age. Molecular diagnosis was performed by direct sequencing analysis of the factor VII gene. It revealed one homozygous missense mutation which is Gly360Asp substituation. Both parents were totally asymptomatic of blood disorders, but had partial FVII deficiency, with factor VII levels of 38.8 and 40.6% for the mother and father respectively. Family investigation show that this couples are consanguineous marriage. Gene diagnosis results indicated that they were all heterozygous missense mutation carrier. So theoretically this couples will have another congenital factor VII deficiency patient with 25% probability. To avoid conceive another patient fetus, fetal DNA was extracted from 10 to 15 ml amniotic liquid obtained by amniocentesis at 16–21 weeks gestation. Gender detection shows it is a femal fetus and gene analysis for F7 indicated that this fetus have homozygous Gly360Asp missense mutation which is as same as its brother.
Conclusion: To a conclusion, this fetus is a congenital factor VII deficiency female. Use gene diagnosis can help the women from inherited disease family.

PO 380

Inherited factor VII deficiency in three Moroccan pedigrees

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Background: Severe inherited factor VII deficiency is a rare autosomal recessive disorder. The clinical features are variable and there is no relationship between FVII activity and the bleeding tendency which can vary from a modest bleeding to life-threatening hemorrhages.
Many mutations have been already published which are often single base pair substitution.
Aim: To analyze the clinical and biological findings in three Moroccan pedigrees of inherited coagulation factor VII (FVII) deficiency.
Methods: The coagulation function and coagulation factors activity of probands were detected for phenotype diagnosis, all exons and junctions of FVII gene from the family members' genomic DNA were amplified using polymerase chain reaction (PCR), and detected the gene mutation by direct sequencing.
Results: The proband 1 was 37 years old, the proband 2 aged 6 and the proband 3 was 64 years old. They all had consanguineous parents. Proband 1 and 2 had a history of spontaneous bleeding such as epistaxis, menorrhagia and gingival bleeding while proband 3 was asymptomatic.
They all had prolonged prothrombin time (PT) with normal activated partial prothrombin time (aPTT): PT of proband 1 was 45s, for proband 2 it was 33s and proband 3 had a PT of 40s.
The FVII activity was 2% in proband 1, 13% in proband 2 and 10% in proband 3. Homozygous mutation was identified in the proband 1, who had a severe quantitative deficiency, resulting in Arg413Gln. The molecular analysis of proband 2, who carries a moderate qualitative deficiency, showed a homozygous mutation resulting in Arg364Gln. The molecular data aren't available yet for proband 3.
Conclusion: Congenital FVII deficiency is a bleeding disorder that requires optimal hemostatic management for each case due to its wide variety of bleeding symptoms.

PO 381

Effect of social factors on high prevalence of factor XIII deficiency in southeast Iran

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Background: Inherited factor XIII (FXIII) deficiency is a rare bleeding disorder that can present with significant bleeding complications such as umbilical cord Bleeding and life-threatening intracranial haemorrhage. Precise prevalence of the disease is not clear but in countries where consanguineous marriages are relatively high This disorder occur frequently. In Sistan and Baluchistan due to the high rate of consanguineous marriage prevalence of factor XIII deficiency is increased.
Aims: The aim of this study is to assess the role of relatives, ethnicity and residence in prevalence of factor XIII deficiency.
Methods: This study performed on 205 patients with severe factor XIII deficiency. The clot stability test in order to asses the suspected patients was done and in some patients also molecular analysis and factor assay were done. Data of patients was collected by survey of

patient records, designed questionnaire and family tree. Eventually Statistical analysis was done by SPSS software.

Results: The mean age of patients was 12.7 ± 8 years and 47.8% patients were men and 52.2% patients were women. Ninety-three and seven-tenths percent of patients were baluch and a minority of them were Afghan immigrants (1.5%). More than 70 percent of parents of patients were close relative and 11.8% were distant relative while just 10% of parents no family relatives.

Summary/Conclusions: Factor XIII deficiency is rare bleeding disorder in general population while the prevalence of disorder is significantly higher in Sistan and Baluchistan province, southeast of Iran. It seems that ethnicity, religion, residence and consanguineous marriage have crucial role on this high prevalence of factor XIII deficiency.

PO 382

Rare inherited clotting factor deficiencies (RICFD) in pediatric population: King Faisal Specialist Hospital and Research Centre Riyadh Experience

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Background: The rare inherited coagulation factor deficiencies (RICFDs) are deficiencies of factor I, II, V, VII, X, XI, XII, XIII and contact factors.They are generally autosomal recessive disorders or present in homozygous states. Heterozygotes have approximately half-normal levels of coagulation factors and are usually asymptomatic. They have a prevalence of about 1: 500,000 to 1: 2,000,000 in general population. Factor VII and Factor XI deficiencies are the most prevalent world wide. The most typical symptoms in the RICFDs are the occurrence of excessive bleeding at the time of invasive procedures as well as mucosal bleeding. Menorrhagia and postpartum bleeding often occurs in these patients if replacement therapy is not administered.

Aims: To determine the prevalence, clinical profile and treatment outcome of RICFDs in pediatric patients seen at King Faisal Specialist Hospital and Research Centre, Saudi Arabia from 2002 to 2012.

Methods: We are presenting results of a descriptive study where we retrospectively reviewed the charts of the pediatric patients treated at our facility from 2002 to 2012.

Results: Of 22 pediatric (≤ 14 years) patients profiles reviewed, 59% (13) were females. Median age at diagnosis was 5.3 years (0.2–14). Ninety-one percent (20) had a positive family history of RICFD and parents with consanguineous marriages. Family history of RICFD was most common in Factor XIII deficiency (35%). A 27.3% of our patients were from the Northern Region, 18.2% from Western, 13.6% from Eastern, 27.3% from Southern and 13.6% from the Central Region. Median HgB at diagnosis was 119.7(12.3–166), MCV 78.9 (67.2–93) and ferritin 37.9(9–80). Factor XIII deficiency was seen in 8 cases (36.4%), followed by Factor II Deficiency in 4(18.2%) and Factor VII in 3(13.6%). Hepatitis B and C screening was done in 63.6%, all were found to be immune/non reactive. Echymosis was most common bleeding (11.50%) followed by oral in 8(36.4%) and intracranial hemorrhage in 6(27.3%). Eighty-three percent (n = 5) of ICH was seen in patients with Factor XIII deficiency, resulting as the most common serious morbidity. Developmental delay was seen in 2(9%) cases. Five ICU admissions were recorded. Primary reason to ICU admissions was ICH. Treatment modality included topical thrombin in all cases, followed by FFP (17), tranexamic acid (7) and iron supplementation (6). Three were given hormonal contraceptives. All of patients reviewed were alive at the time of last contact.

Conclusion: InSaudi Arabia, the RICFDs are uncommon coagulation problems probably due to under diagnosis of the patients living in remote areas. However in our cohort of pediatric patients, FXIII was the most common presenting with serious bleeding manifestations. Precise knowledge about RICFDs, their clinical presentation, diagnostic work-up and treatment options is vital to accurately diagnosing these patients and providing optimal treatment in pediatric patient population.

PO 383

Handling of a neonate with severe congenital factor VII deficiency and severe bleeding episodes

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Congenital factor VII (FVII) deficiency is a rare bleeding disorder, which manifests with a high phenotypic variability. It seems that the bleeding disposition in patients with FVII < 25% depends more on the variable genetic defect than on the degree of FVII level reduction. Treatment has traditionally involved FVII replacement therapy using fresh frozen plasma, prothrombin complex concentrates or plasma derived FVII concentrates. Recombinant activated FVII (rFVIIa) is actually considered the first-line therapy for replacement therapy in patients with FVII deficiency.

We report the case of an infant with severe congenital FVII deficiency (< 1%). One day after birth, he presented with marked paleness and hemoglobin levels were in the range of 4–5 g/dl. He required phototherapy because of severe icterus based on a severe cephalohematoma. In the context of this bleeding, the diagnosis of a severe FVII deficiency was made. At the age of 6 weeks he suffered from an intraventricular hemorrhage. The administration of rFVIIa stopped the bleeding immediately and the patient survived without any neurological deficit. At the age of 4 month, the infant was referred to our hospital because of a pyelonephritis with macrohematuria, again repetitive dosages of rVIIa (40 μg/kg every 6–8 h) stopped bleeding instantly. Over the course of the next weeks, the child suffered from spontaneous facial and subcutaneous hematoma of the back of his right hand. Based on the several severe bleeding episodes in combination with FVII levels < 1% we started prophylactic therapy with rVIIa 65 μg/kg every second day. No further bleeding episodes were encountered since the initiation of this therapeutic regimen.

Congenital FVII deficiency is a rare autosomal recessive bleeding disorder with an incidence of about 1/500.000. There are only few reports on neonates with intracranial hemorrhage. Lee et al. reported a case of severe intracerebral and intraventricular hemorrhage (IVH) in a neonate with congenital FVII deficiency. At the beginning rFVII was administered in a dose of 20 μg/kg every 4 h. A brain CT 7 days after admission revealed the regression of the IVH. Severe and life threatening hemorrhages are rare (5%) and occur most frequently in the first 6 months of life. In patients with severe hemophilia A prophylactic therapy with factor concentrates is currently the most effective intervention. In patients with severe congenital FVII deficiency prophylactic therapy is uncommon because of the very short half life of infused FVII. Moreover there are no specific guidelines concerning the frequency and dosage of the factor substitution. Authors report about dosages of 20–30 μg/kg once or twice weekly. Other reports have described dosing regimes of 15–90 μg/kg by bolus injection from twice daily to twice weekly. Complicating is that the smallest package available on the market is 1 mg, leading to wastage of a large amount of the very expensive drug.

Congenital VII deficiency is a rare bleeding disorder. NovoSeven is now being used for the treatment of clinically significant bleeding. Despite the low incidence of this illness, further studies are needed for optimizing therapy.

PO 384

Congential afibrinogenemia

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Introduction: Congential afibrinogenemia is a rare bleeding disorder with an estimated prevalence of 1:1,000,000 in the general population. There are about 250 cases reported in the world literature. In department of coagulopathy of Russian National Research Center for Hematology we are observing two patients with this diagnosis.

The aim of investigation: A complex assessment of coagulation system in patients with a different bleeding phenotype of afibrinogenemia.

Materials and Methods: A 22-year-old woman suffers from ecchymosis appearance each 1–1.5 months; twice she has surgery for hemoperitoneum after corpus luteum rupture. A 17-year-old man suffers from intensive epistaxis each 3 months. Clotting tests included: clotting time (CT), activated partial thromboplastine time (APTT), prothrombin time (PT), thrombin time (TT), fibrinogen (Clausse) assay, factors of prothrombin complex and venom tests (echitox time, lebetox time, ancistrodon time). Electrophoresis and immunofixation were used for fibrinogen identification. Global hemostatic tests embrased thrombin generation assay (TGA), thromboelastography (TE), thrombodynamics (TD).

Methods: In both cases CT, APTT, PT, TT, echitox time, lebetox time, ancistrodon time are infinitely prolonged. Fibrinogen is undetectable by Clauss assay. Factors of prothrombin complex are normal. The fraction of fibrinogen is absent by electrophoresis method; there is a trace band of fibrinogen in a γ1 zone by immunofixing. According to TE, TD the clot is not formed. Results of TGA show increase of endogenous thrombin potential (2018 and 3582 nM/min for patients respectively) and thrombin peak (277 and 205 nM) in comparison with controls (1020 nM/min and 150 nM, respectively).

Conclusion: Coagulation system in patients with a different bleeding phenotype of afibrinogenemia is characterized by identical changes according to clotting tests, electrophoresis and immunofixation, global hemostatic tests: TE and TD. TGA shows different increasing of thrombin generation that is individual distinction of patients.

PO 385

Dysfibrinogenemia and pregnancy – a case report

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Background: Congenital abnormalities of the fibrinogen (afibrinogenemia, hypofibrinogenemia, dysfibrinogenemia) have been associated with obstetric complications.

Hereditary dysfibrinogenemias are characterized by the biosynthesis of structurally and functionally abnormal fibrinogen molecules, resulting in decreased clotting activity, despite normal circulating levels of fibrinogen antigen. The obstetric complications observed in dysfibrinogenemias include first-trimester pregnancy loss, haemorrhage, placental abruption, and thrombosis.

In patients with afibrinogenemia and hypofibrinogenemia, it is recommended to maintain fibrinogen levels above 150 mg/dL to achieve a postpartum haemorrhage risk similar to that of healthy women without a fibrinogen deficiency. Management of pregnancy in women with dysfibrinogenemia needs to be individualized, taking into account the fibrinogen level and personal and family history of bleeding and thrombosis. No specific treatment is required in asymptomatic women.

Aims: The aim of this report is to present the case of a patient with diagnosis of hereditary dysfibrinogenemia, without replacement therapy or any treatment to increase her plasma fibrinogen level during pregnancy; she was monitored up the 34th week of gestation without any obstetric complication.

Case report and Results: The patient is a 31-year-old woman with previous personal history of obstetric complications (two anembrionic pregnancies), bleeding after curettage that required transfusion of red blood cells and fresh frozen plasma.

The diagnosis was based on the discrepancy between low fibrinogen clotting activity (C, Clauss Method 145 mg/dL) and normal level of fibrinogen antigen (I, Laurell immunoelectrophoresis: 235 mg/dL). Laboratory tests showed prolonged activated partial thromboplastin time (APTT) (ratio 1.2; RV: 0.87–1.13), that was corrected by the addition of normal plasma (ICA 2; control < 10); prolonged thrombin time (TT) (ratio 2; RV: 0.75–1.25) that was not corrected by the addition of normal plasma (1.6; control 1.2) and borderline value of prothrombin time (PT) (ratio 1.13; RV: 0.82–1.13). Apart from the fibrinogen, other coagulation factor activities were normal; von Willebrand disease was also ruled out.

Hereditary defect was assumed because both her mother (C:110 mg/dL, I: 354 mg/dl) and her brother (C: 60 mg/dL, I: 240 mg/dL), had a previous diagnosis of dysfibrinogenemia.

Five months after the diagnosis she became pregnant. Fibrinogen levels were tested between 12th and 34th weeks of gestation. No significant increase of coagulant activity (range: 140–160 mg/dL) was observed; fibrinogen antigen values changed between 230 and 425 mg/dL, with a maximum at 30th week of gestation.

Due to her previous history of bleeding and the fact that fibrinogen concentrates are not commercially available in Argentina, 6 units of cryoprecipitates were indicated to be infused before delivery and another 6 units if a bleeding complication occurred.

Conclusion: We present a case of congenital dysfibrinogenemia without any fibrinogen replacement therapy; only a frequent control of the fibrinogen was carried out. No obstetric complication was detected up to the 39th week.

PO 386

The laboratory profile of the thrombotic Antiphospholipid Syndrome- Experience from a North Indian tertiary care referral centre

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Background: The antiphospholipid antibody syndrome (APS) is an autoimmune disorder characterized by thrombosis and/recurrent pregnancy losses and positivity for antibodies directed against phospholipids or the proteins attached to them. It may be associated with recurrent arterial or venous thrombosis and is the commonest acquired cause of thrombosis. There is limited data available on the laboratory testing for this disorder in the thrombotic presentation from the Indian subcontinent.

Aims: We attempted to determine the prevalence of APS in patients with thrombosis and to study the pattern of positivity of the lupus anticoagulant (LA), Anticardiolipin antibodies (ACA) and anti beta 2 Glycoprotein 1 (anti β_2 GPI) antibodies in this group of patients.

Methods and patients: Patients with confirmed thrombosis (arterial or venous), referred for workup of the hypercoagulable state were included in the study. Citrated plasma samples were screened and confirmed for LA by the diluted Russel Viper venom time and in- house Kaolin clotting time. Serum samples were screened for ACA and anti β_2 GPI antibodies by EIA. Results of repeat testing after 12 weeks for a positive result, where available, were noted.

Results: In a period of 3 years, 991 cases were tested for the APS. Eight hundred and eighty-nine cases were available for analysis. In 186 (20%) of these, any one of the APS antibodies were positive at least once. In 30 (3.3%) cases, the antibodies persisted for at least 12 weeks

and they qualified for the APS. Eight cases were children. Fifty-four percent cases had venous thrombosis. Fifty percent cases had intracranial thrombosis. A 6.6% cases had recurrent thrombosis. In 21(70%) cases there were no risk factors other than the antibodies tested. Only 3 cases had persistent positivity for all 3 antibodies. In 22 (73%) cases, only 1 antibody was positive. Anti β_2GP 1, ACA and LA antibodies were positive in 28, 9 & 5 cases respectively. Positivity for IgG isotype of anti- β_2GP 1 antibodies were common (70% cases).

Conclusions: APS affects young patients and children form a significant proportion. A majority of APS patients had no other risk factors. Transient antibody positivity was frequent. Positivity for all three antibodies is rare and there is a need to have a wide antibody panel. Anti β_2GP1 antibodies were the commonest ones encountered. Positivity for LA was the lowest among all antibodies studied and this was possibly due to the inability to reconfirm an initially positive positive LA result, if the patient had commenced oral anticoagulant therapy. LA was always positive along with anti β_2GP1 antibodies and this effect was possibly a subset of the anti β_2GP1 antibodies. Anti β_2GP1 antibodies were instrumental in increasing the yield of APS in our population and are a useful addition to the test panel.

PO 387

Anti-annexin V antibodies and clinical manifestations of antiphospholipid syndrome

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Background: The antiphospholipid syndrome (APS) is diagnosed in patients with thromboembolic events and/or recurrent unexplained fetal loss associated with persistent so called antiphospholipid antibodies (aPL). Laboratory diagnosis of APS includes the detection of positive aPL validated by The International Society on Thrombosis and Haemostasis (ISTH): anticardiolipin antibody (aCL) and/or anti-β2 glycoprotein I antibody (anti-β2GPI) of IgG or IgM class, and/or lupus anticoagulant. Some authors have described a potential role of other aPL in the onset of clinical manifestations of APS and in particular anti-annexin V antibodies (AAV), but studies are few and often conflicting.

Aims: In this work, we investigated the place of the detection of AAV in the plasma of patients with thrombosis and/or foetal losses associated or not to positivity for biological markers of APS.

Patients and Methods: We have studied 123 patients (43 males, 80 females) aged 1–81 years (mean 39 years), with confirmed thrombosis: venous (65 cases), arterial (22 cases) or recurrent unexplained fetal loss (36 cases). The control group consisted of 80 healthy subjects. We evaluated aCL, anti-β2GPI and AAV (IgG and IgM isotypes) by an enzyme-linked immunosorbent assay (Orgentec) and for the lupus anticoagulant as recommended by the ISTH.

Results: All normal controls were negative for aCL, anti β2GPI, and AAV. Twenty-three patients (18.7%) had one or more aPL required for the diagnosis of APS. The incidence of isolated positivity for anti-β2GP1, LA and aCL was 9%, 2.4% and 0.8% respectively. The combination of two or three antibodies was observed in 6 (4.9%) and 2 (1.6%) patients respectively. Among these 23 patients, there are two who were also positive for AAV. The presence of AAV was significantly associated with positivity for biological markers of APS ($P < 0.05$). Among 100 patients without conventional biological markers of APS, 4 women (2.5%) had isolated positivity for AAV: 2 thrombotic events (1 arterial and 1 venous) and 2 obstetric events. However there is not a significant association between AAV and clinical manifestations suggestive of APS ($P > 0.05$) in patients with negative classic markers as compared to normal control.

Summary/Conclusion: Our results corroborate those of other studies which report the presence of AAV in patients with thrombosis or obstetrical complications. AAV are not recognized as markers of APS by the ISTH, however their presence associate with classic markers of APS, or isolated in patients with arterial or venous thrombosis, and in women with obstetrical complications suggest a possible diagnosis interest, perhaps in second line when classic markers are negative particularly in case of a strong suspicion of APS. The exact role of AAV in the APS remains uncertain. The results of this study support the complexity of the pathophysiology of APS. Clinical manifestations reflect the interaction of a mix of antibodies. The diagnostic value of AAV deserves to be confirmed on larger studies.

PO 388

Hemolytic anemia and chronic non-healing ulcer on the right foot in a 27-year old female: a case of SLE antiphospholipid antibody positive responsive to cyclophosphamide therapy

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Background: Our patient in discussion was from the Social Service who was asked to sign a consent form that their case can be used as clinical material towards the enrichment of medical education.

SLE is an autoimmune disease in which organs and cells undergo damage initially mediated by tissue-binding autoantibodies and immune complexes. The most common presentation of which are skin and joint manifestations and cardinal symptoms however there are a few who presents differently. The mainstay of therapy is glucocorticoids but to a few who are refractory or resistant, the use of cytotoxic agents can be utilized.

Aims: To present a case of SLE with an atypical presentation.

To present a case of steroid-refractory hemolytic anemia that responded to cyclophosphamide therapy.

Case Report: This is the case of a 27 year-old female who presented with 7-month history of intermittent fever, rash and chronic non-healing right foot ulcer. The right foot ulcer did not improve with regular wound cleaning with Guava leaves and Hydrogen Peroxide. She later on had episodes of dizziness, generalized body weakness and dyspnea on exertion. Consult done showed anemia and thrombocytopenia however diagnosis was unrecalled. Blood transfusions were done but later had ABO incompatibility. A few weeks after discharge, she had muscle and joint pains, discomfort in swallowing, productive coughing and fever with progressive shortness of breath and weight loss of 15Kg over 2 months.

Patient was admitted with anemia of 5.6 mg/dL and thrombocytopenia of 90,000. Peripheral Blood Smear showed hypochromic anemia, slight anisocytosis, and few schistocytes. Coomb's test Direct and Indirect was positive, C3 = 29.8 mg/dL, Anti dsDNA, Anti-Smith, Anti-RNP were strongly positive, ANCA IgM negative, SCL70 Antibody negative, Crossmatching was positive for warm auto-antibodies, both Lupus Anticoagulant and Antiocardiolipin antibody IgG were positive and Duplex Scan of lower extremities was normal for the right lower extremity. She was started on Prednisone 20 mg/tab once daily and was later shifted to Hydrocortisone 40 mg IV once daily then to Pulse Therapy with Methylprednisolone. After 3 days of pulse therapy, her symptoms of fever, easy fatigability, shortness of breath and the thrombocytopenia was improved however the anemia and the right foot ulcer persisted. Patient was started with Hydroxychloroquine 200 mg tab once daily, Prednisone 25 mg after breakfast and 20 mg after lunch and Cyclophosphamide 500 mg IV twice a month for six doses. Anemia was resolved after three doses of Cyclophosphamide and the right foot ulcer completely healed after four doses of Cyclophosphamide.

Conclusion: We presented an SLE patient with antiphospholipid antibody who presented with hemolytic anemia and chronic non-healing ulcer on the right foot who responded to pulse cyclophosphamide therapy.

PO 389

Antyphospholipid syndrome and arterial thrombosis

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Background: Antiphospholipid syndrome (APS) is an acquired autoimmune condition of thrombophilia which predisposes to the development of venous, arterial, microvascular thrombosis and pregnancy complications that have persistently antiphospholipid antibodies (aPL). Arterial thrombosis is the most serious complication of APS and not well known at present that risk factors associated with the development of arterial thrombosis.

Aim: The aim of the present study was to assess the association between risk factors for cardiovascular disease (age, hypertension, dyslipidemia, diabetes, snuff, family history of thrombosis), inherited thrombophilia, oral contraceptives and the presence of arterial and venous thrombosis in patients with APS.

Methods Forty-seven APS consecutive patients were enrolled in a retrospective observational study by San Pedro de Alcántara Hospital (Haematology's Department) between 2000 and 2012. Of them, 22 were men and 25 women aged between 24 and 85 years, classified in multiple or single positive antiphospholipid antibody and obstetric APS. Potential clinical and laboratory risk factor for arterial and venous thrombosis were evaluated. Patients' characteristics were analysed using descriptive statistics in SPSS (Version 19). The level of statistical significance was set to 0.05 (SPSSstatics19).

Results: We observed 23 patients with venous thrombosis, 20 with arterial thrombosis and 4 with obstetrical APS. Lupus anticoagulant present in these patients is the most common (55.3%) being the most prevalent venous thrombosis (57.7%) (OR = 0.38, 95% CI:0.06–2 0.34, $P = 0.001$) compared with arterial thrombosis that is slightly lower (42.3%). Multiple aPL positivity (23.4%) is associated with a more severe course of the disease, increasing significantly the rate of arterial thrombosis (54.5%) compared to venous thrombosis (45.5%) (OR = 1.54, 95% CI:0.3–6.1, $P = 0.001$), whereas the frequency of positive anticardiolipin and ß$_2$glycoprotein-I (4.3% and 8.5% respectively) was the same respectively (50%). Analyzing the distribution of arterial thrombosis in our center a total of 42.6% of APS was observed (25.8% stroke, 9.4% myocardial infarction, 4.7% thrombosis of the central retinal artery, 6.9% peripheral arterial disease) being the most frequent stroke. There wasn'nt a significant relationship between arterial thrombosis and the presence of classic stroke risk factors, inherited and acquired thrombophilia risk factors.

Conclusions: In our group of patients the multiple aPL positivity is associated with a more severe course of the disease, increasing significantly the rate of arterial thrombosis. The most common form of arterial thrombosis in our case series stroke was as described in the literature.

PO 390

Comparison of six dilute Russell viper venom time lupus anticoagulant screen/confirm assay kits

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Background: The normalized dilute Russell viper venom time (DRVVT) ratio provides a robust assay methodology for lupus anticoagulant (LA) detection.

Aims: We evaluated six normalized DRVVT LA screen and confirm systems for inter-method consistency. Reagents were purchased from Diagnostica Stago, Precision BioLogic, Siemens, TCoag, Instrumentation Laboratories, and Sekisui.

Methods: For all assays, we employed the STA-R Evolution® automated coagulometer, adhering to manufacturers instructions. LA-positive and LA-negative plasma controls were purchased from Diagnostica Stago and pooled normal plasma (PNP) was purchased from Precision BioLogic Inc. We computed the mean of the reference interval (MRI) and action limits for all kits using LA-negative aliquots from locally sourced normal subjects ($n = 42$). We then assayed locally sourced LA-positive plasmas ($n = 43$) and using ANOVA compared uncorrected screen/confirm ratios and screen/confirm ratios that were normalized using MRI and mean PNP results.

Results: The grand mean action limit, MRI + 3 SD, derived from the local normal plasmas, was 1.2, confirming the manufacturers' recommended limits; however, limits must be locally computed. The all-sample P value was < 0.001, indicating heterogeneity among ratios. When Sekisui ratios were excluded, the P value was 0.14, thus indicating that this method introduced the major difference among methods. Mean screen/confirm ratios computed from LA-positive specimens were 1.91–2.04 for reagent systems other than Sekisui, which instead yielded a mean ratio of 1.198, indicating that this method was relatively insensitive to LA. A negative bias was recorded by two lots from the Sekisui system for LA-positive specimens. Screen/confirm ratios from combined LA-positive and LA-negative samples generated a combined range of 1.59–1.67 for all reagents except Sekisui, which instead yielded 1.09. All within-run coefficients of variation (CV%) were $< 5.0\%$ using all samples. Between-run CV% using Diagnostic Stago LA-positive and LA-negative controls was $< 5.5\%$.

Conclusion: DRVVT screen/confirm ratios discriminate between LA-positive and LA-negative samples and generally provide acceptable reproducibility. Ratio results may vary among reagent-instrument combinations. In this study, normalization added little to the clinical result interpretation.

PO 391

Prevalence of antiphospholipid antibodies in adults with ischaemic stroke

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Background: Antiphospholipid antibodies are autoantibodies with procoagulant activities. They include lupus anticoagulant, anticardiolipin and glycoprotein I. Their presence is frequently associated with thromboembolic phenomena including ischaemic stroke. The frequency of occurrence of these antibodies in stroke patients in our environment has not been investigated.

Aim: The aim of this study is to investigate the prevalence of antiphospholipid antibodies in adult patients with ischaemic stroke.

Methods: This is a cross sectional case control study. Consecutive established ischaemic stroke patients between 18 and 60 years were recruited as cases and non stroke subjects as controls. Antiphospholipid antibodies were assayed using dilute Russel viper's venom (DRVV) Lupus Anticoagulant kits (LA screen and confirm kits) by TECHNOCLONE DIAGNOSTICS GmbH, Vienna) and total anticardiolipin antibody (ACA) enzyme linked immunosorbent assay (ELISA) based assays (Bioquant Diagnostics, San Diego, USA). Haematological parameters were analyzed with haematology automated analyzer MODEL SYSMEX KN21, Japan.

Data was analyzed using SPSS version 16. The prevalence of antiphospholipid antibodies of the study group and controls were compared using chi-square and Fisher's Exact test. The means of the haematological parameters of both groups were compared using Student T test. P-values < 0.05 was considered significant.

Results: A total of 60 ischaemic stroke patients and 30 controls were recruited. The mean age for the ischaemic stroke patients and controls were 48.5 ± 9.4 years and 42.9 ± 9.5 years respectively. The stroke subjects comprise 36 males and 24 females while the controls included 16 males and 14 females.

Six (10%) of subjects with ischaemic stroke were positive for LA and none of the controls had LA. Twelve (20%) of the stroke subjects were positive for ACA while two (6.7%) of the controls were positive. The prevalence of APLA in ischaemic stroke patients was 28.3% as against

6.7% in the controls. This was statistically significant with a P value of 0.026 and an odd ratio of 5.53. There was no age or sex preponderance in APLA positivity in the ischaemic stroke group and there was no significant difference in the platelet counts of the study groups.
Conclusion: There is a high prevalence of APLA in patients with ischaemic stroke in our environment. Because the presence of these antibodies has therapeutic implications, routine evaluation of APLA is therefore recommended in patients at risk of ischaemic stroke.

PO 392

Incidence of antiphospholipid antibodies in women with preeclampsia seen at the Universty of Benin Teaching Hospital

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Background: Antiphospholipid antibodies (APLA) are heterogeneous group of circulating antibodies against anionic phospholipids. They have been associated with a number of obstetric complications however their role in the pathogenesis of preeclampsia has remained an issue of controversy. There is a dearth of information on APLA in preeclamptic women in our environment.
Aims: The aim of this study is to determine the prevalence of antiphospholipid antibodies in preeclampsia among women presenting to the University of Benin Teaching Hospital (UBTH) Benin City; to determine pregnancy outcome in APLA positive women with preeclampsia. This is with a view to recommending screening for APLA to women at risk of preeclampsia.
Methods: This is a cross sectional case control study. Consecutive pregnant women diagnosed of preeclampsia and age-parity matched normal on-going pregnant women were recruited as participants for the study. Antiphospholipid antibodies were assayed using dilute Russel viper's venom (DRVV) Lupus Anticoagulant (LA screen and confirm kits) and total anticardiolipin antibody (ACA) enzyme linked immunosorbent assay (ELISA) kits.
Results: A total of 100 women were studied comprising 50 pregnant women with preeclampsia and age-parity matched 50 normal ongoing pregnant women as control. The mean ages of the study participants were 31.6 $\pm$ 4.7 years and 30.9 $\pm$ 5.4 years respectively. Thirty of the preeclamptic women were booked in UBTH and twenty were unbooked. Eleven of the preeclamptic women had mild preeclampsia while thirty-nine had severe preeclampsia. The prevalence of APLA in preeclampsia was 10% while none (0%) of the controls was positive. This was not statistically significant P value = 0.056 however the odd of detecting APLA in preeclampsia is infinite. There were no significant differences in the obstetric history, pregnancy complications, maternal and birth outcomes between APLA positive and APLA negative women with preeclampsia. However APLA positive preeclamptic women are at increased risk of abruptio placenta and low birth weight with odd ratios of 5.83 and 1.71 respectively.
Conclusion: The prevalence of APLA though increased in women with preeclampsia there is no association between APLA and preeclampsia in the study participants. Therefore a routine assay of APLA in women at risk of preeclampsia may not be justified however women with preeclampsia with other clinical features of antiphospholipid syndrome should be investigated for APLA.

PO 393

Frequency of antiphospholipid syndrome in 600 of Czech females with venous thromboembolism in association with oral contraceptive use

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Background: Among women in their reproductive age, pregnancy and oral contraceptive use (OC's) are common risk situations of VTE. Because the baseline risk of VTE is low in young healthy women, the absolute risk remains low (3–4 VTE/10,000 women per year using OC. Thirty-three percent of Czech women in their reproductive age are taking oral contraceptives.
The aim of the study: We assessed presence of antiphospholipid syndrome (APS) in a group of 600 women with VTE in association with combined oral contraceptive use (COC's) seen at our clinic since 1997 till the end of 2012. VTE was clinically manifested and objectively documented in all cases. We focused on laboratory work-up of APS. Timing of the detection of lupus anticoagulant (LA) is troublesome, because of influence of many factors: the persisting effect of COC on coagulation, influence of acute thrombosis and also anticoagulation therapy. Diagnosis APS is important for clinical practise, because has impact on: the strategy of anticoagulation therapy, management of further pregnancy, possibility of the onset of autoimmune disease etc.
Methods: LA was examined in all samples at least twice, once at the time of diagnosis and again after anticoagulant therapy. In the case of suspected LA after the cessation of anticoagulation, the examination was repeated after 8 weeks.
Citrated blood samples were centrifuged twice for 15 min at 2700 g and then filtered through a filter with a pore size 0.2 μm. Platelet-free plasma was either analyzed immediately or stored at −70 °C. The laboratory screening of lupus anticoagulant antibodies was examined by following laboratory tests: PT, aPTT, aPTT with high sensitivity to LA, dilute Russell's Viper Venom Time. Thrombin time was done with the aim to rule out the presence of heparin in plasma sample. In the case of a prolongation of one or more of these tests (except TT), the mixing tests were done. As confirmatory tests were used: dilute PT, aPTT and test with hexagonal phospholipids. Anticardiolipin antibodies (ACA) were determined using enzyme-linked immunosorbent assay, as well as beta-2 glycoprotein I antibodies. In the case of positivity, the second evaluation was done again after 6–8 weeks.
Results: The mean age of the females was 26 years, and the average duration of use was 45 months at the onset of thrombosis. APS has been found in 30 females (5%) and in the additional 6 females was combined with F V Leiden mutation in heterozygous form. In 16 females we have found positivity of ACA or beta-2 glycoprotein antibodies, in 10 females only LA and four females were positive both.
Conclusion: APS as single thrombophilia has been found in 5% of females on COC's. The result corresponds to other, mostly smaller studies. We can consider this laboratory work-up as useful for further management of those females.

