



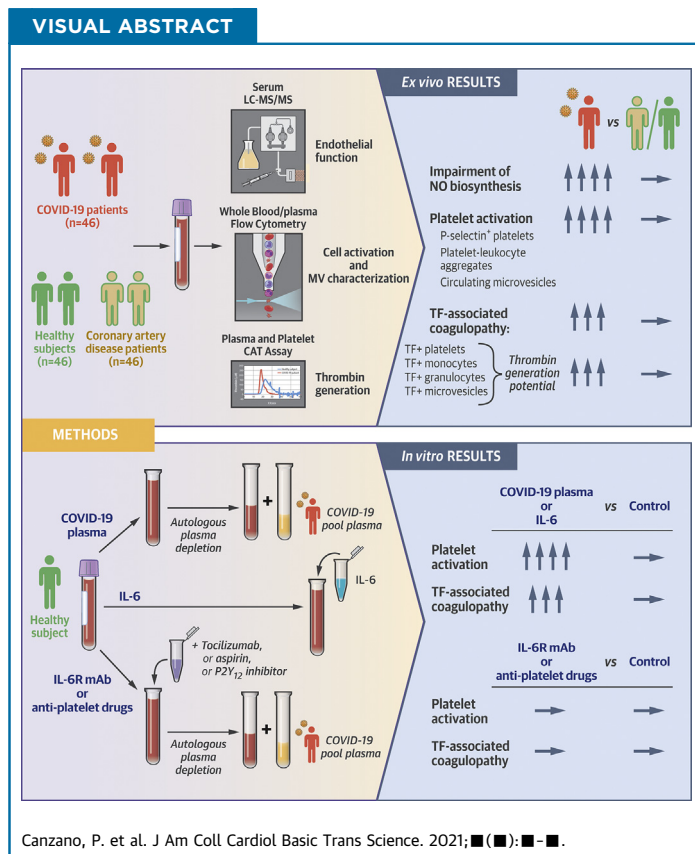
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NEW RESEARCH PAPER

Platelet and Endothelial Activation as Potential Mechanisms Behind the Thrombotic Complications of COVID-19 Patients

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HIGHLIGHTS

- The cytokine storm present in COVID-19 patients induces, together with the imbalance of endothelial functions, a massive cell activation with production of tissue factor, mainly by platelets, granulocytes, and MVs.
- Plasma MV-associated thrombin generation is present in patients despite prophylactic anticoagulation.
- COVID-19 plasma, added to the blood of healthy subjects, induces platelet activation similar to what is observed in vivo. This effect is blunted by pre-incubation with tocilizumab, giving insights into the IL-6-mediated platelet activation that triggers the hypercoagulable state in COVID-19, suggesting the potential effectiveness of anti-IL-6 antibodies and antiplatelet drugs.
- Our data provide the bench-to-clinic rationale behind the ongoing clinical trial assessing the potential effectiveness of antiplatelet drugs and IL-6R antagonists in the treatment of COVID-19 patients.

ABBREVIATIONS
AND ACRONYMS**ADP** = adenosine diphosphate**CAD** = coronary artery disease**COVID-19** = coronavirus disease-2019**CRP** = C-reactive protein**GPA** = granulocyte-platelet aggregates**HS** = healthy subject**IL** = interleukin**IL-6R** = interleukin-6 receptor**LMWH** = low-molecular-weight heparin**MPA** = monocyte-platelet aggregates**MV** = microvesicle**NO** = nitric oxide**NOS** = nitric oxide synthase**PGI₂** = prostacyclin**PLA** = platelet-leukocyte aggregates**PS** = phosphatidylserine**SARS-CoV-2** = severe acute respiratory syndrome-coronavirus-2**TF** = tissue factor

SUMMARY

The authors hypothesized that the cytokine storm described in COVID-19 patients may lead to consistent cell-based tissue factor (TF)-mediated activation of coagulation, procoagulant microvesicles (MVs) release, and massive platelet activation. COVID-19 patients have higher levels of TF⁺ platelets, TF⁺ granulocytes, and TF⁺ MVs than healthy subjects and coronary artery disease patients. Plasma MV-associated thrombin generation is present in prophylactic anticoagulated patients. A sustained platelet activation in terms of P-selectin expression and platelet-leukocyte aggregate formation, and altered nitric oxide/prostacyclin synthesis are also observed. COVID-19 plasma, added to the blood of healthy subjects, induces platelet activation similar to that observed in vivo. This effect was blunted by pre-incubation with tocilizumab, aspirin, or a P2Y₁₂ inhibitor. (J Am Coll Cardiol Basic Trans Science 2021; ■:■-■) © 2021 The Authors. Published by Elsevier on behalf of the American College of Cardiology Foundation. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Since the outbreak of the coronavirus disease-2019 (COVID-19) pandemic, a growing amount of clinical data has documented how severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2) may predispose patients to thrombotic disease, both in the venous and arterial vascular beds (1,2).

Patients with severe COVID-19 pneumonia experience hypoxemia, not only due to inflammatory alveolar involvement, but also to endothelial dysfunction together with a systemic cytokine storm response, which ultimately leads to micro- and macrothrombosis in the pulmonary vessels, as well as in other organs. Abnormal coagulation parameters, that is, a significant rise of D-dimer levels, prolonged prothrombin time, and low platelet counts, reflect the coagulopathy that correlates well with disease severity in COVID-19 patients (3). Interestingly, the severe thrombotic complications may develop despite standard pharmacological prophylaxis with heparin.

It has been well documented that inflammation due to viral and bacterial infections can lead to the systemic activation of coagulation (4). D-dimer, an independent predictor of poor prognosis in COVID-19, is a biomarker of this activation that is triggered by tissue factor (TF), the key activator of the blood coagulation cascade. D-dimer, however, does not

identify per se the underlying molecular mechanisms and/or the dysfunctional cell population involved in its production.

Endothelial injury, a common feature of viral infection, can alter hemostasis directly or indirectly (5). Viral or bacterial infections and inflammatory stimuli can indeed induce TF expression, not only in endothelial, but also in circulating, monocytes and granulocytes. These cells upon activation also release microvesicles (MVs) into the bloodstream. Among the circulating MVs, those expressing phosphatidylserine (PS) and TF are defined as procoagulant MVs and globally contribute to the activation of the coagulation (6).

Severe imbalances of endothelial function during systemic infections with hyperinflammation can further alter hemostasis through the reduced production or action of mediators such as prostacyclin (PGI₂) and nitric oxide (NO), pivotal in the control of platelet activation (7). Platelets participate in inflammation and thrombotic responses in many viral infections (8). The low platelet count often described in COVID-19 patients indeed suggests increased consumption due to a massive platelet activation and thrombus formation. It should be mentioned in this regard that activated platelets also express a functionally active TF (9). Thus, within the hemostatic process, platelets not only aggregate providing the negatively charged phospholipid bilayer for the

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assembly of the coagulation factors, but they are able themselves to trigger the coagulation cascade.

Finally, the cytokine storm associated with severe COVID-19, which is considered a pathological underpinning for disease progression and multiorgan failure in these patients, is characterized by increased plasma concentrations of several cytokines including interleukin-6 (IL-6) (10). Preclinical studies seem to suggest the efficacy of anti-IL-6 receptor (IL-6R) blockade with tocilizumab (11,12). However, the effect of the cytokine release on platelet and endothelial activation in this clinical setting is almost completely unknown. Thus, whereas data are rapidly emerging about COVID-19-associated coagulopathy and thrombosis risk, there is little high-quality evidence to guide antithrombotic management.

On the basis of the findings collected so far, we hypothesized that the cytokine storm taking place in COVID-19 patients, deeply affecting endothelial functions, leads to consistent blood cell activation, resulting, not only in a generalized cell-based TF-mediated activation of blood coagulation and release of procoagulant MVs, but also in a massive platelet activation. Thus, we carried out this study to assess in COVID-19 patients: 1) the level of TF expression among the circulating cells and MVs; 2) the residual plasma thrombin generation capacity despite heparin treatment; and 3) the extent of platelet and endothelial activation.

Finally, through an *in vitro* approach, we verified whether plasma from COVID-19 patients added to blood cells from healthy subjects (HS) induced platelet activation similar to what is observed *in vivo*. In this experimental setting, we also assessed whether IL-6R blockade or antiplatelet drugs reverted platelet activation.

