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Platelet dysfunction and thrombus instability in flow conditions in patients with severe COVID-19

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Highlights

- Severe COVID-19 is associated with significant risks of thrombosis and bleeding
- Platelets from patients with severe COVID-19 show signs of pre-activation at ICU admission
- Platelets from patients with severe COVID-19 are hyporeactive to several agonists
- Platelets hyporeactivity may be related to platelet exhaustion or to a plasmatic factor
- Platelets from patients with severe COVID-19 form smaller and unstable thrombi

ABSTRACT

Severe COVID-19 has been associated with a high rate of thrombotic events but also of bleeding events, particularly when the level of prophylactic anticoagulation was increased. Data on the contribution of platelets to these thrombotic events are discordant between reports, while the involvement of platelets in bleeding events has never been investigated. The objective of the present study was to assess platelet function during the first week of ICU hospitalization in patients with severe COVID-19 pneumonia. A total of 35 patients were prospectively included and blood samples were drawn on day (D) 0, D2 and D7. COVID-19 pneumonia was severe with a median PaO_2/FiO_2 ratio of 91 [68-119] on D0. Platelets from these patients showed evidence of pre-activation and exhaustion with a significant reduction in the surface expression of GPVI, GPIb and GPIIbIIIa, together with a decrease in serotonin content. Platelets from patients with severe COVID-19 were hyporesponsive with a reduced maximal aggregation response to several platelet agonists and decreased adhesion to immobilized fibrinogen. Aggregation of washed platelets and plasma substitution experiments indicated that a plasma factor was at least partially responsible for this hyporeactivity of platelets. Blood flow experiments showed that severe COVID-19 platelets formed smaller, less stable aggregates on a collagen-coated surface, which could explain why some patients develop bleeding events. These findings should prompt us to carefully evaluate the risks and benefits of high-dose prophylactic anticoagulation, and to decrease the level of anticoagulation once the initial phase of the disease has resolved.

Keys words: Platelets, COVID-19, Thrombosis, Bleeding, thrombus instability

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Authorship Contributions

CT, BH and PM contributed substantially to the conception and design of the study and to acquisition, analysis and interpretation of the data, drafted the article and provided final approval of the version submitted for publication.

XD and PMM contributed substantially to analysis and interpretation of the data and provided critical revision of the article and final approval of the version submitted for publication.

CM, CB, AE, SM and LS contributed substantially to acquisition and interpretation of the data and provided critical revision of the article and final approval of the version submitted for publication.

CG contributed substantially to the conception and design of the study, to the interpretation of the data, provided critical revision of the article and provided final approval of the version submitted for publication.

Disclosure of Conflicts of Interest

CT reports personal fees paid by Sanofi for conference attendance.

All other authors have no conflict of interest to declare.

INTRODUCTION

COVID-19 pneumonia has been associated with an increased risk of thrombosis, particularly in the most severely affected patients, hospitalized in intensive care units (ICUs), for whom this risk appears to be higher than in pneumonia from other causes[1-3]. The occurrence of a thrombotic event during severe COVID-19 pneumonia has even been identified as an independent risk factor for mortality[4]. In view of this high thrombotic risk, several medical societies have proposed increasing the dose of prophylactic anticoagulation, sometimes up to therapeutic doses in the most severe cases[5, 6]. While an increased dose of prophylactic anticoagulation appears to reduce the risk of thrombotic events[7], its effect on mortality remains controversial, particularly in critically ill patients for whom the balance between risk and benefit is very tight[8, 9]. A possible explanation for this lack of improvement in mortality is the presence of diffuse microthrombi, as observed in the lungs in autopsy series[10]. These microthrombi contribute directly to the severity of COVID-19 pneumonia and result from interactions between the damaged endothelium, a hypercoagulable state and activated neutrophils and platelets[11]. Thus, anticoagulation alone may not be sufficient to prevent the formation of microthrombi and a therapeutic approach directly targeting blood platelets might be of interest.

The majority of studies of platelet function in severe COVID-19 pneumonia have shown a hyperactivated state in the most severe cases[12-14]. Some authors have even reported a direct infection of platelets by SARS-Cov-2, leading to a state of hyperaggregability[14]. However, most of these investigations used a simplistic model looking at platelet activation and did not focus on more integrated approaches such as perfusion assays, which are particularly informative to determine the ability of platelets to form a plug under shear flow.

These studies were also performed in patients treated with standard-dose prophylactic anticoagulation, a dose that was found to be insufficient to prevent thrombin generation[15]. Thrombin is a potent platelet activator and this could be a confounding factor, masking the direct effect of SARS-CoV-2 infection on platelets. The management of critically ill patients with severe COVID-19 has also evolved, with a wider use of immunomodulatory therapies, such as corticosteroids and anti-interleukin-6 antibodies, which could result in different patterns of platelet activation.

When using an increased dose of prophylactic anticoagulation, several authors have reported the occurrence of bleeding complications[16]. Although the increased dose of anticoagulation was thought to be responsible, it would not be sufficient on its own to explain these bleeding events. The thrombotic and bleeding events were staggered over time[17, 18], while the pathophysiology of the bleeding, and in particular the contribution of platelets, has not yet been properly investigated. Therefore, we decided to conduct this study to assess platelet function during the first week of ICU hospitalization in patients with severe COVID-19 pneumonia.

METHODS

Study population

Consecutive adult patients with severe COVID-19 pneumonia admitted to the intensive care unit of the University Hospital of Strasbourg were prospectively included in this observational study. The protocol was approved by the Institutional Review Board Île-de-France (2020-A00958-31) and registered at ClinicalTrials.gov (NCT04359992). Patients were included in the study within the first 72 h after admission to the ICU, after obtaining informed consent from the patient or the first next of kin. Individuals treated with extracorporeal membrane oxygenation (ECMO) before inclusion, unable to give consent and with no available next of kin, with a life expectancy inferior to 24 hours or with a do-not-resuscitate order, were not included in the study. Patients were considered to have severe COVID-19 pneumonia if they had laboratory-confirmed SARS-CoV-2 infection and required advanced oxygenation (high flow nasal canula, non-invasive ventilation, or invasive ventilation). All patients were managed according to the guidelines in force at the time of the study. Anticoagulant therapy was managed according to the recommendations of the French Working Group on Perioperative Hemostasis (GHIP) and the French Study Group on Thrombosis and Hemostasis (GFHT) issued in April 2020. Follow-up was completed when the patient was discharged from the ICU or at his death. Patients treated with ECMO after inclusion were excluded from the platelet analysis to avoid confounding, but clinical data were still recorded.

Thrombotic and bleeding events

All clinically relevant thrombotic and bleeding events were recorded during the ICU stay. Thrombotic events included pulmonary, deep venous and arterial thrombosis (myocardial

infarction, mesenteric infarction, peripheral arterial thrombosis and stroke). No specific screening protocol was implemented. Bleeding events were recorded according to the ISTH guidelines[19]. The ISTH criteria were originally developed for the evaluation of side effects of anticoagulant drugs, but they are classically used to define bleeding complications, particularly during COVID-19. A patient could only be reported once for each type of event.