PO 394

Lupus anticoagulant and anticardiolipin antibodies in polytransfused beta thalassemia major

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Background: The presence of anti-phospholipid antibodies (lupus anticoagulant, LA and anti-cardiolipin antibody, ACA) has recently been reported in polytransfused patients of beta thalassemia. Moreover,

high prevalence of hepatitis C virus (HCV) infection and thrombotic risk is described in thalassemia.
Aims: We aimed at investigating the prevalence of ACA, LA, and their relation with HCV infection in patients with thalassemia major.
Methods: Presence of anti-HCV antibody, IgG ACA, IgM ACA and LA activity was determined in 36 patients with thalassemia major (male/female: 17/19 aged 12–37 years) registered at China Medical University Hospital, Taiwan. Two patients had thromboembolic events.
Results: LA was seen in 25% (9/36) of cases. The number of transfusions but not age was slightly higher in LA positive patients as compared to LA negative patients, however the results were not statistically significant. Anti-HCV antibody was positive in 15 (41.6%), IgG ACA in 7 (19.4%), IgM ACA in 21 (63%), and LA activity in 9 (25%) patients. A 71.5% of patients positive for IgM ACA had a low titer of ACA. Strong positive IgM ACA were detected in 16.6%(6/36) of patients but no statistically significant correlation was found with age, number of transfusions, and coagulation parameters. No statistically significant difference in the prevalence of LA or ACA was found between HCV-infected and non-infected patients.
Conclusion: A high prevalence of LA and ACA, also the majority in low titers, was detected in patients with polytransfused thalassemia major in Taiwan irrespective of previous history of blood transfusion and presence of HCV infection.

PO 395
Lupus anticoagulants sensitivity of Siemens APTT reagents

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Background: Several studies investigating lupus sensitivity of APTT reagents produced conflicting results with regard to the sensitivity of ellagic acid-based reagents.
Aims: Demonstrate that the activator *per se* does not determine the lupus anticoagulant sensitivity of an APTT reagent.
Methods: The internal study included different APTT methods on the BCS® XP System. One hundred and fifty-four frozen plasma samples from healthy donors (considered negative for LA) and 100 commercially available frozen plasma samples characterized by a positive dRVVT test were investigated. Three samples with prolonged thrombin time were excluded, leaving 97 true dRVVT positive samples for evaluation. All samples were tested by LA1 Screening Reagent, LA2 Confirmation Reagent, LA1 mixing, Pathromtin® SL reagent (PSL); Dade® Actin® FSL reagent (AFSL, low phospholipid content); and Dade Actin FS reagent (AFS, with high phospholipid content). Furthermore, mixing studies with a 1:1 mix of sample pooled normal plasma were performed for PSL and AFSL. While PSL and AFSL were used as screening tests, AFS, with its known low lupus sensitivity, was used as a confirmation test; screen to confirm ratios were calculated for PSL (PSL/AFS) and AFSL (AFSL/AFS). Additionally, the anti-ß2GP1 IgG and IgM and anti-Cardiolipin IgG and IgM antibodies have already been determined.
Results: For the APTT tests, the highest sensitivity was seen for AFSL, with 83% applying the 99th percentile cutoff (96% with 97.5th percentile cutoff), whereas for PSL, the reagent compliant with the SSC recommendation, sensitivity was only 60% (72%). For AFS, the low lupus sensitivity was again confirmed (21%/40%).
In the APTT mixing studies, sensitivities in general were even higher than in the APTT test, as the more narrow distribution of the reference samples and consequently low cutoff resulted in slightly abnormal results for several samples just below cutoff in the initial APTT. In the mixing study, AFSL again showed higher sensitivity than PSL.
The APTT ratio AFSL/AFS shows more moderate sensitivity at 69% (79%) than the APTT mixing with AFSL. For PSL, the corresponding sensitivities are even lower, at about 45%.
The APLA profile shows that one sample of the reference panel was anti-ß2GP1 positive. All 97 dRVVT positive samples included were anti-ß2GP1 positive and 39 of the samples exhibiting strong dRVVT were also anti-Cardiolipin antibody positive (triple positive) and one of the weak dRVVT positive sample.
Summary/Conclusion: The APTT comparison using different activators revealed a significantly higher sensitivity for the ellagic acid-based reagent AFSL vs. the silica-based reagent PSL. Similar results have already been described by others, which demonstrates that the lupus sensitivity of APTT reagents is not determined by the activator used, but by the phospholipid source and content, as well as other ingredients. This fact is further underlined by the difference in lupus sensitivity seen for AFSL and AFS, which both use the same activator but employ phospholipids of different source and concentration. Among the APTT reagents tested in this study, AFSL clearly showed the highest sensitivity, and only slightly lower sensitivity than dRVVT.

PO 396
Overweight, obesity and body composition and risk thrombotic complications

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Background and Aim: WHO estimates 700 million adults will be obese in 2015 at least. There is evidence that obesity and overweight suppose a prothrombotic state associated with greater activation of coagulation and/or less fibrinolysis. Several studies link obesity and overweight with increased VTE recurrence. This has been the aim of our study.
Methods: We included 99 patients with VTE in the study, mean age 51.6 ± 15.8 years. The patient group consisted of 54% female and 46% male. Thrombotic risk factors previously described (PRETE-MED Guide) were collected for each patient. All patients underwent a study of body composition by bioelectrical impedance (Tanita, TBF300a. Arlington Heights, IL) obtaining data for fat mass (FM%) and body mass index (BMI). We apply Fisher's test to assess differences between groups.
Results: The distribution by type of thrombosis was: DVT in legs (48%), PE (17.5%), DVT + PE (12.5%), DVT in arms (0.7%), TV surface (11, 3%) and TV-unusual localization (10%). The distribution by BMI was 1% underweight (BMI < 18.5), 23.3% normal weight (BMI = 18.5–24.9), 50.5% overweight (BMI = 25–30) and 25, 2% obese (BMI > 30). A 54.5% of patients suffered recurrent thrombosis, of whom 80% had BMI > 25, and 70% FM% abnormal. A 38.4% developed post-thrombotic syndrome, of which 79% had BMI > 25, while 68% had abnormal FM%. We studied a possible association of these variables with the risk of recurrence and the post-thrombotic syndrome, by sex and age (< 50 or ≥ 50 years). Men with higher FM % have more recurrent events than those FM% within normal limits ($P = 0.0048$). We do not find differences in FM% in women, but if any in BMI. Women with BMI > 25 have an increased frequency of recurrent events ($P = 0.0001$). In patients < 50 years, recurrence ($P = 0.0001$) and PTS ($P = 0.005$) are more frequently when BMI ≥ 25. In patients ≥ 50 years, recurrence are more prevalent when BMI ≥ 25 ($P = 0.0001$) and FM% are higher ($P = 0.002$).
Summary: In our group being male, aged ≥ 50 years and have a higher FM% relates significantly with thrombotic recurrence. In women BMI ≥ 25 was associated with a higher recurrence of VTE regardless of age. Besides BMI ≥ 25 was associated with post-thrombotic syndrome in young patients (< 50 years). The results of our study support a relationship between obesity and venous thrombosis.

PO 397

Measuring quality of life in acute venous thromboembolism

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Background: Future funding of new diagnostic and therapeutic technologies will be guided in large part by economic analyses where quality of life is combined with survival in a single unified outcome. We recently completed the largest study estimating quality of life in acute deep vein thrombosis and pulmonary embolism, using the 'gold standard' measurement technique: the standard gamble. The results showed high degree of variation which suggested further exploration of the use of the standard gamble technique in this area.
Aims: To explore the validity, reliability, reproducibility and acceptability of the standard gamble (SG) measurement tool for quality of life (QoL) in acute pulmonary embolism (PE) and deep vein thrombosis (DVT).
Methods: Thrombosis clinic patients who had been diagnosed with either DVT or PE in the last year were eligible to participate in the study. After consent, the participants were interviewed by one researcher using multiple techniques: the SG technique, the short form-36 (SF-36), disease specific questionnaires (Veine-Qol and PembQol) and a visual analogue scale. These were performed in random order. The SF-6D score was calculated. Participants were asked to return for a repeat interview 4 weeks later.
Results: Forty-five patients consented to participate. The mean age was 54, 28 (64%) were men, 8 (18%) had cancer and for 32 participants (73%) it was their first diagnosis of DVT or PE. Median time since diagnosis was 86 days (IQR 30–169). Sixteen participants returned for a repeat interview.
Acceptability: 24/44 classified the SG method as 'acceptable' and 10/44 as 'pleasant and comfortable', but 5/44 as 'uncomfortable'. One patient declined to perform the SG.
Responsiveness: The SG QoL estimates increased in 4/16 participants on repeat interview. The visual analogue score increased in 12/16, and the EQ-6D score increased in 7/16.
Test-retest reliability: Initial and repeat SG QoL measurements correlated well (Spearman's Rho = 0.664).
Criterion validity: The only SF-36 scale associated with SG QoL was Role Emotional. The SG estimates correlated poorly with the SF-6D (Spearman's Rho = 0.18). There were no significant associations with the dimensions of the Veine-Qol or the PembQol.
Construct validity: Participants with PE gave SG QoL estimates which were lower than participants with DVT (median 92.5, IQR 81.6–1.0 vs. 98.9 IQR 85.0–1.0) although this was not statistically significant. There was no correlation with days since diagnosis, age, chronic illness and immobility.
Discriminant validity: Participant sex, education and number of dependents did not influence the SG QoL results.
Significantly higher SG QoL scores were found in those who said they had ingested harmful substances ($P = 0.04$) and wear what they choose to ($P = 0.05$). Nonsignificantly lower scores were seen with depression, 'glass half empty' outlook and lack of social support.
The SG technique demonstrated a ceiling effect with 16/44 giving estimates of 1.0.
Summary/Conclusions: Measurement of quality of life in acute thrombosis using the standard gamble technique does not correlate well with the SF-36 or disease specific questionnaires, and may be influenced by lifestyle factors such as risk taking, confidence and depression.

PO 398

Initiation of evidence-based guidelines in hemostaseology in a local hospital in southern germany

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Background: In our academic hospital center (530 beds)- attached to the university of Ulm – with several disciplines like neurosurgery, stroke unit, neonatology, hematology, obstetrics and several other disciplines use of antithrombotics and other drugs was historically dependent on personal experience and preference. Knowlegde concerning treatment and prophylaxis of various hematologic diseases was very different both concerning doctors and nurses. We started in 2008 to adapt international and german guidelines dealing with hematologic, thrombogenic diseases and bleeding disorders to our specific needs.
Aims: We wanted to establish evidence-based medicine and improve patients outcome and apply best medical care to our patients although financial resources are rather limited. Finally we made proposals in our pharmacologic conference and decided about the drugs used in daily routine in our hospital.
Methods: A group of interested and ambitious physicians (anasthesiologists, obstetricians, surgeons, neurologists, hematologists, cardiologists, pharmacists) has been meeting every 3 months to discuss and adapt external guidelines of medical societies. Advanced training of all staff in different departments were performed and prescription of new drugs like DTI or factor Xa- inhibitors were scientifically prepared and controlled by our department of pharmacy. Our own guidelines were distributed both electronically and in print form.
Results: Several new drugs were reviewed and introduced in our daily practise. Treatment was standardised and the quality of treatment improved. The total number of consultations with hemostatic topics has been rising. Economical improvement and big attention to patient care is continually observed.
Summary: We started in 2008 to implement a hemostasiological quality circle in our hospital as to improve patient safety and we applied evidence-based medicine to our daily routine. The advantage for our hospital is both economically and scientifically measurable. We recommend that hospitals being attached to a universitary institution or not must discuss and adapt guidelines for their own issues to improve their patient care.

PO 399

A rare cause of recurrent and serious venous thromboembolism: Klinefelter's syndrome

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Background: Klinefelter's syndrome (KS) is characterized by XXY karyotype, gynecomastia, small testis and hypogonadism with a prevalence of 1/500–1000 in man. There is an increased risk of venous thromboembolism (VTE), arterial thrombus and postthrombotic venous ulcerations. The relatively high serum estrogen concentrations and low androgen levels may be the cause of hypercoagulable state. Obesity that is common in KS patients may serve as an additional risk factor however the exact underlying mechanism is not completely understood yet. Here we report a 14 year old boy who presented with serious and recurrent venous thromboembolism and was found to have Klinefelter's syndrome on follow-up. To our knowledge this is the youngest KS patient reported to have VTE so far.
Case: A 14 year old presented with a sudden swelling and pain in his left leg. There was no family history of venous thrombosis and the

parents were non-consanguineous. On Doppler ultrasonographic evaluation of his lower extremity, there was a large, acute thrombosis starting from proximal saphenous vein spreading to the popliteal vein. D-dimer level on admission was 5.6 mg/L (Normal range: 0–0.5). The patient was diagnosed as deep vein thrombosis and anticoagulant therapy was started with low-molecular weight heparin (LMWH) for 14 days and prophylaxis was given afterwards. On physical examination, he was obese, mildly retarded and had a thickened neck, low neck-line and gynecomastia. He had social and psychiatric problems and was using medication for attention deficit. He was transferred to genetic department for further investigations. One and half month later, he presented again with thrombosis this time at the right leg when he was still on LMWH prophylaxis. Doppler USG showed acute thrombosis from the right iliac vein protruding to the popliteal vein. D-dimer level was 11 mg/L. LMWH dose was increased to the treatment dose. Coagulation tests, plasma FVIII, protein C, protein S and homocystein levels were normal. Genotype analyses for factor V Leiden and MTHFR C677T mutations revealed negative results however, he was heterozygous for G20210A mutation of the prothrombin gene and homozygous for the A1298C mutation of the MTHFR gene. Cytogenetic analysis showed 47, XXY phenotype consistent with KS. The patient is still on LMWH prophylaxis without recurrence.

Conclusion: We want to emphasize the hypercoagulable state and increased risk of recurrent and serious thrombosis in patients with KS. Most patients with KS remain undiagnosed due to heterogenous clinical presentation and unawareness of medical professionals. Early diagnosis might be life-saving. Optimal management of these patients need to be clarified, life-long anticoagulation prophylaxis may be considered in selected cases.

PO 400

Cancer-related thrombosis shows refractory to anticoagulant treatment

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Background: Three cases with refractory, recurrent deep vein thrombosis who were finally diagnosis with respectively gastric cancer, colon cancer or ovary malignant tumor were analyzed and discussed.

Aims: To stress the distinct clinical features and prognosis of cancer-related thrombosis through analysis of the underlying thrombophilic factors, the clinical features of the above cases.

Methods: Clinical courses, treatment protocols and outcomes of the above cases with cancer-related thrombosis had been recorded. Chromogenic substrate methods were used to detect protein C and antithrombin activities. Clotting method was used to detect free protein S and cloting factor VIII and XII activities. ELISA method was used to detect antiphospholipid antibodies.

Results: All three patients had demonstrated with recurrent deep vein thrombosis in bilateral upper or lower extremeties or both, in mesentery vein. Two of them experienced pulmonary embolism. All three cases were refractory to routine anticoagulant therapy including heparin, LMWH, warfarin and riveroxaban. Deficiencies in all the three anticoagulation proteins, e.g., protein C, protein S and antithrombin were excluded. Antiphospholipid antibodies were negative in them. Symptoms and signs were subsidized after removal of tumors.

Summary: Malignant tumors were the only known underlying factors of their refractory and recurrent thrombosis. Patients with refractory recurrent and extensive thrombosis should be examined for malignant tumors. Appropriate treatment for the cancer itself helps to alleviate the symtoms and signs of thrombosis.

PO 401

Automated assay of plasminogen activator inhibitor-1 (PAI-1) activity on STA-R®

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Background: The proteolytic degradation of fibrin clots is mediated by plasmin, an enzyme activated by the urokinase-type plasminogen activator (uPA) or tissue-type plasminogen activator (tPA). The main function of PAI-1 is the inhibition of fibrinolysis by regulation of uPA and tPA activity. Increase of PAI-1 level is associated with many cases of thrombosis, cancer, liver disease and inflammatory syndromes. In contrast, PAI-1 deficiency is associated with risk factors for bleeding diathesis.

Aim: Automation of Stachrom® PAI kit on the STA-R® (Diagnostica STAGO, Asnières, France) to measure elevated levels of PAI-1 and to detect deficient patients. Development of two protocols: standard range for high levels of PAI-1 and low range for PAI-1 deficiency.

Methods: Calibration and samples were measured using chromogenic method at 405 nm for both protocols. Three calibration points were used to obtain a linear calibration: the three calibrators of the kit were ready to use in the standard range protocol while dilutions of calibrator 2 in calibrator 1 were used in the low range protocol. The working range was 3 to 12 AU/mL using low range protocol (depending on the titer of calibrator 2) and 15 to 44 AU/mL using standard range protocol (depending on the titer of calibrator 3).

Results: Using low range protocol: correlation coefficient of calibration was > 0.990, reproducibility within and between assay through automation on STA-R was found to be 10% and 11% C vs. respectively (mean 9.6 AU/mL). Results of normal and low level plasma samples (normals treated with 5 U/mL t-PA) were in agreement with expected values (range from 3 to 11.4 AU/mL and 0.3 to 7.7 AU/mL respectively with a difference mean of 4.2 AU/mL corresponding to uncomplexed PAI).

Using standard range protocol: correlation coefficient of calibration was > 0.995, reproducibility within and between assay through automation on STA-R was found to be 2.2% and 5.6% C vs. respectively (mean 29 AU/mL). Results of normal samples, from cancer patients and pregnant women were in agreement with expected values (< 15 AU/mL in normals, > 26 AU/mL in patients).

Conclusion: The automated assay of PAI-1 activity using the Stachrom® PAI kit on STA-R® showed good performances and allowed to obtain a high sensitivity at low levels. It is easy to improve the measurement of PAI activity in clinical laboratory.

PO 402

Protective anticoagulation effects of peptide Arg-Pro-Gly-Pro and complex heparin with Arg-Pro-Gly-Pro in conditions of immobilization stress

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Background: Some short proline-containing peptides facilitate the activation of the functional state of the anticoagulative system (ACS) and have anticoagulant, fibrinolytic and antiplatelet effects in the bloodstream. At the same time many regulatory peptides play a key role in the maintenance of normal homeostasis and under stressing conditions. We suggested that these peptides would have an anticoagulation effect under the conditions of hypercoagulation during immobilization stress due to their ability to activate ACS.

Aim: The aim of this research was to study the influence of Arg-Pro-Gly-Pro (RPGP) and complex heparin with RPGP on the anticoagulant and fibrinolytic units of haemostasis system under experimental acute immobilization stress and to compare the antistress effects of these drugs.

Methods: The experiments were carry out on the white rats. RPGP and complex heparin with RPGP (with molar ratio 1/1) were used intranasal prior to lasting immobilization. In the blood samples we determined APTT, fibrinolytic activity (FA), plasminogen activator (PA) activity and ADP-platelet aggregation.
It was demonstrated that lasting immobilization stress activated the blood coagulation and promoted the decrease of fibrinolysis. The intranasal injection of RPGP or complex heparin with RPGP to rats prior to immobilization prevented the hypercoagulation, which appeared after lasting immobilization stress. At the same time FA, PA, anticoagulant and antiplatelet activity were increased. Thus the protective effect against hypercoagulation induced by immobilization was shown after administration of study drugs The complex heparin with RPGP were found to have the most significant effects.
Conclusion: Thus our results showed that as RPGP as the complex heparin with RPGP have protective effect against enhanced blood coagulability resulting from immobilization stress. We confirmed that RPGP and complex heparin with RPGP are capable of ACS activation after their administration.

PO 403

Flow conditions modulate fibrillogenesis of plasma fibronectin

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Background: Soluble plasma fibronectin (Fn) with its inactive compact structure needs to be unfolded to assemble to active fibrils. As reported, mechanical forces, assessed by atomic force microscopy, could regulate the transformation of Fn from the compact form to the extended fibrillar state by exposing cryptic binding sites in individual Fn type III repeats.
Aims: This study investigates the conformational changes of Fn in response to flow conditions simulating venous or arterial shear rates and resulting biomechanical forces.
Methods: Fn isolated from fresh frozen human plasma was added, at different concentrations (50 and 100 μg/ml), to the plate pre-coated with 100 μg/ml Fn or BSA and subsequently exposed to shear rates (50–5000 s) using a cone plate rheometer (Haake Rheostress 1). Viscosities of Fn solutions were recorded over 10 min. To quantify the amount of fibril formation, DOC solubility assays and Western blotting were performed. Control experiments were conducted under static conditions.
Results: After exposure to shear rates, the viscosities (mPa s) of Fn solutions at concentration of 50 and 100 μg/ml increased from 1.87 ± 0.87 (mean ± SD) to 5.03 ± 1.86 and from 2.03 ± 1.63 to 9.29 ± 6.27 on Fn surfaces; from 1.55 ± 0.11 to 4.31 ± 1.7 and from 1.61 ± 0.7 to 4.92 ± 1.8 on BSA surfaces, respectively. In addition, the ratios of fibrils to soluble Fn increased significantly from 0.018 ± 0.012 (mean of ratio ± SD) to 0.121 ± 0.08 ($P < 0.05$) (Fn surfaces) and from 0.021 ± 0.009 to 0.059 ± 0.022 ($P < 0.05$) (BSA surfaces) when increasing the concentration from 50 μg/ml to 100 μg/ml. Under static conditions, no fibril formation was detected.
Conclusion: Our results indicate that the formation of Fn fibrils can be monitored by changes in viscosity. The formation of fibrils is dependent on the Fn concentration, but not on the type of the coated surface. In addition, fibrillogenesis of Fn is modulated by flow conditions.

PO 404

Functional constituents in Natto: nattokinase, vitamin K2 and polyamine contents

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Background: Natto is produced through the fermentation of steamed soybeans by using *Bacillus subtilis natto*. In previous papers, we have presented reports on the physiochemical properties of nattokinase, a constituent of natto (Sumi *et al*., *Experientia*, 43, 1110, 1987; Yatagai *et al*., *Pathophysiol. Haemost. Thromb*., 36, 227, 2009). It is now widely recognized in particular that natto is a food rich in polyamine, a substance with anti-aging effects (Soda *et al*., *J. Nutr. Sci. Vitaminol*., 55, 361, 2009).
Aims: We have recently had an opportunity to analyze the natto products that were judged to be superior and received prizes at the official National Awards Ceremony Recognizing Excellence in Natto held over the past few years. For natto products, which are considered to have excellent flavor and aroma, the potency of the constituent nattokinase, the amount of vitamin K_2 produced, and the polyamine content have now been identified.
Methods: Of the 224 natto products entered in the above-mentioned competitions, the 14 types that received prizes were frozen at −30 °C until used in the analysis. The procedures for preparation of rabbit antibody for nattokinase and for double immunodiffusion were carried out as reported previously (Sumi *et al*., ISTH, PP-WE148, Kyoto, 2009; Sumi et al., *New Food Industry*, 54, 12, 2011). Thrombolytic activity was measured by using the fibrin plate method. For vitamin K_2, the following procedures were carried out according to the method previously reported (Sumi *et al., Food Sci. Technol. Res*., 5, 48, 1999). Polyamine was measured through the using HPLC by the on-column method (Saito *et al., Anal. Sci*., 8, 675, 1992).
Results: Nattokinase contained in the 14 types of natto showed strong thrombolytic activity. For each product tested, the thrombolytic activity was neutralized with the antibody for nattokinase. BSB (borate saline buffer) at pH7.8 was used, and the nattokinase showed strong activity for the synthetic substrate I (Bz-Ile-Glu-(OR)-Gly-Arg-*pNA*). Because activity declined to less than half the level of the synthetic substrate II (Suc-Ala-Ala-Pro-Phe-*pNA*) used for comparison, the true enzymatic activity of nattokinase is believed to be at work. Potency was calculated at 1.12 ± 0.46 IU/g. Sublitisins, enzymes produced by *Bacillus subtilis* including ones for such industrial uses as cleaning and textile processing, show particularly strong activity for synthetic substrate II. The measured value of vitamin K_2 content was 12.19 μg/g on average, a little higher than expected. Polyamine content in natto was 4.06 ± 0.86 mg/100 g and in *Bacillus subtilis natto* was 66.53 ± 20.71 mg/100 g.
Summary/Conclusion: The production of nattokinase was shown through the lytic ability for the synthetic substrate Bz-Ile-Glu-(OR)-Gly-Arg-*pNA*. Each natto tested contained a large amount of vitamin K_2. The polyamine content in *Bacillus subtilis natto* was quite high at about 10 times the content in natto. Content as high as this was not observed in soybean paste or soy sauce, which are produced by a different microbe.

PO 405

Thrombotic risk factors in cirrhotic patients

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Background: Cirrhosis results in a complex pattern of defects in haemostatic functions with reduced synthesis of pro and anticoagulant

factors. As possible complication of coagulation disorders in cirrhosis, could be the development of arterial and venous thromboembolism (AVTE). The purpose of our study was to determine thrombotic risk factors in cirrhotic patients.

Methods: Fifty one cirrhotic patients were enrolled into a case control study. The presence of personal and familial history of AVTE were investigated. White blood cells, platelet count, prothrombine time, INR, albumin, urea, procoagulant factors (VIII, XII, VII, II, V) were determined. Level of antithrombin, protein C and protein S were measured (respectively STACHROM AT, STACLOT PC, STACLOT PS; DIAGNOSTICA STAGO). Search for factor V Leiden and prothrombin gene mutation (G20210A) were performed with PCR-RFLP. Anticardioloipin and antiB2glycoprotein antibodies were also investigated.

Results: Mean age was 56.8 years old (range16–86 years old). Sex ratio was 0.9. Among the 51 cirrhotic patients, 7 patients (13.7%) had experienced AVTE after cirrhosis diagnosis: deep venous thrombosis ($n = 2$), pulmonary embolism ($n = 1$), Budd Chiari syndrome ($n = 1$), portal thrombosis ($n = 3$). They were compared to 46 cirrhotic patients without thrombosis. No patient with AVTE had neither personal nor familial history of thrombosis. In an univariate analysis, white blood cell count and platelet count were significantly higher in patients with AVTE than other cirrhotic patients (respectively, 8795 vs. 5032/mm^3, $P < 0.018$ and 91133 vs. 154375/mm^3, $P = 0.03$) However, In a multivariate analysis only the platelet count was independently predictive of AVTE in cirrhotic patients ($P = 0.05$). White blood count was not an independent predictive factor of thrombosis in cirrhotic patients ($P = 0.07$). Moreover, prothrombin time, INR, albumin, urea, level of pro and anticoagulant factors were not statistically different in both groups. There was no link between the presence of Factor V Leiden, prothrombin gene mutation (G20210A), anticardiolipin and antiB2glycoprotein antibodies to thrombosis.

Conclusions: Approximately 13.7% of cirrhotic patients resulted in a thromboembolic event. Platelet count was predictive of increased risk of AVTE as it was supported by other studies. Understanding the factors predisposing to thrombosis in cirrhotic patients could play a role in identifying a subgroup of patients at high risk of thrombosis and making decisions regarding the utility of anticoagulation therapy.

PO 406

Unusual thrombosis and thrombophilia: a difficult problem to address: 4 years experience

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There are sites called vascular territories can present unusual thrombotic events: venous sinus, upper limbs and splanchnic with clinical consequences can be catastrophic.

Objective: To describe the frequency of thrombophilia in patients who present with thrombosis in unusual sites treated at the hematology service of Hospital San Jose, between January 2007 and December 2011. They describe the population in terms of demographics, gender, age, recurrence, and complications associated and treatments used.

Methods: This is a descriptive case series, patients older than 18 years with first thrombotic event that involves site vascular unusual, data were extracted of medical records retrospectively.

Results: Seventy-three patients entered the study, most women (65.8%), with a location in venous sinuses (47.9%), splachnic (24.0%), upper limb (27.4%), with risk factors for pregnancy frequently (12.5%), puerperium (4.2%), oral anovulatories consumption (10.4%), smoking (9.6%), thrombophilia was found in 57% of patients with thrombosis in unusual sites, the most frequent diagnosis of antiphospholipid syndrome.

Conclusions: There is a high frequency for thrombophilia in patients with thrombosis in unusual site, antiphospholipid syndrome being the most common in our series.

PO 407

New automated chromogenic assay for Protein C activity in Q Hemostasis Analyzer

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Background: Deficiency of the Protein C (PC) anticoagulant pathway is associated with an increased risk of venous thrombosis.

It is usually recommended to evaluate PC activity as a first step in the screening for thrombophilia. For this purpose, Grifols has developed a new automated PC chromogenic assay for use in Q *Hemostasis Analyzer*.

Aims: Evaluate the performance, robustness and sensitivity to interfering substances of a new chromogenic PC assay (DG-Chrom PC).

Methods: DG-Chrom PC assay is based on the activation of PC in plasma by a specific activator (extract from the *Agkistrodon contortrix contortrix* snake venom). The amount of functional PC in the sample is determined by the hydrolysis of a specific chromogenic substrate for APC.

Precision, Trueness, Linearity, Limit of Detection, Limit of Quantitation and Interferences have been performed in Q *Hemostasis Analyzer* following CLSI EP5-A2, CLSI EP15-A2, CLSI EP6-A, CLSI EP17-A and CLSI EP7-A2, respectively.

Results: For DG-Chrom PC no clinically significant interference was detected up to 2 IU/mL of heparin, 450 mg/dL of hemoglobin, 990 mg/dL of triglycerides and 30 mg/dL of bilirubin.

Robustness of the test DG-Chrom PC at different incubation times was demonstrated within a range between 150 and 360 s. Moreover, variations at different incubation temperatures (between 35 and 39 °C) did not affect PC results.

Precision (in terms of CV%) was assayed at three different levels ($n = 80$). For normal control plasma (100% PC) a within-lot precision of 2.3%, repeatability of 1.2% and a between-lot precision of 2.3% were obtained. For abnormal control plasma (40% PC) a within-lot precision of 2.5%, repeatability of 2.0% and a between-lot precision of 2.9% were obtained. For very low levels of PC (22% PC) a within-lot precision of 4.2%, repeatability of 3.0% and a between-lot precision of 3.6% were obtained.

DG-Chrom PC showed a good linearity between 0 and 150% of PC activity ($n = 5$) with a mean $r^2 = 0.9995$.

Trueness of DG-Chrom PC was evaluated ($n = 5$) using 2nd SSC plasma analysed at three dilutions. Undiluted 2nd SSC plasma (89% PC) generated a mean of 89.5% PC. At 44.5% PC level, the mean obtained was 47.1% and at 8.9% PC level, a mean of 8.9% was generated.

The limit of detection of DG-Chrom PC determined with a PC deficient plasma (Technoclone) was 2.3%. The limit of quantitation of the method was evaluated using 2nd SSC plasma ($n = 10$). DG-Chrom PC accurately measured PC level at 5.6% of PC.

Conclusion: The results obtained support the intended use of DG-Chrom PC assay in Q *Hemostasis Analyzer* as a valuable tool for measuring PC activity in citrated patient plasma samples.

PO 408

The level of β-thromboglobulin and thrombophilia

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Background: B-thromboglobulin – is a protein located in the α-granules of platelets and is released into the bloodstream during platelet activation. β-thromboglobulin is a marker of activation of platelet hemostasis. Thrombophilia is defined as an inherited feature of an organism, which increases susceptibility to intravascular thrombus

formation in the absence of other diseases. It is known that the presence of thrombophilia affect plasma haemostasis. Influence of thrombophilia on platelet part of haemostasis has not studied yet.
The aim of our study was to investigate the effect of thrombophilia on platelet part of haemostasis.
Methods and Results: The study included 41 people with the presence of one or a combination of the following mutations: mutation Leiden, prothrombin gene mutation 20210A, a mutation in the genes of MTHFR and PAI-1. Of these, 29 women and 12 men. The control group included 11 healthy individuals with no of these mutations, including 7 women and 4 men. Mean age 35 ± 7.5 years. Inclusion criteria was the lack of thrombotic history and absence of inflammatory diseases at the time of blood sampling. Blood and urine in all subjects were normal.
All people have been identified β-thromboglobulin level (ELISA method, Stago Diagnostica).
Results: The level of beta-thromboglobulin was increased in 56.09% ($P < 0.05$) of people with thrombophilia. In the control group, increasing β-thromboglobulin was observed in 17.1% ($P < 0.05$).
Conclusion: Thus, thrombophilia affect platelet part of haemostasis.

PO 409

Thrombophilia as a cardiovascular risk factor for acute myocardial infarction in a young patient-case report

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Background: Hereditary and acquired abnormalities in blood coagulation (hypercoagulation) may predispose for venous and arterial thrombosis. Arterial thrombotic pathology is currently the most important cause of mortality in adult patient; thrombotic arterial occlusion can involve very different territories: coroanarian, limb vessels, mesenteric arteries, cerebrale arteries.The most common is coronary thrombosis that causes acute myocardial infarction. Etiology is diverse, being hipercoagulability are relatively frequent, but rarely found.
Aim: We report a case of 27 years old male patient, with obesity grade 3, smoker of one packs of cigarettes per day, who was admited in our clinic at 30 h of onset of constrictive chest pain radiating to interscapulo-vertebral and left upper member and dyspnea.
Methods: For this case we use laboratory testes, ultrasound methods and coronary angiography.
Results: At admission:blood pressure 130/80 mmHg, heart rate 120b/min, enzymes of myocardial necrosis elevated, on electrocardiogram sinus rhythm, Q wave in V1-V5, ST segment elevation 3–4 mm in V2-V6 and 1–2 mm in DI, AVL.
Cardiac ultrasound: Left ventricle with normal size and significant changes in kinetics, akinesia of half-apical of interventricular septum, apical third of the inferior and anterior wall, basal hypokinesia of interventricular septum, EF = 40%;mild ischemic mitral regurgitation, left atrium dilated.
Angiocoronarography releved left coronary system dominant, left main coronary artery, left circumflex artery, right coronary artery without significant lesions, occlusion of proximal left anterior descending artery without visualization of distal territory; during intervention we observed massive thrombosis on left anterior descending artery until the apex, on a distance of 15–20 centimetres. Thrombus was aspirated repeatedly with an aspiration catheter, with a small amount of thrombus staying in the proximal left anterior descending artery. Then we stented the proximal left anterior descending artery. After stenting thrombus formation was observed proximal and distal to the stent, for which was performed proximal and distal stenting with good result. After five min we observed thrombus formation in the left main coronary artery, for which we performed thrombus aspiration, without succes, after which we stented from the left main coronary artery to the circumflex artery, with good result, without proximal or distal dissection, without residual stenosis, with miocardial blush grade 3.
Due to massive thrombosis in the left anterior descending artery and subsequent thrombosis occurred during the procedure, in the first day after stenting the pacient was investigated for hypercoagulation status. Screening of thrombophilia related hyperhomocysteinemia (homocysteine level = 19.12 μm/L), heterzozygote mutation of methylenetetrahydrofolate reductase A1298C and low level of antitrombin III.
Summary: Evolution under terapy was very well, wiht significant decrease of the markers of myocardial necrosis after 3 days. The patient was discharged after 8 days with dual antiagregation and oral anticoagulation therapy.
Conclusions: Inherited thrombophilia is a congenital tendency to thrombosis which may be manifested episodically usually in the presence of another predisposing factor. Thrombophilia induce a hypercoagulable state, which, with another cardiovascular risk factors, explain repeatedly arterial thrombosis during the interventional procedure at this young patient.

