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Curative anticoagulation prevents endothelial lesion in COVID-19 patients

Brief title: Coagulopathy and endothelial lesion in COVID-19

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Authors contributions:

DMS, TM and JLD interpreted data, conceived and supervised the study. LK and NG interpreted data and drafted the manuscript. RC analyzed the data and supervised statistical analysis. LK, NG, GG, RC and BD analyzed the data and reviewed all patients' characteristics. All authors' interpreted data, drafted and revised the manuscript, and approved the final version.

Essentials section:

- Coronavirus disease-2019 (COVID-19) has been associated with coagulopathy and endotheliitis
- Coagulopathy and endothelial dysfunction in COVID-19 patients at admission need to be precisely determined
- Adding D-dimers to gender and pneumonia at CT scan significantly increased specificity of COVID-19 diagnosis
- Curative anticoagulation could prevent COVID-19-associated endothelial lesion.

Abstract

Background: Coronavirus disease-2019 (COVID-19) has been associated with cardiovascular complications and coagulation disorders.

Objectives: To explore the coagulopathy and endothelial dysfunction in COVID-19 patients.

Methods: Study analyzed clinical and biological profiles of patients with suspected COVID-19 infection at admission, including hemostasis tests and quantification of circulating endothelial cells (CECs).

Results: Among 96 consecutive COVID-19-suspected patients fulfilling criteria for hospitalization, 66 were tested positive for SARS-CoV-2. COVID-19 positive patients were more likely to present with fever (p=0.02), cough (p=0.03) and pneumonia at CT-scan (p=0.002) at admission. Prevalence of D-dimer >500 ng/mL was higher in COVID-19 positive patients (74.2% vs. 43.3%; p=0.007). No sign of disseminated intravascular coagulation were identified. Adding D-dimers >500 ng/mL to gender and pneumonia at CT scan in ROC curve analysis significantly increased AUC for COVID-19 diagnosis. COVID-19 positive patients had significantly more CECs at admission (p=0.008) than COVID-19 negative ones. COVID-19 positive patients treated with curative anticoagulant prior to admission had less CECs (p=0.02) than those without. Interestingly, patients treated with curative anticoagulation and ACEi or ARBs had even lesser CECs (p=0.007).

Conclusion: Curative anticoagulation could prevent COVID-19-associated coagulopathy and endothelial lesion.

Keywords: SARS-CoV-2, COVID-19, circulating endothelial cells, D-dimers, coagulopathy

Abbreviations list:

ACEi = angiotensin-converting enzyme inhibitors
ACE2 = angiotensin-converting enzyme 2
ARBs = angiotensin receptor blockers
AUC = area under the curve
CECs = circulating endothelial cells
DIC = disseminated intravascular coagulation
ISTH = international society of thrombosis and haemostasis
ROC = receiver operating characteristics

Introduction

In December 2019, an epidemic pneumonia caused by a new coronavirus occurred in Wuhan (Hubei Province) spread rapidly throughout China, evolving into a global pandemic (1). Originally called new coronavirus 2019 (2019-nCoV), this virus was then officially named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) by WHO (3). On January 30, 2020, the WHO declared the SARS-CoV-2 epidemic (COVID-19) a public health emergency of international concern. Compared to SARS-CoV which caused a SARS epidemic in 2003, SARS-CoV-2 has a higher transmission capacity with a higher mortality (2). With a rapid increase in confirmed cases, there is an unmet need of prevention and therapeutic strategies of COVID-19. Although COVID-19 clinical manifestations are dominated by respiratory symptoms, some patients have severe cardiovascular damages (3, 4) and kidney disease participating to a multiple organ failure. Patients with cardiovascular comorbidities may also be at higher risk of death (4). For example, COVID-19 patients with hypertension have an increased mortality and morbidity with hazard ratio ranging from 1.7 to 3.05, depending of studies (3, 5). Based on this, raising concerns emerged regarding angiotensin-converting enzyme inhibitors (ACEi) and angiotensin receptor blockers (ARBs). Indeed, angiotensin-converting enzyme 2 (ACE2) has been shown to be a co-receptor for viral entry for SARS-CoV-2, and it has been demonstrated that ACEi and ARBs could enhance ACE2 expression (6) that may therefore jeopardize patient susceptibility to viral host cell entry and dissemination.