Blood analyses

Samples (20 mL) of blood were drawn at three different time points: on day 0, within the first 72 h after ICU admission, then on days 2 and 7. The samples were directly transferred to the laboratory for analysis. Blood samples from healthy donors were obtained from the French National Blood Donor Service (EFS Grand Est) with the informed consent of the donors. Healthy donors were not taking any anticoagulant or antiplatelet medication and had no history of venous thromboembolic or cardiovascular disease. Healthy controls were not matched with patients with severe COVID-19. Blood was drawn into 3.8% (v/v) sodium citrate (1:9) (for preparation of citrated PRP), acid-citrate-dextrose (ACD) (1:6) (for preparation of washed platelets), or hirudin (525 anti-thrombin activity units (ATU).mL^{-1}) (for blood flow assays) as the anticoagulant. Not all tests could be performed on all patients due to the amount of blood required for each test. Hematological parameters, PF4 and serotonin levels were assessed in all patients. Platelet P-selectin expression, GPIb, GPVI, GPIIbIIIa expression, cPRP platelet aggregation measurements, and scanning electron microscopy analysis were performed mostly on the same patients. Similarly, the majority of patients evaluated for thrombi disaggregation under flow conditions or washed platelet aggregation studies were also evaluated for P-selectin exposure and Annexin V binding after whole blood stimulation with various agonists. The majority of patients evaluated for platelet thrombus

formation under flow conditions were also evaluated for platelet adhesive properties on fibrinogen or VWF.

Preparation of citrated platelet-rich plasma and platelet-poor plasma

Citrated platelet-rich plasma (PRP) was prepared by centrifugation of citrated (3.8%) whole blood at 250 *g* for 10 min at room temperature (RT). To prepare platelet-poor plasma (PPP), citrated blood was centrifuged at 3,500 *g* for 5 min. The supernatant was then centrifuged again at 12,000 *g* for 5 min and the resultant PPP was stored at -80°C until use.

Platelet washing procedure and aggregation studies

Aggregation assays were only performed at RT. Washed platelets were prepared as previously described[20]. Briefly, platelets were isolated by differential centrifugation and washed twice at 37°C in Tyrode's buffer (137 mM NaCl, 2 mM KCl, 12 mM $NaHCO_3$, 0.3 mM NaH_2PO_4, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5.5 mM glucose, 5 mM HEPES, pH 7.3, 295 $mOsm.L^{-1}$) containing 0.35% purified human serum albumin (HSA) and 0.5 µM prostaglandin I2 (PGI_2). Platelets were finally suspended in Tyrode's buffer containing 0.35% HSA and 0.02 $U.mL^{-1}$ of the adenine nucleotide scavenger apyrase, a concentration sufficient to prevent desensitization of platelet responses to ADP[21]. The final suspension contained no PGI_2 and was adjusted to 3 x 10^5 $platelets.\mu L^{-1}$. Washed platelets were kept at 37°C throughout all experiments. Aggregation was measured at 37°C for 5 minutes by a turbidimetric method in an APACT aggregometer. A 270 µL aliquot of platelet suspension was stirred at 1,100 rpm and activated by addition of 30 µL of the appropriate agonist. The extent of aggregation was estimated quantitatively by measuring the maximum curve height above baseline.

Flow cytometry

Hirudinated (100 $U.mL^{-1}$) whole blood from healthy donors or patients with severe COVID-19 was incubated for 10 min at RT with a mouse anti-human GPVI (3J24), GPIbα or GPIIb monoclonal antibody (10 $\mu g.mL^{-1}$) or with control mouse IgG1. FITC-conjugated polyclonal anti-mouse antibodies were then added for 10 min at RT. α-granule secretion was evaluated with a FITC-conjugated mouse anti-human CD62P (P-selectin) monoclonal antibody. The externalization of phosphatidylserine (PS) was measured with Alexa 488-conjugated annexin V. Glycoprotein and P-selectin expression were quantified by flow cytometry using a Platelet Calibrator kit for GPVI and PLT Gp/Receptors kit for P-selectin, GPIb and GPIIbIIIa with calibrated beads, as described by the supplier (Biocytex, Marseille, France).

Citrated whole blood from healthy donors or patients with severe COVID-19 was stimulated for 5 min at RT with CRP (1 $\mu g.mL^{-1}$) or U46619 (2 µM) and P-selectin exposure was detected by co-staining for 20 min with a FITC-conjugated mouse anti-human CD62P monoclonal antibody and an Alexa 647-conjugated anti-GPIbβ monoclonal antibody (RAM.1) (1 $\mu g.mL^{-1}$). Samples were analyzed using a Gallios flow cytometer and Kaluza software (Beckman Coulter). The light scattering and fluorescence intensity of 10,000 platelets were collected with a logarithmic gain. Results were expressed as the number of sites per platelet or the mean fluorescence intensity (MFI).

Platelet morphology

To evaluate the morphological changes of platelets, a morphology score was devised using a modification of the method of Kunicki[22]. Washed platelets were fixed by addition of an equal volume of fixative solution (2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer containing 2% sucrose, 305 $mOsm.L^{-1}$, pH 7.3) and prepared for scanning electron

microscopy (SEM, Helios 500i, FEI, Eindhoven) as previously described[23]. The morphology score was based on the following aspects: 1) disc-platelets which had retained their discoid shape, 2) filopodia-platelets which had developed filopodia and 3) contracted platelets displaying a spherical shape. The percentage of each morphological type was multiplied by an arbitrary factor as follows: disc x 4, filopodia x 2 and contracted x 1. The morphology score was defined as the sum of the resulting three numbers. One hundred platelets were quantified and the investigator was blinded to the type of platelet suspension. The highest score indicating the best preservation of platelet morphology was 400.

ELISA assays

Plasma and serum concentrations of PF4 (Quantikine® ELISA human CXCL4/PF4, R&D Systems™) and serotonin (Serotonin ELISA Fast Track, LDN®) were measured by enzyme immunoassay using ELISA kits according to the manufacturers' instructions.

Blood flow assays

Microfluidic flow chambers were coated with the indicated adhesive proteins overnight at 4°C and blocked with PBS containing HSA (10 mg.mL^{-1}) for 30 min at RT. Hirudinated (525 ATU.mL^{-1}) whole blood from patients with severe COVID-19 or healthy human volunteers was perfused through the coated capillaries with a syringe pump (Harvard Apparatus, Holliston, MA, USA) at 37°C and the indicated flow rates. Platelet adhesion and/or aggregation were monitored in real time by differential interference contrast (DIC) microscopy (Leica DMI4000B) using a 40x, 1.25 numerical aperture oil objective and a Hamamatsu CMOS ORCA FLASH-4 LT camera (Hamamatsu Photonics, Massy, France). Images were analyzed with ImageJ software (National Institute of Health, Bethesda, MD, USA).

Statistics

Statistical analyses were performed with the GraphPad Prism program, version 9.2.0 (Prism, GraphPad, La Jolla, CA, USA). Values are indicated as the median [interquartile range]. Groups were compared using non-parametric test (Mann-Whitney and Kruskal-Wallis). The statistical analyses are described in the legends to the figures.

RESULTS

Study population

Between April 2020 and June 2021, 35 patients were included in the study. No patients were lost to follow-up during their ICU stay. The demographic characteristics and medical history of the study population are described in **Table 1**.