PO 410

Inherited thrombophilia – accidental discovery at patients with neoplastic diseases from III rd Pediatric Clinic, Department of Hematology Oncology and Bone Marrow Transplantation Timisoara

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Background: Inherited thrombophilia is a genetic tendency to venous thrombosis. Many thrombotic episodes occur in association with other identifiable risk factors (pregnancy, oral contraceptives, surgery, cancer). Screening for thrombophilia is recommended for people at high risk of thrombotic events.
Aim: We report four cases of patients aged 6–19 years with inherited trombophilia and cancer.
Methods: Hypercoagulation status was provided with laboratory testes and imagistics methods (Doppler ultrasound, angio magnetic resonance imaging).
Results: Case 1: Boy,19 years, acute lymphoblastic leukemia phenotype B; during chemotherapy he developed deep vein thrombosis on common femoral vein over a distance of 10–15 centimetres, 3 weeks after insertion of tha central venous catheter on the right femoral vein. Anticoagulant therapy was initiated with heparin sodium. In the present is under oral anticoagulant. Screening for thrombophilia: heterozygous mutation for methylenetetrahydrofolate reductase C677T and A1298C, moderate hyperhomocysteinemia (Homocysteine = 21.74 mM).
Case 2: Girl, 6 years, acute lymphoblastic leukemia phenotype B.Cardiological evaluation showed on electrocardiogram sinus arrhythmia, ST segment depression in V5-V6, T wave negative in DII, DIII, aVF and all precordial leads, EF = 46%, with markers of myocardial necrosis negative. Screening for thrombophilia: heterozygous mutation for methylenetetrahydrofolate reductase C677T and A1298C, moderate hyperhomocysteinemia. (Homocysteine = 12.71 mM). Chemotherapy was initiated and was started heparin therapy prophylaxis. A few h after starting heparin, the massive macular hemorrhage preretinian in left eye appeared. We stopped the heparin; in evolution -without acute thrombotic events, currently maintains branches of the catheter with alternative heparin administration in very low doses, daily.
Case 3:Girl 19 years, Hodgkin lymphoma stage IV, second relapse, with deep venous thrombosis on left subclavian and cephalic vein, under anticoagulation therapy. We performed two apheresis procedures, central venous catheter was mentained on right femoral vein for thirty h, under anticoagulant therapy. After removal of the catheter,

she developed partial thrombosis on the left femoral vein, full thrombosis on the right femoral vein.Screening for thrombophilia: heterozygous mutation for methylenetetrahydrofolate reductase C677T and A1298C, moderate hyperhomocysteinemia. (Homocysteine = 15, 0.27 mM).Therapy was initiated with heparin sodium;in the present she is at six mounths after autologous bone marrow transplantation, under oral anticoagulant therapy, in well condition.

Case 4: Boy,11 years, Hodgkin lymphoma stage III A, second relapse and autologous bone marrow transplantation. Three months he had catheter on right subclavian vein with prophylactic heparin therapy. Fourteen days after removal the catheter, Doppler ultrasound showed partial thrombosis of left internal jugular vein. Screening for thrombophilia:, methylenetetrahydrofolate reductase A1298C heterozigot, moderate hyper homocysteinemia (Homocysteine = 15.27 mM).

Summary/Conclusion: Three of four patients developed deep vein thrombosis and we evaluated the genetic risk for thrombosis. All the patients have hyperhomocysteinemia and heterozygous mutations of methylenetetrahydrofolate reductase and they received anticoagulant therapy. The presence of central venous catheter in all the patients was a risk factor for the occurrence of thrombosis, in association with hypercoagulability status of the neoplastic disease and L-asparaginase.

PO 411

The intensity of the intravascular coagulation and thrombophilia

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Background: Intravascular coagulation in the human body is constantly. Change in the intensity of blood coagulation is observed in various pathological conditions. Thrombophilia is defined as a feature of an organism that determines its increased risk of thrombosiss in the intravascular normal situations, in the absence of other diseases and often are inherited. Currently it's unknown what changes are observed intravascular coagulation in patients with thrombophilia.

Aim: The aim of our study was to determine the intensity of the intravascular microcoagulation in healthy individuals with thrombophilia.

Methods: The study included 41 healthy people, including 29 women and 12 men. All people had a mutation or a combination of mutations such as Leiden mutation, prothrombin gene mutation 20210A, a mutation in the genes of MTHFR and PAI-1. The average age was 35 ± 7.5. As inclusion criteria ware the lack of a history of thrombosis and the absence of inflammatory disease at the time of blood sampling. Blood and urine in all subjects were normal.

By studying the intensity of intravascular blood microcoagulation to determine the degree of platelet activation we measured the level of beta-thromboglobulin (ELISA method, Stago Diagnostica). To determine the level of procoagulant activity and fibrin formation we measured the level of D-dimer (ELISA method, Technoclone). Was also investigated ADAMTS-13 (ELISA method, Technoclone) as a possible factor in increasing the intensity of the intravascular coagulation.

Results: All people had a normal ADAMTS-13 and D-dimer levels. As for beta-thromboglobulin, it was increased in 56.09% ($P < 0.05$) of person with thrombophilia.

Conclusion: Thus, we can conclude that thrombophilia increase the intensity of intravascular coagulation, which is manifested by increased activity of platelet hemostasis. The fact that the final link of blood coagulation, in particular D-dimer remained normal, confirms the assertion that for the manifestation of thrombophilia person need to have additional triggers.

PO 412

Plasminogen activator inhibitor type 1 (PAI-1) level and activity at patients with PAI-1 polymorphism

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Plasminogen activator inhibitor type 1(PAI-1 or SERPINE-1) is an important component of the coagulation system that functions as the principal inhibitor of tissue and urokinase plasminogen activators (tPA, uPA) and hence fibrinolysis that degrades blood clots. It is possible, that PAI-1 plays an important role in determining predisposition to cardiovascular disease.

The PAI-1 gene is located on chromosome 7 (7q21.3-q22). There is a common polymorphism known as 4G/5G in the promoter region. The 5G allele is slightly less transcriptionally active than the 4G. The man has two copies of each gene (one from the mother and one from the father) in a population and as a result three different genotypes are possible: 5G/5G, 5G/4G, 4G/4G. It was found that people with 4G/4G variant has much higher concentration of PAI-1 than people with 5G/5G and 5G/4G, which may increase the risk of blood clots.

Objective: To assess PAI-1 level and activity at healthy patients with PAI-1 polymorphism.

Materials and Methods: The study included 39 practically healthy people. PAI-1 polymorphism was found at 32, among them 8 men and 24 women. The middle age was 32.3 years. The PAI-1 antigen and activity was assessed by ELISA method, Tehnoclone (Austria). The *PAI-1 polymorphism* was determined by polymerase chain reaction (*PCR*).

Results: Thirty-two (82%) of investigated people had PAI-1 polymorphism 5G/4G, 4G/4G, among them 12 homozygotes and 20 heterozygous. There was no high antigen level in both groups. Only three individuals (15%) among heterozygous had high activity of PAI-1. Among homozygotes there was no increasing of it.

Conclusions: It is possible to think, that 5G/4G, 4G/4G PAI-1 polymorphism doesn't increase the antigen level and activity of PAI-1 and doesn't lead to thrombosis. So we think, that some other triggers and conditions influence on the intensity of the intravascular coagulation and increase it.

PO 413

Observational analysis of genetic risk factors in patients with a documented diagnosis of cerebral sinus vein thrombosis

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Background: In industrialized countries, the cerebral sinus vein thrombosis (SCVT) is most often aseptic and it can be observed in all the following settings: surgery, obstetric-gynecology and medicine. SCVT can particularly occur in postoperative period and puerperium, both contexts characterized by hyperfibrinogenemia and thrombocythemia. In addition, in SCVT the use of birth control pills is often imputed as a risk factor. However, the etiology of this disease remains indefinite in 25–35% of cases.

Aims: The objective of this study was to retrospectively analyze the relationship between factor V Leiden, prothrombin G20210A mutation (PT 20210A), MTHR and the occurrence of SCVT.

Methods: The study has included 20 patients with a diagnosis of SCVT instrumentally performed between 2002 and 2012. We determined the mutational status of factor V Leiden, Prothrombin (PT) G20210A and C677T and A1298C of MetilenTetraHydropholateReductaase (MTHFR) in each enrolled patient. We performed a comparative analysis of mutations found in patients with a diagnosis of cerebral sinus

thrombosis compared with a population of unselected patients without venous thrombosis.

Results: The distribution of mutational status in the genetic analysis performed in the enrolled patients was as follows: Wild type for Factor V Leiden, Factor II and MTHFR: 19%.Factor VLeiden: 15% heterozygote and 0.5% homozygote in cases and only 2- 3% and 0.02% respectively in control group (European unselected patients for venous thrombosis).Heterozygous for PT G20210A mutation: 20% in patients and 3–5% in control group. Mutation of the MTHR C677T and A1298C: 45% heterozygote for C677T and 10.5% double heterozygote for C677T and A1298C in cases. In the control group, the mutation C677T was not statistically different from cases, on the contrary a double heterozygote mutation for C677T and A1298C was 1.5%.

Conclusions: The presence of factor V Leiden and PT 20210A are known risk factors for SCVT. The single mutation of C677T of MTHFR does not represent a risk factor for the disease because it has the same prevalence in the normal population. Instead, the coexistence of a double mutation in MTHFR (C677T and A1298C) can be considered a risk factor, as the mutations of the factor V and factor II. The coexistence of a double mutation of MTHFR is associated an increased relative risk for venous thromboembolism in the examined population.

PO 414

About hemostasis system genes polymorphisms in patients with acute ischemic stroke

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At the modern stage of clinical medicine development a reasonable view on multifactorial nature of congenital predisposition to rapid atherothrombosis progression is formed.

Materials and methods. The research was performed on group of patients (n = 117) with clinical manifestations of ischemic stroke against the background of multifocal atherothrombosis, who underwent surgical brain revascularization (carotid endarterectomy). SPSS for Windows (13.0) software was used for mathematical processing of the research results.

Methods: Cluster analysis of studied genes was performed to investigate the proximity degree of studied polymorphisms. We have studied different mathematic models describing different combinations of hemostasis system genetic polymorphisms. The first cluster united homozygous polymorphisms PAI-1 -675 4G/4G, FGB 455 A/A and heterozygous polymorphism ApoE 28 L/P. It's important that 72% of patients with acute ischemic stroke in anamnesis belonged to the second cluster which united 5 gene variants: MTHFR 677 C/T, PAI-1 675 4G/5G, FBG 455 G/A, GpIIIa PIA1/A2 and ApoE. The third cluster included 22% of patients with acute ischemic stroke in anamnesis and united 6 genotype variants: MTHFR 677 C/T, PAI-1 675 4G/5G, FBG 455 G/A, GpIIIa PIA1/A2, ApoE and FV 1691 G/A.

Conclusion: We revealed significant spread of prothrombotic polymorphisms against the background of atherothrombotic affection of brachiocephalic artery in MTHFR, PAI-1, APOE and GpIIIa genes. It is associated with high risk of atherothrombosis and its complication (brachiocephalic artery thrombosis). At the modern stage of clinical medicine development a reasonable view on multifactorial nature of congenital predisposition to rapid atherothrombosis progression is formed.

Materials and methods. The research was performed on group of patients (n = 117) with clinical manifestations of ischemic stroke against the background of multifocal atherothrombosis, who underwent surgical brain revascularization (carotid endarterectomy). SPSS for Windows (13.0) software was used for mathematical processing of the research results.

Methods: Cluster analysis of studied genes was performed to investigate the proximity degree of studied polymorphisms. We have studied different mathematic models describing different combinations of hemostasis system genetic polymorphisms. The first cluster united homozygous polymorphisms PAI-1 -675 4G/4G, FGB 455 A/A and heterozygous polymorphism ApoE 28 L/P. It's important that 72% of patients with acute ischemic stroke in anamnesis belonged to the second cluster which united 5 gene variants: MTHFR 677 C/T, PAI-1 675 4G/5G, FBG 455 G/A, GpIIIa PIA1/A2 and ApoE. The third cluster included 22% of patients with acute ischemic stroke in anamnesis and united 6 genotype variants: MTHFR 677 C/T, PAI-1 675 4G/5G, FBG 455 G/A, GpIIIa PIA1/A2, ApoE and FV 1691 G/A.

Conclusion: We revealed significant spread of prothrombotic polymorphisms against the background of atherothrombotic affection of brachiocephalic artery in MTHFR, PAI-1, APOE and GpIIIa genes. It is associated with high risk of atherothrombosis and its complication (brachiocephalic artery thrombosis).

PO 415

VKORC1 gen polimorphisms distribution features in native population of European Russia

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The aims of the research are: reveal VKORC1 gen polimorphisms distribution features in native population of European Russia Far North; determine incidence of C1173T and G3730A polymorphisms of VKORC1 gen in Nenets Autonomous District; compare received data with C1173T and G3730A polymorphisms of VKORC1 gen incidence in population of European North of Russia.

Materials and methods. The number of investigated samples was 88. The object was genomic DNA received from peripheral venous blood leukocytes. Genotyping was held with the use of polymerase chain reaction with allel-specific primers and electrophoretic detection in agarose gel.

Results and discussion. Polymorph allel 1173T of VKORC1 gen was found in 76.1% of participants and allel 3730A – in 53.2%. VKORC1 gen polymorphisms incidence in native population of European Russia Far North differs from those in population of European North of Russia. The most widely spread allel is 1173T (76.1%). In people with T/T genotype synthesis speed and concentration of vitamin K epoxide reductase are minimal. In people with VKORC1 1173 C/C genotype the enzyme activity is the highest and hypercoagulation risk is increased. Polymorphism 3730 A/A also increase enzyme activity and hypercoagulation tendency.

Investigation of VKORC1 gen polymorphisms incidence in particular populations and thrombosis risk analysis in different regions will allow more efficient prevention of thrombosis-associated diseases and pregnancy problems.

PO 416

The difference of homocysteine plasma in acute myocardial infarction depending on genetic polymorphisms of the enzyme methyltetrahydrofolatreductaza

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The risk of acute myocardial infarction (AMI) depending on hyperhomocysteinemia (HHC) increases by 3–4 times. The main reason for HHC is a genetic defect of the enzyme MTGFR.

Materials and Methods: To study the difference of HC plasma at the 1th and 14th days of AMI, depending on the genetic polymorphism MTGFR. Patients of 70 years old and younger (n = 40) suffering from AMI. To process the received data the SPSS 18.0 software package for Windows has been used.

The results. The level of HC at the first day of AMI was Me 24.4 [15.7, 28.6] mM. It was higher than normal values at the all patients. A 27.5% of patients had heterozygous polymorphism (GT) in the gene for the enzyme MTGFR, 12.5% of patients had homozygous polymorphism (TT). The level of HC in the group with allele (TT) was higher than that of other patients at the 1th ($P = 0.028$) and at the 14th days of AMI ($P = 0.001$). The correlation was detected between (TT) allelic variant and the level of HC at the 1th day ($r = 0.534$, $P = 0.001$) and at the 14th day ($r = 0.314$, p = 0.048).

Conclusions: Patients with homozygous polymorphisms MTGFR have higher levels of HC in both acute and subacute stage in AMI, which is an additional risk factor for adverse thrombotic events.

PO 417

MTHFR C677T and A1298C as a hyperhomocysteinemia risk factor in native population of Nenets Autonomous District

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In the metabolic processes study and many common diseases preventing a human ethnic origin should be taken into consideration. Cardiovascular diseases risk factors study and the development of early preventive measures are of particular topicality.

Aim: to investigate the prevalence of methylenetetrahydrofolate reductase gene polymorphisms MTHFR C677T and A1298C (associated with hyperhomocysteinemia risk), the level of homocysteine in blood and lifestyle of the Nenets Autonomous District (NAD) population.

Materials and methods.

Design: One-moment population-based investigation of native population (nenets) from different settlements. Inclusion criteria: nenets ethnicity; constant residence in NAD; presence of informed consent. The study approved by the NSMU local ethics committee (protocol №6, 8.06.2011). Questioning – data on diet, smoking, alcohol intake from survey respondents ($n = 226$). The homocysteine level in the blood serum ($n = 90$) was determined by solid-phase ELISA using reagents Axis Shield Diagnostics Ltd. Identification of polymorphisms MTHFR C677T ($n = 121$) and A1298C ($n = 90$) was performed in CSRL of NSMU. Genome DNA received from peripheral venous blood leukocytes was used in PCR molecular genetic analysis with allele-specific primers with detection by agarose gel electrophoresis.

Results: The age of the study sample Me = 42 [30;51] years, Me = 46 [35;54] for female and Me = 41 [37;49.5] for male.

Among the modifiable risk factors of hyperhomocysteinemia smoking (25%) and alcohol intake (51%) were present. In smoking men 52% smoke more than one cigarette pack per day. In smoking women 36.7% smoke more one cigarette pack and 41.7% half of a pack per day.

Alcohol is taken more often than once a month by 22% of women and 60.8% of men (average age 42.75 ± 11.59 and 42.91 ± 10.15 years respectively).

Homocysteine level was determined in 90 nenets. By to the results they were divided into three groups according to thrombosis risk (Shmeleva V, 2008): < 10.5 nkM (normal amount), 10.5–13.5 mkM (thrombosis risk increased 1,3–3 times) and more than 13.5 mkM (thrombosis risk increased 3–5 times). The results were following: 8.07 [7.2;9.3] for the 1st group ($n = 28$), 11.5 [11.2;12.0] for the 2nd group ($n = 22$) and 17.2 [14.6;19.8] for the 3rd group ($n = 40$).

Frequency of T677T polymorphism in nenets population significantly less than in Russian population in Tomsk ($\chi 2 = 10.59$, $P = 0.001$). A1298C polymorphism frequency significantly differ in compared populations ($\chi 2 = 6.17$, $P = 0.01$).

Assessment of interlinkages of homocysteine level in the blood serum with different allelic MTHFR gene variants carriage was performed using Kruskal-Wallace test ($P = 0.05$). No significant differences were revealed: 0.066 for MTHFR C677T and 0.531 for MTHFR A1298C.

We revealed a high percentage of hyperhomocysteinemia in nenets population. It should be in connection with external modifiable factors.

PO 418

The value of hyperhomocysteinemia on the severity of acute myocardial infarction

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The risk of acute myocardial infarction (AMI) depending on hyperhomocysteinemia (HHC) increases by 3–4 times. The main reason of HHC is genetic defects of enzymes responsible for the metabolism of HC, in particular methyltetrahydrofolatreductaza (MTHFR).

The Objective: To study the impact of hyperhomocysteinemia and genetic polymorphisms in the gene methyltetrahydrofolatreductaza (MTGFR) on the severity of acute myocardial infarction.

Materials and Methods: Patients of 70 years old and younger ($n = 40$) suffering from AMI who were hospitalized at the cardio intensive care unit of the 1stCityClinicalHospital from 09.01.2010 to 31.12.2010. Diabetes, rheumatism, systemic diseases, blood diseases were the exclusion criteria. Plasma levels of HC by enzyme immunoassay were determined at 1 and 14 days of AMI. Genetic polymorphism of the enzyme were identified in the laboratory MTGFR human genom NSMU. To process the received data the SPSS 18.0 software package for Windows has been used. The age span of the examined patients was Me 55 [45.0;60.25].

Methods: The level of HC at the first day of AMI was Me 24.4 [15.7, 28.6] mM. The level of HC at the 14th day was Me 21,4 [15;27]. It was higher than normal values. A 27.5% of patients ($n = 11$) had heterozygous polymorphism (allele GT) in the gene for the enzyme MTGFR, 12.5% of patients ($n = 5$) had homozygous polymorphism (allele TT). The level of HC in the group with allele (TT) was higher than that of other patients at the 1th ($P = 0.028$) and at the 14th days of AMI ($P = 0.001$). The correlation was detected between (TT) allelic variant and the level of HC at the 1th day ($r = 0.534$, $P = 0.001$) and at the 14th day ($r = 0.314$, $P = 0.048$). Long-term results were studed during the period of 1 year after AMI. The end points was: the development of unstable angina, ischemic stroke, re-infarction, recurrent episodes of revascularization, death from coronary heart disease. The correlation was detected between (CT) allelic variant and frequency of repeat stenting in the second half of the observation ($r = 0.453$, $P = 0.003$). The correlation was detected between (TT) allelic variant and frequency of repeat stenting in the first half of the observation ($r = 0.466$, $P = 0.005$). The correlation was detected between (CT) allelic variant and the incidence of unstable angina pectoris in the 2nd half-year of observation ($r = 0.640$, $P = 0.0001$). The correlation was detected between (TT) allelic variant and the development of re-infarction during the first 6 months of observation ($r = 0.466$, $P = 0.002$).

Conclusions: Patients with homozygous polymorphisms MTGFR have higher levels of HC in both acute and subacute stage in AMI, which is an additional risk factor for adverse thrombotic events. Relationship was found between the presence of polymorphism in the MTHFR gene and the incidence of recurrent cardiovascular events. Thus, in patients with allele (CT), there is a high incidence of repeated revascularization, mostly in the 2nd half-year of observation, while having the allele (TT) – in the first half.

PO 419

Allicin inhibits tissue factor expression in thrombin-induced endothelial cells by blocking MAPK and NF-?B

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Background: Inflammation and resultant oxidative stress play great roles in cardiovascular disease. Regulation of inflammatory reactants is an important strategy to improve the outcome of cardiovascular patients. Allicin is the major biologically active component of garlic and manifests hypolipidemic, antiplatelet, and procirculatory effects.
Aims: The objectives of this study were to investigated mechanisms underlying the modulatory effects of Allicin on tissue factor (TF) in endothelial cells.
Methods: HUVECs were treated with or without Allicin for 24 h before exposure to thrombin for 4 h. TF gene and protein expressions were determined by RT-PCR and ELISA. Cell viability was determined by methyl thiazoyltetrazolium (MTT) assay. Phosphorylation of ERK, JNK and p38 were examined by western blotting.
Results: Preincubation of endothelial cells with Allicin dose-dependently inhibited thrombin-induced mRNA and protein expression of TF. Thrombin-induced phosprylation of mitogen-activated protein kinase (MAPK), including ERK, JNK, and P38, were mitigated by Allicin. Activation of NF-κB stimulated by thrombin were also suppressed by Allicin.
Conclusions: Allicin can modulate the responsiveness of endothelial cells to thrombin through inactivation of NF-κB and MAPK pathways, which may explain the ability of Allicin to suppress inflammation in cardiovascular diseases.

PO 420

At equivalent biologic units, commercially available prothrombin complexes are not the same: differential generation of thrombin and factor Xa upon tissue factor mediated activation

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Introduction: Commercially available prothrombin complex concentrates (PCCs) are used in the management of bleeding in patients with both oral anticoagulant associated and deficiency of the synthesis of Vitamin K dependent coagulation factors (II, VII, IX, X) associated bleeding. Currently, several PCCs such as Beriplex, Cofact, Octaplex, Prothromplex, and Profiline are available. An activated PCC preparation (FEIBA) is also available. The purpose of this study was to compare the TF mediated generation of thrombin and factor Xa in various.
Methods: Commercially available PCCs were diluted in saline and 5% human albumin to obtain working solutions of 1.25–10 U/ml. A recombinant thromboplastin preparation (Innovin) was used to trigger protease generation which was measured using chromogenic substrate and fluorogenic substrate based assays.
Results: Each of the PCCs produced varying amounts of factor Xa and thrombin in a concentration dependent fashion. Cofact and Octaplex produced relatively stronger effects. In the fluorometric assays, wide variations in the generation of thrombin were noted among various PCCs. Both Cofact and Octaplex produced much higher amounts of thrombin (> 500 nM/unit), where as Prothromplex and Profiline produced < 100 nM/unit. Beriplex was also weaker and produced 200 nM/unit.
Discussion: These results demonstrate that despite unit equivalence for factor IX, various PCCs are capable of generating different amounts of factor Xa and thrombin on TF activation. The marked differences in the generation of thrombin may be due not only to composition but also to the presence of adjunctive antithrombin and heparin. Each of the different PCCs exhibits its own homeostatic profile and therefore these homeostatic agents are not interchangeable for such an indication. Each agent should be considered individually and pre-clinical and clinical data may be needed to demonstrate their relative efficacy in bleeding complications.

PO 421

Influence of D-1208I gene polymorphism on tissue factor expression in healthy and influenza

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Background: Tissue factor (TF) plays the key role in the development of thrombosis. Influence of D-1208I polymorphism on the function of TF is poorly studied.
Aim: Was to study the effect of D-1208I polymorphism on TF expression by blood monocytes and lung macrophages.
Methods: there were 84 healthy volunteers under observation (42 men and 42 women). The mean age was 37 ± 11 years.
To assess the expression of TF by lung tissue macrophages a material of 20 patients who died of influenza A H1N1 in 2009 was used. The mean age of these patients was 40 ± 14 years (6 men and 14 women). Infection was laboratory confirmed by PCR. The cause of death in these patients were pulmonary embolism (8 people) and respiratory distress syndrome (12 people). All subjects of research were Russian, who was born and resident in the Transbaikalia. Genetic polymorphisms were determined by PCR. To study the TF expression the whole blood samples were cultured during 4 h in presence of LPS and without it and their re-calcification time were measured by impedance coagulometer. The TF expression was estimated by the degree of reduction of coagulation time, expressed as a percentage according to the formula: (t1–t2) × 100%/t1, where: t1 – unstimulated blood clotting time; t2 – stimulated blood clotting time. Lung macrophage TF expression was studied The lung tissue, that was obtained at autopsy from patients who died of complications of influenza A (H1N1) in October-December 2009 in the Transbaikalia, was used as a material. Assessment of TF expression by lung macrophages were determined by immunohistochemistry (TF9-10H10, Santa Cruz Biotechnology, USA). The lung tissue, that was obtained at autopsy from patients who died of complications of influenza A (H1N1) in October-December 2009 in the Transbaikalia, was used as a material. Statistical processing was performed using *t*-test and *U*-test.
Results: It was revealed that the prevalence of TF D-1208I polymorphism among healthy individuals and in patients with lethal influenza A H1N1 did not differ. A 27.4% of the healthy volunteers and 35.0% of patients had DD genotype. A 52.2% and 40.0% of healthy patients – DI genotype. A 21.4% and 25.0% of healthy patients – II genotype. It was established that monocytic TF expression was 179.4 ± 66.2% in a group of volunteers. TF expression of heterozygous was a decreased in comparison with homozygous (198.6 ± 72.9% and 160.1 ± 53.0%, respectively, *t*-test, $P = 0.007$). It was revealed that the TF expression by lung macrophages at D-1208I heterozygous and homozygous patients died from influenza complication differed. The average value of TF expression index was 6 points in the group of heterozygous patients, while in the group of homozygous – 11.1 points (*U*-test, $P = 0.016$).
Conclusion: D-1208I polymorphism heterozygous have shown to reduce the levels of macrophagic and monocytic TF expression, compared to homozygous.

PO 422

Mycophenolate mofetil for treatment of relapsed, refractory thrombotic thrombocytopenic purpura

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Background: Thrombotic thrombocytopenic purpura (TTP) is a rare life-threatening disease. Plasma therapy has dramatically decreased the mortality rate from 90% to less than 10% for TTP patients. In many TTP patients, prolonged Plasma therapy and Immunosuppressive agents (corticosteroids, rituximab, and high-dose intravenous immunoglobulin) is required to achieve remission, however, about one-third of acquired TTP patients still relapse with ADAMTS13 deficiency. Mycophenolate mofetil (MMF), an immunosuppressive agent, has been recently reported to treat for relapsed, refractory TTP patients. The efficacy and safety of MMF is still unknown.
Aims: our study is aim to observe the efficacy and safety of MMF for relapsed, refractory TTP patients.
Methods: We present a case of multiply relapsing TTP which was refractory to many of the treatment modalities mentioned above; and failed a course of rituximab. Then mycophenolate mofetil (100 mg per day) was used with plasma therapy for the patient, the patient were continuing 22 monthly follow-up. ADAMTS13 activity, inhibitor of ADAMTS13, platelet counts and LDH levels were monitored at each visit in conjunction with routine laboratory parameters.
Results: Remission was achieved with mycophenolate mofetil aiming to maintain remission. Seven months later, the patient relapsed after reduction in MMF dose (50 mg per day), platelet counts dropped to 48×10^9/L, A second remission was achieved by plasma therapy combination with MMF (100 mg per day). MMF was continued concomitantly with dose of 100 mg per day. Then the patient was again sustained remission. MMF was very well tolerated in the patient. The patient remains relapse-free survival for 1.5 years. However, it is interesting that ADAMTS13 activity is always less then 5%, and an inhibitor of ADAMTS13 is also detected Whether or not the patient is in remission. And platelet counts was always normal when remission.
Conclusions: Our study suggests that MMF may be a choice for relapsed and refractory TTP patients. Further studies are still needed to confirm the efficacy, while the mechanism of long-term remission for MMF in the patient is remain unclear.

PO 423

Diagnosis of Von Willebrand disease: use of bleeding score

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Background: The bleeding score was proposed, to standardised a collection of the bleeding history and use them in diagnosis of bleeding disorders.
Aims: The aims were to assess the bleeding score, in patients previously diagnosed with Von Willebrand disease (VWD).
Methods: The bleeding symptoms of 37 patients with VWD and 30 subjects without any bleeding disorders (15 women and 15 men) were assessed retrospectively using a standardised bleeding questionnary (Tosseto, 2006).
The bleeding score ranged from a minimum of -3 (no symptoms) to a maximum of 45 (blood transfusion or replacement therapy).
Results: In normal subjects, the mean bleeding score was 0.7 (range 0 to 3).
In 37 patients with VWD, the mean bleeding score was 6.9 (range 3 to 15).
In 15 patients with VWD type 1, the mean bleeding score was 5.8 (range 3 to 9).
In 13 patients with VWD type 2, the mean bleeding score was 5.7 (range 3 to 10).
In 09 patients with VWD type 3, the mean bleeding score was 9.4 (range 6 to 15).
The use of bleeding score greater than 3 as a screening test for VWD has a sensitivity of 81% and specificity of 100%.
Conclusion: The bleeding score is a useful clinical tool for the identification of a possible VWD and evaluated a bleeding tendency to indicate a prophilactic therapy.

PO 424

A simplified assay for von Willebrand factor multimers analysis

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Background: Von Willebrand Disease (vWD) is the most common bleeding disorder, affecting up to 1% of some selected populations. Analysis of von Willebrand Factor (vWF) multimers is essential to classify vWD subtypes. The procedure remains highly specialized, labour-intensive, and inaccessible to most clinical laboratories.
Aims: Our objective was to develop a simple vWF multimers assay for routine analysis, with no compromise on quality.
Methods: Plasmas from patients with type I, II or III vWD and a pool of normal plasmas were used as qualitative internal controls.
We used 1.1% or 1.4% agarose in TRIS HCl pH 8.8, SDS 0.1% for resolving gels and 0.8% agarose in TRIS HCl pH 6.8, SDS 0.1% for staking gels. Gels were sequentially poured into an horizontal DNA electrophoresis unit, and plasma samples in TRIS buffer, EDTA 8 mM, SDS 1.6% urea 6.4 M, pH 6.8 were subjected to electrophoresis at 35V overnight at room temperature, in TRIS 0.05M, Glycine 0.384 M, SDS 0.1%, pH 8.35). The gels were then washed in TRIS buffer Saline containing Tween 20, and proteins were transferred onto nitrocellulose using a semi-dry high-speed transfer apparatus (Trans-Blot® Turbo[TRADEMARK] Bio-Rad) for 10 min at 12V with ready-to-use transfer packs. vWF multimers were revealed directly on nitrocellulose membranes using a rabbit polyclonal anti-human vWF-HRP antibody and 4-chloro-1-naphthol as substrate in TRIS0.05 M, pH 7.4, ethanol 15%.
Results: The assay was completed within 24 h, including overnight electrophoresis. The use of an inexpensive horizontal DNA electrophoresis system was particularly convenient to promptly cast the gels, and load up to 44 plasma samples (within 2 h). Semi-dry high speed transfer with ready-to-use transfer pack and direct staining of the nitrocellulose membrane further shortened the assay procedure and enabled a surprisingly good recovery of high molecular weight vWF multimers (HMW vWF) on the membrane, with a resolving power of up to 20 mers. Consistent intra- and inter-assay reproducibility was obtained, enabling unambiguous detection of HMW vWF deficiency in the plasma of type IIA and IIB VWD patients and normal distribution but reduced concentration of vWF multimers in Type I vWD patients.
Summary/Conclusions: By choosing simple technical options, we developed a rapid, reliable and cost-effective vWF multimers assay easily transposable to clinical laboratories.

PO 425

A de novo type 2A von Willebrand factor mutation (Ser1517Arg)

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Background: De novo heterozygous mutations have been rarely reported in von Willebrand disease (VWD).
Aim: We report here a novel de novo VWF mutation.
Methods: A 3-year old boy was referred for severe bleeding from a tonsil. A severe reduction of FVIII and von Willebrand factor was observed (FVIII:C 15 U/dL; VWF:Ag 15 U/dL; VWF:RCo < 3 U/dL). An abnormal VWF:RCo/VWF:Ag ratio was evident suggesting the diagnosis of type 2. Bleeding promptly stopped after administration of VWF/FVIII concentrate. A few months later a severe post-traumatic epistaxis occurred, again controlled with replacement therapy. The parents were asymptomatic and had clearly normal FVIII and VWF measurements. All exons and intron-exon boundaries of VWF gene were sequenced in the propositus and a novel heterozygous c.4551 C>A mutation was identified predicting a Ser1517Arg mutation in the A2 domain of VWF protein. This change was not present in the parents, although the coinheritance of VWF haplotype was evident. The mutation was subsequently identified also in a single patient from Southern Italy, with significant bleeding history and similar FVIII/VWF levels.
Conclusions: De novo mutations are important to be identified by VWF gene sequencing since the typical autosomal dominant inheritance is not evident in the family and the phenotype is not in keeping with a true recessive disorder. This also is relevant to predict the risk of additional affected offspring in the same family.

PO 426

The first successful treatment of trauma in von Willebrand disease with a specific von Willebrand Factor Concentrate (VWF) in Paysandú, Uruguay – Treatment of fractured unstable lumbar vertebrae

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Background: Achieving hemostasis in von Willebrand Disease (VWD) presents a challenge as not all concentrates used for haemophilia A correct the bleeding defect in these patients. The development and approval for the market of specific VWF concentrates represents an important advance.
Aims: We report a case of a female patient, 16 years old, with Type 1 VWD defined, diagnosed in 2002 following multiple episodes of epistaxis and gingival bleeding. Two brothers had Type 1 VWD, one of whom died in a traffic accident.
Methods: Presentation: Trauma with maximum impact on the lumbar region of the back, following a fall of 1.5 metres, while under the influence of inhaled substances. A 20 × 20 by 10 cm height mass at the back of the lumbar spine was detected. Pain was reported around paravertebral muscle masses. Abdominal and right flank hypogastrium pain was elicited without peritoneal tap. Investigation by CT scan revealed unstable fracture of L1 lumbar vertebra at the right lamina, pedicle and facet joint. There were no hematomas in the abdomen pelvic region, and ultrasound revealed no free intra-abdominal fluid. Investigations: Hemostasis Tests: APTT: 34.6 s, Prothrombin time 11.9 s, fibrinogen 372. A 4 mg/dl. Platelet adhesiveness: 26% (normal range 25–75); FVIII coagulant activity 113% (normal range 60–150%), von Willebrand Factor 30% (normal range 50–150%) von Willebrand antigen 41% (normal range 58–132%). Treatment: A specialist Transfusion Medicine consultation was performed 2 days after admission. Haematological investigations (Table).
Factor therapy: Commercial Factor VIII concentrate (Immunate®, Octapharma) loading dose 1000 IU I/V bolus, followed by commercial von Willebrand Factor concetrate (Wilate®, Octapharma) 900 IU loading dose followed by 450 IU intravenously every 12 h for 6 days.
Results: Bleeding continued until the initiation of substitution therapy, after which the patient was stabile (Table). The hematoma was resolved and plaster immobilization with a thoracolumbar brace was possible, stabilizing the lumbar vertebrae and preventing further damage. All objective clinical and preclinical criteria indicated complete remission after initiation of replacement therapy. Excellent response and tolerance to the treatment was observed.
Conclusion: Use of a specific VWF concentrate allowed treatment and resolution of this complex case. The provision of such products is an essential part of modern care for VWD. The importance of assessing the patient in a comprehensive and interdisciplinary treatment to achieve timely, safe and effective resolution was evident. Rapid communication between the admitting area and the specialist service is essential for optimal and cost-effective care.