During Chinese epidemic, coagulopathy has been reported in severe COVID-19 patients. D-dimer levels above 1000 ng/mL were an independent risk factor of in-hospital death (5). Coagulopathy was also found in fatal cases of COVID-19 patients, including a significant higher proportion of patients with D-dimers above 500 ng/mL and prolonged prothrombin time (PT) in non-survivors (7). This study was replicated in a second Chinese population where D-dimers were still associated with in-hospital mortality (8). The hypothesis of microthrombi in kidney was also suggested in COVID-19 patients since high creatinine level was correlated with D-dimers above 500 ng/mL (9). Finally, endothelial dysfunction might also play a role in the incidence of severe respiratory symptoms and viral systemic dissemination. Indeed, SARS-CoV-2 receptor ACE2 is strongly expressed on endothelial cells (10). We hypothesized that endothelial cell infection may induce endothelium damage and dysfunction/activation that triggers coagulation activation (11). Circulating endothelial cells (CECs) are considered as relevant markers of

endothelial lesion or dysfunction (12) and were used to explore the potential vascular dysfunction in COVID-19 patients.

The aim of our study was to identify biological markers related to COVID-19 diagnosis and severity. We also aimed at better characterizing subpopulations of patients at risk of coagulopathy and/or endothelial dysfunction to target COVID-19 patients at risk for the worst outcomes.

Methods

Study design and population

This is a single-center, prospective observational cohort study conducted in a university hospital in Paris (France). From March 14, 2020 to March 20, 2020, all consecutive patients aged over 18 years, presenting to the emergency room of the Georges Pompidou European hospital and fulfilling hospitalization criteria or direct in-patient referral, with an infectious syndrome suspect of COVID-19 were included. Hospitalization criteria were based on local guidelines as described in **Table 1.** Suspicion of COVID-19 was defined by the presence of at least one of the followings: fever, headache, myalgia, cough, dyspnea, rhinorrhea or digestive symptoms. All COVID-19-suspected patients had a clinical evaluation, blood test, CT-scan and were tested for SARS-CoV-2 infection by nasopharyngeal swabs before they were transfer to dedicated hospitalization units: medical department or intensive care unit (ICU). The study was performed in accordance with the Declaration of Helsinki. All patients provided written informed consent before enrollment (CPP 2020-04-048 / 2020-A01048-31 / 20.04.21.49318). For all patients, baseline characteristics (demographic, treatment, clinical, cardiovascular risk factors and body mass index), biological data and CT-scan results were retrieved from the medical records using a standardized data collection.

Laboratory confirmation of SARS-CoV-2 infection

Nasopharyngeal swabs were collected in universal transport medium (Xpert® nasopharyngeal sample collection kit). SARS-CoV-2 was detected using Allplex™ 2019-nCoV Assay (Seegene), a multiplex Real-time PCR assay that detects three target genes (E gene, RdRP gene and N gene) in a single tube. Data were automatically analyzed using Seegene viewer software. Only qualitative data were available.

Routine blood examinations

All samples were collected on EDTA, sodium heparin and 0.129 M trisodium citrate tubes (9NC BD Vacutainer, Plymouth, UK). Routine lab tests were complete blood count, creatinine, C-reactive protein (CRP) and high-sensitive troponin I (Hs-TnI). Coagulation tests were PT ratio, fibrinogen, soluble fibrin monomer, (STA®-Liatest FM; Stago) and antithrombin levels

(Stachrom® AT III 6, Stago) explored on a STA-R® Max (Stago) coagulometer as previously described (13). D-dimer concentrations were determined using the Vidas D-Dimer assay (BioMérieux) according to the manufacturer's instructions.

CECs quantification

Peripheral venous blood samples were collected on EDTA after having always discarded the first milliliter of blood to avoid presence of endothelial cells dislodged by puncture. CECs were isolated by immunomagnetic separation with mAb CD146-coated beads and staining with the fluorescent probe acridin orange as previously described (12, 14-16).

Statistical analysis

Continuous data were expressed as median [interquartile range: (IQR)] and categorical data as proportion. In the univariate analysis, we determined differences in median using the unpaired t-test (Mann-Whitney U test) for continuous variable and in proportions we were using the Chi-square test or Fischer exact test if necessary. We generated receiver operating characteristics (ROC) curve with the regression models that included variables with significant difference in the univariate analysis (17, 18). The model included gender, pneumonia at CT scan and D-dimers above 500 ng/mL. This model helped assess the extent to which the level of D-dimers influenced the predictability of COVID-19 diagnosis. We compared area under the curve (AUC) of each ROC curve using the Delong test. All analyses were 2-sided and a p-value of $p<0.05$ was considered statistically significant. Statistical analysis was performed using R studio software (R Development Core Team (2019). R: A language and environment for statistical computing. R Foundation for Statistical Computing,Vienna, Austria).