Variables	**Patient cohort (N = 35)**
Demographic data	
Age (years)	64 [57-71]
BMI (kg.m^{-2})	29 [26-33]
Sex ratio (M/F)	1.2 (19/16)
Medical history	
Arterial hypertension	18 (51)
Diabetes	9 (26)
Smoking	1 (3)
Alcohol	3 (9)
COPD	6 (17)
Heart failure	3 (9)
Coronary artery disease	3 (9)
Atrial fibrillation	3 (9)
Stroke	3 (9)
Chronic kidney disease	2 (6)
Venous thromboembolism	3 (9)
Active cancer	2 (6)
Malignant hemopathy	1 (3)
Chronic medication	
Aspirin	3 (9)
Clopidogrel	2 (6)
DOAC	2 (6)
ICU management	
Non-invasive ventilation	10 (29)
High flow nasal canula	27 (77)
Mechanical ventilation	18 (51)
Duration of invasive ventilation (days)	20 [7-26]
Tracheotomy	6 (17)
Dexamethasone	35 (100)
Tocilizumab	18 (51)
ECMO	1 (3)
In-ICU death	5 (14)
Delay ICU admission – death (days)	20 [4-30]

Table 1: Demographic characteristics, medical history of the study population and Intensive care unit (ICU) management of patients with severe COVID-19. Results are expressed as n

(%) or median [interquartile range]. BMI: body mass index; COPD: chronic obstructive pulmonary disease; DOAC: direct oral anticoagulant therapy. ECMO: extracorporeal membrane oxygenation.

SARS-CoV-2 infection was confirmed by RT-PCR in all patients. COVID-19 pneumonia was diagnosed within a median of 2 [0-6] days after the appearance of the first clinical symptoms. Patients were hospitalized within a median of 7 [5-9] days after the onset of the first symptoms and were transferred to an ICU within a median of 1 [0-2] day after admission to hospital. The patients in our cohort had severe pneumonia with a median PaO_2/FiO_2 ratio of 91 [68-119] on day 0. Eighteen (51%) of them required invasive mechanical ventilation and 5 (14%) died during their ICU stay. No patient had disseminated intravascular coagulation. Only one patient had an ISTH score of 7 but had active massive aortic thrombosis. The management of our study population during intensive care is described in **Table 1** and the clinical evolution and the evolution of the anticoagulation treatment are detailed in **Table 2**. The median duration of follow-up was 11 [5-28] days.

Variables	Day 0 (n=35)	Day 2 (n=34)	Day 7 (n=25)
Severity			
PaO_2/FiO_2 ratio	91 [68-119]	93 [76-124]	139 [108-177]
SOFA score	5 [4-7]	9 [4-11]	10 [4-11]
SIC score	2 [2-3]	2 [2-2]	2 [2-2]
ISTH DIC score	2 [2-3]	2 [2-3]	2 [2-3]
CRP ($mg.L^{-1}$)	82 [52-166]	41 [15-139]	N/A
Patients on RRT	1 (3)	2 (6)	1 (4)
Patients on norepinephrine	7 (20)	14 (41)	11 (44)
Max dose of norepinephrine ($\mu g.kg^{-1}.min^{-1}$)	0.14 [0.06-0.25]	0.11 [0.05-0.19]	0.12 [0.04-0.18]
Anticoagulation			
Patients on UFH/LMWH	4/31	6/28	6/19
Daily dose of UFH ($IU.kg^{-1}$)	30 [17-255]	386 [185-443]	338 [90-378]
Daily dose of LMWH ($IU.kg^{-1}$)	61 [44-85]	133 [89-156]	121 [86-144]
Anti-Xa activity ($IU.mL^{-1}$)	0.34 [0.17-0.60]	0.52 [0.30-0.61]	0.46 [0.33-0.75]

Table 2: Evolution of the study population within the first week of hospitalization in intensive care. Results are expressed as n (%) or median [interquartile range].

DIC: disseminated intravascular coagulopathy; ISTH: international society on thrombosis and haemostasis; LMWH: low molecular weight heparin; N/A: not available; SIC: sepsis-induced coagulopathy; SOFA: sequential organ failure assessment; RRT: renal replacement therapy; UFH: unfractionated heparin.

Anticoagulation and thrombotic and bleeding events

All patients received increased-dose pharmacological thromboprophylaxis during the first week of ICU hospitalization according to the guidelines available at the time of the study. **Figure 1** shows the proportions of patients who experienced at least 1 event during their ICU stay. Five patients (14%) presented a total of 7 thrombotic events, including 3 pulmonary embolisms, 1 aortic thrombosis, 1 limb artery thrombosis, 1 vena cava thrombosis and 1 stroke. The median time from ICU admission to the first thrombotic event was 3 [0-20] days. Eleven patients (31%) presented a total of 55 bleeding events according to ISTH criteria, including 50 requiring transfusion of 2 or more units of packed red blood cells (PRBCs), 2 hemorrhagic shocks, 1 arm hematoma requiring surgical debridement, 1 hemostatic

splenectomy and 1 hemostatic lobectomy. The median time from ICU admission to the first bleeding event was 10 [7-13] days. Thus, severe COVID-19 pneumonia was associated with significant risks of thrombosis and bleeding, the bleeding risk arising later than the thrombotic risk.