METHODS

PATIENT SELECTION. This prospective study recruited 46 consecutive COVID-19 patients admitted to San Luca Hospital, Istituto Auxologico Italiano IRCCS, Milan, between April 1st and 30th, 2020. Patients with a positive severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2) polymerase chain reaction test and requiring oxygen supplementation were included. Criteria for hospital admission were defined as those requiring inpatient care as a result of the severity of illness based on laboratory and radiological parameters, as well as clinical findings. Study patients were divided into 2 groups according to their oxygen supplementation:

patients needing low-flow oxygen supplementation only (with nasal cannulae or Venturi mask, group 1) and patients needing mechanical ventilation (invasive or noninvasive, group 2). Following admission, all patients received supportive care in line with best international practice. In particular, hospitalized COVID-19 patients were treated with: 1) lopinavir/ritonavir or darunavir/cobicistat; 2) with/without hydroxychloroquine; 3) with/without antibiotic prophylaxis; or 4) with/without steroids. Moreover, hospitalized patients for COVID-19 received low-molecular-weight heparin (LMWH) thromboprophylaxis according to their body weight and renal function, unless contraindicated, as part of standard of care.

Biochemical variables, including inflammatory and thrombotic parameters (IL-6, C-reactive protein [CRP], lactate dehydrogenase [LDH], fibrinogen, D-dimer, procalcitonin) and arterial blood gas analysis (partial pressure of oxygen/fraction of inspired oxygen [pO_2/FiO_2] ratio, oxygen saturation), were recorded at hospital admission and immediately before or soon after oxygen supplementation, concomitantly with the blood sampling needed for the analysis described in the following text. Data from 46 HS (35% male, mean age 42 ± 11 years) and 46 stable coronary artery disease (CAD) patients (91% male, mean age 67 ± 10 years) with recent (<6 months) percutaneous coronary revascularization, and on dual antiplatelet therapy, previously recruited at Centro Cardiologico Monzino IRCCS, Milan, were analyzed for comparisons. The study was approved by the Ethical Committee of the Institution (number 2020_06_16_18), and informed consent was obtained from all participants according to the principles of the declaration of Helsinki.

STATISTICAL ANALYSIS. Quantitative variables are presented as median with 25th and 75th percentiles, being mostly log-normally distributed; within-group comparisons were made with the Wilcoxon signed rank test, and between-group comparisons were made with the Wilcoxon rank sum test. The normality of the variable distributions was assessed by the use of the D'Agostino-Pearson omnibus K2 test. Categorical variables are presented as n (%) and were compared by chi-square or Fisher's exact test, when appropriate. Associations between variables were assessed by Spearman's correlation coefficient (rs). A p value <0.050 was considered statistically significant. p Values presented in this report have not been adjusted for multiplicity to protect type I error, and

TABLE 1 Demographic and Clinical Characteristics of Enrolled COVID-19 Patients

	All (N = 46)	Oxygen Therapy [Group 1] (n = 20)	Mechanical Ventilation [Group 2] (n = 26)	p Value
Age, yrs	72 (58-84)	67 (58-81)	74 (63-84)	0.288
Male	28 (61)	11 (55)	17 (65)	0.182
In-hospital mortality	10 (21.7)	0 (0)	10 (38.5)	0.031
Length of hospitalization, days	40.5 (29-43.5)	40 (29-47)	41 (29-43)	0.862
Interleukin-6, pg/ml	41 (27-77)	32 (24-37)	70 (39-107)	0.028
CRP, mg/dl	8.8 (4.6-12.3)	4.7 (1.5-7.4)	11.8 (7.1-17.1)	<0.001
D-dimer, µg/l	1,422 (774-2,139)	1,229 (774-1,727)	1,612 (981-2,787)	0.192
Arterial gas analysis				
Oxygen saturation	94.5 (92-97)	96 (93-97)	94 (88-97)	0.149
Respiratory rate	20 (18-24)	18 (18-20)	22 (18-27)	0.035
pCO ₂	36.5 (33-44)	36.5 (35-39.5)	37.5 (32-44)	0.871
pO ₂ /FiO ₂ ratio	177 (132-343)	362 (324-397)	160 (130-180)	0.002
pH	7.5 (7.4-7.5)	7.5 (7.5-7.5)	7.5 (7.4-7.5)	0.269
Lactate	1.3 (0.9-1.6)	1.4 (0.7-1.5)	1.3 (1-1.7)	0.478
Cardiovascular risk factors				
Smoking status				
Active smoker	3 (8)	2 (10)	1 (4)	0.577
Former smoker	6 (16)	3 (15)	3 (11)	
Dyslipidemia	2 (5)	0 (0)	2 (8)	0.979
Hypertension	32 (69)	13 (65)	19 (73)	0.348
Diabetes	4 (11)	0 (0)	4 (18)	0.138
Past medical history				
Cardiovascular diseases	33 (87)	13 (65)	20 (77)	0.979
Cerebrovascular diseases	7 (18)	2 (10)	5 (8)	0.681
Respiratory diseases	10 (27)	4 (20)	6 (23)	0.968
Endocrinologic diseases	8 (21)	3 (15)	5 (22)	0.897
Renal diseases	2 (5)	0 (0)	2 (8)	0.240
Pharmacological therapy				
LMWH treatment, dose				
4,000 U	1 (2.2)	1 (4)	0 (0)	0.623
6,000 U	5 (11.1)	3 (12)	2 (10)	
4,000 U ×2	17 (37.8)	10 (40)	7 (35)	
6,000 U ×2	11 (24.4)	6 (24)	5 (25)	
8,000 U ×2	2 (4.4)	2 (8)	0 (0)	
Antiplatelet agents	5 (11)	5 (25)	0 (0)	<0.001
Hydroxychloroquine	4 (12)	3 (15)	1 (4)	0.087
Cortisone	13 (30)	4 (20)	9 (34)	0.922
Biochemical parameters				
Ferritin, µg/l	857 (498-1,686)	498 (358-1,758)	946 (612-1,356)	0.634
AST, U/l	35 (23-44)	30 (18-40)	38 (29-46)	0.314
ALT, U/l	30 (20-57)	24 (16-33)	31 (23-66)	0.117
LDH, U/l	326 (249-383)	256 (181-314)	348 (317-390)	0.027
S-Creatinine, mg/dl	0.9 (0.7-1.0)	0.8 (0.6-0.9)	0.9 (0.8-1.1)	0.016
PT/INR	1.22 (1.15-1.30)	1.25 (1.13-1.34)	1.19 (1.15-1.24)	0.938
PTT ratio	1.35 (1.18-1.47)	1.39 (1.19-1.56)	1.33 (1.18-1.44)	0.152
TnT, ng/l	26 (13-48)	26 (17-37)	26 (11-49)	0.700
ProBNP, ng/l	197 (97-315)	159 (60-207)	315 (172-521)	0.059
Procalcitonin, ng/ml	0.1 (0.1-0.2)	0.1 (0-0.1)	0.2 (0.1-0.3)	0.118
Fibrinogen, mg/dl	546 (412-637)	424 (379-627)	546 (474-647)	0.302

Continued on the next page

therefore, the reproducibility (statistical power) of the inferences drawn from these statistics could be questioned. All analyses were performed using GraphPad Prism version 9.0 software (GraphPad

Software, San Diego, California) and SAS version 9.4 software (SAS Institute, Cary, North Carolina).

See the [Supplemental Appendix](#) for extended experimental procedures.

TABLE 1 Continued

	All (N = 46)	Oxygen Therapy [Group 1] (n = 20)	Mechanical Ventilation [Group 2] (n = 26)	p Value
Blood cell count				
WBC, 10 ³ /μl	10.1 (6.6-12.3)	9.7 (7.2-10.8)	10.5 (7.0-14.6)	0.515
Neut, 10 ³ /μl	6.8 (4.0-9.8)	6.4 (4.4-8.3)	8.7 (4.7-12.2)	0.283
Lymph, 10 ³ /μl	1.3 (0.9-2.1)	1.7 (1.2-2.1)	1.1 (0.6-1.9)	0.035
Mono, 10 ³ /μl	0.7 (0.5-0.9)	0.7 (0.6-0.9)	0.6 (0.5-0.9)	0.457
RBC, 10 ⁶ /μl	3.7 (3.2-4.0)	3.9 (3.6-4.2)	3.4 (3.1-3.7)	<0.001
Hgb, g/dl	10.7 (9.8-11.9)	11.2 (10.6-12.0)	10.1 (9.3-11.3)	0.019
HCT, %	33.8 (30.9-36.9)	35.4 (33-37)	31.8 (28.9-35.8)	0.010
PLT, 10 ³ /μl	267 (202-314)	295 (269-362)	232 (128-289)	0.001
PDW, fl	12.9 (11.6-14.2)	12.3 (11.2-13.4)	13.7 (12.6-15.5)	0.008
MPV, fl	11 (10.4-11.6)	10.7 (10.1-11.2)	11.3 (10.9-12.1)	0.004
P-LCR, %	32.5 (27.2-36.3)	30.7 (25.3-34.2)	35.1 (31.3-40.8)	0.004
IPF, %	3.7 (2.7-5.7)	2.8 (1.8-4.3)	4.8 (3.3-8.2)	0.003

Values are median (interquartile range) or n (%).