Results

High level of D-dimers is a discriminant factor during COVID-19 suspicion

Among the 96 COVID-19-suspected patients included, 66 were positive for SARS-CoV-2. COVID-19 positive patients were more likely to be males (N=44, 66.7%) than COVID-19 negative patients (N=13, 43.3%) (p=0.05). Otherwise, the two populations were strictly comparable in terms of time from illness onset to hospital admission, age, body mass index, cardiovascular risk factors, medical history and treatments (**Table 2**). Considering clinical features at admission, COVID-19 positive patients were more likely to have fever (p=0.02), cough (p=0.03) and interstitial pneumonia at CT-scan (p=0.002). In terms of biological features (**Table 3**) COVID-19 positive patients had a significantly lower white blood cell count, including neutrophil count (respectively p=0.008 and 0.02). Regarding hemostasis, the proportion of COVID-19 positive patients with D-dimers above 500 ng/mL was significantly higher (74.2% vs. 43.3%; p=0.007). No difference in PT ratio or fibrin monomers was observed between groups. In the context of COVID-19-associated coagulopathy with high levels of D-dimers, fibrinogen and CRP at admission, the low level of fibrin monomers and normal antithrombin levels allowed us to exclude a disseminated intravascular coagulation (DIC). When adding D-dimers above 500 ng/mL to gender and pneumonia at CT scan, ROC curve area (**Figure 1**) significantly increased from AUC 0.73 (95% CI 0.61–0.85) to AUC 0.82 (95% CI 0.69–0.95) (p=0.02), and confirm the relevance of D-dimers in diagnosis of COVID-19 at admission in hospital. The ROC curve with D-dimers above 500 ng/mL yielded a high sensitivity of 98.1% (95% CI 50.0–100.0), a low specificity of 28.6% (95% CI 21.0–85.8), a high positive predictive value of 77.9% (95% CI 65.3–90.0) and a high negative predictive value of 85.7% (95% CI 55.0–100.0).

Anticoagulated COVID-19 positive patients have a significant lower CECs count

CECs were quantified as markers of endothelial lesion, using the reference method (14) as detailed in the method section (12). Using this assay, the upper limit of normal range at 10 CECs per mL of whole blood was previously determined and confirmed in several studies, including ours (12, 19-21). Among COVID-19 positive patients, 64% were above this threshold, suggesting a SARS-CoV-2-induced endothelial lesion. For comparison, COVID-19 negative population had less CECs (p=0.008) (**Table 3**) with only 27 % above the normal range (p=0.012). Since the coagulopathy observed in COVID-19 patients could be related to this endothelial lesion, we analyzed whether anticoagulation could impact CECs level. Patients treated with curative anticoagulation prior to admission for any medical reason (83% for atrial fibrillation; 17% for venous thromboembolism) had indeed a lower CECs level than those without curative anticoagulation: 9 [8, 17] vs. 24 [14, 42] CECs per mL (p=0.02) respectively (**Figure 2**). Interestingly in patients treated with ACEi or ARBs the effect of curative anticoagulation on the CECs level was more pronounced with 10 [5, 14] vs. 30 [17, 43] CECs per mL (p=0.007) in those without curative anticoagulation (**Figure 2**).

Discussion

The originality of this study was to evidence an endothelial lesion during SARS-CoV-2 infection, as witnessed by increased levels of CECs. Second, we show that this endothelial damage is thwarted by curative anticoagulation.