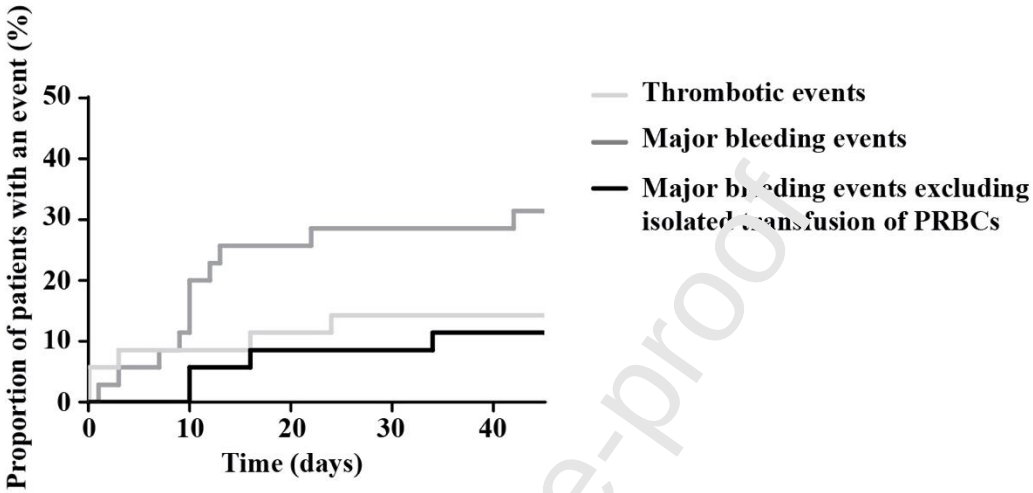
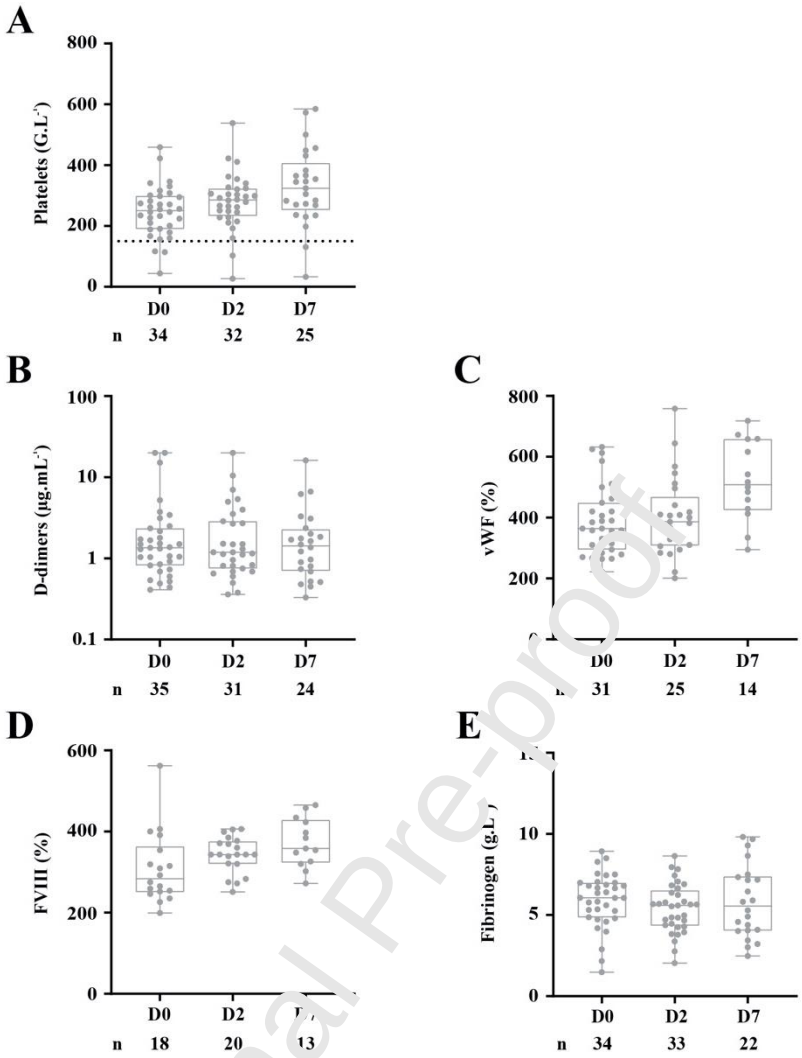


Figure 1: Proportions of patients experiencing at least one thrombotic or bleeding event during their stay in intensive care.

PRBCs: packed red blood cells.

Hematological parameters of patients with severe COVID-19

Platelet counts remained within the normal range (150-400 $G.L^{-1}$) during the first week of ICU hospitalization in most patients. Only 3 (9%) displayed thrombocytopenia during the study period (**Figure 2A**). D-dimer, fibrinogen, VWF antigen and FVIII levels were elevated at ICU admission at 1.35 [0.82-2.35] $\mu g.mL^{-1}$ (normal < 0.5 $\mu g.mL^{-1}$), 6.1 [4.9-7.0] $g.L^{-1}$ (normal 2-4 $g.L^{-1}$), 364 [295-449] $IU.dL^{-1}$ (normal 50-150 $IU.dL^{-1}$) and 284 [251-383] $IU.dL^{-1}$ (normal 60-150 $IU.dL^{-1}$), respectively, and remained elevated during the study (**Figure 2B-E**). All parameters were not measured in each patient to allow functional tests as volume sampling was limited.



<u>Figure 2:</u> Evolution of biological markers in patients with severe COVID-19 during the first week of ICU hospitalization. A, platelet counts. **B**, D-dimers. **C**, von Willebrand Factor antigen (VWF). **D**, factor VIII (FVIII). **E**, fibrinogen.

Platelet activation markers in patients with severe COVID-19

Several reports have indicated that platelets from patients with COVID-19 were hyperreactive, notably on the basis of their increased P-selectin exposure. We observed only a slight non-significant increase in P-selectin exposure on the platelets of our patients with severe COVID-19 on day (D) 0 (**Figure 3A**). Levels of platelet GPVI, GPIb and GPIIbIIIa were slightly but significantly reduced in our patients on D0, suggesting that the platelets were

exposed to conditions promoting a certain degree of activation (**Figure 3B**). This level of pre-activation was not associated with a marked change in the morphology of circulating platelets, as evidenced by their discoid shape under the scanning electron microscope (**Figure 3C**) and further analysis of the Kunicki scores (**Figure 3D**), although activated platelets might have been trapped in blood vessels. The presence of a pre-activated state of platelets in the blood of patients with severe COVID-19 was further supported by a significant increase in levels of PF4, a specific marker of platelet granule secretion, in the plasma of the patients on days 0, 2 and 7 post-ICU admission (**Figure 3E**), whereas their serum PF4 remained in the normal range (**Figure 3F**). Interestingly, levels of serotonin were also increased in the plasma of the patients, but only after 7 days (**Figure 3G**). Serum serotonin levels were decreased at ICU admission as compared to healthy controls (69 [43-91] vs. 111 [96-128] $ng.mL^{-1}$, $p<0.01$) and remained low during the entire study period (**Figure 3H**). Overall, the blood of patients with severe COVID-19 presented signs of platelet activation even if the level of activation of circulating platelets was low.