ALT = alanine aminotransferase; AnnV, annexinV; AST = aspartate aminotransferase; COVID-19 = coronavirus disease-2019; CRP = C-reactive protein; FiO₂ = fraction of inspired oxygen; HCT = hematocrit; Hgb = hemoglobin; INR = international normalized ratio; IPF = immature platelet fraction; LDH = lactate dehydrogenase; LMWH = low-molecular-weight heparin; Lymph = lymphocyte; Mono = monocyte; MPV = mean platelet volume; Neut = neutrophil; pCO₂ = partial pressure of carbon dioxide; PDW = platelet distribution width; P-LCR = platelet large cell ratio; PLT = platelet; pO₂ = partial pressure of oxygen; ProBNP = pro-B-type natriuretic peptide; PT = prothrombin time; PTT = partial thromboplastin time; RBC = red blood cell; TnT = troponin T; WBC = white blood cell.

RESULTS

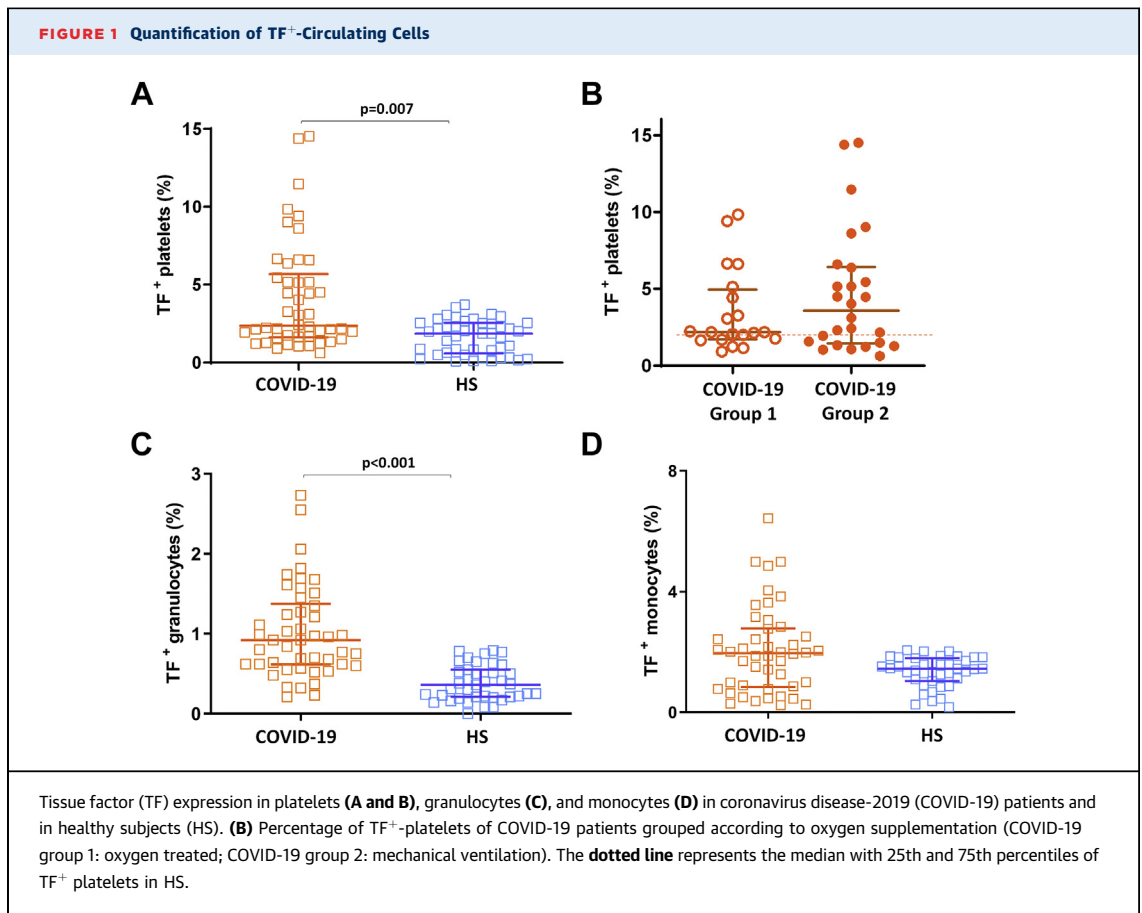
PATIENT CHARACTERISTICS. Baseline characteristics of the enrolled patients are summarized in [Table 1](#). The median age was 67 [58 to 81] years and 74 [63 to 84] years, for patients on oxygen therapy (group 1) and invasive or noninvasive mechanical ventilation (group 2), respectively, with a predominance of men in both groups (55% and 65% in group 1 and group 2, respectively). Chronic diseases, including cardiovascular and cerebrovascular, respiratory system, endocrinologic, and kidney diseases were comparable between the 2 groups. In the patients requiring mechanical ventilation, the arterial blood gas analysis parameters, in particular the pO₂/FiO₂ ratio, were significantly worse. Ferritin, LDH, serum creatinine, procalcitonin, D-dimer, fibrinogen, CRP, and IL-6, measured at hospital admission, were higher in group 2 patients. At the time of blood sampling for the analysis described in the following text, group 2 patients also had lower platelet, lymphocyte, and erythrocyte counts, and lower hemoglobin levels. Conversely, the platelet-related parameters platelet distribution width, mean platelet volume, platelet large cell ratio, and immature platelet fraction were all significantly greater compared with group 1. During hospitalization, LMWH at prophylactic (1 per day) or therapeutic (2 per day) regimen, hydroxychloroquine and cortisone treatment was comparable between the 2 groups, whereas antiplatelet drugs were administered in only 5 group 1 patients (25%). No symptomatic

venous thromboembolisms were diagnosed during hospitalization.

CHARACTERIZATION OF TF-EXPRESSING CELLS AND MVs. We first assessed TF expression among the circulating cells and MVs. Whole-blood flow cytometry analysis of TF⁺ cells showed that the median level of TF⁺ platelets in COVID-19 patients was significantly higher than that found in HS (p = 0.007), with a trend toward greater values in group 2 ([Figures 1A and 1B](#)). Interestingly, 40% of patients in group 1 had TF⁺ platelets far above the median value of HS.

Granulocytes were also ~3-fold greater in COVID-19 compared with HS (p < 0.001) ([Figure 1C](#)), whereas only a trend increase was observed for TF⁺ monocytes ([Figure 1D](#)). Distribution of TF⁺ granulocytes and monocytes was not different between COVID-19 patients. Notably, TF⁺ platelets and granulocytes were also significantly higher in COVID-19 than in CAD patients on dual antiplatelet therapy, whereas TF⁺ monocytes were similar ([Supplemental Figure 1](#)).

MVs were assessed in plasma, taking into account: 1) their total number; 2) the procoagulant phenotype in terms of annexinV (AnnV) binding to PS and TF expression; and 3) their cell origin, focusing on those derived from platelets, granulocytes, monocytes, erythrocytes, and endothelium. Total and TF⁺ MVs were significantly higher in COVID-19 patients compared with HS, whereas the AnnV⁺ MVs were lower ([Table 2](#)). All the parental cells released a double amount of TF⁺ MVs, the most abundant coming



from platelets and erythrocytes, followed by those from endothelium, granulocytes, and monocytes. Despite a similar MV distribution in the 2 COVID-19 groups, the number of procoagulant MVs (AnnV⁺/TF⁺) was greater in group 2 patients (Supplemental Figure 2). Notably, almost all the TF⁺ MVs were significantly higher in COVID-19 than in CAD patients (Supplemental Table 1).

Considering the massive expression of cell- and MV-associated TF measured, we then tested the residual plasma thrombin generation capacity. At the time of our sampling, all but 20% of patients were on LMWH (Table 1). When no exogenous TF was used to trigger the reaction, the few patients that were not on LMWH generated thrombin much faster than HS (lag time 11.3 [10 to 22.3] min and 23.3 [18.7 to 30.2] min, respectively). Among those on LMWH, thrombin generation was observed in 20% of patients. The time needed for thrombin formation was as high as that of HS (lag time 21 [18.3 to 22] min and 23.3 [18.7 to 30.2] min in COVID-19 patients and HS, respectively) and they accounted for 30% of those treated with prophylactic LMWH.

When the TF-FVIIa complex formation was no longer the limiting step in the system (by adding excess of exogenous TF), residual thrombin formation was observed in all patients treated with 6,000 U/day and in 40% of those with 8,000 to 16,000 U/day LMWH, being mainly (70%) in group 2.