PO 427

Severe periodical menorrhagia, corpus luteum rupture, haemoperitoneum with acute abdomen, laparoscopic ovariectomy, WILFACTIN® substitutive therapy in young female with type IIA Von Willebrand disease

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Von Willebrand disease (vWD) is the most common hereditary bleeding disorder affecting both males and females. It arises from quantitative or qualitative defects of von Willebrand factor and causes mucocutaneous and visceral haemorrhages, as well as rarely hemartroses.
The aim of treatment is to correct the dual defect of hemostasis caused by the abnormal/reduced vWF and the concomitant deficiency of Factor VIII. In the females, especially in those in fertile age, the menorrhagia, the severe anaemia and haemoperitoneum remain of big concern. So, the individual treatments is often difficult.
We here report the case of a young female, aged 25 years, HBV, HCV and HIV negative, affected by type IIA of von Willebrand disease; the parents are cousins.
The patient had heavy menorrhagias, treated for long time with plasma products containing vWF, started at the onset of bleeding, tranexamic acid, iron oral therapy, with sufficient control of the bleeding.
In December 2010, she was hospitalized for an excessive bleeding, with severe anaemia (hemoglobin 5 g/dl), due to the rupture of a corpus luteum, subsequent haemoperitoneum with acute abdomen.
Surgical removal of one ovary, RBC packed units ($n = 4$), antifibrinolytic agents and replacement therapy with plasma products containing vWF at the dosage of 40 IU/Kg b.w. every 8 h were carried out.
Then, she was discharged with iron therapy and oral contraceptives. Despite the therapy, she had excessive bleeding during the menstrual cycles, severe iron deficiency anaemia, with a poor quality of life.
Then we decided to try therapy with Wilfactin©, a highly purified plasma vWF concentrate containing very little FVIII, at the dosage of 40 IU/Kg b.w./day for 3 days, administered during menstrual cycle, with a remarkably reduction of this, from 8 to 10 days to 4–5, with a good response to the therapy, without collateral effects and normalization of the values of hemoglobin. The patient had no need to continue iron therapy, with an excellent outcome. Now the patient has normal value of hemoglobin and her quality of life is good.
We conclude that Wilfactin® may be considered for short-term prophylaxis in elective surgery or for long-term secondary prophylaxis in women with dangerous menorrhagia.

PO 428

Management of severe coronary artery disease in a patient with type Vicenza von Willebrand disease

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Background: Von Willebrand disease (VWD) is a congenital bleeding disorder caused by von Willebrand factor (VWF) deficiency or abnormalities. Although high levels of VWF are referred to play an important role in the onset of atherothrombosis, a lifelong hypocoagulable state does not seem to protect VWD patients against atherosclerosis. As previously reported, moreover, long-term prophylaxis with FVIII/VWF could increase the thrombotic risk due to excessive circulating FVIII levels. The literature on cardiovascular disease management in VWD patients is limited and recommendations on the type and duration of antithrombotic therapy for such patients are unavailable.
Case report: A 69-year-old diabetic male with type Vicenza VWD presented with a recent worsening of a known stable angina. Basal blood tests showed factor VIII (FVIII:C) levels of 34% (normal range 60–160%), von Willebrand factor antigen (VWF:Ag) of 17.8% (normal range 60–160%), and von Willebrand factor ristocetin cofactor (VWF:RCo) of 16.6% (normal range 60–130%). He had a past history of several muco-cutaneous bleeding episodes, chemotherapy for a nasal non-Hodgkin lymphoma, a surgically-treated testicular lymphoma, and HCV-related hepatocellular carcinoma treated with transarterial chemoembolization (TACE). He had also undergone coronary artery by-pass surgery 9 years earlier for severe coronary artery disease (CAD).
After admission, coronary angiography via radial artery puncture demonstrated severe and extensive CAD. During procedure unfractionated heparin (1000 IU) was administered intra-catheter. Prophylaxis with FVIII/VWF concentrates (40 IU/kg) was administered before and for 3 days after the endovascular procedure. No endovascular or surgical treatment for CAD was proposed.
Considering the high cardiovascular risk and worsening coronary status, clopidogrel was administered for secondary anti-platelet prophylaxis. Since the patient had never taken antiplatelet drugs before, and considering his lifelong bleeding history, we planned a long-term prophylaxis with VWF concentrates. To avoid excessive FVIII concentrations and the correlated risks of arterial and venous thrombosis, we preferred a highly-purified plasma-derived VWF concentrate (Wilfactin®, LFB). The patient received 60 UI/kg twice weekly to maintain VWF:Ag and VWF:RCo above 25–30% and FVIII below 80–100%. Anti-hemorrhagic prophylaxis, in association with antiplatelet treatment, was given for 7 months. No bleeding or thrombotic complications were observed during a further 7-month follow-up.
Conclusions: The appropriate antithrombotic management of VWD patients presenting with coronary syndromes is still unclear. Our experience suggests that antiplatelet drugs might be used in association with VWF concentrates to reduce the bleeding risk. Administering highly-purified VWF concentrates might be helpful, as anti-hemorrhagic prophylaxis, in type 2 VWD patients at high thrombotic/cardiovascular risk, in order to avoid excessively high concentrations of circulating FVIII.

PO 429

Dental invasive procedures in von willebrand disease (VWD) outpatients treated with high purity VWF/FVIII complex concentrate: experience of a single center

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Background: Dental procedures in Von Willebrand Disease (VWD) patients are associated with a risk of bleeding during and after surgery. Replacement prophylactic therapy, and local measures, reduce the risk of complications in this kind of surgeries. VWF/FVIII concentrate is the treatment of choice in VWD type 3 and type 2b, but is also indicated in other VWD subtypes when there is no response or contraindication to Desmopressin. Aim. To describe the dental procedures performed in a group of VWD outpatients utilizing systemic prophylactic therapy with a high purity VWF/FVIII and local hemostatic control measures.
Methods: During 6 years, in our Dental Department, we performed 23 dental procedures on eight patients (4M, 4F; median age 63 years, range 49–78) affected by VWD: 2 type 1, 3 type 2b, 3 type 3. All patients were treated with a high purity VWF/FVIII concentrate. All the surgeries were performed under local and loco-regional anesthesia. Local hemostasis was ensured applying gelatine packing, fibrin glue, absorbable suture, 15-min compression with tranexamic acid saturated gauze. In the post-operative period patients were treated with antibiotics. Tranexamic acid mouthwashes (three times a day for 3 days) were prescribed; acetaminophen was used as the only pain-relief treatment.
VWD type 1. One patient has undergone 3 roots extractions and one other, 2. They have been treated with high purity VWF/FVIII concentrate according to the subsequent scheme: 1 h before surgery (t_0), 60 IU/Kg; from the 12th to 48th h (t_{12h} and t_{48h}) after surgery, 30 IU/Kg every 12 h; from the 3rd to 7th post-operative day, 30 IU/Kg/day.
VWD type 2b. One patient has undergone 5 roots extractions according to the subsequent scheme: VWF/FVIII 50 IU/Kg t_0; $t_{12\ h}$à$t_{60\ h}$ 25 IU/Kg, every twelve h, then 25 IU/Kg every 24 h, until 7th post-operatory day. One other patient has undergone 1 tooth extraction while treated with VWF/FVIII 50 IU/Kg t_0, t_{24}. The last one has undergone 1 extraction, 1 scaling and root planning, 1 excisional biopsy under the administration of VWF/FVIII 30 IU/Kg t_0; then he continued treatment with tranexamic acid orally, at a dosage of 40 mg/kg/day until 7th post-operatory day. VWD type 3. One patient has undergone 2 wisdom teeth extractions according to the subsequent scheme: VWF/FVIII 50 IU/Kg t_0; $t_{12\ h}$à$t_{48\ h,}$ 30 IU/Kg every twelve h, then tranexamic acid orally, 40 mg/kg/day until 7th post-operatory day. One other patient has undergone 3 roots extractions, 2 dental extractions and 1 wisdom tooth extraction under treatment with VWF/FVIII 60 IU/Kg t_0; $t_{12\ h}$à$t_{48\ h}$ 50 IU/Kg every twelve h, then 30 IU/Kg/day until 7th post-operatory. The last one has undergone 1 scaling and root planning while treated with VWF/FVIII 50 IU/Kg t_0, t_{24}. We didn't observe any hemorrhagic or local abscesses. All patients completed the post-surgery home-treatment.
Conclusions: A tailored prophylactic treatment with high purity VWF/FVIII concentrate ensures a good hemostasis in VWD patients undergoing dental procedures. Multidisciplinary approach allow to manage coagulopathic patients without bleeding complications; home-treatment limits patient discomfort and reduces management cost.

PO 430

Successful percutaneous coronary intervention in a patient with von Willebrand disease

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Background: The prolonged life expectancy in coagulopathic patients has increased the risk of age-related morbidites, such as cardiovascular disease. There are no standard treatment guidelines for coronary artery disease in patients with von Willebrand disease (vWD), especially in the field of coronary intervention. We describe a successful percutaneous coronary intervention (PCI) in a patient with vWD.
Case Report: The patient is a 63 year-old man with type I vWD and several cardiovascular risk factors (obesity, hypertension, dislipidaemia, diabetes mellitus). He was hospitalized for unstable angina and a coronary angiography was planned. He was treated with a factor von Willebrand (vWF) concentrate with low factor VIII (FVIII) content (Wilfactin®, LFB) infused the day before the procedure at the dose of 40 IU/kg and with the same dosage one h prior the angiography. The

procedure was performed by radial venipuncture and evidenced a critical coronary disease whose indication was a surgical revascularization, refused by the patient. Alternatively, he underwent a PCI with implantation of a bare metal stent in the middle portion of the left ascending coronary artery. The angioplasty was performed one h after the infusion of 40 IU/Kg of Wilfactin®. Furthermore, according to the standard practice, the patient received a preloading dose of clopidogrel (300 mg) and during the procedure he was treated with 70 IU/Kg of unfractionated heparin and 250 mg of acetylsalicylic acid. The PCI was uneventful and bleeding complications were not observed. After the procedure he continued the dual antiplatelet therapy (clopidogrel 75 mg/die and aspirin 100 mg/die) for 2 months and in this period he received a prophylactic treatment with Wilfactin® at the dose of 40 IU/kg twice a week, without presenting any haemorragic or thrombotic event. With clopidogrel discontinuation he stopped Wilfactin® prophylaxis, carrying on long-term aspirin treatment (100 mg/die).

Conclusion: Little data are available regarding the management of coronary artery disease in vWD patients. In our case Wilfactin® showed a good efficacy and safety profile in providing an adequate hemostatic cover during the PCI. A vWF concentrate with low FVIII content may be more suitable in vWD patients with cardiovascular risk factors, because it ensures the haemostasis avoiding persistently high FVIII plasma levels, which could represent an additional thromboembolic risk factor.

PO 431

Acquired von Willebrand syndrome secondary to a functional defect of von Willebrand factor

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Background: The acquired von Willebrand syndrome (AVWS) is a bleeding disorder that is frequently unrecognized or is misdiagnosed as von Willebrand disease.

Aims: To describe a case of a patient with Acquired von Willebrand syndrome.

Methods: Case report.

Results: Female patient, who from her 12 years began to have episodes of gingival brushing associated with joint pain in knees and ankles. The PTT was above the reference range and the initial study for coagulation disorders showed deficiency of factor XII associated with positive antinuclear antibodies (1:160 speckled pattern) and C3 low, no other conclusive criteria for systemic lupus erythematosus (SLE) or for antiphospholipid syndrome. One year later she started her menstrual cycles although the bleeding was not abnormal, she presented exacerbation of abdominal pain, diagnosed with dysmenorrhea associated with polycystic ovary syndrome. New studies evidenced the persistence of prolonged PTT, ANAS positive 1:320 homogeneous pattern, decreased complement C4 fraction and deficiency of coagulation factor XII. Four years later she starts to have episodes of metrorrhagia, with iron deficiency anemia motive why she start with drospirenone/ethinyl estradiol regulating the cycles and bleeding. Two years later she consulted the emergency department with acute abdomen, finding hemoperitoneum without a clear cause, new immunological profile revealed ANAS 1:1320 homogeneous pattern, all other tests were negative. She was transfused with fresh frozen plasma presenting allergic reaction. One year later she consults due to metrorrhagia, bruising and right hip pain with functional limitations, hemarthrosis was discarded by MRI. Given the evidence of hemorrhagic manifestations with prolonged PTT is was considered that it was not the clinical behavior of a deficiency of factor XII. Mixing test is performed and PTT was corrected with plasma, indicating deficiency of factor. Paraclinical input is sought: platelet aggregation curve was normal with ADP, collagen, epinephrine and ristocetin, von Willebrand factor 52%, ristocetin cofactor 31.9%, clotting factor VIII 62.8%, clotting factor IX 61%, inhibitors against factor VIII and IX negative. The patient continued with metrorrhagia and spontaneous ecchymosis increase. Further studies were performed that showed a progressive decrease of ristocetin cofactor activity (16%). She was valued by rheumatology, anticromatinm antibodies were negative. Given the findings of bleeding and some symptoms of connective tissue disease diagnosis was proposed as an acquired von Willebrand disease mediated by autoantibodies, the treatment was defined by low doses of prednisone (20 mg/day) associated with hydroxychloroquine 200 mg/day, which results in the disappearance of spontaneous ecchymosis, the remission of musculoskeletal symptoms, normalizing menstruation and the absence so far of new bleeding episodes.

Summary/Conclusion: This case is interesting because it is a prolonged TTP patient suggestive of an acquired bleeding disorder. Initially it raised the possibility of deficiency of factor XII, but the patient always presented bleeding manifestations WITH prolonged PTT which indicated that there was another clotting defect, hemophilia A and B acquired were ruled out and finally it was documented progressive decrease ristocetin cofactor activity, which is an unusual presentation of an acquired von willebrand syndrome.

PO 432

Bleeding score and fibrinolysis after DDAVP in possible type 1 VWD patients

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Background: VWD is rarely associated with spontaneous bleeding, but may result in a possibly severe bleeding after a haemostatic challenge such as trauma, delivery or surgery. Nevertheless, incomplete VWD penetrance and low specificity of the bleeding symptoms make type 1 VWD a diagnostic challenge, especially in the subjects with low VWF (30–50 IU/dl) and mild bleeding symptoms who are currently diagnosed as 'low VWF' or 'possible' type 1 VWD.

DDAVP is the main treatment in most of VWD pts, although the enhanced response of fibrinolytic could add a bleeding risk factor in these pts. In addition of the VWF, DDAVP also produces the release of fibrinolytic activators from the endothelial cells.

Fibrinolytic activity is evaluated by euglobulins lysis time (ELT).

Aims: Our purpose was to evaluate the changes in plasmatic fibrinolytic activity induced by DDAVP infusion, and to correlate these changes with the bleeding score (BSS), in 471 possible type 1 VWD adult pts (358 females, 113 males).

Methods: BSS was calculated. Blood samples were drawn before (B), and after 1 (1 h) and 2 (2 h) the DDAVP infusion (i.v. 0.3 μg/kg). ELT was done in each sample.

Pts were grouped according to gender and the BSS being < 5 and ≥ 5 for females, and < 3 and ≥ 3 for males. Difference (DIF) between ELT-1 h and 2 h vs. before was calculated.

Results: Females: BSS < 5 ($n = 276$): ELT-B = 190.6 ± 91.6 min; 1 h =73.3 ± 34.8 min; DIF = 3.08 ± 1.6 min; 2 h = 93.1 ± 46.4 min; DIF = 2.3 ± 0.9.

BSS ≥ 5 ($n = 84$): ELT-B = 177.7 ± 65.7 min ($P = 0.23$); 1 h = 63.9 ± 28.5 min ($P = 0.025$); DIF = 3.5 ± 1.5 min ($P = 0.03$); 2 h = 85.1 ± 29.6 min ($P = 0.137$); DIF = 2.3 ± 0.7 ($P = 1$).

Males: BSS < 3 ($n = 95$): ELT-B = 177.1 ± 72.5 min; 1 h = 78.6 ± 44.9 min; DIF = 2.7 ± 1.3 min; 2 h = 89.4 ± 46.6 min; DIF = 2.3 ± 1.4.

BSS ≥ 3 ($n = 18$): ELT-B = 168.9 ± 67.5 min ($P = 0.661$); 1 h = 67.5 ± 39.1 min ($P = 0.329$); DIF = 3.1 ± 1.9 min ($P = 0.238$); 2 h = 89.3 ± 43.2 min (0.993); DIF = 2.0 ± 0.8 ($P = 0.38$).

Conclusions: In females with a BSS ≥ 5 there was a significantly higher fibrinolytic activity in the first h post DDAVP. In contrast, there were no differences in males. Given these results, it is possible to consider that some enhanced fibrinolytic activity associated with pathophysiological stress situations, could be influencing the severity of the clinical

profile observed in possible type 1 VWD patients. Gender-related differences in endothelial response to stimuli such as stress or drugs (DDAVP) could explain both the differences found in the fibrinolytic activity, and the clinical expression of VWD in women.

PO 433

Changes in von Willebrand parameters during the menstrual Cycle

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Introduction: Von Willebrand disease is most common bleeding disorder with a prevalence of 1–2% of the population. Nevertheless to diagnose a von Willebrand Syndrom Typ 1 is still a challenge. In a newer publication (1) 30 studies about the haemostatic variables during the menstrual cycle were compared. Eleven studies were focused on the von Willebrand parameters but only in one study they observed these parameters in patients with von Willebrand disease. We like to investigate if there are cyclic variations in women with menorrhagia which can lead to a diagnosis of a coagulation disorder.
Samples and Methods: We conducted a laboratory workup in 94 women sent to our lab for menorrhagia. The following tests were conducted: Blood count, VWF:RCo, VWF:Ag, VWF:CB, Fibrinogen (Clauss), activities of FII, FV, FVII, FVIII (clotting and chromogenic), FIX, FX, FXI, FXII, FXIII during the menstrual cycle on predefined time points (day 1–6, day 7–11, day 12–18, day 19–23, day 24–28).
Results: In 47 (50%) patients a von Willebrand disease could be detected, 41% hat other coagulation disorders like p. e. factor-VII-deficieny and factor-XIII-deficiency. In 8.5% no coagulation disorder could be found.
In patients with von Willebrand disease we found cyclic variations especially in the VWF:Ag ($P = 0.026$). They showed the lowest level during the ovulation. The same was found for VWF:RCo and F VIII but without significance.
Conclusion: There are cyclic variations in von Willebrand parameters. To investigate women on predefined time points during the menstrual cycle can be useful to diagnose a von Willebrand disease particularly in mild cases in which no other suspicious bleeding symptoms exists.
Literature: Knol H.M., Kemperman R.F.J., Kluin-Nelemans C., Mulder A.B., Meijer K., Haemostatic variables during normal menstrual cycle, Thrombosis and Haemostasis 107.1/2012.

PO 434

Feno- and genotyping von Willebrand disease type 2A patient because of spontaneous tonsilliar haemorrhage

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Background: A 14-year-old female patient was admitted to local haemophilia treatment center due to repeated bleeding from the tonsils. Spontaneous tonsillar haemorrhage could not have been controlled by increasing dose of von Willebrand factor (VWF) – FVIII concentrate.
Aim: In order to precise diagnosis and treatment, the patient has been transferred to our hospital.
Methods: Platelet function testing: Bleeding time, PFA-100; aggregation; VWF level and activity measurement using VWF:Ag, VWF:RCo, VWF:CB assays, VWF multimer analysis by SDS-agarose electrophoresis; genetic analysis by direct sequencing of exon 28 (primers were designed at the basis of Penas et al. 2005).
Results: Diagnosis was confirmed in our laboratory as type 2A VW disease (VWD): PFA-100 closure times were > 300s, VWF:Ag = 18–35%, VWF:RCo = 13%, VWF:CB = 3–15%, FVIII = 33%, RIPA test revealed no aggregation for 1.2 mg/mL, nor for 0.6 mg/mL ristocetin and lack of high molecular weight multimers (HMW) was shown by SDS-agarose electrophoresis. DDAVP increased the amount of the secreted VWF; still no HMW multimers were found. Platelet suspension did not result in any change in bleeding tendency. HMW multimers appeared in patient plasma after intravenous administration of FVIII concentrate and platelet count increased from 316 to 336; however, this treatment did not decrease PFA-100 closure time, or bleeding time. Detailed platelet aggregation and secretion analysis revealed a 36; 39 and 44% decrease in ATP secretion elicited by ADP, adrenalin and collagen, respectively. Intravenous test dose of VWF-FVIII concentrate (2000 IU), platelets (4 U) and rFVIIa (90 μg/kgbw) resulted in a decrease in bleeding time from 14.5 to 9.0 min. Thus, the patient was given a combination of the above factors what was accompanied by the stop of tonsillar bleeding and afterwards tonsillectomy was performed without any complication. Molecular genetic analysis revealed mutation type 2A in exon 28; nucleotide change: 4628C>T, amino acid substitution: S1543F. Two more siblings have the same mutation, with the same bleeding disorder. A 3692 A>G polymorphism was detected in the three children of the family with the VWD and in the one healthy sibling too.
Summary/Conclusion: This report suggests that in surgeries like tonsillectomy, prophylactically administered rFVIIa together with VWF-FVIII and platelet supplementation seems effective to avoid bleeding in type 2A VWD patient with mild secretion defect. We provide three further cases with 4628C>T mutation to the two listed in the ISTH-SSC VWFdb and four more to the 3692 A>G polymorphism data.

PO 435

Treatment of a high thrombosis risk von Willebrand disease type 3 patient with high-purity von Willebrand factor during last trimester, cesarean delivery and post partum – a Case Report

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Background: Patients with von Willebrand disease (VWD) type 3 may have increased risk of venous thrombosis during surgery while using von Willebrand factor (VWF)/Factor VIII (FVIII) replacement therapy due to an elevated concentration of factor VIII. Patients with previous venous thromboembolic events may benefit from high-purity VWF substitution treatment containing very low levels of FVIII. This case report describes the treatment and clinical features of a high thrombosis risk VWD type 3 patient receiving replacement therapy with high-purity VWF during last trimester, cesarean delivery and puerperium of her first child. To enable rapid adjustments of VWF activity levels during the cesarean procedure, an approach with a loading dose followed by continuous infusions (CI) was selected.
Case presentation: A VWD type 3 patient with pronounced bleeding tendency and previous venous thrombosis after dental surgery while using a FVIII containing VWF product was given prophylactic treatment with a high-purity VWF through the last trimester, cesarean delivery and puerperium. One bolus infusion (4000 IU) was administered before start of surgery, followed by CI (1500–2000 IU/24 h) during delivery until 5 days post partum. Prophylactic therapy of 1000 IU daily as bolus infusions was started 4 months before planned delivery and was continued after the end of the CI. Tranexamic acid 1 g × 3 was started at day of delivery and continued for 15 days. Low molecular heparin (Dalteparin 2500 IE × 1 s) was given from day 1–5.
Before start of surgery VWF antigen (VWF:Ag) was 58%, VWF activity (VWF:RCo) was 24% and FVIII activity (FVIII:C) was 81%. At start of surgery VWF:Ag was 211%, VWF:RCo was 77% and FVIII:

C was 79%. During the whole procedure and until 5 days post partum, VWF:RCo was kept > 50%. FVIII:C increased to a maximum level of 139% at day 3 post partum, and gradually decreased to 90% on day 7 post partum. A good haemostasis was achieved using the substitution therapy, and no signs of thrombosis were observed.
Conclusion: Treatment with high-purity VWF during last trimester, cesarean delivery and puerperium successfully controlled any bleeds in the patient. There was no indication of any thromboembolic events.

PO 436

Automated ristocetin cofactor assay on STA-R®

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Background: von Willebrand disease (VWD) is the most inherited bleeding disorder, caused by either a quantitative or qualitative deficiency of von Willebrand factor protein (VWF). The ristocetin cofactor assay (VWF:RCo) is mandatory for the diagnosis and the monitoring of patients with VWD. Quantitative analysis of VWF:RCo in human plasma can be determined by the agglutination on an aggregometer of formalin-fixed platelets via the GPIb-V-IX receptor with VWF patient plasma and ristocetin. Since this method is time consuming, requests an experimented operator and is poorly reproducible (within and between-assay), automation of VWF:RCo assay can be helpful. Moreover, it tends to improve these drawbacks.
Aim: Automation of VWF:RCo assay on STA-R® (Diagnostica STAGO, Asnières, France) and its evaluation in VWD diagnosis.
Methods: The ABP Ristocetin co-factor assay kit (American Biochemical & Pharmaceutical Ltd., Epsom, UK) was automated using immunoturbidimetric method at 540 nm. The concentrations of platelets and ristocetin were optimized to improve the assay sensitivity at low level. Moreover the software was changed to discriminate the absence and presence of agglutination using kinetic curves. The reconstitution of ABP platelets was modified. Results of normal and VWD plasmas with automated assay were compared to those obtained with the reference aggregometric method.
Results: Order 3 polynomial regression was used for calibration with a coefficient of correlation > 0.950 and a range from 0.10 to 0.60 IU/mL (from 0.10 to 1.80 IU/mL with redilution). Reproducibility within and between-assay was improved through automation on STA-R®: 5% and 8% CV respectively, threshold 0.27 IU/mL. The ratio calculation (VWF:RCo/VWF Ag) using automated assay allowed VWD typing in agreement with the reference method.
Conclusion: The automated VWF:RCo assay using ABP-RCof kit showed good performances and offers a greater sensitivity at low levels compared to Chronolog aggregometric method. It is easy to improve the VWF:RCo measurement in clinical laboratory.

PO 437

Case study: rare laboratory presentation of type IIB von Willebrands disease

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Background: An adult female patient was referred to her local haematology clinic requiring a surgical procedure with a historical definitive diagnosis of von Willebrands disease (VWD) possibly Type IIB. Original investigations had been performed overseas and clinical documentation detailing results and diagnosis were unavailable. She had a bleeding phenotype going back to early childhood associated with nose bleeds, post trauma and surgical procedures on three occasions. At the last of these procedures she was treated with DDAVP and Haemate P. Since then the diagnosis of VWD has been questioned.
Aims: To perform diagnostic investigations to clarify the presence or absence of a congenital bleeding disorder in this female patient.
Methods: Samples were collected for initial diagnostic laboratory investigations for a suspected congenital bleeding disorder. Analysis performed included prothrombin time (PT), activated partial thromboplastin time (APTT), fibrinogen and platelet function screening (PFA), factors VIIIC (FVIIIC), XI and VII activity, von Willebrand factor (VWF) activity (VWF:RCo), VWF antigen (VWF:Ag) and collagen binding assay (VWF:CB), full blood count (FBC) and liver function tests (LFT's). Further investigations were subsequently performed on freshly collected samples including repeat VWD screening, VII activity, ristocetin induced platelet agglutination (RIPA) and VWF and VII genetics studies.
Results: There was no evidence to support a diagnosis of VWD from initial investigations. PT = 15.1 s (s) and APTT = 39.2 s which were both mildly prolonged with a normal fibrinogen (2.6 g/L). Factor XI = 59.1 IU/dL, FVIIIC = 97.8 IU/dL, VWF:Ag = 100.2 IU/dL, VWF:RCo = 90.7 IU/dL and VWF:CB = 117.0 IU/dL were all within normal range. PFA screening was normal with closure time COLL/EPI = 99s and COLL/ADP = 72s. Factor VII was measured due to prolonged PT and found reduced at 37.7 IU/dL. FBC indices showed evidence of mild iron deficiency and LFT's were normal. These findings raised the question of possible vitamin K deficiency. Investigations were repeated on fresh citrate samples with consistent results, FVIIIC = 88.8 IU/dL, VWF:Ag = 96.1 IU/dL, VWF:RCo = 92.9 IU/dL and FVII = 40.3 IU/dL. RIPA performed at ristocetin concentrations of 1.25 and 0.5 mg/ml demonstrated a hyper-agglutination response to low dose ristocetin. Genetic studies identified a c.3797C>T mutation on exon 28 VWF gene which replaces proline with leucine and is associated with VWD type IIB and c.1304G>A mutation on exon 9 factor VII gene which is associated with mild FVII deficiency.
Summary/Conclusions: A comprehensive repertoire of specific laboratory diagnostic assays routinely performed to identify VWD unexpectedly failed to detect the VWF abnormality in this patient. This case highlights the importance of the clinical history in considering and identifying patients were further investigation may be warranted to elucidate a rare bleeding disorder when an initial extensive diagnostic assessment is normal. It illustrates the pivotal role of platelet and genetics studies which are not always readily available in the confirmation of type IIB VWD diagnosis in this patient.

PO 438

Von Willebrand disease a common inherited bleeding disorder after haemophilia A in Pakistan

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Background: Von Willebrand disease is one of the common autosomal recessive inherited bleeding disorders globally. It may be due to quantitative or qualitative defect of Von Willebrand factor secondary to mutation in Von Willebrand factor gene located on chromosome 12. The current study was carried out in patients with a history of spontaneous bleeding with primary objective to determine the frequency of hemophilia A, B and Von Willebrand disease in patients with bleeding disorders and with secondary objective to categorize Von Willebrand disease into various its types.
Material and Method: This study was carried out from January 2010 to December 2012 in NIBD (Karachi), Children Hospital & Chughtai Lab (Lahore), PAEC (Islamabad) and Hayatabad Medical Complex (Peshawar) after approval of institutional review board. Patients with

history of spontaneous bleeding were selected, blood samples were collected and tested for Platelets counts, PT, APTT, Bleeding Time, Factor VIII, Factor IX, Von Willebrand factor & Ristocetin cofactor after written consent from the patients or relatives..

Results: A total of 470 patients were evaluated with inherited bleeding problems, among them 165 (35.1%) had Haemophilia 'A', 28 (06%) Haemophilia 'B' & 114 (24.3%) were diagnosed as Von Willebrand disease. There were 63 (55.26%) males & 51 (44.74%) females with the mean age of 10.32 ± 7.81 years. Out of 114 patients of VWD, 5 (4.4%) were categorized as Type 1 (with mean APTT 51.64 ± 25.62 s, F-VIII 27.4 ± 9.44%, VWF 23.2 ± 7.46% & Ricof 14.73 ± 19.39%), 3 (2.6%) as Type 2 (with mean APTT of 37.36 ± 5.35 s, F-VIII 62.66 ± 13.01%, VWF 37.66 ± 4.5% & Ricof 20.3 ± 13.62%) & 106 (92.9%) patients were diagnosed as Type-3 (with mean APTT 61.51 ± 8.63 s, F-VIII 3.99 ± 3.14%, VWF 3.48 ± 5.56% & Ricof 2.89 ± 3.39%). The patients were categorized into various types of VWD according to Nichols WL et al., 2008.

Conclusion: Our study has found VWD as 2nd most common inherited bleeding disorder in Pakistan. For accurate & precise diagnosis not only well equipped laboratory is required but there is also a need of awareness campaign among the treating physicians & diagnostic workshops should be arranged for healthcare professionals early diagnosis and management of these patients.

PO 439

Update on laboratory findings in a prospective cohort of childbearing women with bleeding history

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Background: Menstruation and childbirth affect excessively women with bleeding disorders. Previously (ISTH Congress 2011), we described that the most frequent disorder in women with bleeding complaints was low von Willebrand factor level.

Aims: To investigate the prevalence of hemostatic and platelet functional disorders we studied a prospective cohort of childbearing women with bleeding complaints.

Patients and Methods: Three-hundred women (mean age 30 years, range 10–50, 54% blood group O) were included. Gynecological and obstetric history (menorrhage defined by PBAC), as well as other bleeding symptoms were registered. Bleeding was defined as major if it was associated with either a decrease in the hemoglobin level of at least 2 g/dL or the need for transfusion of 2 or more units of red cells or hospitalization or surgical procedure. Laboratory parameters: platelet count, bleeding time (BT), PT, aPTT, fibrinogen, factor XIII, euglobulin lysis time, FVIII:C, VWF:Ag, VWF:RCo. Platelet aggregation and ATP release were done as platelet functional studies.

Results: Sixty-nine percent of women had moderate bleeding that required medical intervention, and 61 patients (20.3%) had a major bleeding. The frequency of symptoms was: 87% muco-cutaneous; 68% menorrhage; 5% hemorrhagic ovarian follicle; 51/118 bleeding after delivery. The diagnosis were: 16 mild thrombocytopenia; possible VWD type 1: 37 (2 associated to storage pool disease-SPD); VWD type 1: 26; VWD type 2: 9; Normandy phenotype: 8; Abnormal platelet function: 15; Immune Thrombocytopenia: 2; abnormal ELT: 7; Factor deficiencies: 7.

Discussion: In 300 consecutive women at childbearing age with bleeding symptoms, 41.7% had an hemostatic and/or platelet disorder. VWF abnormalities (62.9%) were the most frequent findings. Platelet function, factor levels and ELT should be studied as well in every patient.

PO 440

De novo mutation in von willebrand disease type III in two siblings of surinamese origin

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Introduction: Von Willebrand Disease (vWD) is a bleeding disorder caused by inherited defects in the concentration, structure or function of Von Willebrand Factor (vWF). VWD is an under diagnosed disease in Suriname, a country with approximately 500,000 residents. The prevalence of vWD is 1 in every 100 cases but only 1 of 10,000 cases has clinical significance. We recently started a systematic analysis to identify the incidences of bleeding disorders in Suriname. So far we have identified only one family, which we suspect is affected by vWD. Two female members showed to have an increased bleeding phenotype, abnormal Ivy bleeding time and prolonged APTT. PT and fibrinogen levels were normal. The screening tests of the mother were normal. No other family members were included.

Methods: vWF confirmation assays and vWF genotyping were performed to confirm the diagnosis in both siblings and their mother.

Results: Both sisters had factor VIII activity levels of 1%. VWF ristocetin cofactor activity (vWRiCoF) levels were below the cut off levels (< 5%) and vWF antigen (vWF: Ag) levels 1–2%. The mother had 45% factor VIII activity, 38% vWRiCoF and 44% of vWF: Ag levels. Genotypic analysis showed that mother is heterozygous for a small deletion in exon 32 (c.551delG) of the vWF gene resulting in a frame shift and premature stop. The two sisters were carrying the mothers small deletion as well as a nonsense mutation in exon 5 (c.477C>A; p.Cys159X). Both mutations have not been described in vWD databases.

Conclusion: These results show that the two sisters have vWD type 3 due to a compound heterozygous vWF genotype. Their mother is a heterozygous carrier of type 3 vWD. Clinically she will be categorized as type I vWD, but with an autosomal recessive inheritance pattern and not autosomal dominant as seen for other vWD type I patients.

PO 441

Clinical presentation and laboratory findings of Von Willebrand Disease in a tertiary center in Saudi Arabia

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Background: Von Willebrand Disease (VWD) is the most common inherited bleeding disorder affecting up to 1% of the population. It is characterized by mutations that lead to an impairment in the synthesis or function of Von Willebrand factor (VWF).

Aims: To identify the incidence of VWD in tested samples in our institute.

Methods: We conducted a retrospective review of all investigated samples for VWD antigen and activity from 2006 to 2011. Out of 100 patients only 60 patients had complete data and were analyzed. There were 28% males & 71.6% females with mean age 20 years range (6–47 year). Information about the following laboratory values were collected including, PT, PTT, platelet count, PFA 100, factor VIII activity, VWF:RCo, VWF:CB, VWF:Ag, & blood group. Clinical data for bleeding were collected and analyzed.

Results: Out of the 60 patients, 15 (27.7%) were having mucous membrane bleed, 14 patients (25.9%) were having ecchymosis & mucus membrane bleed, 9 patients (16.7%) were having only ecchymosis, 5 patients (9.26%) were having genitourinary bleed, 1 patient (1.85%) was having GIT bleed & 10 patients (18.5%) were having other type of bleeds not included in the data collection.

The nature of bleeding was spontaneous in 28 patients (60.8%).it was spontaneous & post trauma in 13 patients (28.3%), post surgery in 3 patients (6.53%), spontaneous & post surgery in 1 patient (2.17%) & post trauma in 1 patient (2.17%),26 patients (48.1%) were having

positive family history, 17 patients (31.5%) were having negative family history and in 11 patients (20.4%) family history was not available. PT & PTT were found normal in 57 (95%) of patients & prolonged in 3 patients (5%) Platelet count was normal in 46 patients (77.9%), high in 12 patients (20.3%), & low in 1 patient (1.69%). PFA was found to be abnormal in 36 patients (78.2%) & normal in 10 patients (21.7%). According to laboratory results and after correction for ABO blood group we hah classified the patients to, 29 (48.3%) were having type I VWD, 24 (40%) were having type III, 4 patients were classified as type II A (6.7%),2 patients (3.3%) having type IIB & 1 patient (1.67%) has type IIN.

Conclusion: Majority 48% of our patients were type 1 and type 3 were 40%.The clinical presentation varies depend on the type of VWD. PFA was found to be the screening test of choice. The high incidence of type III likely to be due to referral bias and probably due to racial difference.

PO 442

Operative management and outcomes in patients with von willebrand disease

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Background: Surgery in patients with von Willebrand Disease can present a range of challenges to physicians.

Aims: To determine the perioperative management and outcome of patients with von-Willebrand disease during surgical procedures.

Methods: Data pertaining to surgeries from 2003 to 2012 at our center were retrospectively analysed. All operations, except one appendectomy, were elective and a plan for management of hemostasis was prepared for each patient. This study approved by medical ethic committee.

Results: During this period 31 surgeries were performed in 22 patients with vWD (6 with type 1, 4 with type 2 and 12 with type 3). The surgeries were divided three cathegories: major, minor and dental procedures. Ten major, 12 minor and 9 dental procedures were performed. Major surgeries were 2 orthopedical (resection of radius head, bilateral hip prothesis), 2 urological (both were hypospadias surgeries), 1 urogynecological surgeries (hysterectomy and transobrurator tape), 2 seasons sex change operation (including bilateral orchiectomy, penectomy, vulva/vaginal construction, vaginoplasty, bilateral breast implantation, partial resection of the thyroid cartilage, rhinoplasty), 1 paratyroidectomy, 1 appendectomy and 1 adeno-tonsillectomy. Minor procedure were mostly orthopedical (5 radioactive synovectomy, 1 knee cast and 1 cyst excision from elbow), others were 3 circumcisions, 1 endoscopy and 1 testis biopsy. The patients median age was 21 (4–47), 14 males and 8 females. All patients were treated with intravenous bolus factor replacement therapy pre- and post-operatively and antifibrinolytics. DDAVP were used only half of the type 2 patients. No severe life threating bleeding and thromboembolic event occured in any of the patients. Only 2 patients had mild bleeding after surgery (hysterectomy and hypospadias surgery) and those 2 paitenst were consanguineous. Factor replacement was continued a for 3 months in one patient who underwent bilateral hip prothesis, no bleeding was seen.

Conclusion: Establishing haemostasis for surgical procedures in patients with inherited bleeding disorders is challenging. Providers are often hesitant to undertake surgeries in patients with bleeding disorders out of fear of bleeding complications. However our results show a good outcome in surgery of patients with vWD with a good hemostasis plan and adequate factor replacement therapy. Bleeding remains a problem in a subset of patients and requires ongoing haematological involvement and oversight.

PO 443

Resistance to Anti-platelet therapy in Saudi patients with coronary heart disease

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Background: It has repeatedly shown that in a significant number of patients on anti-platelet therapy, no therapeutic benefit could be gained whether by the laboratory detection of platelet function or the prevention of further cardiorovascular events, so-called *resistance to anti-platelet therapy*. Extensive coverage in the literature on *resistance to anti-platelet therapy*, comes predominantly from developed countries and very scanty information, if any, comes from developing countries.

Aims: To document the prevalence of *aspirin* and *clopidogrel* resistance in Saudi patient with coronary heart disease and to probe the possible mechanism(s) responsible for aspirin and *clopidogrel* resistance.

Materials and Methods: A 238 patients with CHD were enrolled from the outpatient clinic and wards of King Khalid University Hospital Riyadh.

The following platelet function tests were undertaken:

Agonist-induced platelet aggregation: was undertaken in platelet rich plasma in response to arachidonic acid (as a test of aspirin response) and ADP (to test for clopidogrel response).

PFA100 Closure time using both the collagen/epinephrine and the collagen/ADP cartridges.

Results: The prevalence of resistance to *aspirin* (as reflected by the residual response to arachidonic acid-induce platelet aggregation) was detected in 12.6% (30 out of 238 patients). As for resistance to *clopidogrel* (as reflected specifically, by the residual response to ADP-induce platelet aggregation) it was detected in 45 out of 179 (25.1%) patients.

PFA100 closure times: Detection of closure times within the limits of the normal (Laboratory Reference) range was taken to indicate lack of response (or resistance) to anti-platelet therapy. Non-responders were 31 out of 101 patients (30.7%) as detected by the collagen/epinephrine cartridge closure time and in 33% (35 out of 106) patients using the collagen/ADP closure times.

There was no significant effect of diabetes mellitus, smoking, dyslipedidaemia, or consumption of non-steroidal anti-inflammatory drugs (NSAIDs) and the detection of resistance to antiplatelet therapy.

All Patients were followed up for no less than 6 months for outcome, recurrence of angina or MI, re-occlusion of stents or death. A total of 38 episodes were recorded and with significant difference between patients with or without resistance to antiplatelet therapy.

Conclusion: The prevalence to antiplatelet therapy among Saudi CHD patients is comparable with similar figures from most published studies. We feel strongly for the routine use of laboratory testing of platelet function at least as a pharmacological evidence to support the efficacy of administration of antiplatelet drugs.

PO 444

Frequencies of CYP2C19 in mexican-mestizo patients with coronary or cerebral atherosclerosis, and their association with high on-treatment platelet reactivity to adenosine diphosphate

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Background: There exists a wide variability of individual response to clopidogrel, dependent on CYP2C19 polymorphisms as well as intrinsic and extrinsic platelet factors. Clopidogrel resistance has been related to an adverse evolution in patients with coronary artery disease.

Aims: Obtain the prevalence of CYP2C19 polymorphisms in Mexican mestizos patients and its relationship with clopidogrel platelet response.

Methods: One hundred and sixty cardiovascular patients and 11 stroke patients were analyzed for CYP2C19 alleles *1, *2, *3 and *17, that were determined by allelic discrimination assay with Taqman probes (Applied Biosystems) and TaqMan GT Master Mix (Applied Biosystems) in a 7900HT Fast Real-Time PCR System thermal cycler (Applied Biosystems). Of them, 83 were tested for clopidogrel resistance by 10 microM ADP-induced platelet aggregation according to North American Consensus Guidelines for medical laboratories; high-on-treatment residual platelet reactivity bas when maximal platelet aggregation > 60%. CYP2C19 polymorphism was expressed as ultrarapid-, normal-, rapid-, normal/intermediate, intermediate and poor metabolizers.

Results: Allelic frequencies were 9.6%, 0% and 11.7% for CYP2C9*2, *3 and *17 respectively. Genotypes were expressed as normal (CYP2C19 *1/*1), intermediate (CYP2C19 *1/*2), normal/intermediate (CYP2C19 *2/*17), rapid (CYP2C19 *1/*17), ultrarapid (CYP2C19 *17/*17) or poor metabolizer (CYP2C19 *2/*2). Frequencies of CYP2C19 genotypes were: CYP2C19*1/*1: 62.6%; CYP2C19*1/*17: 19.9%; CYP2C19*1/*2: 12.3%; CYP2C19*2/*17: 2.3%; CYP2C19*2/*2: 2.3% and CYP2C19*17/*17: 0.6%. Of 83 patients with platelet aggregation, 54.2% were responsive and 45.8% were resistant to clopidogrel. Genotypes in clopidogrel resistant patients were: 46.8% for CYP2C19*1/*1, 18.8% for CYP2C19*1/*17, 66.6% for CYP2C19*1/*2, 0% for CYP2C19*17/*17, 66.6% for CYP2C19*2/*17 and 75% for CYP2C19*2/*2. In addition, patients were divided into two groups: Group A (n = 64; 77.1%): normal, rapid and ultrarapid metabolizers, and Group B (n = 19; 22.9%): intermediate, normal/intermediate and poor metabolizers. Responsiveness vs. resistance to clopidogrel was 60.9% vs. 39.1% and 31.6% vs. 68.4% respectively. Clopidogrel resistance was more associated with unfavorable genotypes for metabolism (CYP2C19 *1/*2, *2/*17 and *2/*2) than with favorable genotypes (CYP2C19 *1/*1, *1/*17 and *17/*17), OR = 3.38 (CI 95%: 1.13–10.05). Maximal platelet aggregation in each genotype group, expressed in median and quartiles (p25-p75) was: CYP2C19*1/*1: 56.5% (27.3–72.1%); CYP2C19*1/*17: 49.1% (44.2–58.4%): CYP2C19*17/*17: 38.0%; CYP2C19*1/*2: 62.9% (52.8–74.9%); CYP2C19*2/*2: 83.4% (64.1–90.2%); CYP2C19*2/*17: 73.2% (43.1–84.9%).

Conclusion: Prevalence of CYP2C19 in Mexican mestizos patients is similar to other populations. Clopidogrel resistance is a complex *in vitro* phenomenon that is not explained by CYP2C19 polymorphisms alone, like inflammation mediators or acute phase proteins that were not studied in these patients.

PO 445

Comparative effectiveness of the verifynow P2Y12 Test and light transmittance aggregometry for detecting the antiplatelet effect of clopidogrel

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Background: The VerifyNow P2Y12 Test is a rapid, point-of-care platelet function test that has been extensively validated as a tool for measuring the antiplatelet effect of P2Y12 receptor inhibitors. The VN P2Y12 Test reports results as P2Y12 Reaction Units (PRU) and device-reported percent inhibition of platelet reactivity (%I), based on using thrombin receptor-induced platelet aggregation as a substitute for a baseline, P2Y12 inhibitor naïve PRU result. The PRU result is highly specific for P2Y12 receptor blockade due to the effect of a P2Y12 inhibitor, and is more specific than light transmittance aggregometry (LTA) due to the presence of PGE1 in the assay, which minimizes the effect of the platelet P2Y1 receptor. The present analysis evaluates the comparative effectiveness of the VN P2Y12 PRU and%I results and LTA (%AGG) for the detection of a P2Y12 inhibitor effect, measured as reduced platelet reactivity to ADP. The sensitivity of detecting the antiplatelet effect of a P2Y12 inhibitor can be affected by 1) the time since the last dose, 2) the potency of the P2Y12 inhibitor therapy, and 3) inter-individual variability in the response to the drug. Factors such as genetics, concomitant disease, antecedent medication, and compliance can all influence the individual response to antiplatelet therapy. The ability to specifically detect the antiplatelet effect of P2Y12 inhibitors is important whenever the physician wishes to identify the presence of an antiplatelet effect in their assessment of the patient.

Aims: The objectives for this study were to: (1) compare the discriminatory ability of each method for detecting the P2Y12 inhibitor effect, and (2) determine the optimal cutoff for each test from ROC analysis.

Methods: Participants eligible for the study had to (1) have a clinical indication to receive a P2Y12 inhibitor, (2) be taking aspirin at least 2 days prior to enrollment, and (3) have at least two risk factors for developing vascular disease: family history of vascular disease; sedentary lifestyle; diabetes mellitus; hypertension; morbid obesity; known history of hypercholesterolemia; postmenopausal women; and smoking. VN P2Y12 and LTA measurements were performed from blood samples collected prior to clopidogrel ingestion and either 24 h after ingestion of ≥ 300 mg clopidogrel loading dose or 7 days after starting a 75 mg/day clopidogrel maintenance. ROC curve analysis, sensitivity and specificity calculations were based on the ability to correctly identify the presence of a P2Y12 inhibitor.

Results: The area under the ROC curve for VN P2Y12 PRU and%I results was significantly greater than%AGG (0.95 vs. 0.90, P = 0.0067; 0.98 vs. 0.90, P < 0.0001 respectively). The optimal cutoff for detecting the presence of a P2Y12 inhibitor was VN P2Y12 Test PRU < 208,%I > 18%, and%AGG < 62%. PRU and%I had greater sensitivity and specificity than%AGG at these cutoffs.

Summary/Conclusion: The VN P2Y12 Test is superior to LTA for detecting the presence of a P2Y12 inhibitor, with significantly greater specificity. VN P2Y12 is suitable for use in clinical settings where it is necessary to identify a measurable effect of a platelet P2Y12 inhibitor.

PO 446

An optimal approach to selecting the appropriate cutoff for platelet function tests

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Background: Various platelet function tests (PFT) have been described for their ability to identify patients at increased risk for future cardiovascular events. The association between on-treatment platelet reactivity and incidence of cardiovascular events is largely attributed to the

level of pharmacodynamic effect of the antiplatelet medication. The optimal cutoff for describing the prognostic utility of PFT has been frequently determined through ROC analysis and selection of the cutoff with the highest J statistic. This approach has resulted in variability in the reported 'optimal' cutoff.

Aims: The objective of this study was to evaluate the variability in 'optimal' cutoff selection based on ROC curve analysis of a prognostic evaluation (Px) dataset compared to a diagnostic evaluation (Dx) dataset.

Methods: A dataset comprised of naïve and on-treatment PRU measurements from 147 subjects was used for the Dx dataset. ROC analysis was used to characterize the ability of the PRU result to distinguish on-treatment samples from naïve samples after pooling the naïve and on-treatment PRU results. A dataset comprised of on-treatment PRU measurements form 3059 subjects was used for the Px dataset. ROC analysis was used to characterize the ability of the PRU result to distinguish subjects that had a future cardiovascular event from those that remained event-free during long-term followup. The J statistic was determined for each cutoff according to the formula J = sensitivity+specificity-1, and the standard deviation (SD) of the J statistic was calculated for each cutoff. A 2 × SD range was used to describe the bounds of the J statistic. Uncertainty in cutoff selection for each dataset was described by determining the range of PRU cutoffs where the upper bound of the J statistic was greater than the J statistic for the optimal cutoff. To neutralize the effect of differences in sample size and the associated differences in average SD, the uncertainty analyses was repeated using a fixed variability of 0.05 in the J statistic at the optimal cutoff, representing a 5% absolute difference in the combination of sensitivity and specificity.

Results: The J statistic range for the Px dataset was 0–0.192, compared to a 0–0.769 range for the Dx dataset. The range of PRU cutoffs with a J statistic upper bound greater than the 'optimal' cutoff J statistic was 160–271 for the Px dataset compared to 182–266 for the Dx dataset. When imposing a fixed variability of 0.05 units to the J statistic, the range of PRU cutoffs with a J statistic upper bound greater than the 'optimal' cutoff J statistic was 167–261 for the Px dataset compared to 196–260 for the Dx dataset.

Summary/Conclusion: The use of datasets evaluating prognostic performance introduces greater uncertainty in optimal cutoff selection compared to datasets evaluating diagnostic performance. This uncertainty is largely attributed to differences in the range of the J statistic, which is typically much lower for datasets evaluating prognostic performance. Cutoff selection for PFT should be performed based on diagnostic performance for detecting the drug effect and the diagnostic cutoff should be confirmed on the basis of prognostic performance.

PO 447

Urinary levels of 11-dehydro-TxB2 are not an accurate marker of inhibition of platelet thromboxane A2 production by aspirin

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Background: Aspirin is used in the prevention of coronary artery disease (CAD) and other clinical manifestations of atherothrombosis. It inhibits the synthesis of the platelet agonist thromboxane A_2 (TxA_2) from arachidonic acid, by acetylating the platelet cyclooxygenase-1 (COX-1). Some studies suggested that incomplete inhibition of platelet TxA_2 biosynthesis by aspirin is associated with heightened cardiovascular risk, based on the finding that high urinary levels of the TxA_2 metabolite, 11-dehydro-TxB_2, are associated with increased risk for cardiovascular events. However, urinary 11-dehydro-TxB_2 reflects systemic TxA_2 formation, which not exclusively occurs in platelets. About 30% of urinary 11-dehydro-TxB_2 derives from extra-platelet sources and this fraction increases in inflammatory conditions. Inflammatory cells produce thromboxane A_2 via COX-2, which is mimimally affected by the low doses of aspirin that are used to prevent CAD. Therefore, urinary 11-dehydro-TxB_2 is not an accurate parameter for monitoring the effects of aspirin on platelets. Because atherothrombosis is an inflammatory disease, high urinary levels of 11-dehydro-TxB_2 may reflect an increased generation of COX-2 dependent eicosanoids by monocytes/macrophages in severely inflamed atherosclerotic plaques, which are at high risk of thrombotic complications. If this hypothesis were true, urinary levels of 11-dehydro-TxB_2 should correlate with urinary levels of 8-isoPGF2α, a product of the free radical-mediated oxidation of arachidonic acid, which induces inflammatory vascular responses that are involved in the pathogenesis of atherosclerosis.

Aims: To test whether, in aspirin-treated subjects, the urinary levels of 11-dehydro-TxB_2 correlate with the degree of inhibition of platelet COX-1 (reflected by the levels of serum TxB_2) and/or with the urinary levels of isoprostane 8-isoPGF2α.

Methods: We measured the levels of serum TxB_2, urinary 11-dehydro-TxB_2 and 8-isoPGF2α 24 h after the last dose of aspirin in 50 patients with CAD and diabetes mellitus (CAD-DM), 50 patients with CAD on chronic treatment with aspirin (100 mg od), and in 25 healthy subjects who had been on aspirin treatment (100 mg od) for 4 days.

Urinary 8-isoPGF2α levels were higher in CAD and CAD-DM patients, compared to healthy controls; a similar trend was observed for urinary 11-dehydro-TxB_2 levels, but differences did not reach statistical significance. There was no difference in serum TxB_2 across the 3 groups of subjects. There was no correlation between urinary 11-dehydro-TxB_2 and serum TxB_2 levels in each study group and in all subjects considered together ($r = 0.04$, $P = 0.68$). In contrast, there was a statistically significant correlation between urinary 11-dehydro-TxB_2 and 8-isoPGF2α levels in each study group and in all subjects considered together ($r = 0.4$, $P < 0.0001$). Conclusion. The lack of correlation between urinary 11-dehydro-TxB_2 and serum TxB_2 in subjects on aspirin treatment is compatible with our hypothesis that urinary 11-dehydro-TxB_2 levels are not an accurate marker of inibition of platelet COX-1 by aspirin. The positive correlation between urinary 11-dehydro-TxB_2 and isoPGF2α levels in subjects on aspirin treatment supports our hypothesis that urinary 11-dehydro-TxB_2, in subjects whose platelet COX-1 has been inhibited by aspirin, reflects the production of arachidonic acid metabolites by inflammatory cells, which are implicated in the pathogenesis and progression of atherosclerotic lesions.

PO 448

Usefulness of light transmission aggregometry to optimize aspirin regimen in patients with left ventricular assist devices

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Background: Left ventricular assist devices (LVADs) are mechanical blood pumps used for the treatment of severe heart failure (e.g., as a bridge to heart transplantation, myocardial recovery, or as long term destination therapy). Foreign surfaces of LVADs altere rheologic conditions and blood stasis within the chambers of the native heart induce activation of coagulation, requiring adequate anticoagulation to prevent thromboembolic events. Because platelet function is generally assumed to play a key role in these deleterious complications, antiplatelet therapy is considered crucial for such patients. However, they are also exposed to increase bleeding tendencies due to impaired platelet function and/or acquired von Willebrand disease in relation to high shear stress. Therefore, monitoring of platelet function and aspirin response is necessary to manage postimplantation LVADs bleeding risks in those patients.

Aims: We evaluated platelet function and aspirin response in the early, intermediate, and late phase after LVAD implantation.
Methods: We measured induced platelet aggregation to different agonists (ADP, collagen, arachidonic acid, ristocetin and TRAP) using an APACT-4004 aggregometer in 18 patients on LVAD support. Aspirin therapy was administered for 24–48 h after device placement when platelet functions were considered almost normal. Then, aspirin regimen was progressively increased until arachidonic acid-triggered platelet aggregation dropped to levels below 20%.
Results: Eighteen patients, 34–74 years old, with acute or end-stage heart failure were included in this study. Seven of the patients underwent LVAD placement under emergency conditions, and 11 were electively scheduled for surgery. Patients were closely monitored with light transmission aggregometry (LTA) to optimize the dose of aspirin. In the early phase after implantation, initial platelet functions were almost normal in all patients. Using the cut-off of 20% LTA response to arachidonic acid (AA), an aspirin dose of at least 160 mg/day was progressively required in 78% of the patients and a dose of at least 300 mg/day in 39% of the patients. In postoperative period, ristocetin-induced platelet agglutination was impaired in three patients and this condition was associated with a lack of high molecular weight VWF multimers, confirming an acquired von Willebrand disorder which led us to optimize the dose of aspirin. Using this strategy, four patients suffered from major bleeding complications, but among them, only one received 300 mg of aspirin daily. One patient had a cerebral thromboembolic event leading to death, although he received an aspirin dose of 500 mg/day.
Conclusion: We successfully used LTA to optimize the dose of aspirin in 18 patients supported with LVADs and showed that 39% of them required high dose of aspirin to reduce the maximum AA-induced platelet aggregation less than 20%. This strategy allowed us to avoid major thrombotic complications that can often be fatal in such patients. However, whether these patients are resistant to aspirin still remains unclear and needs to be further explored.

PO 449
Antithrombotic activity of synthetic compound LASSBio-752

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Background: Cardiovascular disease is the leading cause for mortality and morbidity in the world.
Aims: Searching for new compounds that have potential antithrombotic activity, the synthesis of a new series of N-acylhydrazones was recently described (Lima *et al*., *Eur. J. Med. Chem*. 2008).
Methods: In this work we test the compound LASSBio-752 in thrombosis models in rat. In a thrombosis model that combines stasis and hypercoagulability the control group received tissue thromboplastin doses of 3 mg/kg and the mean thrombus weight was 11.2 ± 0.1 mg (n = 8).
Results: Orally administration (O.V.) one h before of LASSbio-752 (100 μM/kg) decreased thrombus weight by 37 ± 0.2%. The antithrombotic action this compound (100 μM/kg) showed a time-dependent pattern, showing 87.5 ± 2.1% of inhibition after 24 h of administration. When tested in the arterial thrombosis model of the compound also showed inhibitory effect, reducing the time of vascular occlusion. From the interval of 1 h there is already an extension in time of total occlusion was 34 ± 2.4 min and the intervals between 6 and 15 h of administration there was no occlusion of the artery. The occlusion begins to be observed in 24 h, where the occlusion time is 23.8 ± 2.3 min, close to the time control 17.6 ± 2.0 min. In the model of induced bleeding was observed that the effect of bleeding was not too intense for any of the intervals tested (1–48 h).
Conclusion: Up to now our results indicate that compound LASSBio-752 is potential candidates for utilization in the treatment of cardiovascular diseases.

PO 450
First European performance evaluation of the VerifyNow II System

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Background: The VerifyNow System has been well-characterized for its ability to provide accurate and rapid information about the antiplatelet effect of aspirin and P2Y12 inhibitors such as clopidogrel, prasugrel and ticagrelor. The results from the VerifyNow Aspirin Test and VerifyNow P2Y12 Test have been clinically validated to identify patients at increased risk for thrombosis and bleeding on the basis of their platelet reactivity. The VerifyNow II System is a next-generation test system that uses the same reagents as the VerifyNow System, but incorporates several new features to improve the user experience, including the ability to use various commonly-used blood collection tubes and a reduced sample wait time prior to performing measurements of response to aspirin therapy.
Aims: The objectives for this study were (1) to show equivalence between the VerifyNow II System results and the VerifyNow System results, (2) to demonstrate the suitability of alternative blood collection tubes, and (3) to evaluate the blood sample wait time prior to performing the VerifyNow II Aspirin Test.
Methods: A total of 23 subjects receiving treatment with a P2Y12 inhibitor and aspirin were enrolled. All testing was performed according to the manufacturer's instructions. Results obtained from the VerifyNow II Aspirin and P2Y12 Tests were compared to results from the same samples tested with the VerifyNow Aspirin and P2Y12 Tests. Evaluation of alternative blood collection tubes was performed using the VerifyNow System-recommended Greiner Bio-One partial fill Vacuette tube compared to the standard BD Vacutainer blood collection tube with 1.8 cc and 4.5 cc fill volume. All blood collection tubes contained 3.2% sodium citrate. The sample wait time prior to VerifyNow II Aspirin testing was evaluated by comparing VerifyNow II Aspirin measurements performed after a 30 min waiting period (as required with the VerifyNow Aspirin Test) to measurements performed after a 10–15 min waiting period. Data were analyzed using Deming regression and Lin's concordance correlation coefficient.
Results: PRU,% inhibition, and ARU results obtained with the VerifyNow II System were equivalent to the VerifyNow P2Y12 System, with Lin's concordance correlation coefficients of 0.98, 0.96, and 0.96, respectively. VerifyNow II System PRU,% inhibition, and ARU results obtained with standard blood collection tubes were equivalent to partial-fill blood collection tubes, with Lin's concordance correlation coefficients of 0.97, 0.94, and 0.98, respectively. There was no difference between 1.8 cc and 4.5 cc fill volumes for the standard blood collection tubes. VerifyNow II Aspirin results obtained after a 10–15 min sample wait time were equivalent to results obtained after a 30 min wait time (Lin's r = 0.95).
Summary/Conclusion: The results of this investigation confirm that the VerifyNow II System produces results that are equivalent to the original VerifyNow System, with the added benefits of allowing use of standard blood collection tubes and a reduced sample wait time prior to VerifyNow II Aspirin testing.

PO 451

Effects of platelet reactivity on long-term clinical outcomes and bleeding events in Japanese patients receiving aspirin therapy

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Background: Aspirin is a widely-used for prevention of cardiovascular diseases while it is also known to increase bleeding events. It is controversial whether a low response to aspirin is associated with poor prognosis or not.
Aims: The aim of this study was to evaluate the impact of the antiplatelet activity of aspirin on cardiovascular and bleeding events in Japanese patients.
Methods: In the study the University ethical committee approved, we analyzed the clinical course of 239 Japanese patients undergoing antiplatelet therapy with aspirin alone for 5 years in a prospective observational study where all participants provided written informed consents. Their residual platelet reactivity was examined at enrollment and after 2 years by the optical aggregometer and whole blood aggregometer with screen filtration methods using collagen as an agonist. The co-primary endpoints were the occurrence of major adverse cardiac and cerebrovascular events (MACCEs) and bleeding events.
Results: The results revealed that the annual incidence of MACCEs and major bleeding events was 3.7% and 0.48%, respectively. With a cut-off value of platelet reactivity measured by the optical aggregometer at enrollment, 67 patients (28%) were classified as low-responders. Low response to aspirin was not associated with increase of MACCEs, while it was clearly associated with increased MACCEs in patients less than 70 years old (low-responders 36.9% vs. responders 14.8%, log rank $P = 0.008$). On the other hand, low responders determined at enrollment by the whole blood aggregometer (27%) did not show increased risks. Major bleeding occurred in 5 patients, who were all responders although the difference was not statistically significant ($P = 0.07$).
Summary: Thus, low response to aspirin was not associated with increase of long-term MACCEs, while it increased MACCEs in patients less than 70 years old; however, it tended to decrease major bleeding events in Japanese patients.

PO 452

Coagulation markers and platelet reactivity after dual antiplatelet therapy in patient with acute myocardial infarction treated with primary percutaneos coronary inervention

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Background: Intracoronary thrombosis is almost a synonym for acute coronary syndrome. An impaired response to antiplatelet therapy with aspirin and clopidogrel has been associated with major adverse cardiovascular events (MACE) in patients with acute myocardial infarction. Elevated levels of coagulation markers, especially fibrinogen and von Willebrand factor increase platelet reactivity and predispose arterial thrombosis.
Aims: We tried to establish a correlation between low response to aspirin and clopidogrel measured by impedance aggregometry and level of blood coagulation markers (aPTT, PT, INR, fibrinogen, FVII, FVIII, vWF, FXII, AT-III, FXII, plasminogen, PAI).
Methods: Between November 2010 and October 2011, patients with acute myocardial infarction submitted to primary percutaneous coronary intervention were enrolled in this prospective study. Patients were consecutively recruited from the Coronary Unit at Clinical Centre of Serbia. A total of 126 patients fulfilled inclusion criteria and their diagnostic and clinical parameters were collected and analyzed. On the basis of platelet functional analysis, patients were divided in two groups: those with good and others with low response to dual antiplatelet therapy.
Results: Of 126 patients, good clopidogrel response was found in 66 (52.4%), while weak clopidogrel response was present in 60 (47.6%) patients. Good aspirin response was recorded in 108 (85.7%) patients, while weak aspirin response was found in 18 (14.3%) patients. Those with weak response to dual antiplatelet therapy and high platelet activity had significantly higher levels of fibrinogen and vWF, in comparison to patients with sufficiently inhibited platelet function. Chances to suffer MACE were significantly higher among weak responders. In patients with MACE, significantly elevated fibrinogen levels and highly significantly elevated vWF levels were recorded.
Summery/Conclusion: Weak responders to dual antiplatelet therapy had elevated levels of fibrinogen and vWF. Weak responders to dual antiplatelet therapy and high residual plaelet activity were significantly correlated with risk of MACE.

PO 453

Non-invasive method for study aggregation properties of the platelet, leukocyte, erythrocyte and hemostasis state

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Aims: The aim of our research is the study the aggregation activity of blood cells using non-invasive method of speckle-analysis.
Summary. Non-invasive method for an assessment the thrombocyte and leukocyte-thrombocyte complexes activity and some other parameters of the haemostasis is proposed. This method is based on the analysis of the speckle pattern behavior that is originated by the coherent light that is scattered from the skin surface.
A significant correlation between the measured optical indexes and spontaneous, ADP-, adrenalin and collagen induced platelet aggregation as well as with the leukocyte-erythrocyte and platelet-erythrocyte aggregates, fibrinogen, soluble fibrin-monomer complex, and some other related parameters was found.
The obtained results indicate that the newly proposed non-invasive method might be usable for an assessment of a tendency of the intravascular clotting.

PO 454

Comparison of P2Y12-receptor blockade in cardiovascular disease patients undergoing antiplatelet therapy by Innovance PFA P2Y assay and light transmission aggregometry using ADP agonist

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Background: In the recent years platelet function testing has expanded to include the assessment of the effectiveness of antiplatelet therapy in both research and clinical settings. Light transimission aggregometry (LTA) is still regarded as the gold standard for platelet function testing, despite its technical limitations. Development of point-of-care platelet function analyzers, such as Platelet Function Analyzer (PFA-100 system, Siemens, Germany) has allowed simpler and rapid assessment of platelet function using whole blood sample and thus presenting the potential for widespread clinical use. The recently available P2Y cartridge (Innovance PFA P2Y) of the PFA-100 system is specifi-

cally designed for monitoring clopidogrel therapy by measuring ADP-induced platelet aggregation.

Aim: The aim of this study was to evaluate the PFA-100 P2Y assay by measuring on-treatment platelet reactivity in parallel by LTA method using ADP agonist. We aimed to establish the agreement between results assessed by the two methods.

Methods: Twenty (7 females, 13 males) stable cardiovascular patients under antiplatelet therapy were included in the study. Fifteen patients were treated with clopidogrel alone (75 mg/day) and the remaining five patients were on acetylsalicylic acid (ASA, aspirin, 100 mg/day) alone. Clopidogrel-induced inhibition of platelet aggregation was assessed by PFA-100 P2Y assay and by LTA method using ADP agonist on Behring Coagulation Timer (Siemens, Germany). The PFA-100 P2Y is an assay in which citrated whole blood is aspirated through disposable cartridge containing ADP thus measuring the antiplatelet effect of clopidogrel due to P2Y12-receptor blockade. Platelet aggregation is determined by the time taken for the occlusion of blood flow. The cut-off value for PFA P2Y was defined at 106 s according to the manufacturer¢s recommendations and cut-off for LTA using 20 μM ADP was defined as value < 60% according to the literature data.

Results: The clopidogrel treatment target value for PFA P2Y (> 106s) was achieved in all 15 patients on clopidogrel therapy, while ADP-induced LTA found 14 of 15 patients on clopidogrel to be responsive (< 60%). Using cut-off values of 60% (LTA) and 106s (PFA P2Y), the aggrement between the two methods was 19/20 (0.95), while 1 of 20 (0.05) results showed discrepancy. The only discordant result showed reduced platelet aggregation by P2Y (> 300s) and LTA ADP-induced aggregation of 64%. All five patients on ASA therapy alone had P2Y values below 106s.

Conclusions: Comparison of the results obtained by the two methods has shown very good agreement. Sensitivity of PFA P2Y assay in detecting responsiveness to clopidogrel was comparable to LTA method using ADP agonist. The results suggest PFA P2Y assay to be suitable for detection of P2Y12-receptor blockade and monitoring the antiplatelet effect of clopidogrel. Cut-off value (> 106s) given by manufacturer can be considered as suitable for the defining the responsiveness to clopidogrel. The results in patients on ASA therapy alone showed that this therapy has no effect on the PFA P2Y assay. Compared with the LTA method, PFA P2Y assay is much easier to perform, requires smaller amount of whole blood sample and results are accessible in a shorter period of time.

PO 455

Identification patients less sensible to Aspirin treatment through arachidonic acid stimulated platelet marker

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Background: Arachidonic Acid (AA) induced platelet aggregation > 20% is currently used to identify patient less sensible to aspirin treatment.

Frelinger et al. (Circulation 2009) demonstrated that residual COX-1 function measured by serum TXB2 assay correlates with subsequent major adverse cardiovascular events, but not with AA-stimulated platelet markers.

Aims: In this study, we investigated if platelet aggregation induced by Arachidonic Acid is useful to identify patients less sensible to aspirin treatment.