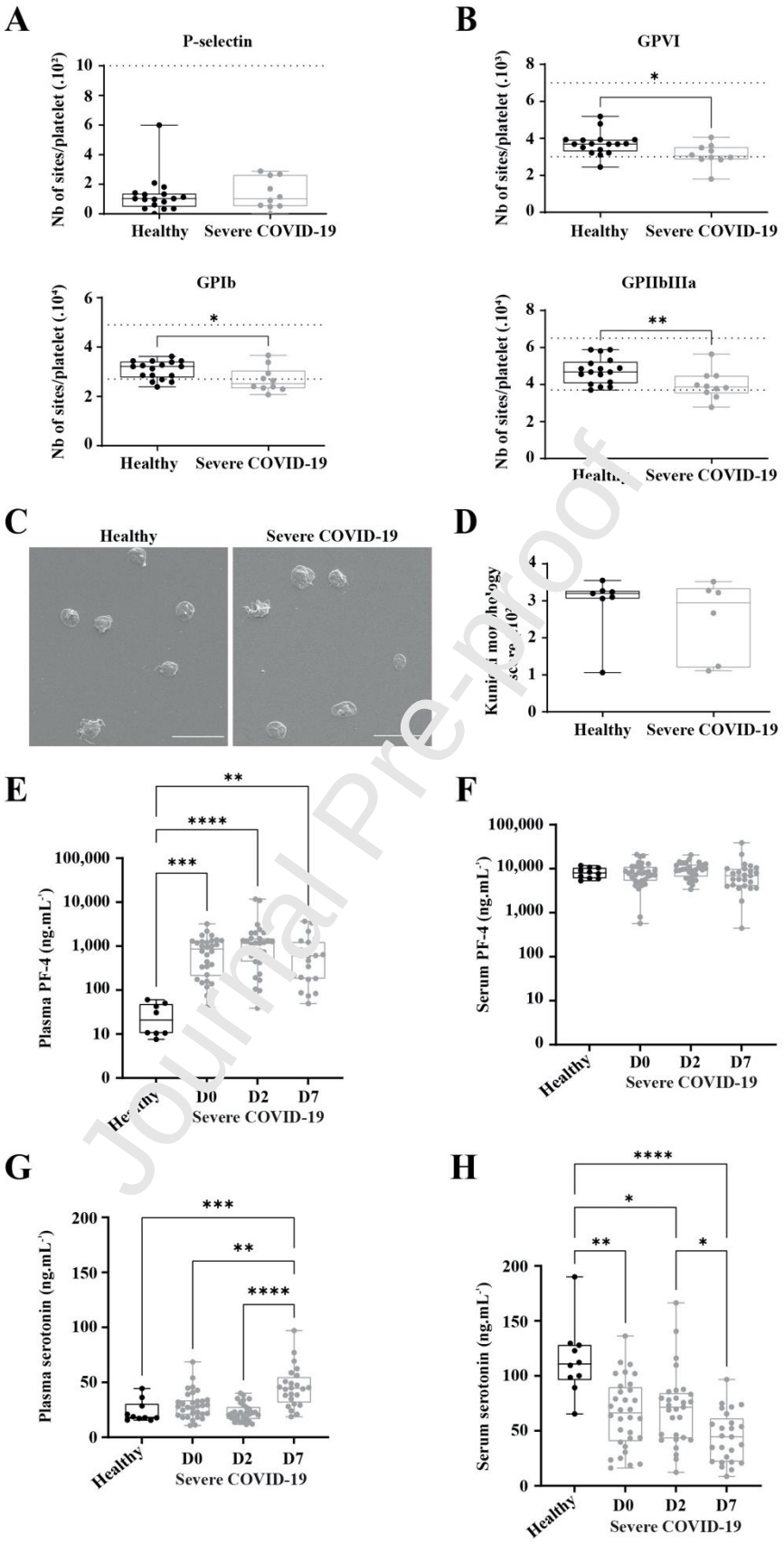


Figure 3: **Platelets from patients with severe COVID-19 show signs of pre-activation. A and B,** the expression of platelet P-selectin (A), GPIb, GPVI and GPIIbIIIa (B) in hirudinated whole blood from healthy donors (n=17) or patients with severe COVID-19 (n=10, D0) was evaluated by flow cytometry. Results are expressed as the median with first and third quartiles, maximum and minimum and were compared by the Mann-Whitney test (*p<0.05; **p<0.01). **C**, representative scanning electron microscopy (SEM) images of platelets from healthy donors and patients with severe COVID-19; scale bars: 10 µm. **D**, morphological changes of washed platelets from healthy donors (n=7) and patients with severe COVID-19 (n=6) were evaluated by SEM according to a modification of the method of Kunicki. Results are expressed as the median with first and third quartiles, maximum and minimum and were compared by the Mann-Whitney test. **E, F, G and H**, concentrations of PF4 and serotonin in plasma and serum from healthy donors (n=10) or patients with severe COVID-19 on D0 (n=32), D2 (n=28) and D7 (n=23) post-ICU admission. Results are expressed as the median with first and third quartiles, maximum and minimum and were compared by the Kruskal-Wallis test (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001).

Severe COVID-19 platelets display reduced functions

While we observed a trend towards increased P-selectin exposure on severe COVID-19 platelets under resting conditions, this increase was not observed after stimulation of whole blood with either CRP or the TxA2 analogue U46619, which even induced a trend towards reduced P-selectin exposure (**Figure 4A left panel**). Annexin V binding following combined stimulation of whole blood with 1 µg.mL^{-1} CRP and 10 µM TRAP (thrombin receptor-activating peptide) tended to be reduced in patients with severe COVID-19 (**Fig. 4A right panel**). Stimulation of citrated platelet-rich plasma (PRP) with 5 µM ADP, 2.5 µg.mL^{-1}

collagen, 2 µM U46619 or 50 µM TRAP resulted in significantly lower maximal aggregation responses in D0 PRP from patients with severe COVID-19 as compared to healthy controls (**Figure 4B and C**). We next looked at whether the plasma from these patients could contribute to their diminished platelet aggregation responses. Stimulation of D0 washed platelets from patients with severe COVID-19 with several platelet agonists showed that the maximal aggregation responses were no longer reduced in comparison with healthy controls, suggesting that a plasmatic factor could contribute to the hyporesponsiveness of platelets (**Figure 4D**). To test this hypothesis, citrated plasma from patients with severe COVID-19 or healthy donors was mixed with washed platelets isolated from healthy donors (3:1, v/v) and stimulated with 2.5 µg.mL^{-1} collagen. As shown in **Figure 4E**, D2 or D7 plasma from these patients inhibited the platelet aggregation induced by collagen, confirming that a factor contained in the plasma of our patients with COVID-19 contributed to their reduced platelet aggregation responses. Altogether, platelets from patients with severe COVID-19 seemed to present defective activatory functions as compared to those from healthy controls.