In addition, despite the administration of comparable daily-dose LMWH, thrombin was more than 3- to 5-fold higher in group 2 than in group 1 patients (endogenous thrombin potential [ETP] 1,064 [1,040 to 1,240] nmol/l × min vs. 584 [529 to 638] nmol/l × min, respectively; $p < 0.001$; peak high 165 [156 to 169] nmol/l vs. 40.7 [35 to 46.4] nmol/l thrombin, respectively; $p < 0.001$), evidencing a higher prothrombotic risk in patients with reduced respiratory capacity. Interestingly, the ETP correlated with the number of procoagulant MVs ($r = 0.495$; $p = 0.020$).

Overall these data, underscoring the marked prothrombotic phenotype associated with both TF-bearing cells and MVs, provide insights into the potential mechanisms that trigger the hypercoagulable state, especially in severe COVID-19 patients.

CHARACTERIZATION OF PLATELET ACTIVATION.

Platelet-associated TF expression and circulating platelet-derived MVs are only 2 among the markers of platelet activation. For a more comprehensive analysis, we also assessed P-selectin exposure and platelet-leukocyte aggregate (PLA) formation. The percentage of P-selectin⁺ platelets was 10-fold higher in COVID-19 patients compared with HS ($p = 0.006$) (Figure 2A). This was paralleled by a marked increase in the frequencies of granulocyte-platelet aggregates (GPA) and monocyte-platelet aggregates (MPA); $p < 0.001$ (Figures 2B and 2E), as well as in TF⁺ aggregates ($p < 0.001$) (Figures 2D and 2G). All these parameters were also higher compared with CAD patients (Supplemental Figure 3). Of note, IL-6, CRP, and D-dimer levels negatively correlated with GPA ($r = -0.38$; $p = 0.022$; $r = -0.45$; $p = 0.002$, and $r = -0.47$; $p = 0.003$, respectively) and MPA ($r = -0.51$; $p = 0.002$; $r = -0.53$; $p < 0.001$, and $r = -0.35$; $p = 0.033$, respectively). Indeed, GPA and MPA were significantly lower in group 2 than in group 1 ($p = 0.003$ and $p = 0.004$, respectively) (Figures 2C and 2F).

All together, these results sustain the concept of a very high platelet activation state in COVID-19 patients. Moreover, the negative correlation of PLA with inflammatory and thrombotic parameters, along with the lower platelet count of group 2 patients, suggests their possible consumption even in COVID-19 patients considered to be at low risk.

CHARACTERIZATION OF ENDOTHELIAL ACTIVATION.

Perturbed endothelium, as occurs during systemic infection, affects platelet activation (7). Thus, we evaluated the extent of endothelial dysfunction by assessing NO and PGI₂ production. COVID-19 patients showed an impairment in NO biosynthetic pathway, being almost one-half the concentration of arginine and twice the levels of asymmetric dimethylarginine (ADMA), the specific NO synthase inhibitor, compared with HS ($p < 0.001$) (Figures 3A and 3B). Global arginine bioavailability ratio, calculated as arginine/(ornithine+citrulline), was significantly reduced in COVID-19 patients as a result of the higher levels of ornithine compared with HS (Figures 3C and 3D). Similar data were observed comparing COVID-19 to CAD patients (Supplemental Figure 4). Of note, ornithine concentrations were also significantly lower in group 2 compared with group 1 patients ($p = 0.048$) (Figure 3E), and citrulline, which is produced in equimolar amounts to NO, behaved similarly (Figures 3F and 3G). COVID-19 patients also had higher concentrations of 6 ketoprostaglandin F1 alpha (6ketoPGF_{1α}), the main plasma metabolite of COX-

TABLE 2 MV Characterization in COVID-19 Patients and in HS

MV/ μ l	COVID-19 (n = 46)	HS (n = 46)	p Value
Total	1,036 (853-1339)	657 (571-792)	<0.001
TF ⁺	435 (276-633)	184 (136-229)	<0.001
AnnV ⁺	87 (68-132)	152 (128-194)	<0.001
Platelet-derived, CD41 ⁺	274 (217-367)	205 (159-247)	<0.001
Erythrocyte-derived, Glyco ⁺	278 (219-411)	216 (169-280)	<0.001
Monocyte-derived, CD14 ⁺	76 (45-117)	45 (39-55)	<0.001
Granulocyte-derived, CD66 ⁺	145 (103-256)	62 (52-84)	<0.001
Endothelium-derived, CD146 ⁺	141 (98-179)	125 (91-141)	0.092
CD41 ⁺ TF ⁺	193 (117-279)	85 (63-111)	<0.001
Glyco ⁺ TF ⁺	190 (132-277)	90 (67-120)	<0.001
CD14 ⁺ TF ⁺	41 (29-60)	18 (14-24)	<0.001
CD66 ⁺ TF ⁺	85 (63-157)	40 (32-50)	<0.001
CD146 ⁺ TF ⁺	89 (81-107)	48 (37-80)	<0.001

Values are median (interquartile range).
AnnV = annexin V; COVID-19 = coronavirus disease-2019; Glyco = glycoprotein; HS = healthy subjects; MV = microvesicle; TF = tissue factor.

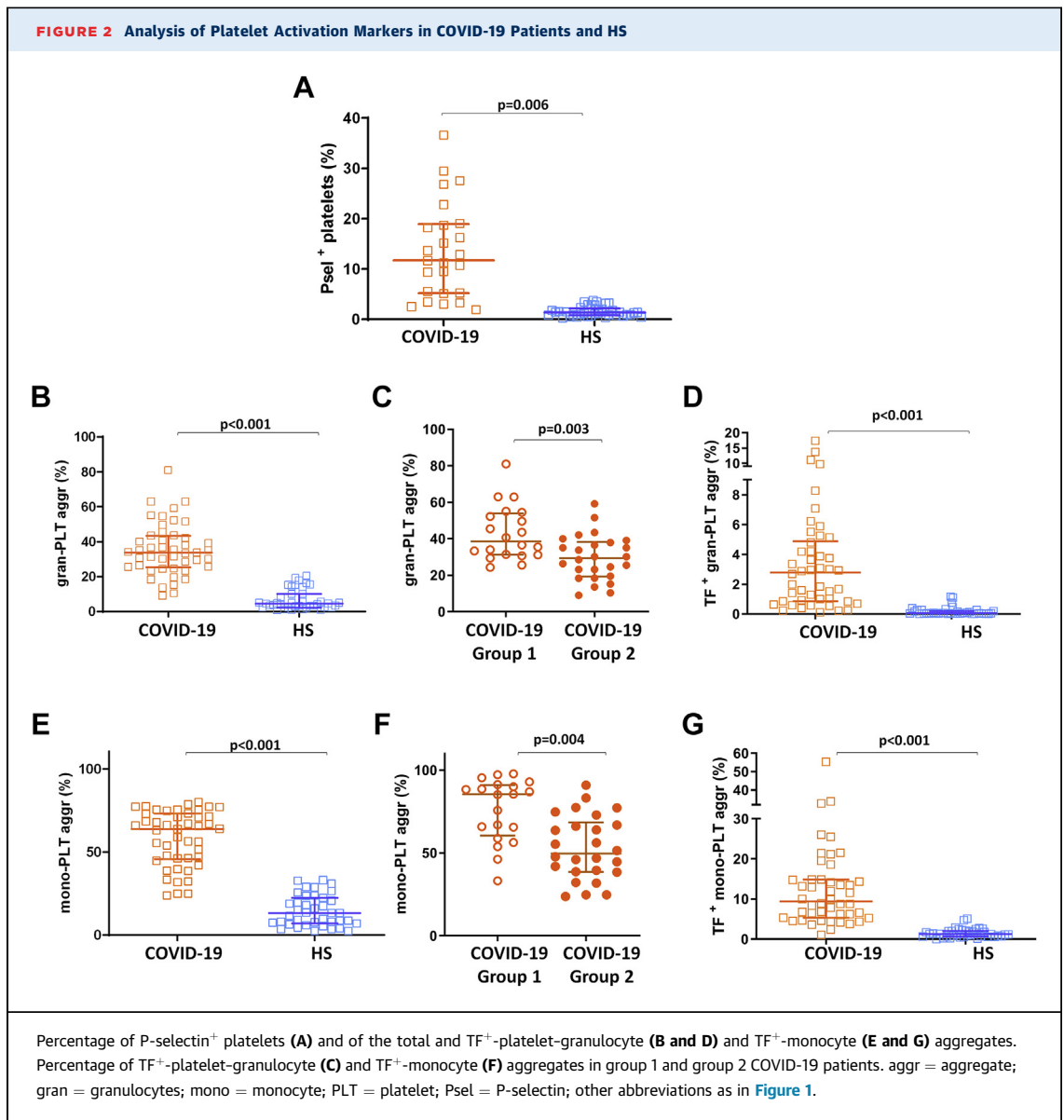
2-produced PGI₂, than those of HS ($p = 0.010$) (Figure 3H) with higher levels in group 2 compared with group 1 patients ($p = 0.035$) (Figure 3I).

The relation between L-arginine/NO pathway impairment and the systemic inflammatory response was supported by the negative correlation between CRP and both arginine and citrulline ($r = -0.34$; $p = 0.037$; $r = -0.42$; $p = 0.009$, respectively) and IL-6 with both citrulline and ornithine ($r = -0.45$; $p = 0.014$; $r = -0.43$; $p = 0.012$, respectively).

Taken together, these data highlight the endothelial impairment that could affect platelet activation.

IN VITRO MODEL OF COVID-19-INDUCED PLATELET ACTIVATION.

In consideration of the relevance of the cytokine storm in the pathogenesis of severe COVID-19, we tested whether the cytokine content of plasma from COVID-19 patients reproduced, in cells from HS, the massive platelet activation observed in vivo. Blood from HS was plasma-depleted and reconstituted with plasma pools from COVID-19 patients or from HS. Analysis performed after 30-min incubation showed that only COVID-19 plasma significantly increased the number of TF⁺ platelets (Figure 4A) and their TF-dependent thrombin generation capacity (lag time 13.4 [13.2 to 14.1] min and 15.7 [14.8 to 16.4] min for COVID-19 and HS plasma, respectively; $p < 0.003$) (Supplemental Figure 5C). Plasma COVID-19 also induced MV release, as well as expression of the other platelet activation markers (Figures 4B to 4G), but activated glycoprotein (GP) IIb/IIIa and PS exposure (Supplemental Figures 5A and 5B). Interestingly, this activation was not due to a direct effect of SARS-CoV-2 because digital PCR

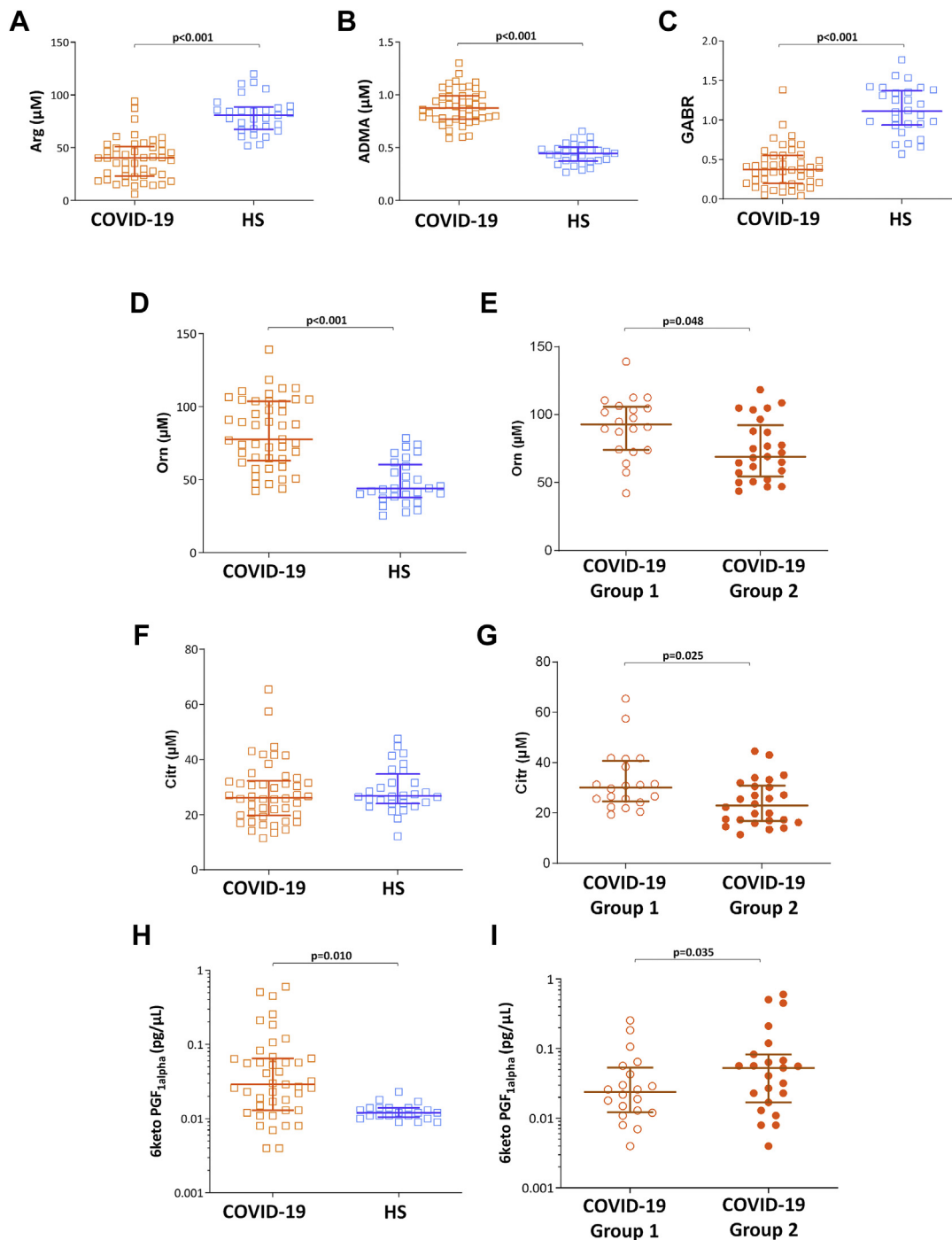


analysis ruled out its presence in patients' plasma (Supplemental Figure 6).

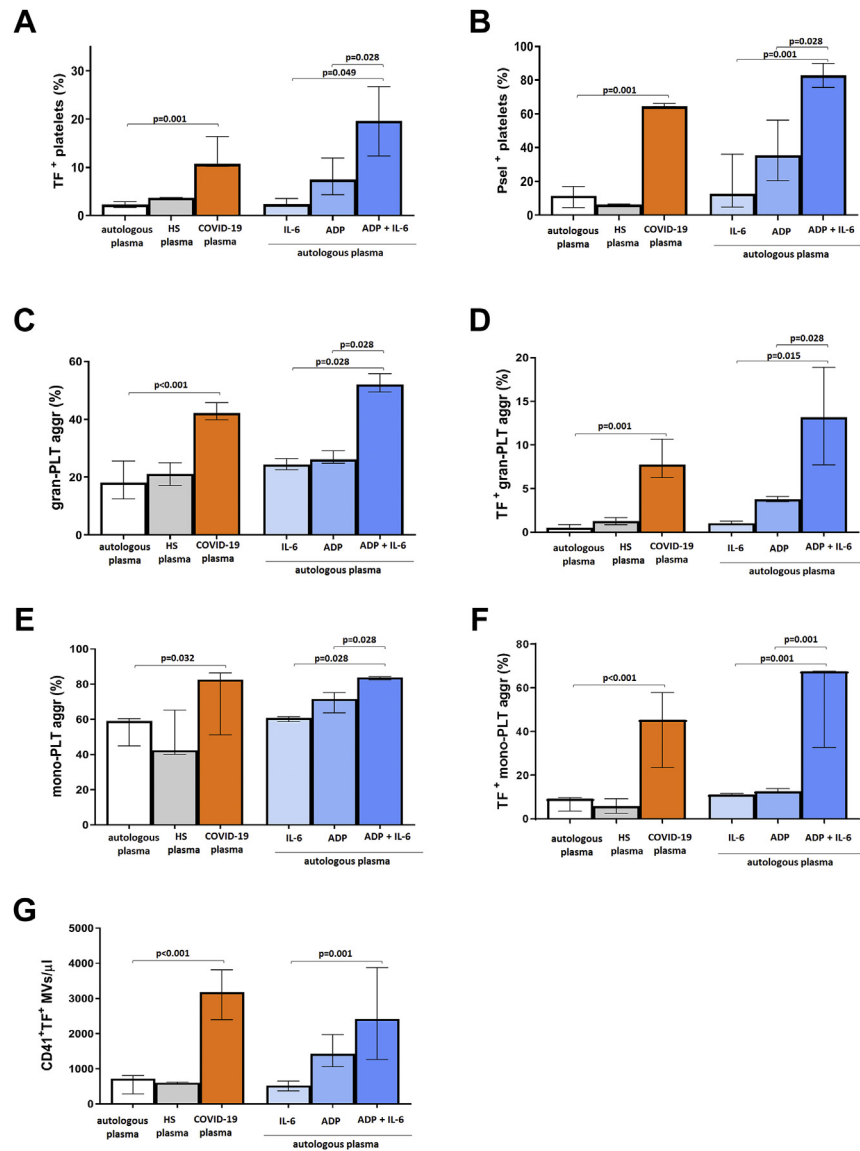
Among the mediators released during the cytokine storm, IL-6 seems to play a major role because efficacy of anti-IL-6R blockade with tocilizumab has been reported (13). Thus, we explored whether IL-6 added to blood from HS could recapitulate the effect observed with COVID-19 plasma. Interestingly IL-6, at concentrations comparable to those found in COVID-19 patients, was ineffective in inducing cell activation. Conversely, it significantly potentiated the effect of low-concentration adenosine diphosphate (ADP) or thromboxane A₂ (stable analogue U46619), endogenous platelet agonists released on activation,

resulting in cell stimulation resembling that observed with COVID-19 plasma for all the parameters assessed (Figure 4, Supplemental Figure 7).

On the basis of these findings, we then assessed the effect of blocking IL-6 signaling. To this aim, plasma COVID-19 and blood from HS were treated with tocilizumab at 2 concentrations close to those achieved in therapy. The drug concentration dependently blunted the effect of COVID-19 plasma on the expression of TF⁺ platelets, TF⁺ aggregates, and TF⁺-platelet-derived MVs (Figures 5A, 5F, 5H, and 5I) and significantly reduced platelet-associated thrombin generation (Figures 5B and 5C), P-selectin expression (Figure 5D), and GPA, and MPA formation (Figures 5E

FIGURE 3 Evaluation of Endothelial Activation Markers in COVID-19 Patients and HS

Plasma levels of L-arginine (Arg) (A), asymmetric dimethylarginine (ADMA) (B), global arginine bioavailability ratio (GABR) (C), L-ornithine (Orn) (D), L-citrulline (Citr) (F), and 6-keto- $\text{PGF}_{1\alpha}$ (H). L-ornithine (E), L-citrulline (G), and 6-keto- $\text{PGF}_{1\alpha}$ (I) in group 1 and group 2 COVID-19 patients. Abbreviations as in Figure 1.

FIGURE 4 Ex Vivo Effect of Plasma From COVID-19 Patients on HS Platelets

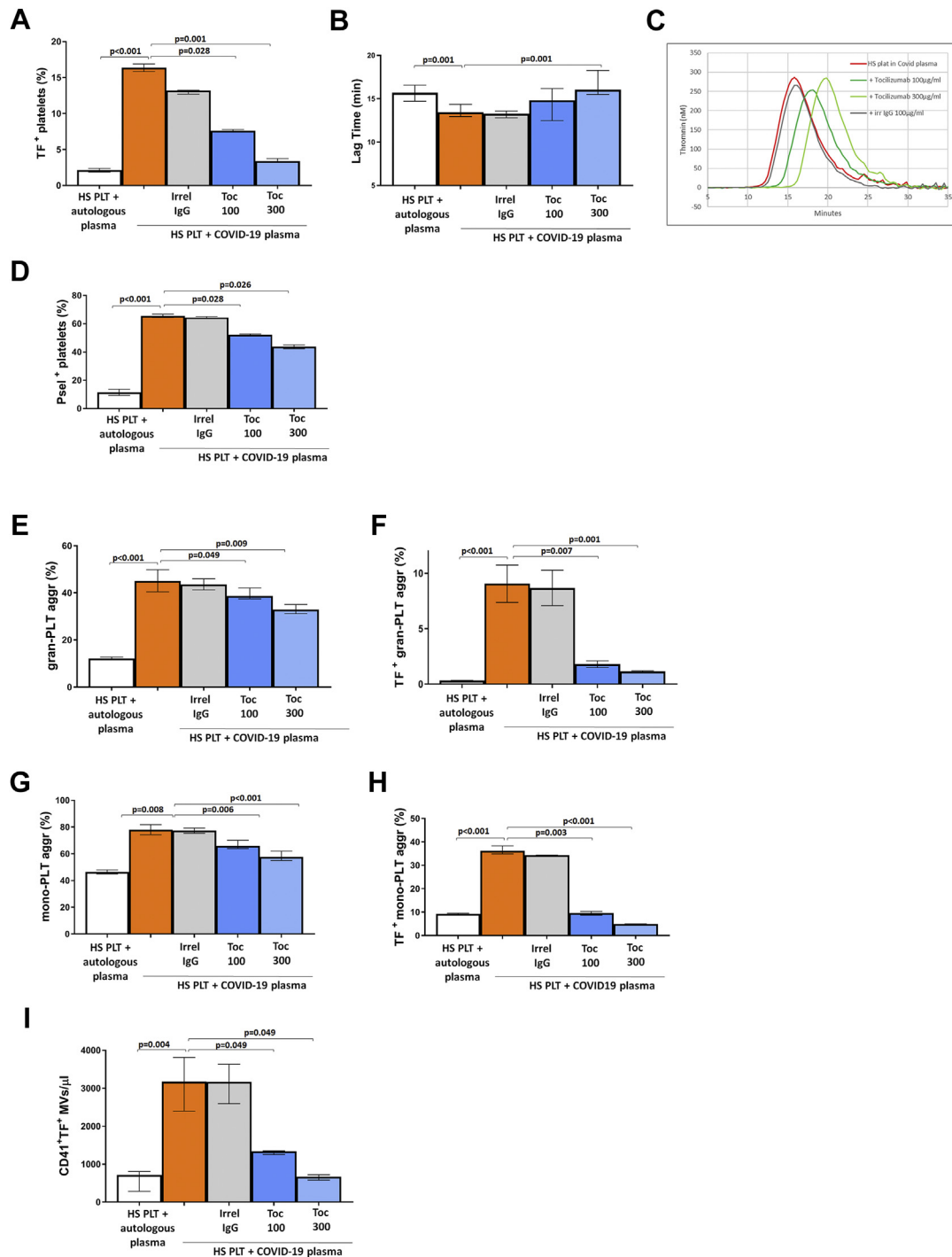
Plasma-depleted blood from HS (n = 5) reconstituted with autologous plasma (white bars), a plasma pool from HS (grey bars), or with a plasma pool from COVID-19 patients (orange bars). Percentage of TF⁺ and P-selectin⁺ platelets (A and B), CD41⁺TF⁺ MVs (G), total and TF⁺ granulocyte-platelet aggregates (C and D) and monocyte-platelet aggregates (D and F). The effect of whole blood incubation with interleukin (IL)-6 (100 pg/ml), ADP (0.1 μmol/l), or ADP+IL-6 on the same parameters is reported (n = 5). ADP = adenosine diphosphate; other abbreviations as in Figures 1 and 2.

and 5G). No effect of tocilizumab on blood reconstituted with autologous plasma was observed (Supplemental Figure 8).

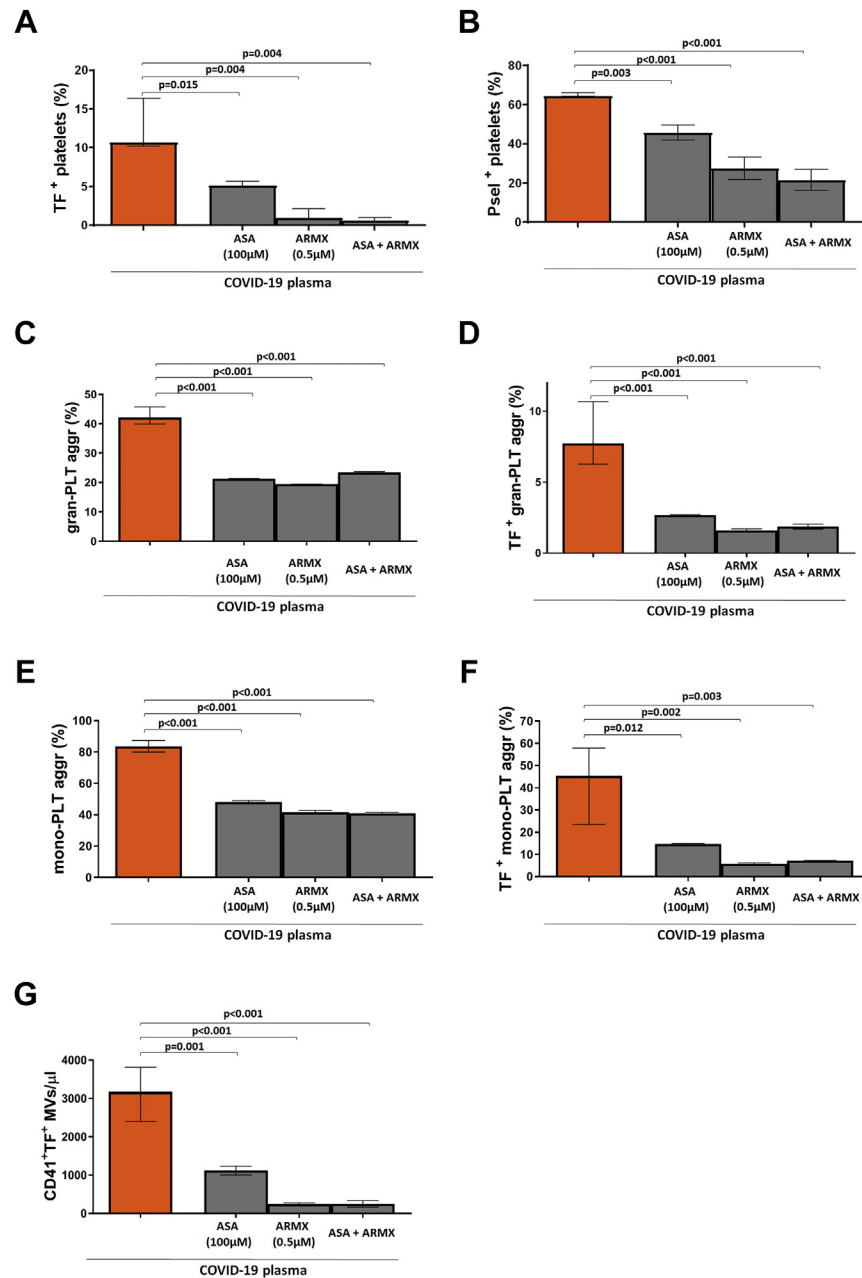
Overall, results obtained with this approach suggest that the platelet activation observed in COVID-19 patients is not directly caused by SARS-CoV-2. Furthermore, these data support the clinical

evidence pointing toward a key role of IL-6 in the coagulopathy described in severe COVID-19 patients.

IN VITRO MODULATION OF COVID-19-INDUCED PLATELET ACTIVATION BY ANTIPLATELET DRUGS. Finally, because in vitro data confirm the sustained platelet activation documented in vivo, we evaluated whether aspirin and the P2Y₁₂ inhibitor AR-C69931MX

FIGURE 5 In Vitro Effect of Tocilizumab on Platelet Activation Induced by COVID-19 Plasma

Plasma-depleted blood from HS ($n = 5$) has been reconstituted with COVID-19 plasma pool (orange bars) or COVID-19 plasma pool preincubated with tocilizumab (Toc; 100 or 300 $\mu\text{g}/\text{ml}$; blue and light blue bars, respectively) or with an irrelevant (Irrel) IgG (300 $\mu\text{g}/\text{ml}$; grey bars). Blood reconstituted with autologous plasma is reported for comparison (white bars). Percentage of TF⁺ and P-selectin⁺ platelets (A and D), CD41⁺TF⁺ MVs (I), total and TF⁺ granulocyte-platelet aggregates (E and F) and monocyte-platelet aggregates (G and H). (B) Time needed for platelet-associated thrombin generation (TG) (lag time). (C) Representative curves of TG were reported. Abbreviations as in Figures 1 and 2.

FIGURE 6 In Vitro Effect of Aspirin and AR-C69931MX on Platelet Activation Induced by COVID-19 Plasma

Blood from HS (n = 5), pre-incubated with aspirin (100 µmol/l) or with AR-C69931MX (0.5 µmol/l) or both, was plasma-depleted and reconstituted with COVID-19 plasma pool. Percentage of TF⁺ and P-selectin⁺ platelets (**A and B**), CD41⁺TF⁺-MVs (**G**), total and TF⁺ granulocyte-platelet aggregates (**C and D**) and monocyte-platelet aggregates (**E and F**). Abbreviations as in **Figures 1 and 2**.

prevented it. Both antiplatelet agents significantly inhibit COVID-19 plasma-induced platelet activation and MV release, AR-C69931MX being more effective than aspirin in reducing TF⁺ platelets and TF⁺-platelet-derived MVs, as well as P-selectin.

Association of these drugs did not result in any additive effect (**Figure 6**). These data suggest the potential benefit of antiplatelet agents in the management of the thrombotic complication of COVID-19 patients.

DISCUSSION

The present study provides, for the first time to our knowledge, evidence that the coagulopathy reported in COVID-19 patients is supported by: 1) a sustained TF expression by virtually all the cells in contact with blood and by the derived MVs; and 2) a massive platelet activation characterized by the formation of PLA that may be involved in the pulmonary microthrombi found in autoptic specimens. All these events occur on the background of a dysfunctional endothelium in terms of NO and PGI₂ production. Moreover, the study highlights the role of IL-6 in sustaining blood cells activation and TF expression, and how the pharmacological modulation, not only with tocilizumab, but also with antiplatelet drugs, turns off PLA and MV formation, and TF expression (Visual Abstract).

COVID-19 is caused by the SARS-CoV-2 infection. Despite hemostatic changes, indicative for a coagulopathy that may predispose COVID-19 patients to thrombotic events, having been consistently reported in the published reports, very little is known on the pathophysiological mechanisms involved. Another knowledge gap is whether the hemostatic abnormalities are directly caused by SARS-CoV-2 or are the results of endothelial dysfunction together with the cytokine storm, as previously reported for other infections (5).

Our data show that the high D-dimer levels often measured in COVID-19 patients could be due to a cell- and MV-associated TF-dependent activation of blood coagulation. The number of circulating TF⁺ platelets and granulocytes is 2- to 3-fold greater than that found in HS or CAD patients. Also TF⁺ MVs doubled compared with HS. Interestingly, TF⁺ platelets and procoagulant MVs both correlate with the disease severity, being higher in COVID-19 patients requiring mechanical ventilation for maintaining adequate arterial oxygenation. Moreover, procoagulant MVs also positively correlated with the residual plasma thrombin generation capacity that, despite comparable LMWH daily dose, was higher again in COVID-19 patients necessitating mechanical ventilation. It is worth mentioning in this regard that, in an attempt to prevent microvascular thrombosis, empiric use of parenteral anticoagulation at a prophylactic or therapeutic dose has been applied to COVID-19 patients worldwide. While this study was conducted, the optimal dosing of anticoagulant agents was still unknown, and further prospective investigation was claimed (14).

Viral infections, bacterial infections, and inflammatory stimuli are well-known inducers of TF expression in endothelial cells, monocytes, and granulocytes (4,15). By adding an ex vivo and in vitro approach to the snapshot of the in vivo situation, this study clearly highlights how platelet TF is also fundamental in the thromboinflammation triggered by SARS-CoV-2. Even numbers come in handy in pathophysiology: when the absolute number of TF⁺ events is computed, taking into account the blood cell concentration, the number of circulating TF⁺ platelets is ~100 times higher than that of granulocytes.

Although the research on TF modulation in platelets is relatively more recent than that on endothelium and leukocytes, several studies have documented by now that, upon platelet activation by the common agonists, TF translocates from the cytoplasm to the cell membrane where it can trigger thrombin generation (9,16,17). More recently, we have shown that also angiotensin II (ATII) stimulates platelets to express TF (18). This mechanism may have direct implications in COVID-19 coagulopathy where ATII levels increase as a result of the down-regulation of angiotensin-converting enzyme 2 (ACE2), the main host cell receptor of SARS-CoV-2, following virus binding (19). In view of the cytokine storm present in COVID-19 patients, the finding that TNF α elicited platelet activation and TF expression, which in turn prompted thrombin generation and clot formation, has also to be considered (15). Interestingly, it has been previously reported that HIV-infected patients, who have an increased risk for thrombotic and cardiovascular events, also have significantly higher levels of TF⁺ and P-selectin⁺ platelets and MVs compared with noninfected subjects (20). Furthermore, in these patients, the risk of death, including that due to cardiovascular events, was linked to higher plasma levels of IL-6, CRP, and D-dimer, similar to what observed in COVID-19 (21).

In these patients, we also observed a very strong up-regulation of platelet P-selectin (10-fold higher than HS). Platelets amplify TF expression on leukocytes via the interaction of P-selectin with PSGL-1, its counter-receptor on monocytes and neutrophils (22). Moreover, P-selectin plays a key role also in the formation of PLA, important elements for the regulation of the immune response and the clearance of infectious agents. By binding and activating leukocytes, platelets promote their effector functions. Platelet-neutrophil complexes have more activated adhesion molecules, greater phagocytic ability, and greater toxic oxygen metabolites than noncomplexed

neutrophils (23). Interestingly, we found a negative correlation between circulating PLA and IL-6, CRP, and D-dimer levels, being indeed lower in group 2 patients. These data, together with the observation that patients on mechanical ventilation were also characterized by a significant lower platelet count compared with those treated with only low-flow oxygen, led us to speculate that these cells could be consumed in the pulmonary microthrombi found in autopsies (24–26).

Several mechanisms can promote platelet activation in COVID-19, and among them, our data point to endothelial dysfunction. Besides the release of TF⁺ MVs and the role of IL-6 (see later in the text), we further highlight that the synthesis of the 2 major molecules controlling platelet activation, NO and PGI₂, is deeply affected. Our data showed a marked reduction of arginine, an essential amino acid whose metabolism has already been recognized as important in several viral infections (27). Arginine can be converted to NO and citrulline by nitric oxide synthase (NOS). The negative correlation between citrulline levels and IL-6 concentrations together with the reduced levels of citrulline in more compromised COVID-19 patients corroborate the role of this metabolic pathway in disease progression. In addition, the increased levels of ADMA, the specific NOS inhibitor, observed in COVID-19 patients further highlight the impaired endothelial function. As expected, ornithine levels were increased in COVID-19 patients as the result of the immunity-mediated up-regulation of arginase, which competes with NOS for the same substrate arginine (28). Interestingly, patients requiring mechanical ventilation showed lower levels of ornithine, which is the precursor of polyamines, a group of small, positively charged molecules, involved in several biological processes. Mounce et al. (29) have recently shown the importance of polyamine biosynthesis in the replication processes of RNA viruses, including Middle East respiratory syndrome coronavirus. In addition, the de novo synthesis of arginine from ornithine could be a vicarious mechanism to integrate low arginine levels. The endothelial activation in COVID-19 patients was further documented by the significantly increased levels of prostacyclin. As previously reported in other clinical settings, the increased endothelial biosynthesis of this antithrombotic prostanoid reflects the homeostatic response to inflammation and platelet activation (30).

The experimental ex vivo/in vitro approach offers several considerations. First, the massive platelet activation observed in COVID-19 patients is not a

direct effect of the virus because SARS-CoV-2 has been detected in blood in a low percentage of infected patients and at very low levels, and no documented cases of transfusion transmission have been reported in the published reports. Conversely, platelet activation is consistent among all patients in this study; and ACE2 is mainly expressed in the lung, intestine, kidney, and blood vessels (31). No evidence for its presence in platelets has been reported so far; SARS-CoV-2 was not detected in the COVID-19 plasma pools used to test their effects on platelets of healthy subjects in vitro. In this setting, platelets are readily activated, and the effect of plasma, especially on TF expression, is blunted by tocilizumab, pointing to IL-6 as 1 of the mediators involved. Second, in COVID-19 patients, platelets are likely pre-activated due to the endothelial dysfunction and the lack of NO bioavailability. In this scenario, the presence of IL-6 within the cytokine storm enhances the effect of mediators released by the activated platelet. This finding is supported by the fact that IL-6 alone does not activate platelets in vitro, but it potentiates the effect of ADP or TxB₂. These data are consistent with a previous study that showed how IL-6 trans-signaling has no effect on platelet degranulation and aggregation by itself (32). Second, aspirin and P2Y₁₂ inhibitors prevent platelet activation induced by COVID-19 plasma. Indeed, aspirin and P2Y₁₂ inhibitors have been previously shown to be able to reduce heterotypic PLA, with the effect of the P2Y₁₂ inhibitors being more potent and consistent, throughout the studies, compared with that of aspirin (33). Moreover, inhibition of P2Y₁₂, not only blocks ADP, but also affects the release of sIL-6R, the specific soluble receptor needed for IL-6 signaling through the membrane-bound gp130 (32). Interestingly, Viecca et al. (34) recently showed that the combined use of several antiplatelet therapies in severe COVID-19 patients with a thrombophilic profile resulted in improved gas exchange efficiency and increased arterial oxygenation. Third, the platelet activation documented in COVID-19 patients has a peculiar feature to fight the virus. The strong induction of TF and P-selectin expression, and PLA formation was observed in the absence of GPIIb/IIIa activation, lacking, therefore, the *conditio sine qua non* to form platelet-platelet aggregates and confer a thrombotic risk. It is conceivable, however, that when the balance between the mechanisms that drive a robust antiviral immune response and regulators that function to limit an excessive immune response are lost, an overt immune-mediated pathology takes over. Thus, although our data should be confirmed in a larger study population, they provide

the scientific rationale to support the ongoing interventional clinical trials aiming at assessing whether the use of well-known antiplatelet agents ACTCOVID19 (Anti-Coronavirus Therapies to Prevent Progression of Coronavirus Disease 2019 (COVID-19) Trial [NCT04324463](#)); C-19-ACS [Preventing Cardiac Complication of COVID-19 Disease With Early Acute Coronary Syndrome Therapy: A Randomised Controlled Trial]; [NCT04333407](#); COVID-PACT [Prevention of Arteriovenous Thrombotic Events in Critically-Ill COVID-19 Patients Trial]; [NCT04409834](#); CORONA [Evolution of COVID-19 in Anticoagulated or Antiaggregated Patients (CORONA STUDY)]; [NCT04518735](#) or COVACTA [IL-6R antagonists (A Study to Evaluate the Safety and Efficacy of Tocilizumab in Patients With Severe COVID-19 Pneumonia)]; [NCT04320615](#); FMTVDM [The Fleming ((FMTVDM)) Directed CoVid-19 Treatment Protocol]; [NCT04349410](#); COVIDOSE-2 [Low-dose Tocilizumab Versus Standard of Care in Hospitalized Patients With COVID-19]; [NCT04479358](#); ARCHITECTS [Trial of Tocilizumab for Treatment of Severe COVID-19]; [NCT04412772](#)) may blunt the coagulopathy occurring in COVID-19 patients, with the ultimate goal to markedly improve their overall prognosis. This intriguing hypothesis is further supported by the finding that severe COVID-19 patients may develop pulmonary embolism/small local thrombi despite prophylactic and even empiric treatment-dose anticoagulation (14), suggesting that an other antithrombotic therapy may be necessary for this therapeutic goal.

STUDY LIMITATIONS. Our findings should be interpreted in the context of their limitations. First, data on the cell activation status found in COVID-19 patients are based on a small sample size. Nevertheless the results appear consistent and are further corroborated by the *in vitro* data. Furthermore, it is worth mentioning that, considering the emergency status existing at the time of patient enrollment and blood withdrawal, and the need to handle blood samples according to more stringent safety procedures, a great effort has been made to carry out such a thorough analysis. Second, our data suggest that an elevated baseline hemostatic potential, despite the anticoagulant treatment, could be responsible for the high thrombotic risk of COVID-19 patients; however, we

cannot exclude a potential role of heparin resistance (35) in the high hypercoagulable state of COVID-19 patients. Likewise, we cannot rule out an off-target effect of LMWH on platelet activation; however, the 2 groups of patients were similarly treated even after adjustment for LMWH U/kg. Third, no systematic search for venous thromboembolic events was performed in our study population, given the risk of transmitting the infection to health care personnel and admitted patients. Thus, future studies should confirm our findings and assess whether they do correlate with the clinical and imaging observation of symptomatic or asymptomatic thromboembolic events. Fourth, due to the small sample size, no correlation of cell-activation parameters with clinical outcome(s) has been performed.

CONCLUSIONS

All together our findings revealed how the cytokine storm present in COVID-19 patients induces, in concert with the imbalance of the endothelial functions, a massive cell activation with production of TF, mainly by platelets, granulocytes, and MVs, these latter responsible for the residual thrombin generation capacity measured in plasma of all patients treated with prophylactic anticoagulation. Furthermore, COVID-19 patients are characterized by a sustained platelet activation with formation of PLA that may be involved in the microthrombi found in autoptic specimens. Finally, these results provide insights into the IL-6-mediated platelet activation that triggers the hypercoagulable state in COVID-19, suggesting the potential effectiveness of anti-IL-6 antibodies and antiplatelet drugs.

AUTHOR DISCLOSURES

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: Patients with COVID-19 are characterized by a generalized cellular activation—involving endothelial and blood cells—that leads to a marked TF expression, mainly by platelets, granulocytes, and MVs. Indeed, residual thrombin generation is measurable in 40% of patients on therapeutic anticoagulation. A massive platelet activation, with formation of platelet-leukocyte aggregates, also contributes to the coagulopathy and to the clinical manifestation of the disease. The evidence that, besides tocilizumab,

aspirin and P2Y₁₂ inhibitors can turn off the IL-6-mediated cell activation could be of guidance for patient care, suggesting the use of these drugs to optimize the pharmacological treatment of COVID-19 patients.

TRANSLATIONAL OUTLOOK: Future studies should confirm our results and address whether the implementation of pharmacological treatment with antiplatelet drugs could improve hard outcomes in COVID-19 patients.

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KEY WORDS antiplatelet drugs, circulating microvesicles, COVID-19, IL-6, platelet activation, tissue factor

APPENDIX For an expanded Methods section as well as supplemental figures and table, please see the online version of this paper.