Methods: We assessed a retrospective study in which serum TXB2 and platelet aggregation (Born's method) induced by Arachidonic Acid 0.5 mg/ml were analysed in 116 patients receiving a daily dose of apirin (100 mg/die > 1 mount) and in 27 patients receiving aspirin on alternate days, on prescription by doctor.

Moreover, it was measured AA induced TXB2 formation in 42 patients of our population.

Results: We assessed cut off for residual platelet COX-1 function TXB2 > 3.1 ng/ml according to Frelinger (Circulation 2009). While, we used TXB2 > 100 ng/ml to estimate patients non compliant to therapy.

According to TXB2 values, we divided our population into three groups: (1) TXB2 < 3.1 ng/ml (n = 83); (2) TXB2 > 3.1 ng/ml < 100 ng/ml (n = 28); 3) TXB2 > 100 ng/ml (n = 5).

In the first group (Tx < 3.1 ng/ml), patients having platelet aggregation induced by Arachidonic Acid > 20% were 8 (tot = 83) In the second group (Tx > 3.1 < 100 ng/ml), patients having platelet aggregation induced by Arachidonic Acid > 20% were 2 (tot = 28); none of the patients in both groups showed platelet aggregation induced by Arachidonic Acid > 30%.

In the third group, patients having platelet aggregation induced by Arachidonic Acid > 20% < 30% was1 and 4 patients were > 30%. All such patients were found non compliant, as plasma salicylates were absent.

Moreover, we divided 27 patients receiving aspirin on alternate days into two soubgroup:

patients receiving aspirin the day before performing platelet aggregation (n = 12).

patients who didn't receive aspirin the day before performing platelet aggregation (n = 15).

The patients who showed platelet aggregation induced by Arachidonic Acid > 20% were similar in both groups (8% and 13% respectively).

In 42 patients out of our daily aspirin assumption population, we performed both AA induced TXB2 formation and serum TXB2 assays. The results obtained showed a good correlation (r = 0.8258) between the two methods.

Conclusions: Platelet aggregation induced by Arachidonic Acid > 20% isn't sensibile enough to identify patients less sensible to aspirin treatment. Moreover, we found good correlation AA induced TXB2 formation and serum TXB2. AA induced TXB2 formation may be a useful test to identify patients less sensible to aspirin treatmnet when it isn't possible to treat blood for 1 h at 37 °C immediately after collection.

PO 456

Platelet – dependent thrombin generation assay provides new insights into the monitoring of antiplatelet therapy effectiveness

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Background: Patients underwent percutaneous coronary interventions have an increased risk of recurrent ischemic events. Using of antiplatelet agents can reduce the occurrence of clinically relevant stent thrombosis. But the individual assessment of the treatment effect is still complicated. Thrombin generation test (TGT) in platelet-rich plasma (PRP) reflects contact pathway activation and may be useful as a global method to assess the procoagulant potential of platelets and its changes induced by antiplatelet agents.

Aims: The aim of our study was to evaluate a possibility of using TGT for the assessment of efficacy of antiplatelet therapy.

Methods: Venous blood was obtained from 25 patients after the successful coronary stent placement. All of them were given clopidogrel and aspirin (75 and 150 mg per day, accordingly). The control group comprised 29 healthy volunteers. TGT was performed in fresh PRP with the platelet count adjusted to 150 × 10^9/L by dilution with autologous platelet poor plasma. Thrombin generation was measured in a Fluoroscan Ascent® fluorometer at 1 pM tissue factor concentration

using dedicated software. The following parameters were analyzed: lag time of thrombin generation (LT, min), endogenous thrombin potential (ETP, nM/min), maximum concentration of thrombin (Peak, nM), time to reach the peak (TTP, min), velocity index (VI = Peak/[TTP – LT], nM/min). Statistical analysis of the results was performed by non-parametric methods using the median (Me), 95% confidence interval (95% CI) and Mann–Whitney *U* test (Statistica 6.0).

Methods: There were no differences in ETP and LT parameters between patients and controls (1854,0 (1781,7–2001,6) vs. 1911,0 (1865,9–2043,8) for ETP and 17,8 (16,1–20,0) vs. 14,6 (14,1–17,9) for LT). The estimation of Peak, TTP and VI in patients vs. controls showed the statistically significant ($P < 0.003$) decreasing of Peak, 112,7 (103,1–122,1) vs. 138,5 (123,6–143,6), and VI, 7,6 (6,4–8,8) vs. 12,1 (10,9–13,7), and prolongation of TTP, 33,3 (30,8–35,5) vs. 26,8 (25,2–29,3).

Summary: Thrombin generation measurement in fresh PRP can be suggested for the monitoring of antiplatelet therapy effectiveness. Nevertheless more research is required.

PO 457

Possibilities to assess compliance with low-dose aspirin treatment using a highly sensitive assay for salicylic acid in plasma?

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Background: Aspirin, i.e., acetylsalicylic acid (ASA), is a widely used antiplatelet drug at low dosages (75–325 mg daily) but is also used at higher doses for its analgesic and antiinflammatory effects. ASA irreversibly inhibits platelet cyclooxygenase and the antiplatelet effect persists 7–10 days. After administration of ASA, it is rapidly hydrolyzed to salicylic acid (SA) which has a longer persistence in plasma. Compliance is an important factor for treatment results and an objective measure of compliance with low-dose ASA treatment would be valuable.

Aim: To establish a highly sensitive method to determine low concentrations of SA in plasma after administration of low-dose ASA for possible use to monitor compliance.

Methods: Citrated plasma samples from 43 patients included in two different studies were used. In the first study, each patient ($N = 25$) took three dosages of ASA (75 mg OD, 75 mg BID and 320 mg OD) for at least 2 weeks in a randomized order. After each treatment period, samples were collected 12 (BID) or 24 (OD) h after the last intake of ASA. In the second study treatment with ASA was not allowed which provided samples from non-ASA treated patients.

For the analysis, 90 μL of patient plasma, standards (0–5000 ng/mL) or quality control samples were aliquoted and 10 μL of internal standard (salicylic acid-d_6; Isotec) was added. After precipitation using MeCN with 0.1% formic acid the tubes were centrifuged (16000 × *g*, 10 min). Supernatants were evaporated under nitrogen and resuspended in MeCN:formic acid 1:1 for analysis using Waters Aquity Quattro Premier XE UPLC and an Aquity BEH SHIELD, Waters column, 1.7 μm 50 × 2.1 mm.

Results: The median (interquartile ranges) concentrations of SA were 1.24 (0.44–4.95) ng/mL in non-ASA treated patients. In patients taking ASA the plasma SA concentrations were 7.84 (4.95–9.63) ng/mL with 75 mg OD, 24.22 (14.41–38.56) ng/mL with 320 mg OD, and 71.56 (44.55–109.51) ng/mL with 75 mg BID. Thus, there was a dose- and time-dependent persistence of SA in plasma during ASA treatment. However, there were overlaps between the dosages, and, most importantly, also an overlap between samples from ASA treated and non-ASA treated patients. Occasional unexpectedly high levels may be due to non-compliance with instructions to take the last dose of ASA 12 or 24 h before sampling.

Conclusion: Low levels of SA can be detected in patients treated with low-dose ASA 12–24 h after intake of the last dose of ASA. However, SA levels above 5 ng/mL could be detected also in non-ASA treated patients. This may be related to uncontrolled intake of ASA and/or the presence of SA in nutrients or from endogenous sources. Thus, salicylate analyses in plasma may be helpful to detect compliance during low-dose ASA treatment but measurements do not seem to be reliable at the lowest end of the concentration spectrum. Further improvement of the analytical technique and better understanding of possible sources of SA may provide a useful method for the monitoring of compliance with ASA treatment.

PO 458

Anti-platelet aggregation activity observed in Honkaku shochu

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Background: We have previously reported that the intake of various types of alcoholic beverages (Sumi *et al*., H., *Alcohol & Alcoholism*,23, 33, 1988; Sumi *et al*., *Jpn. J. Alcohol & Drug Dependence*, 33, 263, 1998) brings about changes in the coagulation and fibrinolytic system, and that drinking *Honkaku shochu* (distilled through a pot still) results in promotion of fibrinolysis in blood for a rather long period. In another report, we demonstrated that the distillation fractions of *shochu* have the effect on cells to release t-PA (tissue plasminogen activator) and that the *shochu* aroma is effective in inhibiting platelet aggregation (Sumi *et al*., 21st ISFP, p.18, Brighton, UK, 2012).

Aims: We have now conducted a comparative study of the effects of various types of Japanese *Honkaku shochu*on platelet aggregation. The results are reported here as new information.

Methods: *Honkaku shochu* is classified by the laws of Japan into 2 types: *shochu* and *awamori*. For the tests, 7 types of *awamori* and 24 types of *shochu* were purchased as specimens, and then diluted by deionized water to achieve an ethanol concentration of 25%.

The platelet aggregation rate was then measured with an aggregometer (PAT-4A). Inhibition rate against platelet aggregation of each aromatic component specimen in the dilution series was calculated and the 50% inhibition value (IC_{50}) was determined.

Results: Approximately half of the *Honkaku shochu* tested exhibited anti-platelet aggregation activity. Under typical concentration conditions of 25% ethanol concentration, direct inhibition against aggregation was observed to be 20.5% on average. The *shochu* specimens exhibiting the strongest inhibitive activity against aggregation were made from such raw materials as brown sugar (S-1), sweet potatoes (S-11), rice (S-6), and barley (S-12). It is presumed that the activity does not difference on the raw materials. Aspirin is a well kwon anti-platelet agent. A-6 and S-11 showed the strongest effects, the inhibitive capacity has an equivalent value to 50–200 mM Aspirin.

Summary/Conclusion: A total of 31 types of *Honkaku shochu* were tested, and it was found that 4 out of 7 types of *awamori* and 12 out of 24 types of *shochu* inhibited the aggregation induced by the use of ADP or collagen. It is believed that the inhibitive capacity observed is not the effect of the materials used in producing *Honkaku shochu*, such as sweet potatoes, rice and barley, but rather the result of the fermentative production process. We foresee that if inhibitive effects against platelet aggregation can be achieved by merely smelling the given material instead of eating it, then these types of material could prove to be functional materials in a completely new category not thought of before.

PO 459

The effect of clopidogrel with and without aspirin on hemostatic system activation *in vivo* in man

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Background: Dual anti-platelet inhibition with clopidogrel (C) and aspirin (A) is a cornerstone in the management of patients with acute coronary syndrome, but confers a considerable risk of bleeding.
Measurement of platelet and coagulation activation indicators in blood emerging from a local injury site of the microvasculature made to determine bleeding time (called 'shed blood') is a method to investigate hemostatic system activation under close to *in vivo* conditions.
Aim: To investigate if combining C and A has a more pronounced inhibitory effect on hemostatic system activation *in vivo* than C alone.
Methods: In a prospective, randomized, double-blind, placebo controlled study, 44 healthy male volunteers (26 years, range 19–43) were treated with C (loading dose 600 mg, maintenance dose 150 mg/d) and A (100 mg/d) or C (same dose) and placebo for 7 days. Shed blood was obtained at baseline, 2 h and 7 days. ß-thromboglobulin (TG) and thromboxane B2 (TxB2) (reflecting platelet activation) as well as D-Dimer (dd) and prothrombin fragment 1.2 (f1.2) (reflecting coagulation activation) were measured by ELISA. The number of microparticles (MP) was determined by flow cytometry. The sample size was calculated to detect a 20% difference in the main outcome variable (TG) with an alpha of 0.05 and a power of 80%. To test the differences between the treatment groups, two sample *t*-tests were performed. Repeated measures ANOVA was applied for comparisons considering repeated measurements in time. The study was approved by the local ethics committee and informed consent was obtained from all volunteers.
Results: Compared to baseline, both CA and C caused a decrease in TG at 2 h [from 3.3 ± 1.6 to 1.7 ± 0.8 ($P < 0.001$) and from 3.0 ± 1.8 to 1.8 ± 1.4 mg/L ($P < 0.001$), respectively) and at 7 days [from 3.3 ± 1.6 to 1.8 ± 1.2 ($P < 0.001$) and from 3.0 ± 1.8 to 2.1 ± 1.7 mg/L ($P < 0.001$), respectively)]. At all time points, no difference in the concentration of TG was found between CA and C. CA (and to a lesser extend C, data not shown) inhibited TxB2 formation: by 80% at 2 h and by 96% at 7 days. Compared to baseline, CA and C caused a decrease in dd at 2 h [from 0.45 ± 0.55 to 0.22 ± 0.08 ($P = 0.05$) and from 0.39 ± 0.29 to 0.22 ± 0.10 mg/L ($P = 0.02$), respectively] but not at 7 days [0.45 ± 0.55 vs. 0.29 ± 0.37 ($P = 0.3$) and 0.39 ± 0.29 vs. 0.45 ± 0.64 mg/L ($P = 0.7$)]. At all time points, no difference in dd was found between CA and C. Neither CA nor C had an effect on f1.2 or MP counts.
Conclusion: In healthy volunteers, both CA and C inhibited microvascular hemostatic system activation. No difference in platelet and coagulation inhibition was found between subjects treated with CA or C indicating that combining C and A does not result in a more pronounced inhibitory effect on hemostatic system activation *in vivo* than C alone.

PO 460

Sulforaphane induces ubiquitination of p85 and PDK1 and inhibits phosphatidylinositol 3-kinase signaling in human platelets

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Sulforaphane, a dietary isothiocyanate found in cruciferous vegetables, has been shown to exert beneficial effects in animal models of cardiovascular diseases. In the present study, we show that sulforaphane inhibited human platelet aggregation caused by different receptor agonists, including collagen, U46619 (a thromboxane A_2 mimic), protease-activated receptor 1 agonist peptide (PAR1-AP), and an ADP $P2Y_{12}$ receptor agonist. In exploring the underlying mechanism, we found that sulforaphane specifically prevented phosphatidylinositol 3-kinase (PI3K)/Akt signaling, without markedly affecting other signaling pathways. The inhibition of the PI3K/Akt pathway by sulforaphane may result from causing ubiquitination of two important components, i.e. PI3K p85 subunit and PDK1, in this pathway. The ubiquitination of p85 and PDK1 can lead to impaired function or protein degradation. In conclusion, we have demonstrated that sulforaphane can prevent platelet aggregation, which may explain its protective effect in cardiovascular diseases; our data also support that the inhibition of the PI3K/Akt pathway by sulforaphane contributes it antiplatelet effects.

PO 461

Could von Willebrand's disease be overlooked in women using combined oral contraceptives?

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Background: Von Willebrand's disease is the most common inherited bleeding disorder. It is caused by a quantitative or qualitative deficiency of von Willebrand factor, a plasma protein involved in primary haemostasis. A common symptom of von Willebrand's disease in women is menorrhagia. For this reason, many women suspected of having von Willebrand's disease are treated with combined oral contraceptives (COCs) and are receiving COC treatment at the time of investigation. Few studies have examined the effect of COCs on von Willebrand factor plasma levels, and results are conflicting. However, if COCs can cause an increase in plasma level of von Willebrand factor, this could interfere with test results, and the disease could be masked in women receiving treatment with COCs.
Aim: The aim of the study is to investigate whether COCs induce an increase in von Willebrand factor plasma levels.
Methods: A clinical longitudinal study is conducted with two groups (each $n = 30$) of healthy participants; A) women wishing to begin p-pill treatment and B) women not receiving p-pill treatment (control group). A baseline blood sample is taken at the time of inclusion, after which the participants in group A begin COC treatment. Blood samples are obtained again 3 and 6 months after baseline. Von Willebrand factor plasma levels at baseline and at 3 and 6 months are compared within the group, and potential differences in von Willebrand factor levels before and after p-pill treatment are compared with the control group.
The following analyses, which are all routinely employed in the diagnosing of von Willebrand's disease, are carried out: von Willebrand factor (vWF) antigen and vWF activity employing immunoturbimetric assays, vWF ristocetin cofactor assay, vWF collagen binding assay (ELISA), and factor VIII Clot.
Results: The project is currently still in the recruitment phase, but we will be able to present preliminary results at the time of the conference.
Summary/Conclusion: The study examines the effect of combined oral contraceptives (COCs) on von Willebrand factor plasma levels in healthy women. Our results will hopefully help in improving the diagnosing of von Willebrand's disease in women treated with COCs.

PO 462

Association of methionine synthase and thymidylate synthase genetic polymorphisms with idiopathic recurrent pregnancy loss

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Background: One-carbon metabolism is important for maintaining pregnancy and the enzymes codified by these genes are relevant in this metabolic pathway.

Objective: To investigate the association between one-carbon metabolism and recurrent pregnancy loss (RPL), we examined polymorphisms in four genes: methionine synthase (*MTR*); methionine synthase reductase (*MTRR*); methylenetetrahydrofolate dehydrogenase 1 (*MTHFD1*); and thymidylate synthase (*TS*).
Methods: A cohort of 353 RPL patients (3.09 ± 1.65 pregnancy losses) and 226 controls. Genotyping was assessed by polymerase chain reaction-restriction fragment length polymorphism assay.
Results: The *MTR* 2756AA polymorphism was associated with RPL. Gene-gene interaction analysis revealed that the frequency of the *MTR* 2756A-*TS* 6 bp allele combination was significantly higher in RPL.
Summary: Based on these results, we propose that the *MTR* 2756AA genotype and *MTR* 2756A-*TS* 6 bp allele combination are possible predisposing factors for RPL development in Korean women.

PO 463

Association study of Paraoxonase-1 gene polymorphisms and Homocysteine Levels in Patients with Ischemic Strokes

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Background: Paraoxonase-1 (PON1), a high-density lipoprotein (HDL) associated enzyme, is involved in the lipid metabolism and detoxification of insecticides and pesticides.
Aims: The aims of this study were to evaluate the role of Paraoxonase-1 gene polymorphisms in patients susceptible to ischemic stroke, and to determine the relationship between PON1 gene polymorphisms and plasma total homocysteine (tHcy) levels.
Methods: Five hundred and eighty-seven patients with ischemic stroke and 476 controls were genotyped for the PO01L55M, Q192R polymorphisms. The PON1 L55M and Q192R genotypes were determined by the polymerase chain reaction-restriction fragment length polymorphism method. Logistic regression analyses, adjusting for multiple comparisons, were used to determine the association between the minor allele of each of the polymorphisms and the risk of ischemic stroke.
Results: The LL and LM+MM genotype frequencies of the L55M polymorphism were significantly different between the stroke and control group. When stratified by the size of the occluded vessel, the PON1 Q192R polymorphism was associated with patients with multiple small-artery occlusions. When the patients were divided into 3 groups according to the tHcy level, the sum of the control groups and ischemic stroke patient groups, age and gender were found to be significantly associated with an elevated tHcy level. Moreover, the folate level was decreased upon increasing tHcy levels.
Summary/Conclusion: In accordance with these studies, our study indicated that PON1 polymorphisms should be considered as risk factor for ischemic stroke in Koreans.

PO 464

Effects of hypertonic saline hydroxyethyl starch solution on heatstroke-induced damage in a rat model

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Hypertonic saline hydroxyethyl starch solutions have been used for 'small volume resuscitation' in patients with peripheral arterial stenosis, hemorrhagic shock, cerebral and cardiac ischemia. In rats, heat stress leads to arterial hypotension, microvascular injury, thrombosis, inflammation, hypoxia, hypercoagulable state and brain and heart dysfunction. The aim of the present study was to determine whether Hypertonic saline hydroxyethyl starch solution could improve the heatstroke-induced microvascular injury, thrombosis, inflammation, circulatory shock, cerebral ischemia, and hypercoagulable state. When the untreated rats underwent heat stress, the heatstroke rats displayed cerebrovascular dysfunction (evidenced by hypotension, hypoxia, and ischemia), higher levels of thrombomodulin, interleukin-1, activated partial thromboplastin time, prothrombin time, D-dimer, and decreased platelet count and protein C (heatstroke-induced disseminated intravascular coagulation was assessed by plasma markers). However, the heatstroke-induced hypotension, cerebral ischemia, activated inflammation, microvascular injury, thrombosis, and hypercoagulable state can be significantly reduced by hypertonic saline hydroxyethyl starch solution. Our results suggest that hypertonic saline hydroxyethyl starch solution can be used as a beneficial agent for heatstroke. In particular, hypertonic saline hydroxyethyl starch solution may ameliorate the situation of microvascular injury, thrombosis, inflammation, and hypercoagulable state in heatstroke.

PO 465

Children's playbook validation for cardiovascular disease prevention

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Background: The playbook uses the influence of early childhood education in the remodeling of the family habits to prevent cardiovascular disease (CVD).
Aims: The aim of this study was the validation of an infant playbook to be used in cardiovascular health education of children, suggest actions seeking education in order to promote the empowerment of the child population in health issues and citizenship.
Methods: We created a first version of the playbook using a roadmap aimed at children and manuals drawings that could be colored by children. This version was assessed by 4 cardiologists, 4 pediatricians and 4 childhood education professionals through a structured questionnaire. From the contribution of professionals, we created a second version evaluated for 40 children aged between 5 and 10 years. The third version of the playbook was made after the relevant corrections pointed out by children, plus the suitability of the information, illustrations and language.
Results: The first version of the playbook was well accepted by most professionals, 83.3% of the participants found the information correct and appropriate for children. Seventy-five percent strongly agreed with the quality and content in this material. The second version got 80.8% in the field understand, 100% attractiveness, 92.3% of children interacted with the material (coloring or doodling), and 100% of children have responded that claim to help your family to improve habits. The analysis of the material by pediatricians allowed the inclusion of an illustration demonstrating the importance of blood pressure measurement in children from 3 years.
Conclusion: The method used in the validation of the children's playbook contributed to the betterment of itself and wide acceptance of the target audience. The third version of the playbook may act in interface education/health seeking to form a culture of prevention of CVD in the family. However, the final version of the material depends on its use and further studies, which may suggest the need for changes in the script and drawings, to achieve the target of an increasingly effective.

PO 466

Coagulation factor VII gene polymorphisms and cardiovascular diseases in Iran

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Background: The association between coagulation factor VII (FVII) gene polymorphisms and FVII level, activity, and cardiovascular diseases (CVD) has been reported in several studies.

Aim: Determine whether two FVII gene polymorphisms including: −401 G>T and HVR4 influence risk of CVD in Iranian population.

Methods: A total of 110 patients with cardiovascular disease that confirmed by angiography (57 male and 53 female, mean age 55.4 ± 10.8 years old) and 110 age and sex match controls (54 male and 46 female, mean age 53.8 ± 9.7) without cardiovascular disease (confirmed by angiography) were genotyped for these two polymorphisms.

Results: Although TT genotype of −401G>T polymorphism was at a higher frequency among control group compared to patients (18.1% vs. 16.3%), the difference was not statistically significant ($P > 0.05$). Also Carriers of the H7-allele (H7H7) were at a higher frequency among control group in compare with patients group (23.6% vs. 21.8%) but it was not statistically significant too ($P > 0.05$).

Conclusion: We could not found any association between −401 G>T and HVR4 polymorphisms of coagulation FVII and CVD in Iranian population, but future studies with larger study groups are recommended for more evaluation of these associations.

PO 467

Compound folate deficiency, elevated homocysteine and pseudo-homozygosity for MTHFR677T as predisposing factors in TIA: a case study

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We present a patient with multiple transient ischaemic attacks due to intracranial stenosis. Folate deficiency, high homocysteine with associated heritable variation in the *MTHFR* gene were the only identified risk factors which contributed to the atherosclerosis.

A 21 year old male of African extraction presented with a 3 month history of intermittent, transient neurological events, which required admission for clinical investigation. Apart from a history of cerebral vascular events in a maternal aunt he had no significant personal or family history, was previously healthy and not on any medication. An MRI scan showed an area of restricted diffusion in keeping with an infarct, and an angiogram demonstrated an intracranial stenosis in the internal carotid artery, consistent with atherosclerosis. A full range of laboratory investigations were performed and a highly elevated homocysteine of 95.3 μM (reference limit < 15) was detected. Further analysis revealed that both plasma folate and 5-MTHF were reduced, and his plasma methionine was at the lower end of the normal range. This raised the suspicion of a remethylation defect of homocysteine to methionine. The patient was treated with folic acid supplementation for 3 months, and his plasma homocysteine responded, falling to 12.9 μM.

Molecular analysis of a small panel of thrombotic risk factors showed him not to carry either the FVL or PT20210 polymorphisms. He was shown to be heterozygous for the common MTHFR 677C/T variant which in this state is neither a thrombotic risk factor nor sufficient cause of the elevated homocysteine levels. Analysis of the entire coding region of the *MTHFR* gene was undertaken to investigate the proposed re-methylation defect and heterozygosity for a c.3G>C nucleotide substitution was identified. Although this base change predicts the replacement of the initiating Methionine at codon 1 with an Isoleucine, it is probable that loss of p.Met1results in the lack of translation from this allele. Protein analysis would be required to confirm this. It is proposed that the loss of p. Met1, coupled with folate deficiency, may be significant for the remethylation process in this patient, and may contribute to the neurological event and narrowing of his cerebral arteries. If the c.3G>C variant is proven to be on the alternate allele to the *MTHFR* 677T polymorphism, the consequence of this would be pseudo-homozygosity for *MTHFR* 677T. Homozygosity for this polymorphism is associated with an increased thermolability of the MTHFR enzyme, with a reduction in enzyme activity of approximately 50%. It is associated with mildly elevated total homocysteine with an increased risk of cardiovascular events. It is probable that the combination of pronounced dietary folate deficiency, an *MTHFR* 'null allele' and the 677T variant is sufficient to explain both the moderate hyperhomocysteinaemia and the clinical presentation in this patient. This case highlights the need to investigate other possible mutations in the *MTHFR* gene, particularly in the absence of homozygous MTHFR 677C/T status in pre-mature cardiovascular events.

PO 468

Individuals with coronary artery disease at a young age and features of the metabolic syndrome have an increased prothrombotic potential

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Background: The relation between coagulation and atherosclerosis has been extensively described. However, most literature on this issue shows conflicting results. Surprisingly, hypercoagulability is most pronounced in young patients with coronary artery disease (CAD).It is known that obesity and hereby induced metabolic syndrome (MS), a risk factor for CAD, are related to a higher incidence of thromboembolic events. We hypothesized that individualswith CAD at a young age and features of MS have an increased prothtombotic potential. Weanalyzed this by measuring the endogenous thrombin potential (ETP) in patients with CADbefore the age of 51 in men and 56 in women and compared them to their healthy first-degreerelatives. Furthermore, we studied whether the presence of the MS in these CAD patientsfurther increased the prothrombotic potential.

Methodology and Principal Findings: We included 118 patients with CAD at a young age and 50 healthy first-degree relatives. Anadjusted general linear model (GLM) showed that there is a positive association between thepeak thrombin levels and the presence of CAD at a young age (B = 9.4; $P < 0.05$). Based onthe NCEP guidelines we divided our patient group in CAD patients with and without MS, andcompared them to healthy first-degree relatives without MS. The adjusted GLM showed thatCAD patients with MS have increased ETP levels, both in comparison with healthy firstdegree relatives (B = 13.0; $P < 0.05$) and with CAD patients without MS (B = 12.9; $P < 0.05$).There was no difference in ETP levels between CAD patients without MS and healthy firstdegreerelatives (B = 0.1; P = ns).

Conclusion: This study shows that individuals with CAD at a young age have an increased prothromboticstate. Furthermore, we show that the increased prohrombotic state in the young CAD patientsis associated with the presence of the metabolic syndrome.

PO 469

Prevalence of risk factors for cardiovascular disease in Thai rural elderly

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Background: The number of elderly people in the society is steadily increasing, and age is considered as an important risk factor for cardiovascular disease. Investigation of the prevalence of risk factors for cardiovascular disease in the elderly may be a guide to their worthy life and community.
Aim: This study aimed to investigate the prevalence of risk factors for CAD in the elderly in the rural area of Thailand.
Methods: The study was based on data from the academic service project concerning a health check up for the elderly at Ban Non Muang, Khon Kaen Province, all of whom were aged 60 years and above. The data of risk factors for cardiovascular disease were collected from questionnaire responses whilst the physical data of subjects were directly measured. Venous blood was taken from the subjects for laboratory analysis. A total of 177 subjects (79 males and 98 females) with the mean age of 62.9 years were recruited. According to their ages, the subjects were classified into three groups: 60–69, 70–79, and > 80 years.
Results: Weight and body mass index were statistically significantly different among age groups ($P = 0.02$ and < 0.001 respectively) which was in corresponding to a rising the nutritional screening initiative (NSI) value. The prevalence of risk factors for CAD in the subjects was evaluated and found that the most common risk was metabolic syndrome (60.5%) with the higher prevalent in females than in males (71.4 vs. 45.6%, $P < 0.001$). However, the prevalence was not statistically significantly different among age groups. Dyslipidemia (49.7%) and hypertension (48.9%) were also highly prevalent in the study population. The highest prevalence of hypertension was found in the male elderly which was higher than those in females (87.5 vs. 35.7%, $P = 0.039$).
Conclusion: The results of this study serve as the useful information for further preventive planning of cardiovascular disease in the elderly at the rural area of Thailand.

PO 470

Immunization With A Peptide Containing Two Epitopes Derived From Chlamydia Pneumoniae (Cpn) Significantly Reduces Atherosclerotic Lesion In Apobtm2SgyLdlrtm1Her/J Mice Infected With The Cpn Bacteria

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Background: *Chlamydia pneumoniae* (*Cpn*) is an important human pathogen that causes atypical pneumonia and is associated with various chronic inflammatory diseases such as atherosclerosis, a major cause of cardiovascular disease and death in the Western world. However, the theoretical role of *Cpn* in acceleration of atherosclerosis is still controversial.
Aim: To investigate the antigenic effect of a peptide containing two epitopes of *Chlamydia pneumoniae* (*Cpn*) on atherosclerotic lesion formation in mice infected with *Cpn*.
Methods: Six-week-old Apob^{tm2Sgy}Ldlr^{tm1Her}/J mice were immunized using a repetitive immunization multiple-sites strategy with KLH-conjugated peptides derived from the major outer membrane protein and the putative outer membrane protein 5 of *Cpn*. Mice were fed a high-fat diet and infected with *Cpn* twice during the 10-week diet period. Lesions were evaluated histologically; local and systemic immune responses were analyzed by immunohistochemistry of aorta samples and cytokine levels in plasma samples and splenocyte supernatants were measured.
Results: Mice immunized with the combined *Cpn* peptide showed a greater reduction in lesion size compared to mice immunized with either epitope alone [54.7% vs. 39.8% or 41.72%] and was also associated with a significant decrease in lesion area in descending aortas compared with those in controls (88.9% for combined Cpn peptide, 81.9% for MOMP peptide and 75.7% for Omp5, respectively). This effect was associated with a shift in the cellular composition of plaques towards decreased inflammatory cell and increased regulatory T-cell content and increased production of anti-inflammatory cytokines and decreased secretion of proinflammatory cytokines demonstrated in plasma and in supernatant of stimulated spleen cells.
Conclusions: Atherosclerotic lesion formation may be promoted by *Cpn* infection in the presence of a high-fat diet, and reduced by immunization with the combined *Cpn* peptide. The combined peptide has more potential than either epitope alone in reducing atherosclerotic lesion formation.

PO 471

Association of serum gamma-glutamyl transferase (GGT) and other markers with extent of coronary artery disease in angiographically proven patients and its potential use as risk indicator

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Background: Accumulating evidence from epidemiological studies suggests that higher serum GGT is associated with incident cardiovascular disease (CVD), its risk and related death.
Aims: In this study we have assessed the levels of GGT and other biomarkers association to the extent of coronary artery disease (CAD) in angiographically proven patients and their use in risk assessment.
Methods: Four hundred and eighteen age and gender matched subjects (187 CAD affected and 231 unaffected) were selected for a case control study from ongoing Indian Atherosclerosis Research Study (IARS). The information regarding inclusion, exclusion criteria and process of sample collection, and data storage have been published (Indian Heart J, 2010;6:286–95). All the blood samples were collected after overnight fasting of 12–14 h. GGT, matrix metalloproaease-9 (MMP9), oxLDL and myeloperoxidase were measured in the serum samples using ELISA assays. The Gensini scoring system was utilized in the evaluation of CAD severity and was calculated for each patient from the coronary angiography report by assigning a severity score to each coronary stenosis. Reduction in the lumen diameter of the affected arteries were evaluated (reductions of < 50%, 50–70%, 71–90% and > 90% were given Gensini scores of 1, 2, 4 and 8 respectively). Each principal vascular segment was assigned a multiplier in accordance with the functional significance of the myocardial area supplied by that segment: the left main coronary artery×5; proximal segment of left anterior descending coronary artery (LAD)×2.5; proximal segment of circumflex artery×2.5; mid-segment of the LAD×1.5; right coronary artery, distal segment of LAD, posterolateral artery and the obtuse marginal artery×1; and others×0.5.The logistic regression model development was performed using SPSS software version 17.
Results: The mean GGT levels were not significantly different between CAD affected (35.6 U/L) and unaffected (35.1 U/L, *P*-value = 0.81), however after adjustment with levels of MMP9 the difference was significant (affected = 39.9 U/L and unaffected = 30.3 U/L, *P*-value = 0.006). The mean GGT levels were found to be correlating with the extent of disease in arteries such as left main coronary artery, diagonal and obtuse marginal artery. In the Logistic Regression model

for risk assessment, GGT gave an odds ratio of 1.08 (95% CI 0.6–1.91, *P*-value = 0.79) which improved to 3.73 (95% CI 1.28–10.87, *P*-value = 0.02) upon addition of MMP9 and to 4.21 (95% CI 1.3–13.46, *P*-value = 0.02) with lipids (HDL, LDL, Tryglycerides and total cholesterol) for the fourth quartile in comparison to first. The C-statistics analysis show that the Area Under the Curve for GGT alone was 0.514 (95%CI 0.46–0.57, *P*-value = 0.64) increased to 0.762 (95%CI 0.68–0.84, *P*-value = 4.93×10^{-8}) with MMP9 and lipids.

Conclusions: Our study suggests that serum GGT levels in association with MMP9 levels and lipids correlate to the severity of the CAD. However the significance of predicting recurrent disease and death requires larger study and long term follow up.

PO 472

The hemostatic system status in chronic generalized periodontitis in the elderly people with coronary heart disease

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Matter of Current: Chronic generalized periodontitis (CGP) takes its features by concomitant atherosclerosis and disseminated intravascular clotting in elderly people. Local changes in the mouth worsen the thrombogenic status on a background of systemic hypercoagulability. Data on profound changes in the hemostatic system in comorbidity of periodontal and ischemic processes in the cardiovascular system are presented in this study.

Purpose: To evaluate the thrombogenic potential of blood and saliva in chronic generalized periodontitis in elderly people with coronary heart disease.

Materials and Methods: Ninety-six patients with generalized periodontitis of moderate severity were examined. They were admitted to Transbaikalia Hospital for War Veterans on the clinical manifestations of coronary heart disease (CHD) with the diagnosis of stable angina pectoris of I-II functional class, CGP of II-A stage, hypertension of 1- 2 stages.

All patients were administered a local anti-inflammatory therapy for chronic periodontitis for 10 days. Additionally cardiovascular therapy of CHD clinical manifestations was performed. Analysis of blood and oral fluid (mixed saliva) was made, taken in the first days of admission and after it. Hemostasis was estimated in the blood on the following parameters: prothrombin time, thrombin time, fibrinogen, fibrin monomer complexes, euglobulin fibrinolysis by the methods described in the classic manuals.

Procoagulant and fibrinolytic activity of saliva was determined by adding of 0.1 ml centrifuged saliva into the plasma of the same individual with the subsequent registration of the time of its coagulation and fibrinolysis.

The control group consisted of 25 elderly people correlating major group in age, sex, degree of CAD, cardiovascular ongoing therapy, but without obvious signs of periodontal inflammation.

Methods: The obtained data suggested that the procoagulant activity of saliva in patients in relapse was higher than in healthy ones. Prothrombin time, thrombin time was accelerated by adding saliva to the blood plasma of the same patients. Clot lysis was slowed due to the presence of fibrinolysis inhibitors in saliva.

Procoagulant activity was decreased in saliva after comprehensive treatment of periodontal disease but inhibited fibrinolysis was remained.

Hypercoagulation was observed in the blood of patients. Induced coagulation was accompanied by the increase of fibrinogen, inhibition of fibrinolysis and a high concentration of fibrin monomer complexes, which were considered the markers of thrombinemia.

he degree of hypercoagulability was reduced after the treatment. Fibrinolysis was stimulated, but did not reach the control level. Concentrations of fibrinogen and fibrin monomer complex were remained high. It meant that the threat of DIS syndrome was remained. The level of fibrin and depressed fibrinolysis was remained high.

Conclusions: Hypercoagulability was developed in the blood of elderly patients ill with chronic generalized periodontitis and coronary artery disease. In saliva procoagulant activity was increased and fibrinolytic activity was decreased. Basic therapy of CGP did not cause the essential correction of hemostasis.

PO 473

Mucosal Tolerance to Multiple Peptides Ameliorates Atherosclerosis in Rabbits

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Background: Atherosclerosis occurs naturally in humans, few primates, and pigs. Rabbits are very sensitive to cholesterol-rich diets and develop an atherosclerotic condition closely resembling human lesions. This rabbit model is highly reproducible with minimal inter and intra laboratory variation between animals. Mucosal route of antigen administration is an attractive method for inducing antigen-specific peripheral tolerance, the efficiency of which is dependent on the type and dose of antigen. Treatment with high and low dose of antigen results T cells anergy and expansion of antigen-specific Treg cells respectively, while intermediate dosed are not effective in inducing tolerance. We have earlier established the efficacy of a recombinant multi antigenic construct in inducing oral tolerance and preventing development of atherosclerosis in mice model.

Aim: The objectives of the present study was to induce oral tolerance to recombinant multi antigenic construct, expressing the epitopes from apolipoprotein B 100 (ApoB), heat shock protein (HSP60) and Chlamydia pneumonia outer membrane protein (Cpn) in normocholesterolimic rabbits and to evaluate the protective effect of tolerance in preventing development of atherosclerosis.

Methods: Four different doses of multi antigenic construct were given by oral route to New Zealand white rabbits in order to determine the dose required to induce tolerance. Optimum dose for inducing tolerance was determined by reduction in systemic T cell response, secretion of the anti-inflammatory cytokines, and suppression of antibody response. Protective effect of tolerance was studied with the optimum dose of recombinant antigen in groups of New Zealand white rabbits with appropriate controls. Rabbits were given a feed containing 0.5% cholesterol for 16 weeks to induce development of atherosclerosis. Quantification of atherosclerotic lesions was carried out in the aortic sinus sections stained with Elastic van Geison (EVG). Lipid content was analyzed by Oil-Red-O staining. Immunohistochemical analysis were carried out by indirect immunofluorescence.

Results: Rabbits were given five doses of recombinant antigen ranging from 5 to 320 ug per dose to determine the optimum dose. Based on the systemic T cell response, Treg activity and antibody levels to peptides, 80 μg/dose was found to be the optimum dose to induce tolerance in rabbits. During 16 weeks of cholesterol rich diet feed, the total serum lipid content increased in control as well as the treated animals. Tolerance to recombinant antigen resulted in increase in the number of Treg cells in peripheral lymphoid organs with antigen specific functional activity. Development of atherosclerosis was reduced by by 51.3% in rabbit aortic sinus and descending aorta following oral tolerance. Total lesional area in the aortic sinus reduced from 28.78 ± 2.83% in control to 14.78 ± 1.66% in tolerized mice. Protection against atherosclerosis was associated with reduction in lipid accumulation as seen by oil -red O staining, infiltration of inflammatory cells and changes in the expression of cytokines and chemokines in the developing lesion.

Conclusions: Our results suggest that oral administration of multi antigenic construct expressing ApoB, HSP60 and Cpn peptides induces peptide specific tolerance which can prevent early atherosclerotic plaque development of in a rabbit model of diet induced atherosclerosis.

PO 474

A genetic risk score for coronary artery disease in Asian Indians

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Background: Recent discoveries of single-nucleotide polymorphisms (SNPs) showing modest association with coronary artery disease (CAD) have spurred the development of genetic risk prediction models.

Aims: We sought to identify genetic markers of CAD in a representative cohort of Asian Indians and to build a genetic risk score for significant discrimination of CAD in combination with the traditional risk factors.

Methods: An initial panel of 61 variants from 42 genes selected from published genome-wide association studies and internal discovery were genotyped by Taqman assay in 500 CAD patients and 500 matched controls in the 1st stage of a two-stage process. The cohort was selected from the ongoing Indian Atherosclerosis Research Study (IARS)*. Significant SNPs were independently validated in Stage II in 534 cases and 534 matched controls and confirmed in the combined cohort. The main outcome measure was the association of specific SNPs with CAD and the combined ability of significant SNPs to discriminate CAD based on 'c' index.

Results: In Stage I, nine SNPs showed significant association with CAD. In Stage II, seven of the nine SNPs showed persistent association and belonged to two distinct loci: 9p21.3 CAD locus (rs10757278, rs2383206, rs10757274, rs1333049, rs4977574) and *CELSR2-PSRC1-SORT1* cholesterol locus (rs646776, rs599839) with strong linkage disequilibrium among the variants. Two representative SNPs, rs2383206 and rs646776, one from each cluster, carrying high allelic odds ratio, constituted the pilot genetic risk score (GRS). Weighted GRS model ($P = 7.88 \times 10^{-10}$) performed better than unweighted additive model ($P = 1.66 \times 10^{-9}$). Subjects in top weighted GRS quartile showed higher risk of CAD than the bottom quartile (OR 2.59, 1.77–3.80; $P = 1.09 \times 10^{-6}$), after adjusting for potential confounders. Prediction model that included 2 SNPs and conventional risk factors enhanced CAD discrimination (c index 0.787, 0.768–0.807) than conventional risk factors (c index 0.779) or SNPs alone (c index 0.574) ($P = 0.018$).

Summary/Conclusion: A pilot 2-SNP GRS shows 2.6-fold higher risk of CAD in Asian Indians, with modest discrimination, offering scope for further improvement with the addition of newer genetic variants.

* The rationale and design of IARS has been published in *Indian Heart journal* 2010,62:286–95.

PO 475

Non-invasive surrogate markers of coronary artery disease in Asian Indians

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Background: A close correlation has been reported between endothelial dysfunction in the coronary and peripheral circulation. Carotid intima media thickness (CIMT), measured by B-mode ultrasound, is one such marker which is expensive and requires trained personnel to perform the test. On the other hand, pulse wave velocity (PWV), arterial stiffness index (ASI) and ankle brachial index (ABI) markers reflect the extent of atherosclerotic disease in the peripheral arteries and can be measured by Periscope, an oscillometry based monitoring and PC based acquisition and analysis system. This tool is cost-effective, easy to perform and ideal for performing large scale screening of cardiovascular disease in the population..

Aims: We studied the association of CIMT and peripheral vascular resistance markers in an unselected cohort of angiographically proven CAD patients and their unaffected family members, assessed the correlation between the different surrogate markers and tested their ability to discriminate CAD independently as well as in combination with the classical risk factors.

Methods: CIMT was measured by B-mode ultrasound in 380 (64 CAD affected and 316 unaffected) subjects while, ankle and brachial PWV, ASI and ABI were measured using Periscope in 710 (125 CAD affected, 585 unaffected) subjects participating in the ongoing Indian Atherosclerosis Research Study. The incremental value of clinical indices in discriminating CAD beyond the established risk factors was assessed though analysis of area under the ROC curve (A[ROC]).

Results: Both mean CIMT (0.68 ± 0.022 mm vs. 0.52 ± 0.010 mm) and max CIMT (0.84 ± 0.026 mm vs. 0.66 ± 0.012 mm ($P < 0.0001$) were higher in CAD affected as compared to unaffected subjects. Further, CAD patients showed higher PWV (1737.18 ± 51.89 m/s vs. 1465.84 ± 18.26 m/s) ($P < 0.0001$) and ASI (41.36 ± 15.92 mmHg vs. 37.56 ± 13.48 mmHg) ($P = 0.018$) as compared to the unaffected subjects. Subjects in the top tertile of mean IMT showed 20-fold increased risk of CAD (OR 20.42, 6.13–68.04 ($P < 0.0001$) before and 6-fold higher risk (6.02, 1.46–24.80 ($P = 0.013$) after adjusting for potential confounders. Similar comparisons yielded 7-fold increased risk of CAD (OR 7.25, 95% CI 3.87–13.60 ($P < 0.0001$) for PWV; however, statistical significance was lost following covariate adjustment. Males showed higher levels of CIMT and PWV than females. There was robust correlation between mean and maximum IMT ($r = -0.92$, $P < 0.0001$) and between PWV and ASI ($r = 0.13$–0.31, $P < 0.001$). Modest correlation was also observed between CIMT and ASI ($r = 0.14$, $P = 0.01$). Contrary to published reports, ABI did not show significant association with CAD in our study. Mean CIMT was higher in the presence of plaque and lower in CAD patients on statins. The risk prediction model that combined the classical risk factors, IMT and Periscope markers yielded the highest c index (AUC 0.906, 95% CI 0.880–0.932) than any of the markers alone, with 34% reclassification in the intermediate risk group.

Summary/Conclusion: Both CIMT and Periscope markers show significant association with CAD, suggesting that they can complement the established risk factors in predicting the risk of CAD in Asian Indians.

PO 476

Non-invasive assessment of extent of atherosclerotic disease, CVD risk factors and atherothrombotic biomarkers in Asian Indians

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Background: A close correlation has been reported between endothelial dysfunction in the coronary and peripheral circulation. Carotid intima media thickness (CIMT), measured by B-mode ultrasound, is one such marker which is expensive and requires trained personnel to perform the test. On the other hand, pulse wave velocity (PWV), arterial stiffness index (ASI) and ankle brachial index (ABI) markers reflect the extent of atherosclerotic disease in the peripheral arteries and can be measured by Periscope, an oscillometry based monitoring and PC based acquisition and analysis system. This tool is cost-effective, easy to perform and ideal for performing large scale screening of cardiovascular disease in the population.

Aims: We studied the association of CIMT and peripheral vascular resistance markers in an unselected cohort of angiographically proven CAD patients and their unaffected family members, assessed the correlation between the different surrogate markers and tested their ability to discriminate CAD independently as well as in combination with the classical risk factors.

Methods: CIMT was measured by B-mode ultrasound in 380 (64 CAD affected and 316 unaffected) subjects while, ankle and brachial PWV, ASI and ABI were measured using Periscope in 710 (125 CAD affected, 585 unaffected) subjects participating in the ongoing Indian Atherosclerosis Research Study (IARS)*. The incremental value of clinical indices in discriminating CAD beyond the established risk factors was assessed though analysis of area under the ROC curve (A [ROC]).

Results: Both mean CIMT (0.68 ± 0.022 mm vs. 0.52 ± 0.010 mm) and max CIMT (0.84 ± 0.026 mm vs. 0.66 ± 0.012 mm ($P < 0.0001$) were higher in CAD affected as compared to unaffected subjects. Further, CAD patients showed higher PWV (1737.18 ± 51.89 m/s vs. 1465.84 ± 18.26 m/s) ($P < 0.0001$) and ASI (41.36 ± 15.92 mmHg vs. 37.56 ± 13.48 mmHg) ($P = 0.018$) as compared to the unaffected subjects. Subjects in the top tertile of mean IMT showed 20-fold increased risk of CAD (OR 20.42, 6.13–68.04 ($P < 0.0001$) before and 6-fold higher risk (6.02, 1.46–24.80 ($P = 0.013$) after adjusting for potential confounders. Similar comparisons yielded 7-fold increased risk of CAD (OR 7.25, 95% CI 3.87–13.60 ($P < 0.0001$) for PWV; however, statistical significance was lost following covariate adjustment. Males showed higher levels of CIMT and PWV than females. There was robust correlation between mean and maximum IMT ($r = -0.92$, $P < 0.0001$) and between PWV and ASI ($r = 0.13$–0.31, $P < 0.001$). Modest correlation was also observed between CIMT and ASI ($r = 0.14$, $P = 0.01$). Contrary to published reports, ABI did not show significant association with CAD in our study. Mean CIMT was higher in the presence of plaque and lower in CAD patients on statins. The risk prediction model that combined the classical risk factors, IMT and Periscope markers yielded the highest c index (AUC 0.906, 95% CI 0.880–0.932) than any of the markers alone, with 34% reclassification in the intermediate risk group.

Summary/Conclusion: Both CIMT and Periscope markers show significant association with CAD, suggesting that they can complement the established risk factors in predicting the risk of CAD in Asian Indians.

* The rationale and design of IARS has been published in *Indian Heart journal* 2010,62:286–95.

PO 477

Association of periodontal disease, extent of peripheral vascular changes, oral pathogens and risk of coronary artery disease in Asian Indians

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Background: There is growing evidence from published studies to support a close association between oral health and cardiovascular disease. Periodontal disease induces early vascular changes in peripheral circulation, while several obligate oral pathogens have been detected in both sub gingival plaques and atheromatous plaque specimens.

Aims: The association of Periodontal disease with the extent of peripheral vascular disease and the association of two oral pathogens with oral and Coronary Artery Disease (CAD) were tested in two subsets of Asian Indians.

Methods: Individuals with either Gingivitis ($N = 575$) or Periodontitis ($N = 312$) were identified based on standard clinical criteria from the dental out-patient unit from a semi-urban charitable hospital in Bangalore. These subjects were then assessed for the extent of peripheral vascular disease using non-invasive oscillometry based Periscope, which measures pulse wave velocity (PWV), arterial stiffness index (ASI) and ankle brachial index (ABI).

Further, saliva samples were collected from 56 Periodontitis and 29 Gingivitis cases along with 51 angiographically proven CAD patients who were recruited from a Cardiac specialty hospital in Bangalore and analyzed for presence of Porphyromonas gingivalis (Pg) and Aggregatibacter actinomycetemcomitans (Aa). Salivary DNA was extracted by in-house method and relative quantitation (RQ) of Pg and Aa were determined by Taqman assay by amplifying pathogen-specific gene targets, 16srRNA and IktA, respectively, and 16s universal bacterial rRNA as endogenous control.

Results: Periodontitis cases showed greater extent of peripheral arterial disease with higher measures of pulse wave velocity and arterial stiffness index as compared to Gingivitis cases ($P < 0.0001$). Furthermore, there was higher prevalence of Pg in CAD patients based on RQ levels (0.85 ± 0.12) relative to Periodontitis (0.32 ± 0.04) or Gingivitis (0.13 ± 0.03) ($P < 0.0001$) cases, after correcting for expression of endogenous control, age and gender. Top RQ quartile of Pg was associated with nearly 8-fold higher risk for CAD as compared to the bottom quartile, after adjusting for potential confounders (OR 7.85, 95% CI:1.77–34.83). Subjects with Periodontitis showed higher prevalence of diabetes and hypertension compared to Gingivitis while Pg expression was also elevated in the presence of these classical cardiovascular risk factors. In variance with other published reports, Aa was detected only in three individuals in this study.

Summary/Conclusion: Our findings of strong association of Periodontitis with peripheral vascular changes and the association of Porphyromonas gingivalis with higher risk of CAD in this Asian Indian cohort provides new opportunities for reducing cardiovascular burden by spreading awareness of oral hygiene in the population.

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A comprehensive genetic analysis of the 9p21.3 CAD risk locus in Asian Indians

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Background: The 9p21.3 region, commonly referred to as the '*9p21.3 CAD risk interval*', encompasses multiple genetic variants in tight linkage disequilibrium, and is considered to be the best replicated genetic region for coronary artery disease (CAD).

Aims: We aimed to study the association of common variants in the 9p21.3 locus with incident CAD, analyze the expression profile of putative candidate genes and ANRIL, a non-coding RNA and their splice variants, supported by *in vitro* functional studies on Human Aortic smooth muscle cells (HuAoSMCs).

Method: A cohort of 1034 cases with positive family history of cardiovascular disease and 1034 age and gender matched controls were selected from the ongoing Indian Atherosclerosis Research Study (IARS) for the genetic association study, while an independent cohort of 100 cases with family history and 100 healthy controls, without family history of cardiovascular disease were included for the gene expression study. Five 9p21.3 common variants, rs10757278, rs10757274, rs2383206, rs1333049 and rs4977574, were genotyped by TaqMan allelic discrimination assay. Relative expression of following candidate genes and splice variants were measured by Taqman assay – *C9orf5, MTAP 1*, *MTAP 2*, *P16INK4a*, *P14ARF* (*CDKN2A*), *p15INK4b* (*CDKN2B*) and three splice variants of *ANRIL*, a non coding RNA – *NR_003529, NR_003529 + DQ485454 and EU741058*. Absolute copies of *DQ485454* were determined by subtracting copies of *NR_003529* from *NR_003529 + DQ485454*. Mechanism of regulation of ANRIL splice variants and neighboring genes in the 9p21.3 locus was determined by transfecting HuAoSMCs with siRNA that target either exon 1–2 or exon 18–19 of ANRIL.

Results: All five variants showed significant independent association with CAD. rs2383206 showed the highest individual association with CAD (OR 2.02, 95% CI 1.56 −2.62, $P < 0.0001$) and an adjusted OR of 2.55 (1.33–2.88) ($P = 0.004$) when combined with rs10757278. Analysis of area under the RIOC curve (A[ROC]) revealed that the risk prediction model that included conventional risk factors (CRFs), rs2383206 and rs10757278 showed a higher 'C' index (0.790, 95% CI 0.770 −0.810) than either CRFs (0.783, 0.763–0.803) or variants (0.561, 0.536–0.586) alone. The *GAAAA* haplotype showed significant protective association against CAD when compared to the common *CGGGG* haplotype (OR 0.45, 95% CI 0.27–0.77, $P = 0.004$). All the five 9p2.1 variants were in strong LD ($r = 0.90$–98). ANRIL splice var-

iant, *DQ485454,* showed higher expression while *CDKN2A* and *CDKN2B* showed lower expression in cases relative to controls. Subjects carrying the *GG* genotype showed higher expression of *EU741058* and lower expression of *P16INK4a* as compared to those with *AA* genotype. This pattern was consistent across all five variants. The AoSMCs transfected with siRNA specific for exon 18–19 but not exon 1–2 showed lower expression of full-length *ANRIL* (*NR_003529*) and p15INK4b, implying that the activity of this tumor suppressor gene is regulated through epigenetic mechanism via ANRIL.

Summary/Conclusion: This study provides further evidence on the importance of the 9p21.3 variants for risk of CAD in Asian Indians. The risk alleles in this locus appear to modulate expression of ANRIL, which in turn regulate the activity of adjacent tumor suppressor genes through epigenetic mechanisms.

PO 479

The polymorphism Sst? in APOC3 gene is not associated with abnormal lipid profile in Brazilian dyslipidemic children

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Background: Polymorphisms in Apolipoprotein C3 (APOC3) gene have been associated with increased triglyceride levels, which may favor the development of cardiovascular disease.

Aims: This study aimed to investigate the relationship between SstI polymorphism (alleles C and G) in the APOC3 gene and lipid profile in dyslipidemic children from Brazil.

Methods: The study included 49 dyslipidemic children and 56 healthy children (control group), selected in the Academic Hospital at the Federal University of Minas Gerais, Brazil, after informed consent by their relatives. The study was approved by the ethics committee of the respective university. DNA samples were amplified by PCR followed by digestion with restriction enzyme *SstI* and polyacrylamide gel electrophoresis. Statistical analysis was performed using SPSS v.13.0.

Results: As expected, triglycerides ($P = 0.001$), total cholesterol ($P < 0.001$) and LDL-C ($P < 0.001$) levels were higher in dyslipidemic children compared to control group. There was no significant difference in the frequency of SstI polymorphism between case/control groups: 0.88 for the CC and 0.12 for the CG genotype in the case group, and 0.87 for CC and 0.13 for the CG in the control group ($P = 0.78$, OR = 0.74 and CI = 0.21 to 2.54). The difference in the allele frequency between normolipidemic and dyslipidemic groups was not significant. There was no association between the allele G or C and the triglyceride levels ($P = 0.97$), total cholesterol ($P = 0.59$), LDLc ($P = 0.62$), and HDL cholesterol ($P = 0.66$).

Conclusions: The results suggest that the polymorphism SstI is not associated with abnormal lipid levels in a small set of Brazilian dyslipidemic children, however, it is necessary to evaluate a larger sample group to confirm this finding.

Supported by CNPq, CAPES and FAPEMIG.

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Improvement of risk assessment with multi-pathway representative markers for coronary artery disease in the indian population

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Background: Asian Indians exhibit higher risk for developing coronary artery disease (CAD) at a younger age and many risk prediction algorithms underestimate this risk. We assessed multiple biomarkers from different patho-physiological pathways in assessing the risk to CAD in Indian population.

Aims: In this study we evaluated association of markers representative of inflammation (IL6, hsCRP, sPLA2), oxidative stress (Myeloperoxidase), Tumor necrosis factor (TWEAK), renal function (Cystatin C) and macrophage activation (Neopterin) pathways for developing multi-marker risk assessment in Asian Indians.

Methods: Eight hundred and sixteen age and gender matched subjects (408 CAD affected and 408 unaffected) were selected for a case control study from ongoing Indian Atherosclerosis Research Study (IARS). The information regarding inclusion, exclusion criteria and process of sample collection, and data storage have been published (Indian Heart J, 2010;6:286–95). All the blood samples were collected after overnight fasting of 12–14 h. Biomarkers Ilterleukin-6 (IL6), high sensitive C-reactive protein (hsCRP), secretory phospholipoprotein-2 (sPLA2), Myeloperoxidase (MPO), Tumor necrosis factor-like weak inducer of apoptosis (TWEAK), Cystatin-C (Cys-C) and Neopterin were assayed using ELISA kits according to the manufacturer's instructions. Logistic Regression, C-statistics and other statistical analysis were performed using SPSS ver17 software.

Results: A 49.5% of CAD affected subjects were diabetic and 45.8% had hypertension which was almost twice that of the unaffected group. A significant proportion of CAD affected subjects were on Statin (68.9%), Betablockers (60.8%), and Antiplatelet (87%) therapies. Higher levels of four biomarkers (IL6, sPLA2, Myeloperoxidase, Neoptrin) out of the seven were observed in CAD affected subjects in comparison to unaffected. In Logistic Regression models it was observed that individual markers were significantly associated with CAD namely IL6 with odds ratio of 1.74 (95% CI 1.14–2.66, *P*-value = 0.011), Neopterin odds ratio of 1.55 (95% CI 1.02–2.37, *P*-value = 0.041) and MPO odds ratio of 1.71 (95%CI 1.12–2.61, *P*-value = 0.013) for fourth quartile in comparison to first. The adjustment of above models to conventional risk factors did not yield any significant improvement in the odds ratio. However, for IL6, the odds ratio improved to 1.87 (95%CI 1.15–2.97, *P*-value = 0.011) after adjustment with lipids (triglycerides, total cholesterol, LDL and HDL). The C-statistics analysis was performed to understand the contribution of these three biomarkers to risk assessment wherein a combination of IL6, Neopterin and MPO alone gave a AUC of 0.647 (95%CI 0.610–0.685, *P*-value = 0.001) which improved to 0.825 (95%CI 0.797–0.853, *P*-value = 4.43×10^{-53}) with lipids. The net-reclasification analysis suggests that final model of combination of biomarkers reclassify 28 upwardly and 55 downwardly in unaffected and 4 upwardly and 6 downwardly in affected group.

Conclusions: Our data suggests that biomarkers from three different pathways namely, inflammation (IL6), oxidative stress (MPO) and macrophage activation (Neopterin) play a major role in risk assessment. For risk stratification by multimarker analysis, a combination of these three biomarkers add significant value proposition to the standard risk factors.

PO 481

The comparison of efficacy between recombinant activated factor VII (ARYOSEVEN®) and NOVOSEVEN® in patients with congenital factor VII deficiency

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Introduction: Recombinant factor VIIa is approved for the treatment of bleeding episodes in congenital factor VII deficiency, hemophilia

A/B with an inhibitor and Glanzmann's thrombasthenia. Factor VII deficiency is an autosomal recessive bleeding disorder. This study is a comparison between the efficacy of a new biosimilar recombinant factor VIIa concentrate (Aryoseven®) and Novoseven® in patients with congenital factor VII deficiency.

Methods: A multicenter double-blind randomized clinical trial was conducted in comprehensive hemophilia care centers in Iran. Inclusion criteria: 1- congenital factor VII deficiency with more than one episode of bleeding per month, 2- age > 2 years. Exclusion criteria: 1- any other coagulation disease, 2- prophylaxis therapy with rFVIIa during the last month, 3- platelet count < 50,000, 4- presence of a neutralizing anti-factor VII antibody.

Consenting patients who met these criteria were randomly assigned to an intervention or a control group. The patients in the intervention group (Group A) received the biosimilar recombinant factor VIIa (AryoSeven) and the patients in the control group (Group B) received Novoseven®. rFVIIa (Novoseven® or AryoSeven) dosage was 30 μg/kg, intravenously, once per week for 4 weeks.

Primary outcome was plasma FVII: C activity IU/dl 20 min after injection, with a level of FVII: C below 30 IU/dl considered a treatment failure. Secondary outcome was self-reported bleeding frequency. FVIIa was measured by STACLOT® VIIa-rTF made by Diagnostica Stago. FVII activity has been checked by recombinant thromboplastin. Sixty-six patients were enrolled and allocated to the two groups by consecutive balanced block randomization tables (fixed block size of 4) per hemophilia center. Group A (35 patients, 53%) received Aryoseven® and group B (31 patients, 47%) received Novoseven®.

Results: The mean age was 21.2 (range: 2–64 years) and 48.5% (32 patients) were male. Median (IQR) plasma level of FVIIa activity in group A and B was 1.6 (1.1–20.0) IU/ml and 6.0 (1.1–27.0) IU/ml before injection. All patients in the two groups had FVIIa concentrations exceeding 30 IU/ml 20 min after rFVIIa injection. There was no difference in increment of FVIIa activity level 20 min after rFVIIa injection between two groups in any of the 4 weeks (for example in week 4: group A: 222.0 (range 192.7–504.3), group B: 216.5 (range 175.4–486.5), $P = 0.481$).

The severity of bleeding was also similar between the two groups, with a similar reduction in bleeding compared with the month prior to treatment (group A -1.5 bleeding events per month, group B -1.53).

Reported side effects were minor (headache, nausea) and occurred in similar frequency in both groups [Group A: 8 (24.2%), Group B: 3 (10.0%)].

Conclusion: AryoSeven® is similar to Novoseven® in post-injection FVIIa activity, as well as in clinical efficacy. The frequency of side-effects was also similar, although the study was underpowered to detect differences in rare complications between the two compounds.

PO 482

New data on the non-invasive method for the study of the haemostatic system

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Aim: The aim of the study to examine the state of the hemostatic system in health and in various of the disease (coronary heart disease, diabetes mellitus type 1 and 2, hyperthyroidism, etc.) through the analysis of the speckle nature of the scattering of coherent light from the skin surface.

Methods: We, together with the union Elfi-tech Ltd (Israel) developed methods to noninvasively assess the degree of plasma viscosity, kinetics of aggregation of blood cells, cardiovascular system, haemostasis, and endothelial dysfunction. With the proposed method in terms of blood flow and stop it determined the following indicators (both before and after clamping): (1) Perfusion index before clamping (characterizes the intensity of blood flow), (2) The index of blood flow (the product of the velocity on the volume of flowing blood), (3) Index corresponding to the blood flow velocity (relative value), (4) SKF-index, which characterizes the biological age of the vessels, (5) Congestion index – the ratio of blood flow after occlusion of blood flow to occlusion, (6) Diffusion coefficient (DC), the corresponding rate of Brownian motion of red blood cell. Simultaneously the state of the aggregation function corpuscles, blood coagulation, the basic content of natural anticoagulants and fibrinolysis were assessed.

Results: It was established that the index of SKF correlated directly with biological age. After 60 years dependence on age is much less pronounced, which is mainly due to the direct or indirect diseases of the circulatory system, proceeding on a background of atherosclerosis. Patients, especially with endothelial dysfunction, the index was higher than in healthy of the corresponding age.

High correlation relationship between SKF and a DC on one side, and the rate of blood coagulation, platelet aggregation, formation of red blood cell-leukocyte-platelet aggregates, the concentration of SFMC and D-dimer, INR, and protein C.

When receiving antiplatelet (aspirin, trental, clopidogrel, etc.) and especially of indirect anticoagulants (warfarin) SKF index decreases. In the case of resistance to antiplatelet SKF little changed. Therefore, using the proposed non-invasive method for studying the activity of blood coagulation can be controlled receiving of indirect anticoagulants (warfarin), and also to judge the resistance to antiplatelet agents.

When registering speckles, which are the result of the Brownian motion of red blood cells, we developed a new method to determine blood clotting time *in vitro*.

In patients with damage to the cardiovascular system, leading to endothelial dysfunction, SKF index to the action of heat does not change or increases.

Conclusion: The developed methods proved to be highly sensitive and can be introduced in clinical practice to assess the aggregation activity of blood cells, vascular-platelet hemostasis and coagulation, and endothelial dysfunction.

PO 483

Relationship of hla-phenotype with some hemostasis profiles

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In recent years, there were many studies about a single integrated system of protection that is formed by the immune system, hemostasis and nonspecific resistance of the body. The existence of immune regulation of physiological functions including hemostasis is determined. Hence, assume the existence of associations between HLA-specificities and hemostasis.

Aim: The goal of our study was to discover interrelations between HLA-phenotype of healthy people of Buryat nationality and hemostasis profiles.

Materials and Methods: we examined 53 healthy donors of Buryat nationality who live in Aga Buryat Autonomous District of Russia in the age of 18–34 years, who are not in relation with each other, with an equal sex ratio.

Nationality was established by polling on the direct relatives of at least 2 or 3 previous generations.

Coagulation tests included determination of activated partially thromboplastin time (APTT), prothrombin time and international normalized ratio (INR), thrombin time, activity of protein C, and antithrombin-III, fibrinogen concentration and XIIa-dependent fibrinolysis.

Antigenic identity was defined in the standard two-stage microlymphocytotoxic test (Terasaki PI, Bernoco D., Park MS et al., 1978) 19 antigens of locus A and 38 antigen of I locus B I class were identified.

Statistical analysis of the data included calculation of the degree of incidence of HLA-antigens, genes, haplotype frequencies, values of linkage disequilibrium, the authenticity of differences and genetic distance between populations for HLA-alleles (P. Mattiuz et al., 1970 Brownlee, KA, 1997). indetifying specific indicators of HLA-antigens, that mark the level of a particular parameter, set the average value of hemostasis in subgroups of healthy people, selection based on the presence or absence of each of the investigated HLA-antigens, and compared using T. Student's test.

Results: Those with a phenotype B51 typically have reduced platelets count and shortened prothrombin time. However, carriers of B62 have increased platelets count, accelerated XIIa-dependent fibrinolysis and reduced concentration of fibrinogen. At the same time, carriers of antigens HLA-B40 and B5 have lower concentration of protein C, whereas in those with B7 phenotype is increased. Specific inhibition of fibrinolysis is typical for people with HLA-B40, they also tend to have low fibrinogen.

Marker B12 is associated with prolongation of prothrombin time and decreasing the concentration of antithrombin-III (AT-III). The carriers of A2 have shortened APTT, and those with A23 antigen have it prolonged. For people with HLA-antigens A1, A9, B7, B12, B44 characteristic reduction in the concentration of AT-III.

It was found that the value of prevalence of parameters associated with a particular HLA-antigen of the total number of investigated profiles of hemostasis respective to the frequency of HLA-antigen in the population, being expressed by the index F/A, is the highest for phenotypes A3 and B12. These antigens are found with average or below-average rate, but are associated with the greatest number of the studied parameters.

Conclusion: The data presented shows close relationship between antigens HLA – class 1 with hemostasis, and confirms that the immune system, vascular-platelet hemostasis, blood coagulation and fibrinolysis are in single humoral defense system.

PO 484

Variations of coagulation parameters during orthotopic liver transplantation – single center experience

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Background: During liver transplantation values of coagulation parameters show significant changes, while in the postoperative period a gradual normalization in the function of the coagulation system occurs.

Aims: Aims of the study were to laboratory monitor changes in the coagulation system intraoperatively and postoperatively and to evaluate the dynamics of normalization of the analyzed parameters.

Methods: There were 20 orthotopic transplantations performed at the Clinical Centre of Vojvodina (KCV) in the period from June 2008 to November 2012. The research was conducted on a group which included 20 patients, 6 women and 14 men. In the majority of cases the aetiology was liver cirrhosis caused by viral hepatitis B and C, which was diagnosed in 14 patients in total. Laboratory testing was carried out at the laboratory of the Department of Thrombosis, Haemostasis and Haematology Diagnostics of the KCV. The test samples were taken intraoperatively and postoperatively. The following coagulation parameters were tested: platelet count (PLT), activated partial thromboplastin time (aPTT), prothrombin time (PT), thrombin time (TT), fibrinogen level (FBG), euglobulin clot lysis time (ECLT), D-dimer, antithrombin activity (AT) and anti-Xa level.

Results: During transplantation the most pronounced coagulation system changes were: thrombocytopenia (93 ± 62.3), prolonged aPTT (1.73 ± 0.8), PT (1.58 ± 0.4) and TT (1.94 ± 1.4), hypofibrinogenemia (2.08 ± 0.4), accelerated fibrinolysis (34 ± 23.5), increase of D-dimer values (1261 ± 1167.1) and a decrease of AT activity (60 ± 16.9). The most prominent disturbances during transplantation was verified in anhepatic phase, predominantly high fibrinolytic activity. Further laboratory monitoring after transplantation from postoperative day 1 revealed a progressive normalization in the values of the tested coagulation parameters, reaching physiological levels of all parameters of the coagulation system on the postoperative day 14, except for a sustained high value of D-dimer (2516 ± 1077.6). The level of anti-Xa activity was within the prophylactic range (0.25 ± 0) after the introduction of low-molecular-weight heparin thromboprophylaxis.

Summary/Conclusions: Permanent monitoring of the coagulation system parameters in liver transplantations is very important, as it enables the use of optimal substitution therapy during and after transplantation, as well as an adequate *postoperative prevention of venous thromboembolism.*

PO 485

Implication of post-translational modifications on the function of key haemostatic proteins

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Background: Post-translational modifications (PTMs) such as glycosylation and phosphorylation play an important role in the function of haemostatic proteins and are critical in the setting of disease. Such secondary level changes to haemostatic proteins have wide ranging effects on their ability to interact with other proteins. The PTM of haemostatic proteins and the subsequent impact on protein structure and function, specifically via interaction with other proteins as well as with novel anticoagulants that target specific proteins represents an important area of research.

Aims: To summarize the recently investigated PTMs associated with haemostatic proteins and the implications these modification have on protein function.

Methods: We performed a review of literature published between 2005 to 2008, limited to English and human studies, with the following search terms; post translational modification & haemostatic & protein, Fibrinogen, Factor VIII, tissue factor, von Willebrand Factor (vWF), antithrombin (AT).

Results: The main PTM described with vWF is N-Glycosylation that prevents adhesion of the protein to platelets. This PTM occurs in 13% of the adult population and the prevalence in children is not well documented.

Tissue factor can undergo phosphorylation, which causes a change in the long sugar chain where protein kinases transfer phosphate groups to the hydroxyl side chains. This typically occurs in the setting of protease-activated receptor-2 signaling pathways for coagulation diseases. In adults phosphorylation of TF occurs in 90–95% of hydroxyl side chains with the effect on child TF not well described. This event increases platelet affinity to other coagulation factors and changes signaling pathways.

Factor VIII is commonly sulfated. For normal physiological function of Factor VIII 6 tyrosine residues of the protein need to be sulfated. The degree of sulfation at various sites on the protein has varying implications on the function of Factor VIII.

Antithrombin (AT) function is highly sensitive to PTM and this protein commonly undergoes a polymerization where arginine residues are converted to citrulline. Increased citrullination can lead to increased risk of thrombosis. Adults with rheumatoid arthritis and adenocarcinoma show significantly higher levels of citrullinated AT compared to the healthy population.

Tyrosine residues are the main target for PTM of Fibrinogen by nitration. It is shown that fibrinogen polymerizes faster when tyrosine sites 292 and 422 are nitrated. This PTM amplifies viscoelastic properties of Fibrinogen and carries a prothrombotic risk factor particularly for

patients with Coronary Artery Disease who show a 30% increase in nitrated fibrinogen compared to the healthy population.
Summary/Conclusion: Common haemostatic proteins have recently been shown to be susceptible to PTM by citrullination, sulfation nitration, glycosylation and phosphorylation. These modifications have implications on haemostatic protein function. Future studies are required to compare and contrast the presence of these modifications in child haemostatic proteins and the mechanisms by which they arise in adults.

PO 486
Proteomics of defined platelets

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Background: Platelets play an important role not only in the prime of coagulation but also in the final event of fibrin stabilization. Too much number of platelets in circulation is lead to the clot formation obstructing blood vessels and too low is resulted in the bleeding. Thus a homeostatic regulation of platelets is necessary to prevent unwanted bleeding and blood vessel obstruction via uncontrolled clot formation.
Aim: Since platelets are highly important in wound healing event, we decided to perform a proteomics (nLC-MS/MS) analysis of defined and highly pure platelets. The protein content of platelets helps us to gain more knowledge about the cells without nucleus.
Methods: As first step, the highly pure Platelets were isolated by iodixanol-gradient centrifugation. The protein coverage was subsequently assessed by nLC-MS/MS.
Results: Orbitrap LTQ-XL (LC-MS/MS) analysis detected approximately 110 proteins divided in many functional clusters of proteins such as adhesion molecules, growth factors, coagulation factors (FV, vWF and FXIIIA), immune system and many novel proteins.
Conclusion: A collection of defined and highly pure platelets proved to be essential for correct protein content information of these cells. nLC-MS/MS proved to be a powerful tool for the analysis of proteins synthesized by platelets.

PO 487
A dual role for monocytes in coagulation and fibrinolysis cascades: non-canonical coagulation factor production

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Background: Coagulation/fibrinolysis system is essential for wound healing after a vascular injury. In canonical haemostasis, synthesis of coagulation factors is exclusively restricted to liver, platelets and the endothelium.
Aim: We decided to challenge this classical interpretation by measuring coagulation factors production in human primary cells.
Methods: The mRNA levels of prothrombinase complex (FV and FX), in tenase complex (FVIII), FVIII-stabilized protein (vWF), fibrin clot stabilizer (FXIIIA) and lyzing (thrombomodulin = TM) were measured in nine different pure human primary cells with and without LPS treatment.
Results: FX mRNA was expressed by fibroblasts, visceral Preadipocytes/adipocytes and hepatocytes, but not macrophages and other cells. All cells express FVIII except endothelial cells. Fibroblasts, endothelial cells and macrophages synthesize TM, but not FV. Since monocytes and platelets express FV and FXIIIA, both cells prime coagulation activation and stabilize the fibrin clot. Vascular-related cells that express FV, FX was not detected and *vice versa*. We called this phenomenon; *FV/FX expression law*. After wound healing, monocytes-released TM possibly destabilizes and lyzed the fibrin clot via 1-FXIIIA-breakdown and 2-activated protein C respectively.
Conclusion: This study provides evidence for the dual role of monocytes in coagulation/fibrinolysis systems. our data suggest that congenital problems in FV production can be rationally treated by allogeneic bone marrow transplantation.

PO 488
Hemostatic alterations in mice model produced by mapanare snake venom (Bothrops isabelae) from Venezuelan Andes

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Background: Venom from *Bothrops isabelae*, which belongs to the Viperidae family, contains a complex mixture of components with many biological functions that can act on the haemostatic system. In Venezuela, Bothrops snakes are responsible for more than 80% of all recorded snakebites, being the most important species *Bothrops colombiensis, B. venezuelensis, B. atrox* and *B. isabelae*.
Aim: Evaluate the lethal dose (LD_{50}) in C57BL/6 mice and determinate the hemostatic alterations in this experimental model.
Methods: Lethality of *B. isabelae* crude venom was determined by intraperitoneal (IP) injections into C57BL/6 mice and expressed as LD_{50}, a value that was calculated according to the method of Spearman-Karber (1978). Male mice were injected with a sub-lethal dose of crude venom in 200 μL of sterile saline solution and control mice were injected with 200 μL of sterile saline solution. Blood samples, about 1 mL per mouse, were collected by cardiac puncture under anaesthesia in CO_2 chamber. Hematologic (hemoglobin, hematocrit, leukocyte count, differential leukocyte count and platelets count) and hemostatic parameters (PT -Prothrombin Time, aPTT -activated Partial Thromboplastin Time, TT -Thrombin time, fibrinogen and endogenous fibrinolytic activity) were measured at 48 and 96 h after venom injection.
Results: LD_{50} of *B. isabelae* crude venom was 134 μg/mice (6.7 mg/Kg). In comparison at control mice, the sub-letal dose (50 μg/mice) of crude venom at 48 h after IP injection induced a shorter in aPTTa (20.3 ± 1.3 vs. 25.1 ± 1.4 s) and TT (11.9 ± 0.7 vs. 21.1 ± 0.6 s) ($P < 0.01$), without variation in PT (13.1 ± 0.8 vs. 13.2 ± 0.7 s). Furthermore, there was a decrease in fibrinogen concentration (1.7 ± 0.5 vs. 2.71 ± 0.42 mg/dL) and an increased in the endogenous fibrinolytic activity (144.92 ± 34.35 vs. 40.12 ± 24.01 mm^2), ($P < 0.01$). In hematological parameters was observed that venom induced a decrease in hemoglobin concentration (10.7 ± 0.6 vs. 12.8 ± 0.7 g/dL), hematocrit (31 ± 2 vs. $39 \pm 2\%$), leukocytes (2313 ± 185 vs. 3298 ± 698 mm^3), platelets (285 ± 60 vs. $701 \pm 53 \times 10^9$/L) and increased in lymphocyte percentage (84 ± 3 vs. $65 \pm 11\%$), ($P < 0.01$). At 96 h, all the parameters above were similar to those obtained for the controls, except leukocyte, platelets, hemoglobin and hematocrit.
Conclusions: These findings are related with previous *in vitro* assays of coagulant and fibrinolytic activities induced by *B. isabelae*. In this sense, our study is the first report of the effect in mice on hemostasis by this venom. In addition, our results suggest that C57BL/6 mice represent a useful model to understand the hemostatic alterations induced by snake venoms, allowing a deeper understanding of the action mechanisms for future therapeutic at this level.

PO 489

Anaemic comorbidity is associated with coagulation dysfunction in patients with heart failure

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Background: Anaemic comorbidity is associated with an adverse prognosis in the HF population. Anaemia-related malnutrition and vascular inflammation may contribute to coagulation dysfunction.
Aims: This study investigated whether anaemic comorbidity influences plasma procoagulant microparticles (MPs) and MP-mediated thrombin generation (TG) in patients with heart failure (HF).
Methods: According to the WHO criteria for anaemia, 109 HF patients were divided into three groups: non-(N-A, $n = 77$), light-(L-A, $n = 16$), and moderate/several-(M/S-A, $n = 16$) anaemic groups. Dynamic TG, procoagulant MPs, coagulation factors, and peroxide/pro-inflammatory biomarkers in plasma were analysed in these HF patients and 33 normal counterparts (N-C).
Results: All HF groups revealed the reduction of dynamic TG by lengthening lag time, lowering endogenous thrombin potential, and decreasing peak height and rate of TG. M/S-A exacerbated the extent of HF-declined TG, whereas no significant difference in TG between various HF groups occurred following depletion of MPs in plasma. Moreover, N-A, L-A, and M/S-A had lower tissue factor (TF)-rich and phosphatidylserine-exposing MPs that were derived from leukocytes ($CD45^+$), platelets ($CD41a^+$), and erythrocytes ($CD235a^+$), as well as, higher endothelial ($CD144^+$) MPs than N-C. However, M/S-A simultaneously lowered TF activity and tissue factor pathway inhibitor (TFPI) level, which were accompanied by heightened plasma myeloperoxidase and interleukin-6 concentrations, compared to N-A and L-A did. Additionally, both TF activity and TFPI level were negatively correlated with the TG rate in HF patients.
Conclusion: HF decreases blood cell-derived procoagulant MPs, increases vascular endothelial shedding, and reduces dynamic TG in plasma. Furthermore, M/S-A augments HF-declined TG, possibly by depressing TF-related coagulation activity.

PO 490

Counselling by healthcare professional for patients taking oral direct inhibitors as anticoagulant therapy

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Background: The Clinical Leaders of Thrombosis (CLOT) are a specialist interest group made up of approximately 450 multi disciplinary healthcare professionals (HCP) working in the UK who have direct patient contact working in the field of Haemostasis and Thrombosis. They include, nurses, doctors, biomedical scientists and pharmacists in both primary and secondary care. The group's aim is to deliver high quality care to patients by sharing knowledge and experience in order to shape future services and advance the boundaries of healthcare delivery.
Aims: Members of CLOT highlighted the need for written guidance on counselling patients who have been prescribed the newly licensed oral anticoagulant agents. The group felt they have many requests for written guidance on the subject with reference to relevant publications, and want to deliver proactive patient focused care. This group is ideally placed to promote, deliver and review this information.
Methods: At the annual meeting in October 2012 an advancing practice workshop was held. The workshop attendees split into discussion groups looking at national, regional, local and pharmaceutical company recommendations. The recommendations of each were looked at, opinions sort and concerns shared. These were used as the basis for the counselling recommendations given.
Results: As a result the issues to be addressed with this patient group have been identified for use by any HCP. The workshop attendees decided by majority vote that the new anticoagulants should be referred to as oral direct inhibitors (ODI). They identified and agreed what the counselling should consist of. Patients should have an explanation as to the purpose of anticoagulant therapy and how the treatment will work for them. Basic therapy information should be given and counselling should address how to take the medication safely and other medications to avoid, possible side effects of treatment and when to seek further advice and help. It also suggests precautions and risks with this treatment. Finally it gives practical advice on care that needs to be undertaken including laboratory testing, and annual follow up arrangements.
Summary: The CLOT group wants all patients taking ODIs to be given the same safe, unbiased and practical information. They felt that a unified approach with adjustments for local varying arrangements to be the way forward. They intend to continue to review and update this.

PO 491

Subcutaneous injection of enoxaparin in neonates

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Background: Enoxaparin, a Low Molecular Weight Heparin (LMWH), is a frequently used anticoagulant in the neonatal population because of reduced adverse effects, ease of administration and reliable pharmacokinetics. The recommended route of administration is by subcutaneous (SC) injection. Through the 1–800-NOCLOTS, a toll-free service established in 1994 to provide consultation to pediatric professionals globally about the management of pediatric thrombosis, the question of the preferred site of SC injection in neonates is often queried.
Objective: To determine if there are any evidence-based guidelines regarding subcutaneous injection of LMWH in neonates.
Methods: Web of Science, PubMed, Medline CINAHL, Cochrane, DARE and OVID databases including relevant published nursing textbooks were searched for articles specific to subcutaneous administration of LMWH in neonates.
Results: A paucity of articles specific to subcutaneous administration of LMWH in neonates exist. We found only one study that examined the absorption of insulin and growth hormone via SC injection, but this did not include neonates. One study was identified on the administration of LMWH to neonates via an indwelling catheter, but the current recommendations are to rotate SC injection sites. One case study supported intravenous administration of LMWH, citing the lack of SC fat in pre-term and low birth weight neonates. A review of nursing textbooks revealed that while SC sites have remained the same for decades, there is no evidence to support the choice of sites except expert opinion.
Conclusion: Currently we rely on expert opinion or local guidelines when administering LMWH by SC Injection during the neonatal period. There are no evidence-based guidelines for the choice of SC site, and the literature regarding optimum sites is based solely on animal data. Several studies have also noted the need for higher loading and maintenance doses of LMWH to achieve target Xa levels during prophylaxis in neonates and this my be influenced by the injection site, the administration procedure or local mass of adipose tissue. A prospective study is needed to determine the best site and angle of SC Injection of LMWH in neonates.

PO 492

Risk of recurrence of venous thromboembolism assessment following discontinuation of initial treatment of anticoagulation

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Background: Specialist nurses working in Haemostasis and Thrombosis have many roles to undertake including delivering proactive patient focused care and to improve the safety and quality of that care within available resources. Clinical leadership should ensure that new evidence based practice is translated into patient care that is safe, effective and person centred.
Aims: To develop a patient focused rather than disease driven approach to care following discontinuation of the recommended initial treatment with anticoagulation after diagnosis of a venous thromboembolism (VTE).
Methods: All patients who commence anticoagulation after diagnosis of a VTE are seen in the anticoagulant clinic. During the clinic visit a care pathway is completed which identifies all patients who have been diagnosed with a first episode of unprovoked VTE. Following national guidelines these patients are advised to have a finite period of anticoagulation. After stopping their initial treatment of anticoagulation these patients are offered an appointment in the nurse led Thrombophilia clinic. This appointment includes a clinical risk assessment which incorporates a Villalta scoring for patients with the post thrombotic syndrome (PTS), appropriate blood testing (including D-dimer) and patient preference discussion. Each patient then has a further clinic appointment, including the Dash scoring system to aid decision making. A treatment plan is agreed based on the patients individual needs.
Results: Since its introduction over 100 patients have followed this pathway. Patients were concerned that following initial treatment with anticoagulation they did not routinely have follow up, or that follow up varied depending on who they spoke to or where they lived. This care pathway has taken pressure off the medical consultants and has become a standardized care pathway in our service delivery. The benefit for patients is a clear route for them to follow and allows closure following discontinuation of treatment after a first episode of VTE.
Summary: This standardized approach allows high quality care to be offered to all patients. We believe we are the first service in the country to introduce a nurse led standardized approach. It is transferable across Trusts, and should be seen as an extension of the role of the clinical nurse specialist in anticoagulation.

PO 493

Treating pregnant women with high dose immunoglobulin (IVIG) in a single skilled center reduce side effects

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Background: Fetal neonatal alloimmune thrombocytopenia (FNAIT) is a life threatening bleeding disorder in the fetus/neonate caused by maternal alloantibodies directed against fetal human platelet antigen (HPA) inherited from the father. In 85% of NAIT cases the immuno dominant is HPA 1a. In Caucasians the allele frequencies of HPA-1a and HPA1b are 85% and 15% respectively.
Aim: In order to prevent the delivery of a newborn with intracranial hemorrhage, one of the serious complications of FNAIT, treatment with intravenous immunoglobulin G (IVIG) with or without steroids is currently the standard treatment.
During the last 4 years, our Center treated 24 women with high dose IVIG because of FNAIT. We had the impression that treating those women in a skilled Center and as a group could reduce side effects related to treatment and furthermore, keep them adhered to treatment's schedules.
Method: For studying that, we followed prospectively in the last year four pregnant women treated with IVIG for approximately 21 weeks, 3 of those were treated with IVIG at standard dose of 1 g/kg/week and the remaining one at dose of 1.5 g/kg/week and steroids at dose of 0.5 mg/kg/d initiated at 31 weeks until term. Two of the women received IVIG (Gammagard, Baxter U.S.A) and the other two women were treated initially with Gammagard and then switched to IVIG (Gamunex, Talecreis, U.S.A.) because of Gammagard shortage. Three women complained of mild headache 4–5 h following treatment was finished and which relieved by acetaminophen. Fatigue was another complaint which last for at least 24 h after.
Results: The success of IVIG treatment (higher platelet counts in newborn following treatment) was to some extent related to the fact that the women came regularly to treatments without any rescheduled or postponed treatment. All four women claimed that being treated with IVIG in a group for the same purpose and with the same drug, improved their wellbeing in regard to FNAIT diagnosis and treatment. To the same conclusions came 6 more women that came to our clinic 2 years after given birth.
Conclusion: In summary, we do believe that high dose of IVIG that given by skilled nurses and accompanied by group of women treated for the same purpose could reduce dramatically side effects attributable to the drug and augment success to IVIG treatment.

PO 494

Hemostatic markers and sleep quality among shift work and day work female nurses

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Background: The haemostatic system has a significant impact on cardiovascular morbidity. Epidemiologic studies indicate an increased risk of cardiovascular diseases amongst shift workers. Such risk may be attributed to circadian misalignment of haemostatic markers and restricted sleep time.
Aims: To reveal some of the underlying mechanisms of cardiovascular morbidity in the shift-work population, we performed a comparison of the profiles of haemostatic markers among healthy shift working vs. daytime working female nurses. It was hypothesized that alterations in the haemostatic system may underlie the increased risk for cardiovascular illness in shift workers. Accordingly it was predicted, that haemostatic markers would be elevated in rotating shift workers compared to regular day workers, and that sleep quality would serve as a mediator in this relationship.
Methods: Thirty shift working and thirty day working female nurses were recruited at Rambam Health-Care Campus (total $n = 60$). For each participant, blood was drawn at 07:00 in the morning for the measurement of six markers of coagulation, including PAI-1, Heparanase procoagulant activity, tissue factor + heparanase complex, protein C, D-dimer and fibrinogen. Sleep quality was assessed by self report (Pittsburgh Sleep Quality Index, PSQI).
Results: PAI-1 levels were significantly higher among shift work nurses compared to day work group (36.6 ng/ml vs. 24.3 ng/ml, $P < 0.05$). In shift workers, Heparanase procoagulant activity was 2-fold and tissue factor + heparanase complex was 1.5-fold compared to day work nurses (both $P < 0.05$). Sleep quality was significantly lower for shift compared to day workers ($P < 0.001$). No group differences were found for Protein C, D-dimer and fibrinogen.
Concusions: PAI-1 and heparanase markers were significantly elevated and sleep quality reduced in shift-work compared to day-work nurses. Such alterations in healthy shift workers point at disturbances in haemostatic system, which may contribute to cardiovascular morbidity in the future.

PO 495

The PLD inhibitor, 5-Fluoro-2-indolyl des-chlorohalopemide, protects from occlusive thrombus formation and ischaemic stroke without impairing haemostasis

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Background: Phospholipase D (PLD) catalyses the hydrolysis of phosphatidylcholine to phosphatidic acid and choline and is expressed in a wide range of tissues. Recently, we demonstrated that Pld1 is required for GPIb-dependent platelet integrin activation and that Pld1 and Pld2 both contribute to platelet α-granule release. As a consequence, mice deficient in Pld1 or in both PLD isoforms were protected from pathological thrombus formation and ischaemic stroke, whereas haemostasis was not impaired. These findings led to the hypothesis that PLD could serve as target for safe antithrombotic therapy, but *in vivo* evidence in support of this has been lacking.
Aims: We sought to assess whether pharmacologic inhibition of PLD activity with the reversible, small molecule inhibitor 5-Fluoro-2-indolyl des-chlorohalopemide (FIPI) affects haemostasis, thrombosis and thrombo-inflammatory brain infarction in mice.
Methods: Mice received FIPI (3 mg/kg) twice, 12 and 1 h before the experiment, intra peritoneal and were analysed in a tail bleeding assay, arterial thrombosis and the transient middle cerebral artery occlusion (tMCAO, 60 min) model of ischaemic stroke.
Results: In agreement with observations made in Pld1 and Pld1/2-deficient mice, FIPI-treated animals displayed impaired thrombus formation. Upon application of $FeCl_3$ on the carotid artery, 8 of 9 control vessels occluded within 10 min. In contrast, 6 of 9 vessels of FIPI treated mice remained open until the end of the 30 min observation period. Similarly, FIPI treatment resulted in smaller infarct sizes and significantly better motor function and coordination 24 h after tMCAO, and this protective effect was not associated with increased intracerebral haemorrhage. In line with this observation, no differences in bleeding times of control and FIPI-injected mice were observed, demonstrating that PLD inhibition by FIPI does not exert a major effect on normal haemostasis.
Summary/Conclusions: These results provide the first evidence that pharmacologic PLD inhibition might be a safe therapeutic strategy to prevent or treat arterial thrombosis and ischaemic stroke.

PO 496

Brachial artery endothelium-dependent flow-mediated dilation and carotid artery intima-media thickness in the cardiac pacing patients

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Background: After pacemaker implantation in the patients with brady-tachy syndrome, sick sinus syndrome (SSS) and atrial ventricular block (AVB II, III gr.), the left ventricule (LV) function improved. Flow-mediated dilation (FMD) of brachial artery, being a marker of *in vivo* NO bioavailability, is also predictive of future cardiovascular events. Dynamic changes in the FMD of brachial artery in the cardiac pacemaker recipients still require further in-depth research. Also the carotid artery intima-media thickness (CIMT) is a marker of prevalent and incident cardiovascular disease and is widely used as an indicator of generalized atherosclerotic burden.
Aim: To assess the dynamics of endothelium-dependent FMD and CIMT in the patients with two modes of cardiac pacing.
Methods: The study group comprised 92 cardiac pacing subjects (38 F, mean age 71 ± 7.58 years), divided in line with respective modes of cardiac pacing: Group A (n = 64; mean age 70.20 ± 7.70 years) with physiological (DDD) pacing; Group B (n = 28; mean age 72.70 ± 10.20 years) with non-physiological (VVI) pacing. Cardiac picemakers were implanted due to SSS, AVB (II, III gr.), atrial fibrillation cum bradyarrhythmiae, respectively. Transthoracic echocardiography was performed prior and after pacemaker implantation. LV ejection fraction (LVEF) and LV diastolic function were calculated, and left and right ventricular end-diastolic diameters (LVEDD/RVEDD) were measured. Endothelium-dependent FMD of brachial artery in response to reactive hyperemia and CIMT were non-invasively evaluated by high-resolution ultrasound. Both measurements were taken five times: at baseline, after 7 days, and within 1, 3, and 6 months of pacemaker implantation.
Results: At baseline, both groups differed in: LVEF ($P < 0.001$), LVEDD and RVEDD ($P < 0.0001$). Significant elevation of LVEF ($P < 0.001$), decrease of LVEDD/RVEDD ($P < 0.0001$), decrease of the left and right atrium diameter ($P < 0.0001$) were noted only after DDD pacing. At baseline the mean FMD value was non-significantly higher in Group A subjects (4.05 ± 1.70% vs. 3.76 ± 2.28% for Group A and B, respectively; P = ns). After the pacemaker implantation, the improvement of brachial artery FMD in both groups were observed at 7 days ($P = 0.01$ and $P = 0.04$, for Group A and B, respectively), and the first month ($P = 0.03$ and $P = 0.05$, for Group A and B, respectively), and within 3 ($P = 0.01$ for both Groups) and 6 ($P = 0.003$ and $P = 0.02$, for Group A and B, respectively) following months. The mean value of CIMT were similar in both study groups (0.78 ± 0.14 mm vs. 0.79 ± 0.17 mm; $P = 0.89$) and there were no differences for up to 12 months.
Conclusions: Improvement of endothelial function after pacemaker implantation may indicate an improved integrity of re-modeling and the repaired vascular function in the cardiac pacing patients.

PO 497

Talin and Src protein phosphorylation in hyperractive platelets

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Background: Sticky platelet syndrome (SPS) is a disorder distinguished by platelet hyperreactivity in response to physiologic stimuli, in conjunction with clinical arterial or venous thrombotic events, and without coagulation disorders. SPS is associated with transient or permanent vascular occlusions, resulting in acute myocardial infarction, transient ischemic cerebral attacks and strokes, and recurrent venous thromboembolism.
Aim: We aimed here to identify which proteins have an altered association with the outer platelet surface molecules upon thrombin stimulation in SPS patients.
Methods: SPS and normal platelets were activated with thrombin, and then changes in protein expression were analyzed by one-dimensional electrophoresis followed by LC-MS/MS analysis and Western-blotting. This study was approved by the institutional review board, and all individuals gave written consent.
Results: Proteomic analysis of thrombin-activated platelets reveal that talin protein was six-fold more expressed in SPS patients, while Src protein (proto-oncogene tyrosine-protein kinase), was 15-fold more expressed in normal individuals. By Western blotting, the level of talin phosphorylation was 10-fold higher in SPS patients than in normal individuals ($P < 0.05$). Among normal individuals and SPS patients, there was a statistically significant difference on Src expression ($P = 0.02$), however, the activity of Src Y418 was found to be much

higher in patients compared to the protein expression. Analysis of Src phosphorylation in thrombin-stimulated SPS platelets revealed approximately three-fold higher phosphorylation levels in the activating site in Src (pTyr-Src418), with a parallel decline of Src (total) expression, compared to healthy individuals. As we did not observed dephosphorylation of Src Y529 in SPS patients, we conclude that an independent regulatory mechanism, might be involved in Src kinase activation.

Conclusion: A significant increase in tyrosine phosphorylation levels in Src Y418, as well as in talin, suggests that these proteins may play a critical role in controlling integrin activation in SPS patients.

PO 498

Treatment modalities and outcomes in 870 non-surgical bleeds in 184 Glanzmann thrombasthenia (GT) patients. The International Prospective Glanzmann's Thrombasthenia Registry (GTR)

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Background: Standard treatment for GT, a severe autosomal recessive bleeding disorder with defective platelet $\alpha_{IIb}\beta_3$, is platelet transfusion. Recombinant FVIIa (rFVIIa) has been effective in GT with platelet antibodies and/or platelet refractoriness.

Aims: To assess the efficacy of rFVIIa, compared to other systemic hemostatics in GT with or without antibodies and/or refractoriness for surgical prophylaxis.

Methods: Analysis of data from GTR, an international multicenter observational study on the efficacy and safety of rFVIIa and other treatment modalities for bleeding and surgery in GT patients.

Results: From 2004–2011, GTR enrolled 218 patients from 45 sites (15 countries, 4 continents) with 1073 admissions. Out of these, 96 patients (F/M: 52/44 [54%/46%]; age ≥18 year, 69 [72%]) were treated for 204 surgical procedures (minor/major, 167/37 [82%/18%]). Out of 55 patients with known disease type, 48 (87%) had type 1 GT. History of antibodies were reported in 43 patients [45%], refractoriness in 23 [24%], and antibodies + refractoriness in 17 [18%], while 47 [49%] had no antibodies/refractoriness. Treatments analyzed include antifibrinolytics (AF), rFVIIa ± AF (r7), platelets ± AF (P) and rFVIIa + platelets ± AF (r7 + P). The top minor procedures were dental (n = 132, 79%), endoscopy (n = 11, 6.6%) and nasal (n = 8, 4.8%). The top major procedures were GI (n = 9, 24.3%) and orthopedic (n = 9, 24.3%). Most frequent treatment for minor procedures was r7 (n = 120 [72%]); and for major procedures, r7 and r7 + P (13 [35%] each). In GT with no antibodies/refractoriness, r7 was 100% efficacious for both minor (n = 41) and major procedures (n = 7), similar to or better than that for P (minor 11/11 = 100%; major 5/6 = 83%) and r7 + P (minor 4/4 = 100%; major 6/9 = 67%). In GT with antibodies and/or refractoriness, the efficacy of r7 (minor procedures 69/79 = 87%; major 4/6 = 67%) was not inferior to that for P (minor 10/14 = 71%; major 2/2 = 100%) and r7 + P (minor 7/9 = 78%; major 2/4 = 50%). A few patients were treated with AF alone, with an overall efficacy rate of 56% for nine minor procedures, but 100% for three major procedures. rFVIIa was generally given initially at a median of ~90 μg/kg at ~2 h intervals, for both minor (median 2–3 doses) and major (number of doses: higher and variable) procedures. The median (mean ± SD) number of platelet units used in patients with no antibodies and/or refractoriness, was 1 (2 ± 1) for minor and 5 (8 ± 7) for major procedures. In patients with antibodies and/or refractoriness, the median platelet use was higher and variable, up to 60 U for one major procedure. Only one thromboembolic event occurred in an adult female with refractoriness treated with r7 + P for a major procedure. One additional allergic reaction was attributed to platelets.

Summary/Conclusions: (1) The GTR provides the largest experience to date on surgical procedures in GT. (2) GTR results show that the efficacy of rFVIIa ± AF was similar to platelets ± AF and rFVIIa + platelets ± AF in surgical procedures in GT patients, regardless of investigator-reported antibodies/refractoriness status. (3) rFVIIa ± AF was safe for surgical procedures. (4) Platelets were efficacious in GT patients with investigator-reported antibodies and/or refractoriness, but higher doses were reported. (5) The combined use of rFVIIa + platelets could be attributed to the insufficient efficacy of one or the other alone.

PO 499

The significance of TEG and TGA in the evaluation of hemostasis in children with cyanotic and acyanotic congenital heart diseases

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Background: Patients with cyanotic congenital heart disease (CHD) have hemostatic abnormalities, which result in an increased risk of bleeding. The cause is unknown, but recent studies have indicated that an elevated hematocrit, which is present in cyanotic patients, could be an important factor.

Aims: In this study, we aimed to evaluate the hemostatic state of children with cyanotic and acyanotic CHD by using thromboelastography (TEG) and thrombin generation assay (TGA).

Methods: Seventy four patients with CHD (39 acyanotic, 35 cyanotic; ages were between 0 and 18 years) were enrolled in this study. Twenty nine, age matched, healthy children were enrolled as a control group. Hemogram, PT, aPTT, TEG and TGA results were evaluated in all patients before angiography and cardiac surgery.

Results: In the cyanotic group, as expected, hematocrit levels were significantly higher than acyanotic and control groups (P = 0.000). When compared to the acyanotic patients, PT anda PTT results were significantly higher in the cyanotic group (P = 0.011 and P = 0.037 respectively). When TEG parameters were analyzed, TEG-R values were significantly longer in cyanotic patients than acyanotic and control groups (P = 0.000 for both groups). Alpha-angle degree and maximum amplitude (MA) values were significantly lower in cyanotic group than acyanotic (P = 0.001) and control (P = 0,005) groups. When TGA parameters were analyzed, peak thrombin levels were significantly lower in cyanotic group than acyanotic and control groups (P = 0.000, P = 0.000 respectively). When compared to control group, peak thrombin time results were significantly longer in cyanotic and acyanotic groups (P = 0.014, P = 0.028 respectively). Endogenous thrombin production was significantly lower in cyanotic group than acyanotic and control groups (P = 0.000, P = 0.000 respectively).

Conclusion: While it is well known that high hematocrit levels increase the tendency for thrombosis in cyanotic heart disease, TEG and TGA results in our study show that there are also coagulation abnormalities in these patients.

PO 500

Caspase-8 activation can be triggered in platelets through the intrinsic apoptosis pathway

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Background: Since the seminal report of Vanags et al. (Br J Haematol, 1997), programmed cell death, apoptosis, has been demonstrated not only in nucleated cells, but also in anucleate platelets. While the *intrinsic* (mitochondria-dependent) apoptosis pathway in platelets is well-documented,the role of the *extrinsic* (death ligand-death receptor-dependent) pathway in platelets remains unclear.

Aims: To address this question, in this study, we initiated platelet apoptosis through the intrinsic pathway, using BH3-only mimetic ABT-737 and calcium ionophore A23187, agents that trigger the mitochondrial platelet apoptosis. Our particular interest was to examine whether activation of caspase-8, a caspase 'classically' defined as an initiator caspase of the extrinsic apoptosis pathway, can be induced in platelets treated *in vitro* with ABT-737 and A23187 via the intrinsic pathway.

Methods: Platelets were treated with 30 μM ABT-737, 10 μM A23187 and control diluent buffer for 90 min at 37 °C, and platelet apoptosis was analyzed by flow cytometry using three experimental settings: assays of (i) caspase-9 activation, an initiator caspase of the intrinsic apoptosis pathway, (ii) caspase-8 activation, an initiator caspase of the extrinsic pathway, and (iii) caspase-3 activation, an executioner caspase of apoptosis, each in combination with P-selectin (CD62) exposure on the platelet surface for concomitant determination of platelet activation.

Results: Flow cytometric fluorescence histogram analysis demonstrated that treatment of platelets with ABT-737 and A23187 significantly increased activation of caspase-9 and caspase-3 in comparison to diluent buffer ($P < 0.0001$). Notably, both agents also induced activation of initiator caspase-8 of the extrinsic apoptosis pathway ($P < 0.0001$). Mean levels of activation of all three caspases (9, 8 and 3) were not significantly different ($P > 0.05$), indicating that ABT-737 and A23187 did not trigger an additive or synergistic activation of executioner caspase-3 by initiator caspases-9 and -8. Dual-fluorescence dot-plot analysis of caspase-9, -3 and -8 activation versus CD62 exposure shows that ABT-737-treated platelets are predominantly (63.7–72.7%) apoptotic but not activated, whereas A23187-treated platelets are predominantly both apoptotic and activated (64.3–74.3%).

Conclusions: We demonstrated that apoptosis induced by treatment of platelets with ABT-737 and A23187, which is not triggered by the interaction of death ligands with death receptors, yields caspase-8 activation in platelets without participation of the extrinsic apoptosis pathway. These data indicate that caspase-8 activation does not always represent a marker of the extrinsic pathway of apoptosis. Despite caspase-8 activation in ABT-737- and A23187-stimulated platelets occurring via bypass of the death receptors, the major 'final goal' of the extrinsic pathway, i.e. an activation of initiator caspase-8, is achieved. The mechanism of this death-receptor-independent mitochondria-initiated caspase-8 activation may take place in platelets as described in some types of nucleated cells, which are less dependent on the extrinsic pathway of apoptosis and in which active caspase-9 propagates a cascade of caspase activating reactions, including activation of executioner caspase-3, followed by activation of downstream executioner caspase-6 which, in turn, activates caspase-8. Caspase cascade reactions permit caspase-mediated cross-talk between intrinsic and extrinsic apoptosis pathways in platelets and can broaden the repertoire of caspase-cleaved protein substrates.