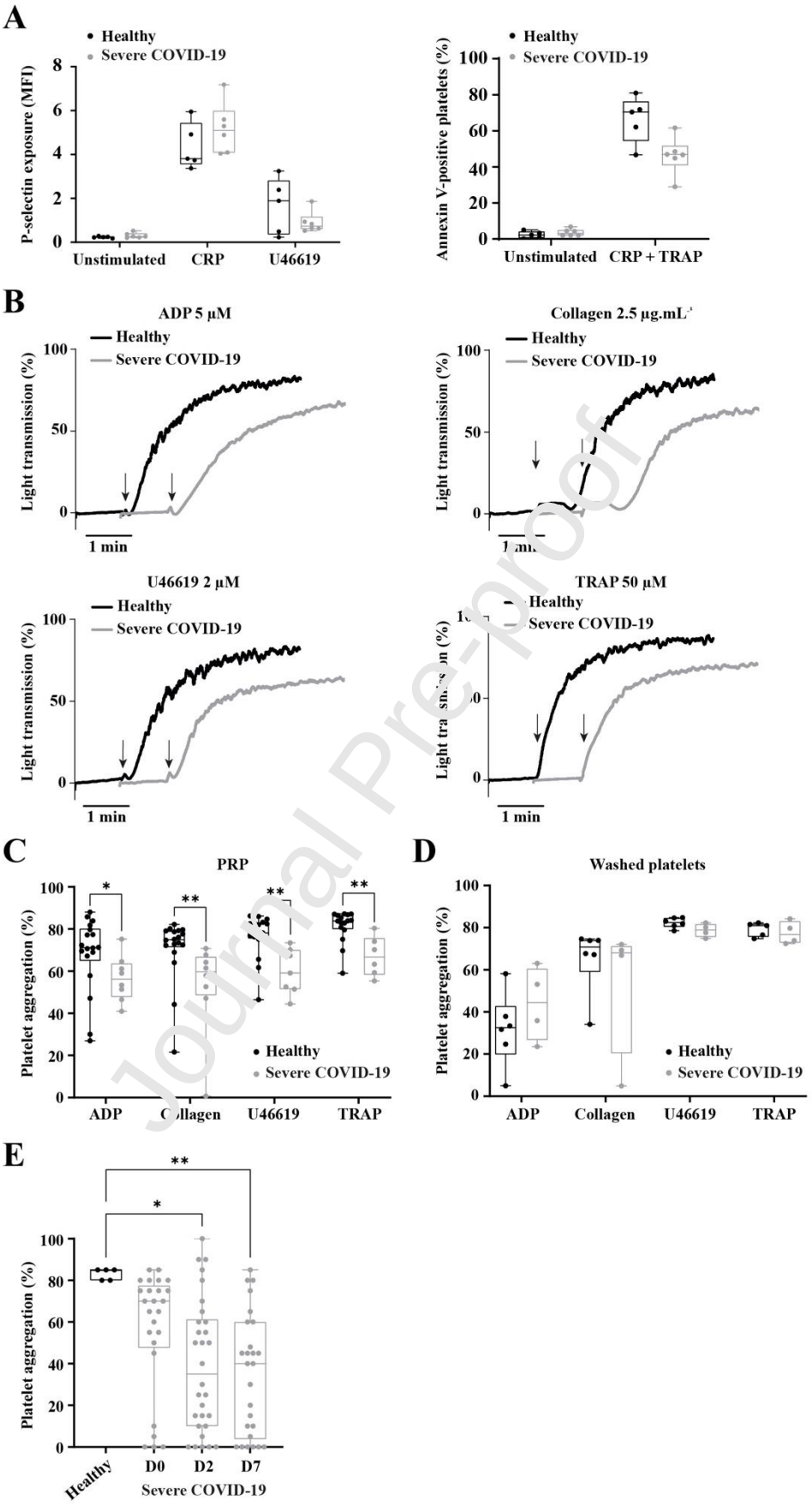


Figure 4: Patients with severe COVID-19 present reduced platelet functions. A, citrated whole blood from healthy donors (n=5) or patients with severe COVID-19 (n=6, D0) was stimulated with CRP (1 µg.mL^{-1}) or U46619 (2 µM) for 5 min, or CRP (1 µg.mL^{-1}) and TRAP (10 µM) for 10 min, and the binding of a fluorescein isothiocyanate (FITC)-conjugated anti-P-selectin antibody or Alexa 488-conjugated Annexin V was detected by flow cytometry. Results represent the mean fluorescence intensity (MFI) (P-selectin, left panel) or percentage of Annexin V-positive platelets (right panel). **B and C,** citrated platelet-rich plasma (PRP) from healthy donors (n=16-18) or patients with severe COVID-19 (n=6-8, D0) was stimulated with adenosine diphosphate (ADP) (5 µM), collagen (2.5 µg.mL^{-1}), U46619 (2 µM) or thrombin receptor-activating peptide (TRAP) (50 µM). **B,** tracings are from one healthy donor and one COVID-19 patient and are representative of the other samples. Addition of the agonist is indicated by the arrow. **C,** the maximal percentage of platelet aggregation was quantified. **D**, washed platelets (3 x 10^5 platelets.µL^{-1}) from healthy donors (n=5-6) or patients with severe COVID-19 (n=4, D0) were stimulated with ADP (5 µM), collagen (2.5 µg.mL^{-1}), U46619 (2 µM) or TRAP (50 µM). The maximal percentage of platelet aggregation was quantified. **A, C and D**, results are expressed as the median with first and third quartiles, maximum and minimum and were compared by the Mann-Whitney test (*p<0.05; **p<0.01). **E,** washed platelets from healthy donors were mixed with citrated platelet-poor plasma (PPP) (3:1, v/v) from healthy donors (n=5) or patients with severe COVID-19 on D0, D2 or D7 post-ICU admission (n=25-30) and stimulated with collagen (2.5 µg.mL^{-1}). The maximal percentage of platelet aggregation was quantified. Results are expressed as the median with first and third quartiles, maximum and minimum and were compared by the Kruskal-Wallis test (*p<0.05; **p<0.01).

Adhesive properties of platelets from patients with severe COVID-19

The adhesive functions of D0 platelets from patients with severe COVID-19 were evaluated using a flow-based assay whereby hirudinated whole blood was perfused over immobilized proteins. We observed a non-significant tendency towards reduced platelet adhesion to immobilized VWF at 1,500 s^{-1} (**Figure 5A and B**), which could possibly be explained by a modest but significant reduction in the surface expression of GPIb in severe COVID-19 platelets (**Figure 3B**). When whole blood from our patients was perfused over immobilized fibrinogen at 300 s^{-1}, a marked drop in platelet adhesion was observed, correlating with the decrease in the surface expression of integrin GPIIbIIIa (**Figure 5C and D**). These results indicated that platelets from patients with severe COVID-19 present defective adhesive functions as compared to platelets from healthy donors.

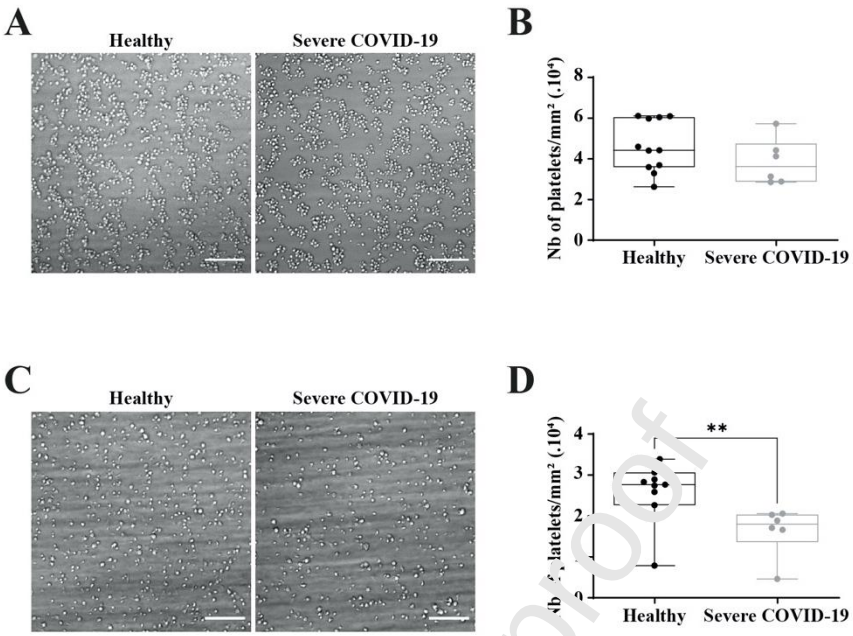


Figure 5: Adhesive properties of platelets from patients with severe COVID-19. A and B, hirudinated whole blood from healthy donors (n=11) or patients with severe COVID-19 (n=6, D0) was perfused through PDMS flow chambers coated with von Willebrand factor (VWF) (100 µg.mL^{-1}) for 5 min at 1,500 s^{-1}. **C and D**, hirudinated whole blood from healthy donors (n=11) or patients with severe COVID-19 (n=6, D0) was perfused through PDMS flow chambers coated with fibrinogen (100 µg.mL^{-1}) for 5 min at 300 s^{-1}. **A and C**, platelets were visualized in random fields by differential interference contrast (DIC) microscopy; scale bars: 30 µm. **B and D**, numbers of adherent platelets were quantified. Results are expressed as the median with first and third quartiles, maximum and minimum and were compared by the Mann-Whitney test (**p<0.01).