Several Chinese studies found that increased D-dimer level correlated with in-hospital mortality (5, 7, 8), suggesting a COVID-19-associated DIC (7, 8, 22). However, in our population, no overt DIC was diagnosed at admission. Indeed, patients had no significant thrombocytopenia, a normal PT ratio and high fibrinogen levels. This was confirmed by a low level of fibrin monomers, which are early markers of DIC. DIC might nevertheless be involved in patient worsening and in particular for those with acute respiratory distress syndrome. Therefore, the increase of D-dimers largely reported in COVID-19 patients is probably not related to DIC but might reflect the microthrombi formation. Indeed, histopathological observations and imaging features of pulmonary lesions associated with SARS-CoV-2 revealed intra-alveolar fibrin deposit in pulmonary samples (23). Moreover, a recent study using standardized protective ventilation settings in COVID-19 patients confirmed that oxygenation was severely compromised with a moderate alteration in the respiratory system compliance. Hypercapnia high prevalence led to the hypothesis of a large amount of ventilated/not perfused alveoli, that could reflect diffuse microthrombi in the pulmonary microvascular bed (24). In renal disease associated to COVID-19, thrombotic lesions were proposed, since high D-dimers were more commonly observed in patients with elevated baseline serum creatinine (9). In this context, the International Society of Thrombosis and Haemostasis (ISTH) has recently recommended measuring D-dimers, PT ratio and platelet count in all COVID-19 patients to help stratifying those who may benefit from hospitalization and a close monitoring (25). Therefore, ISTH, the American College of Cardiology and the French Society of Vascular Medicine suggested the use of prophylactic anticoagulation with low weight molecular heparin (LMWH) for COVID-19 patients, in the absence of any contraindications (25-27). Indeed, preventive LMWH treatment could be associated with better prognosis in severe COVID-19 patients meeting sepsis-induced coagulopathy criteria (28).

We further hypothesized that COVID-19-induced coagulopathy could be a consequence of endothelial injury, based on the rationale that SARS-CoV-2 has an endothelial tropism linked to ACE2 expression. Moreover, recently an endotheliitis has been described in SARS-CoV-2 infection and could be at the origin of impaired microcirculatory function affecting particularly the lungs and kidneys (29). Thus, we explored CECs as a recognized non-invasive marker for

endothelial lesion, as demonstrated in acute cardiovascular conditions such as acute coronary syndrome (30) and pulmonary arterial hypertension (12, 15, 19). In agreement with the consensus protocol from ISTH, we used the reference method for CECs quantification and the threshold of 10 CECs per mL as upper normal value (14). We found that over 60% of COVID-19 positive patients were above this threshold. Interestingly, patients enrolled while they were treated with curative anticoagulation had a significantly lower level of CECs, especially in the hypertensive population treated with ACEi or ARBs. Increased mortality and/or morbidity of COVID-19 in patients with hypertension has been described in China (3). One of the most important concerns is the association between hypertension and treatment with ACEi or ARBs. Indeed, because ACE2 is a receptor for viral entry of SARS-CoV-2, a link between ACEi or ARBs was considered. ACEi or ARBs were described to increase ACE2 expression in the heart, brain, and even in urine after treatment (6, 31). However, main scientific societies of cardiology and more specifically of hypertension took the position not to withdraw ACEi or ARBs therapy in COVID19. Hence, Council on Hypertension of the European Society of Cardiology recommended that "patients should continue treatment with their usual anti-hypertensive therapy because there is no clinical or scientific evidence to suggest that treatment with ACEIs or ARBs should be discontinued because of the COVID-19 infection" (32). Indeed, recent studies did not found any association between ACEi or ARBs therapy and worsening in COVID-19 patients (33).

In our population patients treated with curative anticoagulation had a lower level of CECs, so we hypothesized that curative anticoagulation in COVID-19 patients could decrease thrombotic risk and subsequent mortality. To our knowledge, this is the first time that a potential protective effect of anticoagulant therapy on endothelial dysfunction is described. Besides the coagulopathy associated to endothelial lesion, effect of anticoagulation may act directly on SARS-CoV-2 entrance in endothelial cells. Indeed, cell entry of SARS-CoV-2 depends on the binding of the viral spike (S) proteins to cellular receptors and on S protein priming by host cell proteases. It has been demonstrated that serine protease TMPRSS2 is necessary for S protein priming (34). Thus, a TMPRSS2 inhibitor has been proposed as a treatment option. If virus entrance inside cells is dependent of a serine protease action, anticoagulation by inhibiting thrombin activity and serine proteases of the coagulation cascade could directly limit virus entrance in endothelial but also in other cells. This hypothesis needs to be tested in preclinical models of infection.

Study limitations:

Our study has several limitations. First, we are aware that false COVID-19 negative patients may exist in our study population due to the imperfect sensitivity of the diagnostic test currently used (35). Second, the study population size was necessarily small given the emergency of understanding COVID-19; indeed we decided to investigate the endothelial dysfunction associated to COVID-19 in order to consider new therapeutic alternatives. In addition, due to this small population, our results concerning anticoagulant treatment and ACEi or ARBs therapy generated hypothesis that need to be validated in larger cohorts.

In conclusion, it therefore seems consistent to open the way to curative anticoagulant treatment as part of the management of COVID-19 patients in order to limit associated coagulopathy and endothelial dysfunction. Anticoagulation may not only modify COVID-19 coagulopathy but also pathophysiology of SARS-CoV-2 systemic dissemination. Curative anticoagulation could decrease mortality observed in COVID-19 patient. Further studies should evaluate safety and efficacy of curative anticoagulation in COVID-19 patients to prevent worsening of disease and reduction of admittance in intensive care units.

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Table 1: Hospitalization criteria for COVID-19 suspected-patients

SpO2 for oxygen saturation.

Hospitalization criteria for COVID-19 suspected-patients
Co-morbidities other than respiratory failure **AND** requiring supplemental oxygen <3L/min to obtain SpO_2>96%.
Respiratory failure **OR** dyspnea **OR** patients requiring supplemental oxygen >3L/min to obtain SpO_2>96%
Predominant **AND/OR** decompensated cardiovascular comorbidities

Table 2: Demographic, clinical and treatment characteristics of patients on admission according to COVID-19 viral status. BMI for body mass index; CV for cardiovascular; ACEi for angiotensin conversion enzyme inhibitor; ARBs for antagonist of angiotensin 2 receptor blocker; SpO2 for oxygen saturation; ARDS for acute respiratory distress syndrome; IQR for interquartile range

	COVID-19 negative n=30	**COVID-19 positive n=66**	***p-value***
Male sex – n (%)	13 (43.3)	44 (66.7)	**0.05**
Age - years, median [IQR]	63.0 [55.3, 75.8]	66.0 [54.3, 79.8]	0.690
BMI - kg/m², median [IQR]	24.8 [22.6, 26.3]	26.5 [24.7, 29.1]	0.110
Time from illness onset to hospital admission – days, median [IQR]	4.47 (4.57)	5.45 (3.71)	0.260
CV risk factors, n (%)			
Hypertension	16 (53.3)	31 (47.0)	0.720
Dyslipidemia	6 (20.0)	21 (31.8)	0.340
Diabetes	4 (13.3)	12 (18.2)	0.760
Sedentarity	6 (20.0)	6 (9.1)	0.260
Chronic kidney disease	4 (13.3)	8 (12.1)	1.000
Medical history, n (%)			
Cancer	6 (20.0)	6 (9.1)	0.240
Coronary heart disease	3 (10.0)	7 (10.6)	0.920
Stroke	0 (0.0)	4 (6.1)	0.400
Treatments, n (%)			
Statins	3 (10.0)	13 (19.7)	0.220
Oral antidiabetic agents	2 (6.7)	9 (13.6)	0.510
Insulin	2 (6.7)	5 (7.6)	1.000
β-blockers	5 (16.7)	8 (12.1)	0.770
Calcium channel blockers	7 (23.3)	13 (19.7)	0.890
ACEi or ARBs	9 (30.0)	21 (31.8)	1.000
Diuretics	3 (10.0)	6 (9.1)	1.000
Central acting agent	1 (3.3)	0 (0.0)	0.680
Curative anticoagulation	3 (10.0)	12 (18.2)	0.470

Clinical features, n (%)			
Fever	22 (73.3)	61 (92.4)	**0.020**
Headache	1 (3.3)	12 (18.2)	0.090
Cough	14 (46.7)	47 (71.2)	**0.030**
Productive cough	3 (10.0)	8 (12.1)	1.000
Dyspnea	9 (30.0)	31 (47.0)	0.210
Myalgia	5 (16.7)	21 (31.8)	0.190
Diarrhea	2 (6.7)	7 (10.6)	0.640
Pneumonia at CT-scan	11 (36.7)	48 (72.7)	**0.002**
ARDS	1 (3.3)	9 (13.6)	0.240
SpO2 - %, median [IQR]	96.0 [92.0, 98.0]	95.0 [91.0, 96.0]	**0.050**
Respiratory rate - Breathes per min, median [IQR]	20.0 [16.5, 25.0]	19.0 [16.0, 22.8]	0.550
Pulse - Beats per min, median [IQR]	87.0 [74.0, 100.0]	87.0 [74.5, 103.5]	0.850

Table 3: Biological parameters of patients on admission according to COVID-19 viral status.

	COVID-19 negative n=30	**COVID-19 positive n=66**	***p-value***
White blood cells - x10^9 per L, median [IQR]	7.8 [6.1, 11.4]	6.0 [4.6, 7.4]	**0.008**
Hemoglobin - g/L, median [IQR]	126.0 [110.3, 141.0]	130.5 [112.0, 144.0]	0.490
Platelet count - x10^9 per L, median [IQR]	217.5 [157.0, 278.3]	167.5 [146.3, 223.0]	0.090
Neutrophils - x10^9 per L, median [IQR]	5.7 [4.2, 9.0]	4.0 [3.0, 5.9]	**0.020**
Lymphocytes - x10^9 per L, median [IQR]	1.0 [0.8, 1.7]	0.9 [0.7, 1.3]	0.170
Monocytes - x10^9 per L, median [IQR]	0.6 [0.4, 0.8]	0.4 [0.3, 0.6]	0.300
CRP - mg/L, median [IQR]	55.6 [3.3, 127.2]	74.0 [22.7, 126.3]	0.210
Plasma creatinine level - µmol/L, median [IQR]	99.0 [55.0, 112.0]	79.0 [62.7, 108.7]	0.970
Hs-TNI - pg/mL, median [IQR]	10.5 [3.5, 32.2]	9.5 [5.1, 22.9]	0.740
PT ratio, median [IQR]	0.94 [0.70, 1.10]	0.95 [0.86, 1.00]	0.320
Fibrinogen - g/L, median [IQR]	5.1 [4.3, 5.8]	5.3 [4.7, 6.2]	0.420
D-dimers >500 ng/mL - n (%)	13 (43.3)	49 (74.2)	**0.007**

Fibrin monomers - µg/mL, median [IQR]	7.0 [7.0, 7.0]	7.0 [7.0, 7.0]	0.550
Antithrombin – %, median [IQR]	99.0 [85.3, 102.8]	101.0 [86.5, 106.5]	0.500
CECs per mL, median [IQR]	9 [6, 18]	19 [10, 39]	**0.008**
CECs ≥10 per mL – n (%)	8 (27)	42 (64)	**0.012**

CRP for C-reactive protein; Hs-TnI for high-sensitive troponin I. IQR for interquartile range. PT for thromboplastin time; CECs for circulating endothelial cells.

Figure legends:

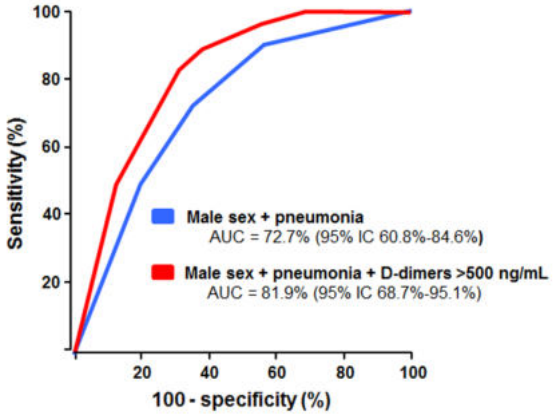
Figure 1: Potential exclusion criteria for COVID-19 diagnosis.

The receiver operating characteristic (ROC) curve including gender and pneumonia with (red line) or without D-dimers threshold at 500 ng/mL (blue line). ROC curve analysis identified the association of female gender, absence of pneumonia at CT-scan and D-dimers below or equal 500 ng/mL as potential exclusion criteria for COVID-19 diagnosis (AUC 0.78, 95% CI 0.66–0.90).

Figure 2: Effect of curative anticoagulation on CECs levels in COVID-19.

Quantification of circulating endothelial cells (CECs) in COVID-19 positive patients at admission. CECs level according to presence or the absence of curative anticoagulation and/or the presence or the absence of angiotensin-converting enzyme inhibitors (ACEi) or angiotensin receptor blockers (ARBs). Red dotted line shows the upper limit of reference values for CECs (<10 CECs per mL).

Figure 3: Curative anticoagulant treatment could be part of COVID-19 management in order to limit associated endothelial dysfunction.



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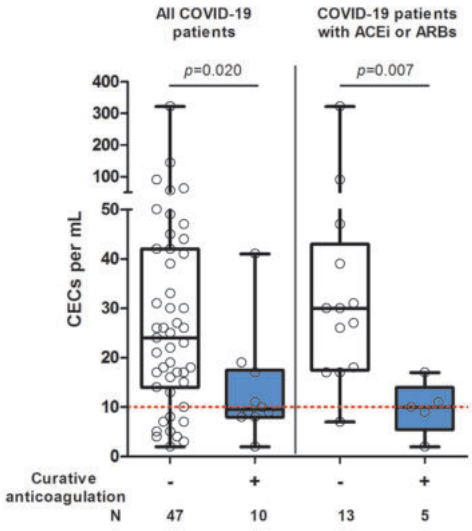

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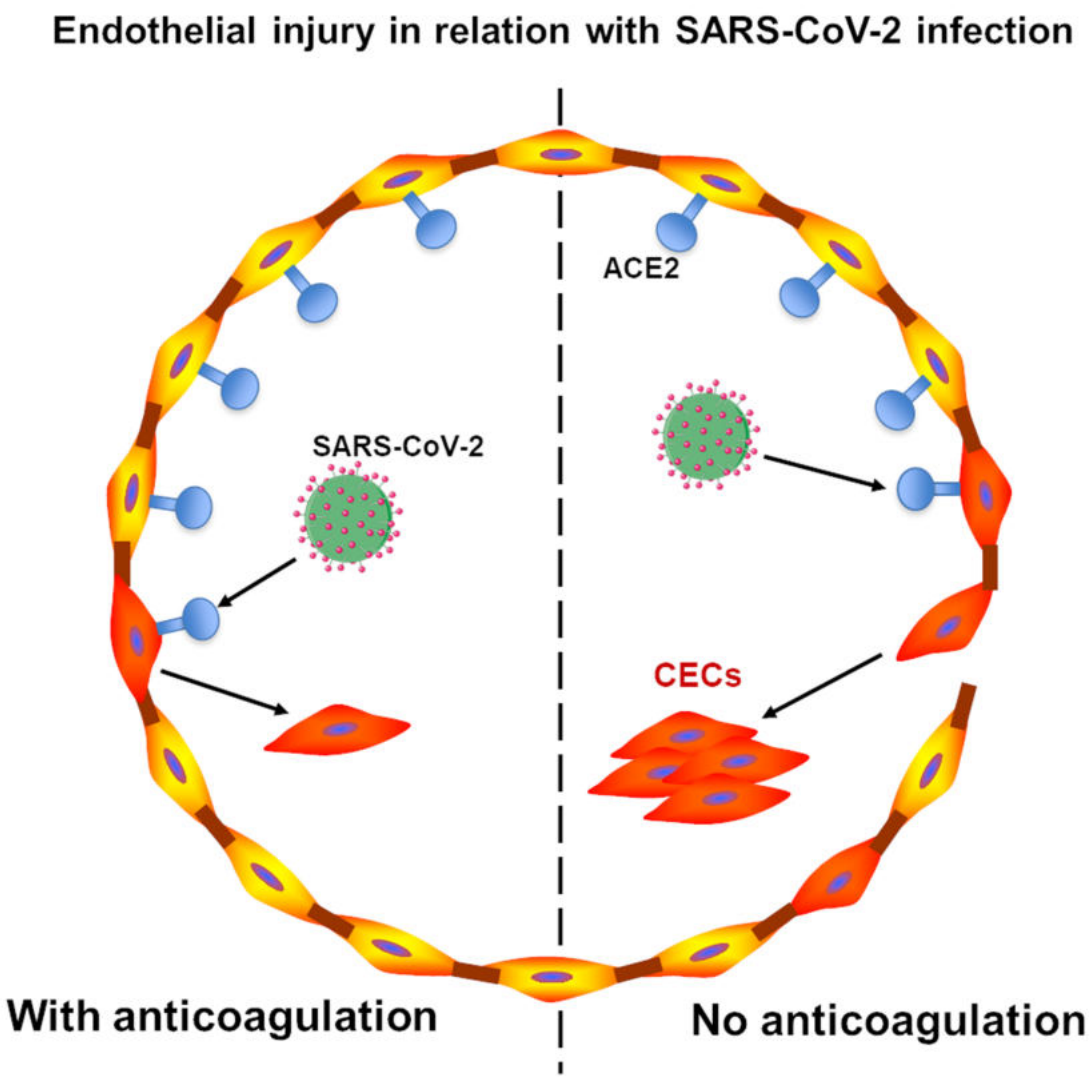

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