Severe COVID-19 platelets form smaller and less stable aggregates

To determine whether the reduced functions of platelets from patients with severe COVID-19 resulted in a defect in platelet plug formation, hirudinated blood was perfused over collagen in a flow-based assay. Real-time video-microscopy showed that smaller thrombi formed on fibrillar collagen using blood from our patients as compared to healthy controls (**Figure 6A**). This observation was confirmed by recording the numbers of stable thrombi in flow experiments performed at 750 (D0), 1,500 and 3,200 s^{-1} (D0 to D7), which all showed a very marked reduction in thrombus formation in blood samples from patients with severe COVID-19 (**Figure 6B**). The small although significant decrease in the expression of GPVI (the main receptor for collagen) on severe COVID-19 platelets would be unlikely to explain this defect (**Figure 3B**). To evaluate the stability of the platelet thrombi of patients with severe COVID-19, we first formed platelet aggregates under very low shear conditions, before perfusing the flow chambers with PBS at 500 s^{-1} and 750 s^{-1}. DIC microscopy showed that the thrombi formed in blood from our patients were highly unstable as compared to those formed in blood from healthy donors (**Figure 6C**). This was confirmed by a very marked increase in the number of disaggregating thrombi using blood from patients with severe COVID-19 (**Figure 6D**). We could conclude that thrombus formation and stability were profoundly impaired in severe COVID-19 platelets.

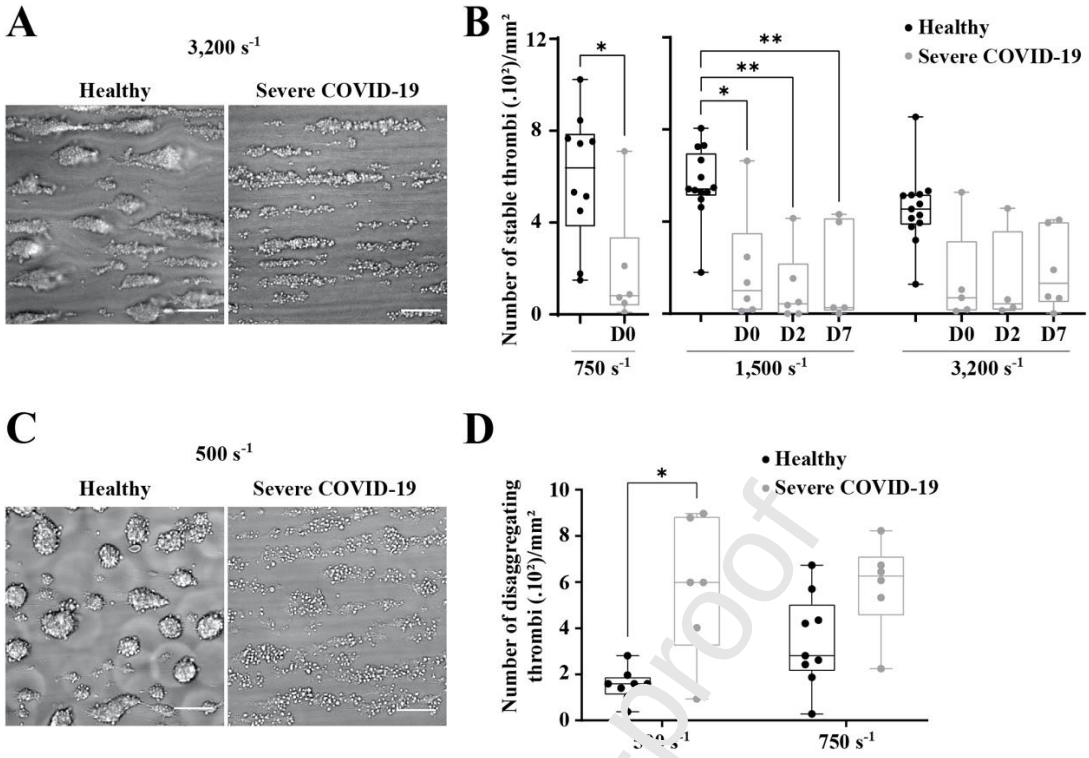


Figure 6: Platelets from patients with severe COVID-19 form smaller and less stable aggregates. A and B, hirudinated whole blood from healthy donors (n=10-13) or patients with severe COVID-19 on D0, D2 or D7 post-ICU admission (n=5-6) was perfused through PDMS flow chambers coated with collagen (200 µg.mL^{-1}) for 3 min at 750 s^{-1} (D0), 5 min at 1,500 s^{-1} (D0 to D7) or for 2.5 min at 3,200 s^{-1} (D0 to D7). **A,** thrombi were visualized in random fields by DIC microscopy; Images were taken after 2.5 min of perfusion at 3,200 s^{-1}; scale bars: 30 µm. **B,** numbers of stable thrombi were quantified. Results are expressed as the median with first and third quartiles, maximum and minimum and were compared by the Kruskal-Wallis test (*p<0.05; **p<0.01). **C and D**, hirudinated whole blood from healthy donors (n=8-9) or patients with severe COVID-19 (n=6, D0) was perfused through PDMS flow chambers coated with collagen (200 µg.mL^{-1}) for 3 min at 750 s^{-1}. PBS was then perfused over the preformed thrombi for 8 min at 500 or 750 s^{-1}. Images were taken after PBS perfusion for 8 min at 500 s^{-1}. **C,** thrombi were visualized in random fields by DIC microscopy; scale bars: 30 µm. **D,** numbers of disaggregating thrombi were quantified. Results are

expressed as the median with first and third quartiles, maximum and minimum and were compared by the Mann-Whitney test (*p<0.05).

DISCUSSION

Our results indicate that (i) severe COVID-19 pneumonia is associated with significant risks of thrombosis and bleeding, the bleeding risk arising later than the thrombotic risk, (ii) platelets from patients with severe COVID-19 show signs of pre-activation at ICU admission, which might reflect a pathophysiological process preceding the clinical worsening of COVID-19 pneumonia, (iii) platelets from patients with severe COVID-19 display reduced adhesive and activatory functions and decreased surface glycoprotein expression, (iv) platelet hyporeactivity may be related to platelet exhaustion or due to the influence of a plasmatic factor (v) platelets from patients with severe COVID-19 form smaller and unstable thrombi on a collagen-coated surface under flow conditions.

Our observations confirm that the thrombotic risk is high in severe COVID-19 infection with 14% of our patients experiencing a thrombotic event. The thrombotic risk associated with severe COVID-19 pneumonia has been extensively described in the literature, pulmonary embolism being reported in up to 25% of ICU patients during the first epidemic wave [3]. Most of these events concerned venous thrombosis, although arterial thrombosis has also been frequently described[16, 24, 25]. Some risk factors for thrombotic events were identified, including age, the severity of lung disease and the need for non-invasive or invasive mechanical ventilation[26]. The thrombotic risk appeared to be particularly high in the acute phase of the disease, with most events occurring within the first week of hospitalization[17].

Circulating platelets from our patients with severe COVID-19 showed evidence of pre-activation at ICU admission, although the level of platelet activation was lower than in other studies[12-14]. Since activated platelets are more likely to be trapped in the circulation, we believe that we preferentially analyzed mildly activated cells. The hypothesis of platelet preactivation is supported by the reduction of surface glycoprotein expression (GPIb, GPVI, GPIIbIIIa), the reduction in platelet serotonin content, as evidenced by the decrease in serum serotonin levels. The elevated PF4 level could also support this hypothesis, although the presence of heparin in patients may be a confounding factor. Increased serotonin levels were measured in the plasma of patients with severe COVID-19, indicating platelet degranulation. The decrease in serotonin levels measured in the serum of patients with severe COVID-19, compared with healthy controls, may suggest an imbalance between serotonin synthesis and consumption. One potential explanation could be related to an alteration by SARS-CoV-2 of the function of enterochromaffin cells in the gut, the main producer of blood serotonin, a phenomenon that is observed in other viral infections [27]. It has also been reported that SARS-CoV-2 causes an upregulation of the enzyme indole 2,3-dioxygenase (IDO), which is responsible for a high consumption of tryptophan, thus limiting its availability for serotonin production and also for the conversion of serotonin to formyl-5-hydroxykynurenamine, thus increasing serotonin metabolism [28]. Another explanation could be related to increased serotonin degradation by monoamine oxidases (MAOs), which are overexpressed by platelets in patients with COVID-19 [29, 30].

While direct interaction of the SARS-CoV-2 virus with platelets might be responsible, endothelial activation or thrombin generation could also have contributed to this activated state[11]. Indeed, several authors have described platelets as being activated during SARS-CoV-2 infection and to thereby contribute to the pathophysiological process of COVID-19-

related thrombotic events[12-14]. Although the ability of SARS-CoV-2 viruses to directly infect blood platelets remains controversial, some studies have shown that the SARS-CoV-2 spike protein can increase the responses of platelets to various agonists and even directly induce platelet activation and aggregation[14, 31]. Other factors such as neutrophil and monocyte activation, or thrombin generation following activation of coagulation pathways during SARS-CoV-2 infection, have also been proposed as possible explanations[11].

Whereas most authors have reported an enhanced platelet response in patients with COVID-19, we observed a reduced platelet aggregation response, regardless of the agonist used and a reduced externalization of phosphatidylserine. Our data also indicate a diminished adhesion capacity, particularly on fibrinogen-coated surfaces under flow conditions, which is in line with two recent studies showing a defect in GPIIbIIIa expression on platelets from patients with severe COVID-19, together with a decreased platelet response to several agonists[32, 33]. Interestingly, we also observed reduced GPIIbIIIa expression on the surface of our patients' platelets, which may have contributed to their defective adhesion.

A possible explanation is that the pre-activation of blood platelets could lead to their exhaustion. The concept of platelet exhaustion has already been evoked in other pathologies such as cancer or hyperhomocysteinemia and more recently in trauma, although the consequence of this exhaustion remains unclear and seems to depend on the causal mechanism[34-36]. However, the normal aggregation responses of washed platelets from our patients with severe COVID-19 indicate that the cells can recover when the plasma is removed and hence do not support the hypothesis of platelet exhaustion. Our aggregation assays using plasma substitution rather suggest that a soluble mediator is at least partially responsible for this platelet hyporeactivity. Interestingly, this inhibitory effect seems to appear secondarily since it is more pronounced on D2 and D7. Proteomic analysis of blood

from patients with severe COVID-19 has shown that the expression of hundreds of mediators, particularly inflammatory mediators such as IL-6 and TNFα, is increased during the initial phase of the disease, while the expression of others is decreased[37, 38]. This makes it particularly difficult to identify the mediator(s) responsible for the observed abnormalities.

We report for the first time abnormal thrombus formation on fibrillar collagen under various flow conditions in blood from patients with severe COVID-19, with smaller, disaggregating thrombi. This is particularly relevant in that several of our patients suffered from bleeding complications requiring surgical hemostasis. The majority of bleeding events described in COVID-19 pneumonia are mild, although intracranial hemorrhage and massive bleeding have been reported and are associated with a significant morbidity[39, 40]. The small size of our cohort and the impossibility of performing a flow analysis in all patients preclude establishing a correlation between our observations and the occurrence of bleeding events. The apparent discrepancy between the small reduction in surface glycoprotein expression and the large reduction in thrombus size and stability under flow conditions may be due to the importance of these glycoproteins, in addition to the reduction in platelet granule content observed in our study, under flow conditions that represent a more realistic approach to platelet function.

Several hypotheses may be proposed to explain the disparities between previously published results and ours. Most of the previous studies showing an increased platelet response were performed in patients of the first epidemic wave, before the publication of recommendations in favor of the use of corticosteroids and immunomodulatory molecules[41, 42]. Hence the use of these molecules was limited while the cytokine storm was particularly intense. In addition, most of the patients in these studies were treated with

standard-dose prophylactic anticoagulation, a dose that did not prevent thrombin generation[15]. The high cytokine levels associated with persistent thrombin generation may have contributed to masking a possible defect in platelet activation and adhesive properties. All patients in our cohort received corticosteroids and an increased dose of heparin for thromboprophylaxis. Half of them were also treated with tocilizumab, an anti-IL-6 molecule with powerful anti-inflammatory properties. The reduction in the cytokine storm in our patients might have unmasked a defect in platelet reactivity. Corticosteroids and tocilizumab could also contribute directly to platelet hyporeactivity and thrombus instability.

The observed effects on thrombus size and stability cannot be explained by the use of an increased dose of prophylactic anticoagulation in our patients because heparin was added to the blood drawn from healthy donors at the same level as measured in patients with COVID-19. Antiplatelet therapy cannot be responsible either since patients on antiplatelet therapy were excluded from the functional analyses.

Our study suffers from several limitations: (i) the size of the cohort was small because of the monocentric nature of this study and the need to ensure that proper preanalytical conditions were respected (maximum delay of 2 hours between blood sampling and analysis); (ii) because of the large amount of blood required for each analysis, not all analyses could be performed for each patient. We therefore lacked power to analyze the correlation between platelet dysfunction and clinical events; (iii) Most of the bleeding events reported in our study did not correspond to clinically identified bleeding but to transfusion of two or more units of PRBCs. Although this criterion is commonly used to define severe bleeding events according to the ISTH criteria[19], anemia is common in ICU patients and multifactorial on account of blood spoliation due to frequent blood tests and reduced

erythropoiesis due to inflammation. Thus, the high rate of bleeding events in our cohort (31%) should be viewed cautiously.

In conclusion, our results indicate that platelets from patients with severe COVID-19 are hyporeactive with reduced surface glycoprotein expression and impaired aggregation leading to the formation of smaller and less stable thrombi. This might help to explain why, after the initial phase of the disease with a strong inflammatory response, neutrophil and monocyte activation and thrombin generation, some patients develop bleeding events. These findings should prompt us to carefully evaluate the risks and benefits of high-dose prophylactic anticoagulation and to decrease the level of anticoagulation once the initial phase of the disease has resolved.

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Declaration of interests

☐ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☒The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: