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ORIGINAL PAPER

Impairment of platelet function in both mild and severe COVID-19 patients

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Summary

Abnormalities of platelet function were reported in patients with severe COVID-19 (severe-C), but few data are available in patients with mild COVID-19 (mild-C) and after COVID-19 recovery. The aim of this study was to investigate platelet parameters in mild-C patients ($n = 51$), with no evidence of pneumonia, and severe-C patients ($n = 49$), during the acute phase and after recovery, compared to 43 healthy controls. Both mild-C and severe-C patients displayed increased circulating activated platelets, low δ-granule content (ADP, serotonin), impaired platelet activation by collagen (light transmission aggregometry) and impaired platelet thrombus formation on collagen-coated surfaces under controlled flow conditions (300/s shear rate). The observed abnormalities were more marked in severe-C patients than in mild-C patients. Overall, 61% (30/49) of mild-C and 73% (33/45) of severe-C patients displayed at least one abnormal platelet parameter. In a subgroup of just 13 patients who showed no persisting signs/symptoms of COVID-19 and were re-evaluated at least 1 month after recovery, 11 of the 13 subjects exhibited normalization of platelet parameters. In conclusion, mild abnormalities of platelet parameters were present not only in severe-C but also, albeit to a lesser extent, in mild-C patients during the acute phase of COVID-19 and normalized in most tested patients after clinical recovery.

KEYWORDS
COVID-19, platelet, platelet aggregation, platelet δ-granules, thrombus formation

INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) causes COVID-19, which is characterized by a highly heterogeneous spectrum of symptoms and complications, ranging from absent or mild clinical manifestations up to severe clinical pictures requiring hospitalization,[1,2] including pneumonia, acute respiratory distress syndrome (ARDS), septic shock and/or multiple organ failure.[3,4] The complex pathogenesis of the life-threatening clinical manifestations of COVID-19 involves a state of strong immune response, hyper-inflammation, endothelial dysfunction and activation of haemostasis, potentially leading paradoxically to both thrombotic and—albeit less frequently—bleeding events.[5] Studies carried out so far have shown that patients with severe SARS-CoV-2 infection have increased levels of cytokines and chemokines,[6,7] features of a 'cytokine storm' and crucial mediators of the adaptive immune response that

strictly correlate with disease severity. In patients with severe disease, this inflammatory state leads to the activation of haemostasis and the subsequent development of venous and arterial thrombosis.[8,9] Several reports have shown the presence of circulating hyper-reactive platelets in patients with severe COVID-19 (severe-C), probably due to the direct action of SARS-CoV-2 and/or the activation of the immune system, with a consequent increase in levels of cytokines and chemokines.[10–14] Theoretically, this phenotype may contribute to the increased thrombotic risk in these subjects. However, until now, haemostasis parameters have rarely been investigated in patients with mild COVID-19 (mild-C).

Objectives of our study were to investigate a series of platelet parameters in a cohort of patients with mild or severe COVID-19 during the acute phase of the infection and, when possible, at least 1 month after recovery.

METHODS

Subjects enrolment

We enrolled 43 healthy controls (HCs) matched by sex and age to 51 mild-C patients and 49 severe-C patients, either admitted or referred to Ospedale San Paolo, ASST Santi Paolo e Carlo, Milan, Italy, between 1 February 2021 and 15 September 2022. The enrolment of 100 COVID-19 patients was non-consecutive, and its timing was based solely on laboratory availability for sample processing. HC were screened for eligibility and included based on their willingness to participate. The COVID-19 diagnosis was confirmed in all patients by a SARS-CoV-2 real-time polymerase chain reaction (PCR) test or a positive rapid antigen test. Enrolled patients were divided into two groups according to the National Institute of Health classification of the disease.[15] Patients not requiring oxygen supplementation and without any evidence of pneumonia at chest X-ray or thoracic computed tomography (CT) scan were classified as 'mild' (mild-C, $n = 51$); patients requiring oxygen supplementation and with evidence of pneumonia confirmed by thoracic imaging were classified as 'moderate or severe' COVID-19 patients (severe-C, $n = 49$). Enrolled patients' demographics, clinical characteristics and biochemical parameters at the time of blood sampling were retrieved from electronic clinical records. Electronic clinical records were also retrospectively reviewed to capture any recorded bleeding or thrombotic events that occurred during the acute phase of COVID-19. Patients with abnormalities of the investigated platelet parameters during the acute phase of the disease were invited to be re-tested at least 1 month after recovery. The participating patients' wellbeing was assessed during a follow-up medical examination prior to blood sampling. Exclusion criteria included treatment with anti-platelet drugs and pre-existing known platelet function defects. The use of non-steroidal anti-inflammatory drugs in the 7 days before blood sampling and ongoing use of selective serotonin reuptake inhibitors (SSRIs) and/or serotonin and norepinephrine reuptake inhibitors (SNRIs) were recorded, and platelet parameters potentially affected by their use (serotonin δ-granule content, platelet aggregation [PA] and thrombus formation) were excluded from data analysis. The study was conducted in accordance with the principles of the Helsinki Declaration and local ethical regulations. All subjects gave their written informed consent to participate in the study.

Blood collection

Venous blood samples were collected into *K2E* EDTA tubes (Greiner) and analysed by Coulter hematology analyzer for blood cell count (Medonic M series 16). The remaining blood samples were anticoagulated with either 109 mmol/L trisodium citrate (9:1; vol:vol) or INN-desirudin 4500 antithrombin activity units (ATU)/mL (9:1; vol:vol) (Canyon Pharmaceuticals). Further details are provided in the Supplemental Methods.

Markers of circulating activated platelets

For measurements of P-selectin expression on the platelet plasma membrane, citrate-anticoagulated blood was immediately fixed after sampling by incubation with Thrombofix (Beckman Coulter) at 4°C overnight, followed by labelling with phycoerythrin (PE) conjugated anti-human CD62P and allophycocyanin (APC) conjugated anti-human CD41a at 4°C for 4 h. For platelet–leukocyte aggregates, citrate-anticoagulated blood was stained within 15 min from sampling with fluorescein isothiocyanate (FITC) conjugated anti-human CD14 or PE anti-human CD66b and APC anti-human CD41a at room temperature (RT) in the dark for 20 min, fixed and red cells lysed. Samples were acquired by a FACS Verse Cytometer (BD Biosciences).

Platelet aggregation induced by exogenous agonists by light transmission aggregometry

Platelet-rich plasma (PRP) was prepared by centrifugation of citrated blood samples at 200*g* at RT for 10 min, as previously described.[16,17] PA was evaluated by stimulation of PRP at 37°C with low concentrations of the exogenous agonists adenosine diphosphate (ADP, 1 μM), collagen (1 μg/mL) or thrombin receptor activating peptide (TRAP, 10 μM), and changes in light transmission were recorded for 6 min. Experiments were completed within 2 h of blood sampling and performed following the recommendations of the International Society on Thrombosis and Haemostasis.[17]

Measurement of platelet nucleotide content

Total platelet ADP and ATP content was measured by the firefly luciferin/luciferase method[18] in a lumiaggregometer

TABLE 1 Demographic and clinical characteristics of the study patients.

(A) Comparison of COVID-19 patients and healthy controls							
	Mild-C ($n=51$)		Severe-C ($n=49$)		HC ($n=43$)		
	Median or n	% or IQR	Median or n	% or IQR	Median or n	% or IQR	p value
Demographics							
Age (years)	69	48–77	59	49–72	63	50–68	0.222
Sex (female)	26	51	20	41	16	37	0.371
Ethnicity (Caucasian)	45	88	42	86	41	95	0.3035

(B) Comparison of mild and severe COVID-19 patients					
	Mild-C ($n=51$)		Severe-C ($n=49$)		
	Median or n	% or IQR	Median or n	% or IQR	p value
Clinical characteristics					
Time from symptom onset to blood sampling (days)	4	3–6	9	7–11	**<0.001**
COVID-19 vaccine	42	82	16	33	**<0.001**
Radiological pneumonia	6	35	49	100	**<0.001**
Comorbidities					
Obesity	10	20	8	16	0.669
BMI, kg/m^2	29	26–38	26.1	24.3–32.2	0.462
Hypertension	17	33.3	20	40.8	0.438
Diabetes	4	7.8	3	6.1	0.736
Cardiovascular disease	16	31.4	2	4.1	**<0.001**
Chronic pulmonary disease	8	15.7	5	10.2	0.415
Chronic kidney disease	5	9.8	2	4.1	0.262
Mild liver disease	2	3.9	2	4.1	0.967
Hepatic cirrhosis	1	2	0	0	0.325
Neurological disease	4	7.8	3	6.1	0.736
Psychiatric disorder	3	5.9	6	12.2	0.266
Autoimmune disease	3	5.9	3	6.12	0.96
Transplant	1	2.0	1	2.0	0.977
Cancer	7	13.7	1	2.0	**0.031**
Haematological disorder	8	15.7	2	4.1	0.053
Age-adjusted CCI	3	1–4	1	1–3	**0.022**
Laboratory values					
Serology (anti-N-SARS-CoV-2)	0.1	0.1–0.2	0.35	0.1–40	0.146
Haemoglobin (g/dL)	12.0	10.3–13.7	12.6	11.9–13.8	0.525
Haematocrit (%)	37.1	31.6–42.8	38.4	36.6–41.2	0.120
Platelets (×10^3/mm^3)	207	177–253	227	162–327	0.439
WBC count (×10^3/mm^3)	5.4	3.2–6.5	6.6	4.5–5.9	**0.044**
Neutrophils (×10^3/mm^3)	3.1	1.6–4.6	4.6	3.1–7.0	**0.001**
Lymphocytes (×10^3/mm^3)	1.1	0.6–1.6	1.0	0.7–1.4	0.596
NL ratio	2.7	1.2–4.9	4.7	2.5–8.5	**0.003**
C-reactive protein (mg/L)	18.6	7.0–52.6	27.1	14.1–51.9	0.151
Prothrombin time (INR)	1.1	1.0–1.2	1.1	1.0–1.2	0.4301
D-dimer (ng/mL)	257	136–335	246	191–359	0.651
Lactate dehydrogenase (U/L)	208	175–256	279	222–348	**<0.001**
PF ratio at admission	304	280–339	249	191–324	0.057

(Continues)

TABLE 1 (Continued)

(B) Comparison of mild and severe COVID-19 patients					
	Mild-C ($n=51$)		Severe-C ($n=49$)		
	Median or n	% or IQR	Median or n	% or IQR	p value
Treatments					
NSAID/COX-ib	4	8	5	10	0.680
LMWH	11	22	46	94	**<0.001**
VKA	3	6	0	0	0.1139
DOAC	4	8	2	4	0.6781
Antivirals anti-SARS-CoV-2	5	10	14	29	**0.017**
Corticosteroids	1	2	46	94	**<0.001**
SSRI_SNRI	4	8	5	10	0.7384
H_1 antagonists	0	0	9	18	**0.0011**
Outcome					
Hospital admission	17	33	49	100	**<0.001**
Transfer to ICU	0	0	6	12	0.130
Discharge	17	100	41	84	0.076
Death	0	0	2	4	0.398
Time from admission to outcome	6	4–16	13	8–20	**0.025**
1 week clinical recovery	21	72	NA	NA	NA
Time from SARS-CoV-2 positivity to viral recovery	9	7–15	NA	NA	NA
30 days mortality	1	2	3	6	0.298
Complication					
Infection other than COVID-19	6	12	8	16	0.511
Bleeding events	2	4	1	2	0.582
Thrombotic events	0	0	1	2	0.305

Note: p-values <0.05 were deemed statistically significant (in bold).

Abbreviations: CCI, Charslon Comorbidity Index; COX-ib, COX-2 inhibitor; DOAC, direct oral anticoagulant; H1-antagonist, histamine$_1$ antagonists; ICU, intensive care unit; LMWH, low-molecular-weight heparin; NL ratio, neutrophil–lymphocyte ratio; NSAID, non-steroidal anti-inflammatory drugs; PF-ratio, PaO_2/FIO_2 ratio; SNRI, serotonin and noradrenaline reuptake inhibitors; SSRI, selective serotonin reuptake inhibitors; VKA, vitamin K antagonist; WBC, white blood cell.

(Chrono-log V400, Mascia Brunelli). ADP and ATP contents were expressed as nmoles/10^8 platelets and the global ATP/ADP ratio was calculated.

Platelet serotonin content was measured by the *o*-phthaldialdehyde fluorometric determination[19] and expressed as nmoles/10^8 platelets.

In vitro thrombus formation

Experiments of in vitro thrombus formation were performed using a microfluidic device as previously described,[20] perfusing desirudin (450 ATU/mL, final concentration) anticoagulated whole blood at a 300/s shear rate for 4 min through a microfluidic channel coated with Horm fibrillar collagen type I (10 μg/mL). At the end of blood perfusion, six images were captured and the percentages of surface coverage (SC), mean fluorescence intensity (MFI) and mean thrombus area (MTA) were calculated.

Further details are provided in the Supplementary Materials under Supplemental Methods.

RESULTS

Characteristics of the subjects enrolled in the study

Demographic and clinical characteristics of the enrolled patients are shown in Table 1. Forty-three HC [median age 63 years (IQR, 51–68); 16 (37%) females] and 100 COVID-19 patients were enrolled (Table 1A). Of the 100 patients, 51 were classified as mild-C. These patients visited the hospital as outpatients to receive anti-SARS-CoV-2 antiviral treatments (34/51) or were admitted to the hospital for indications other than SARS-CoV-2 infection (17/51). The remaining 49 patients were hospitalized with pneumonia, confirmed by

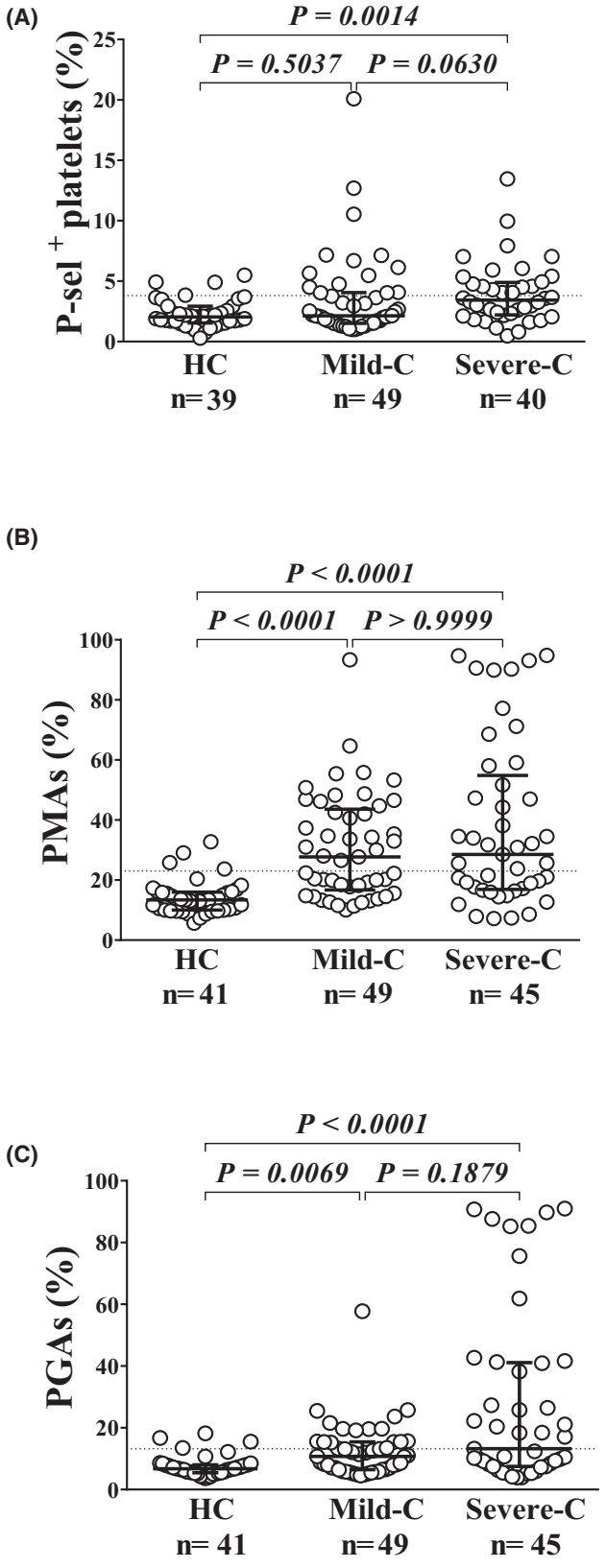

FIGURE 1 Markers of circulating activated platelets in patients with COVID-19 and HCs. Whole blood in 109 mmol/L trisodium citrate (9:1; vol:vol) was immediately fixed after sampling and stained for P-selectin analysis or stained for platelets/monocytes-granulocytes aggregates analysis within 15 min. P-selectin positive platelets (A), PMAs (B), and PGAs (C), in HCs, mild-C and severe-C. Data are represented as dot plot graphs, with medians and 25th and 75th centiles, and analysed by Kruskal-Wallis test and Dunn's post hoc test. The dotted lines indicate the 90th centile measured in HC: 3.8% for P-selectin, 23% for PMAs, and 13.2% for PGAs. HC versus mild-C versus severe-C: $p = 0.0019$ for P-selectin positive platelets; $p < 0.0001$ for PMAs; $p < 0.0001$ for PGAs. Statistical significance was assumed for p-values <0.05. HC, healthy control; mild-C, mild COVID-19; PGAs, platelet-granulocyte aggregates; PMAs, platelet-monocyte aggregates; severe-C, severe COVID-19.

chest imaging, requiring oxygen supplementation and thus classified as severe-C.

Blood sampling was performed as soon as possible during the patients' disease course, at a median of 4 days (IQR, 3–6) since symptom onset for mild-C patients and 9 days (IQR, 7–11) ($p < 0.001$) for severe-C patients. Medical treatments included low molecular weight heparin (LMWH) at prophylactic doses (administered to 11/51 [21.6%] of mild-C patients and to 46/49 [93.9%] of severe-C patients, $p < 0.001$), corticosteroids (1/51 [2%] of mild-C patients and 46/49 [93.9%] of severe-C patients, $p < 0.001$), and anti-SARS-CoV-2 antiviral drugs (5/51 [9.8%] of mild-C patients and 4/49 [28.6%] of severe-C patients, $p = 0.017$) (Table 1B).

Markers of circulating activated platelets

P-selectin expression on the platelet membrane

The median value of P-selectin expression on the platelet membrane of mild-C patients was not significantly different from that of HC (2.1% vs. 2.0%, $p = 0.5037$). However, P-selectin expression was highly heterogeneous among mild-C patients, with 29% (14/49) of them showing a value higher than the 90th centile measured in HC (>3.8%) ($\chi^2 = 4.477$, $p = 0.034354$). In contrast, severe-C patients did show a significantly higher median P-selectin expression compared to HC (3.4% vs. 2.0%, $p = 0.0014$), with 43% (17/40) of them displaying a P-selectin expression above the 90th centile ($\chi^2 = 10.5194$, $p = 0.001$). A close-to-statistically significant difference was observed between mild-C and severe-C patients (2.1% vs. 3.4%, $p = 0.0630$) (Figure 1A).

Platelet–leukocyte aggregates

Platelet–monocyte aggregates (PMAs) were increased in both mild-C and severe-C patients compared to HC, with no statistically significant differences between the two COVID-19 patient groups (mild-C vs. HC: 27.7% vs. 13.4%, $p < 0.0001$; severe-C vs. HC: 28.5% vs. 13.4%, $p < 0.0001$; mild-C vs. severe-C: 27.7% vs. 28.5%, $p > 0.999$). An overall increase of PMAs (>90th normal centile measured in HC of 23%) was observed in 53% (26/49) of mild-C patients ($\chi^2 = 18.8377$, $p = 0.0001$) and 58% (26/45) of severe-C patients ($\chi^2 = 21.780$, $p < 0.0001$). Platelet-granulocyte aggregates (PGAs) were also increased in both mild-C and severe-C patients compared to HC, with no statistically significant differences between them (mild-C vs. HC:

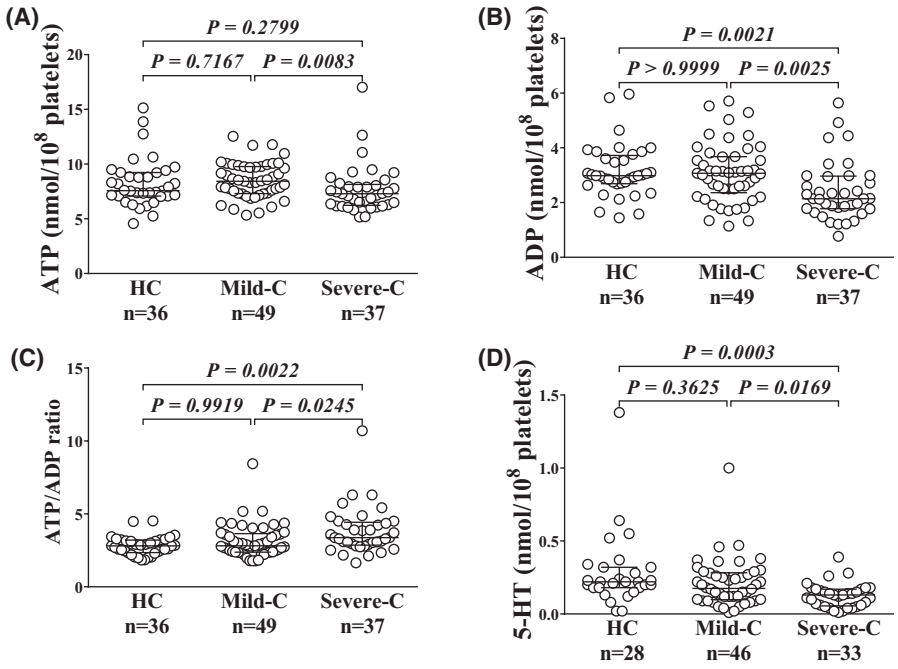

FIGURE 2 Platelet nucleotides and serotonin content. Platelet ATP (A), ADP (B), ATP/ADP ratio (C) and serotonin (5-HT) (D) were measured in healthy controls, mild-C and severe-C. Data are represented as dot plot graphs, with medians and 25th and 75th centiles. HC versus mild-C versus severe-C: $p = 0.0113$ for ATP; $p = 0.0006$ for ADP; $p = 0.0020$ for ATP/ADP and $p = 0.0003$ for 5-HT (Kruskal-Wallis test and Dunn's post hoc test). Statistical significance was assumed for p-values <0.05. HC, healthy control; mild-C, mild COVID-19; severe-C, severe COVID-19.

10.7% vs. 6.7%, $p = 0.0069$; severe-C vs. HC: 13.2% vs. 6.7%, $p < 0.0001$; mild-C vs. severe-C: 10.7% vs. 6.7%, $p = 0.1879$) (Figure 1B,C). Overall, 35% (17/49) of mild-C ($\chi^2 = 7.760$, $p = 0.005$) and 51% (23/45) of severe-C ($\chi^2 = 17.035$, $p < 0.0001$) patients displayed increased PGAs (>90th normal centile measured in HC of 13.2%).

Platelet nucleotide and serotonin content

Median values of platelet ATP (Figure 2A) and ADP content (Figure 2B), ATP/ADP ratio (Figure 2C) and platelet serotonin (5-HT) content (Figure 2D) in mild-C patients were not significantly different from those of HC (ATP: 8.4 vs. 7.6 nmol/10^8 platelets, $p = 0.7167$; ADP: 3.1 vs. 3.0 nmol/10^8 platelets, $p > 0.9999$; ATP/ADP ratio: 2.8 vs. 2.8, $p = 0.9919$; 5-HT: 0.18 vs. 0.22 nmol/10^8 platelets, $p = 0.3625$); 16% (8/49) of mild-C patients had low ADP content ($\chi^2 = 1.177$, $p = 0.278$) with high ATP/ADP ratios; and 11% (5/46) also had low 5-HT content ($\chi^2 = 0.282$, $p = 0.595$). Severe-C patients displayed similar median platelet ATP content, significantly lower median ADP and 5-HT content, and significantly higher ATP/ADP ratios compared to HC (ATP: 7.3 vs. 7.6 nmol/10^8 platelets, $p = 0.2799$; ADP: 2.1 vs. 3.0 nmol/10^8 platelets, $p = 0.0021$, ATP/ADP ratio: 3.3 vs. 2.8, $p = 0.0087$; 5-HT: 0.13 vs. 0.22 nmol/10^8 platelets, $p = 0.0003$); 43% (16/37) of severe-C patients had lower ADP content ($\chi^2 = 11.550$, $p = 0.0007$) with high ATP/ADP ratios; and 33% (11/33) had lower 5-HT content ($\chi^2 = 6.196$, $p = 0.013$).

Platelet aggregation induced by exogenous agonists

The extent of in vitro PA induced by exogenous agonists was heterogeneous in severe-C and mild-C patients, and its median values were not significantly different from those of HC (ADP, 1 μM: 40.4% vs. 29.2% vs. 36.2%, $p = 0.7006$; TRAP, 10 μM: 75.0% vs. 68.3% vs. 69.4%, $p = 0.3221$; collagen, 1 μg/mL: 68.1% vs. 74.7% vs. 74.1%, $p = 0.2002$) (Figure 3A–C). The lag time between platelet stimulation by collagen and the onset of PA was slightly increased in mild-C compared to HC (53 vs. 45 s, $p = 0.09$) and significantly longer in severe-C patients compared to HC (severe-C vs. HC: 80 vs. 45 s, $p < 0.0001$) and in mild-C patients (80 vs. 53 s, $p = 0.0066$) (Figure 3D,E). We found an inverse correlation between lag time and ADP content and a positive correlation with the ATP/ADP ratio, but not with 5-HT (Figure S1). In 16 COVID-19 patients (6 mild-C and 10 severe-C), PA was not performed since the platelet count in PRP was lower than 150×10^9/L.

In vitro thrombus formation under controlled flow conditions

In experiments of platelet thrombus formation on collagen (10 μg/mL)-coated surfaces under controlled flow conditions (300/s shear rate), mild-C patients displayed lower percentages of SC (Figure 4A) and MTA (Figure 4C) compared to HC (SC: 13.14% vs. 18.35%, $p = 0.0058$; MTA:

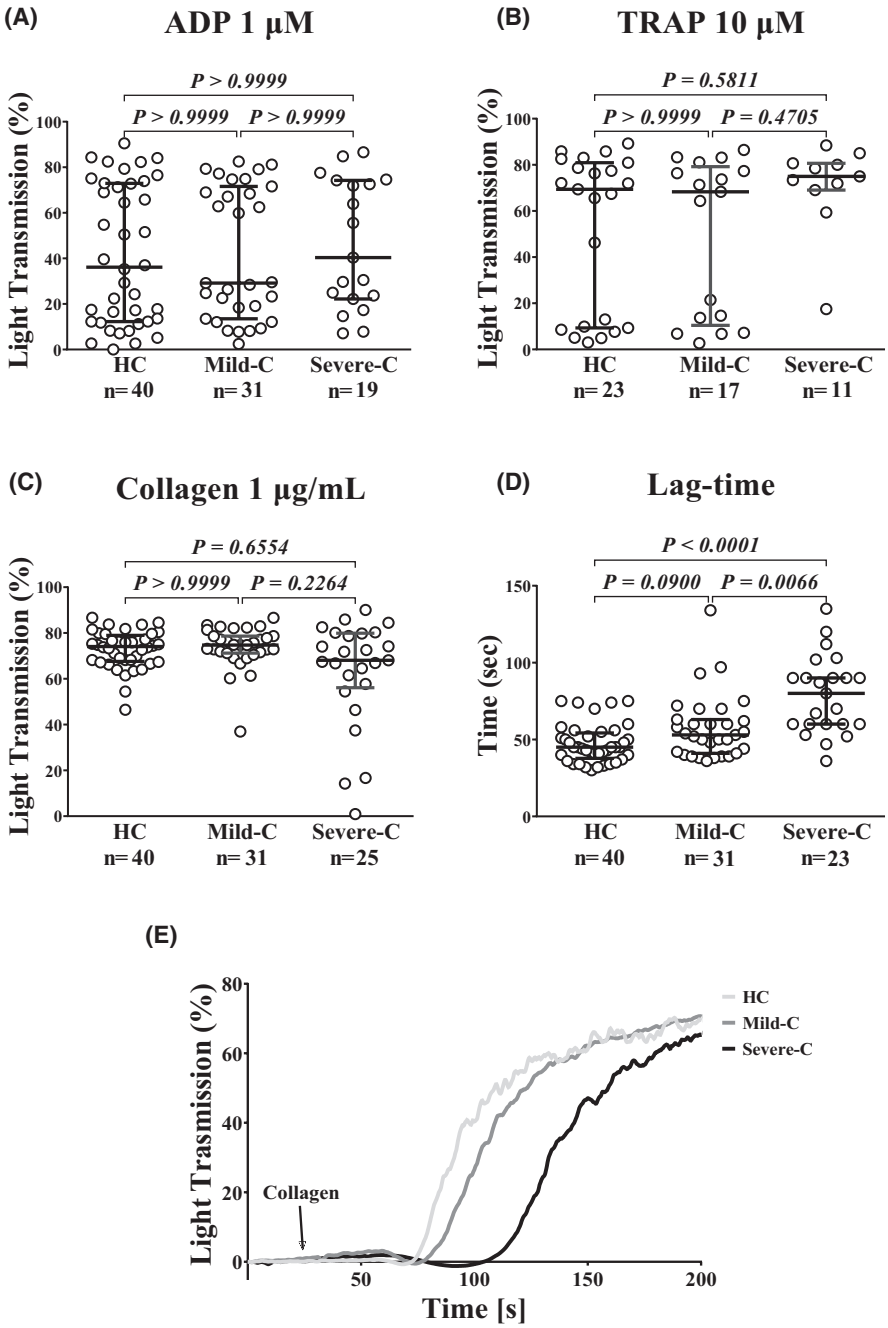

FIGURE 3 Platelet aggregation studies by light transmission aggregometry. 109 mmol/L trisodium citrate (9:1; vol:vol)-anticoagulated platelet-rich plasma (PRP) from healthy controls, mild-C and severe-C was stimulated by ADP 1 µM, thrombin receptor activating peptide 10 µM or collagen 1 µg/mL; the extent of platelet aggregation (A–C) and the lag time (the time elapsed between the addition of collagen and the onset of platelet aggregation) (D) are reported. (E) representative tracings of platelet aggregation induced by collagen 1 µg/mL: the arrow denotes the time of collagen addition to PRP. Data are represented as dot plot graphs, with medians and 25th and 75th centiles. HC versus mild-C versus severe-C: $p > 0.999$ for ADP, 1 µM; $p = 0.5811$ for TRAP, 10 µM; $p = 0.6554$ for collagen, 1 µg/mL and $p < 0.0001$ for lag time (Kruskal-Wallis test and Dunn's post hoc test). Statistical significance was assumed for p-values <0.05. HC, healthy control; mild-C, mild COVID-19; severe-C, severe COVID-19.

80 vs. 139 µm^2, $p = 0.0191$), with normal MFI (771 vs. 799, $p = 0.8653$), reflecting a normal capacity of thrombus growth (Figure 4B).

Severe-C patients displayed lower MFI of thrombi compared to HC (412 vs. 799, $p < 0.0001$) and mild-C patients (412 vs. 771, $p = 0.0004$), reflecting a reduced capacity for thrombus growth (Figure 4B). SC (severe-C vs. HC: 18.88% vs. 18.35%, $p > 0.9999$; severe-C vs. mild-C: 18.88% vs. 13.14%, $p = 0.0606$) and MTA (severe-C vs. HC: 131 vs. 139 µm^2, $p > 0.9999$ and severe-C vs. mild-C: 131 vs. 80 µm^2, $p = 0.1517$) were normal.

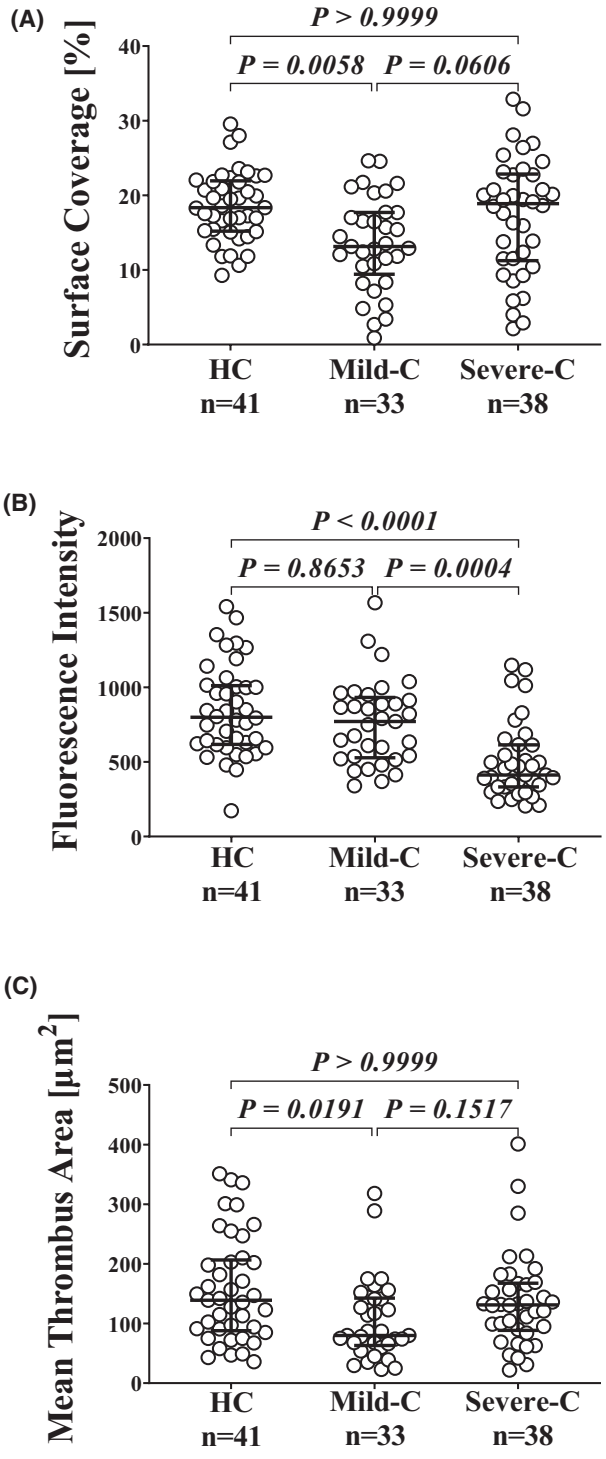

FIGURE 4 Platelet thrombus formation on a collagen (10 µg/mL)-coated microfluidic system at 300/s shear rate. Whole blood anticoagulated with desirudin (450 ATU/mL, final concentration) was labelled with $DiOC_6$ (1 µM) and perfused at 300/s shear rate through a microfluidic system at 37°C for 4 min. Surface coverage (SC) (A), mean fluorescence intensity (MFI) (B) and mean thrombus area (MTA) (C) were evaluated in healthy controls, mild-C and severe-C. Data are represented as dot plot graphs, with medians and 25th and 75th centiles. HC versus mild-C versus severe-C: $p = 0.0061$ for SC; $p < 0.0001$ for MFI; $p = 0.0207$ for MTA (Kruskal-Wallis test and Dunn's post hoc test). Statistical significance was assumed for p-values <0.05. HC, healthy control; mild-C, mild COVID-19; severe-C, severe COVID-19.

Association between platelet ADP content and circulating activated platelets, in vitro platelet aggregation and platelet thrombus formation

Decreased platelet ADP and 5-HT contents reflect the presence of degranulated platelets, likely a consequence of in vivo platelet activation. We hypothesized that degranulated platelets could be responsible for the observed abnormalities in PA and thrombus formation. To test this hypothesis, we analysed our data as a function of the platelet ADP or 5-HT content. We divided mild-C and severe-C patients into two groups based on their platelet ADP content: lower than the 10th normal centile (1.98 nmol/10^8 platelets) (low: ADP/PLT^{low} ≤1.98 nmol/10^8 platelets) and above the 10th normal centile (normal: ADP/PLT^{normal} >1.98 nmol/10^8 platelets). In the ADP/PLT^{low} group compared to the ADP/PLT^{normal} group: (1) the percentages of P-selectin positive circulating platelets (4.3% vs. 2.3%, $p = 0.0224$) and PMAs (34.9% vs. 22.3%, $p = 0.0363$) were higher (Figure 5A); (2) collagen-induced PA was lower (67% vs. 77%, $p = 0.0065$) and lag time for collagen-induced PA was prolonged (68 vs. 52 s, $p = 0.0202$) (Figure 5B); (3) platelet thrombus growth on collagen-coated surface was lower (MFI: 456 vs. 760, $p = 0.0008$), while platelet adhesion (SC: 12.9% vs. 14.9%, $p = 0.8999$; MTA: 80 vs. 109 µm², $p = 0.4953$) was not significantly different (Figure 5C); (4) PGAs were not significantly different (15.3% vs. 10.5%, $p = 0.1165$) (Figure 5A). Similar results were obtained when COVID-19 patients were divided into two groups based on 5-HT platelet content (5-HT/PLT^{normal} group >0.074 nmol/10^8 platelets and 5-HT/PLT^{low} group ≤0.074 nmol/10^8) (Figure S2).

Patients taking SSRIs and/or SNRIs were excluded from the analysis of PA, in vitro thrombus formation and platelet δ-granule content. The exclusion of patients taking SSRIs and/or SNRIs did not significantly alter the results (Table S1).

Bleeding and thrombotic events

Bleeding and thrombotic events recorded in the severe-C and mild-C patient cohorts are shown in Table 1B. Three bleeding events and one thrombotic event occurred in our COVID-19 patient population. One severe-C patient (patient 1) without other comorbidities and on LMWH prophylaxis had an episode of self-limiting haemoptysis during the first day of admission. Two mild-C patients developed bleeding events: one patient with cirrhosis developed gastro-intestinal bleeding (patient 2) and one (patient 3), on treatment with a vitamin K antagonist for atrial fibrillation, developed a haematoma of the *rectus abdominis*. At the time of hospital admission for patient 3, the INR (international normalized ratio) was 2.84 (target values: 2.0–3.0) and the aPTTr (activated partial thromboplastin time ratio) was 1.24 (normal range: 0.8–1.2). The patient's platelet count was within normal limits; no further haemostasis parameters were measured. The δ-granule content was low in patients 2 and 3 and normal in patient 1. One severe-C patient (patient 4) had pulmonary thromboembolism, which was

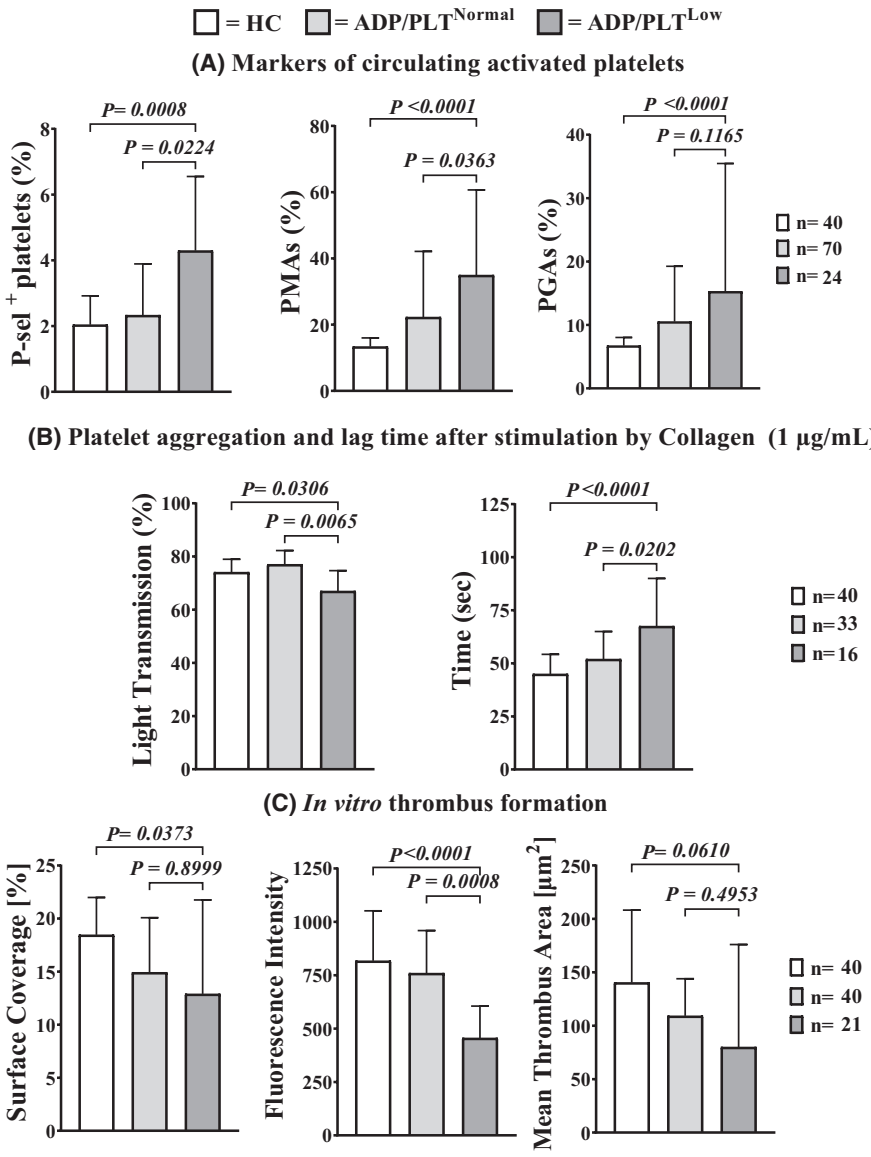

FIGURE 5 Analysis of the association between platelet ADP content and circulating activated platelets, in vitro platelet aggregation and platelet thrombus formation. Platelet parameters in COVID-19 patients, according to whether their platelet ADP content was normal (>1.98 nmol/10^8 platelets, ADP/PLT^{Normal}) or low (≤1.98 nmol/10^8 platelets, ADP/PLT^{Low}). Markers of circulating activated platelets: P-selectin positive ($P\text{-}sel^+$) platelets, PMAs and PGAs (A); platelet aggregation and lag time after stimulation by collagen (1 µg/mL) (B); thrombus formation on a collagen (10 µg/mL)-coated microfluidic system at 300/s shear rate (C). Data are represented as column bar graphs, with medians and 25th and 75th centiles. Statistical significance was assumed for *p*-values <0.05 (Mann–Whitney or unpaired *t*-test as appropriate). HC, healthy control; mild-C, mild COVID-19; PGAs, platelet-granulocyte aggregates; PMAs, platelet-monocyte aggregates; severe-C, severe COVID-19.

diagnosed by angio-CT scan at the time of hospital admission, when she was not yet on thromboprophylaxis with LMWH. During her hospital stay, she was diagnosed with polycythaemia vera. There is no relationship between the laboratory findings and the clinical findings in this study.

Evaluation of platelet parameters at least 1 month after recovery

Platelet parameters were measured only in a small subgroup of 13 patients who showed at least one marker of circulating activating platelets or defective δ-granule content during the acute phase who accepted to be re-investigated. Thus, 13 patients (4 mild-C and 9 severe-C) were re-tested at least 1 month after recovery. None of these patients had persisting signs and symptoms of COVID-19. Median values of markers of in vivo platelet activation and of platelet δ-granule content in convalescent COVID-19 patients were not significantly different from those of HC. All platelet parameters normalized in 11 patients, whereas PMAs were still high in two patients who had severe-C and platelet ADP content was still low in one patient who had mild-C (Figure 6).

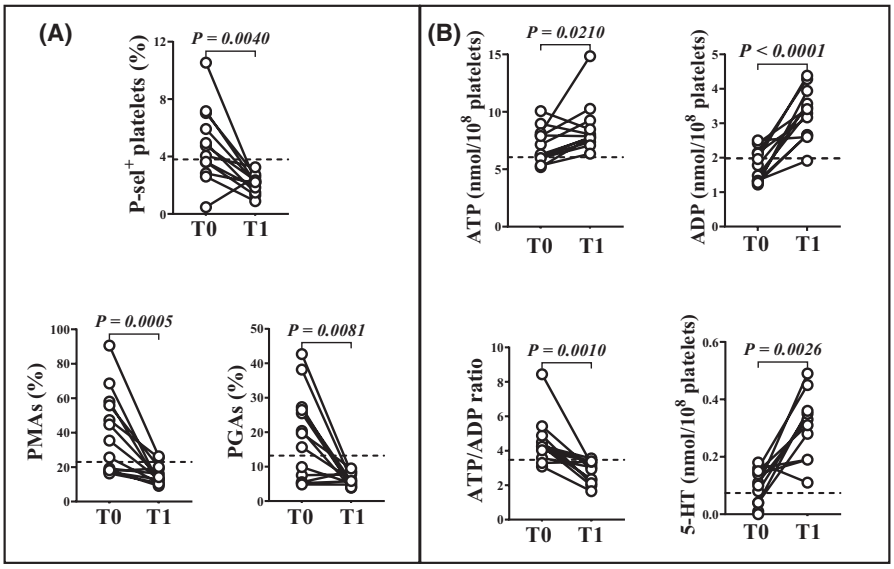

FIGURE 6 Comparison of some platelet parameters in the acute phase of the disease (T0) and at least 1 month after recovery (T1). Markers of circulating activated platelets: P-selectin positive (P-sel$^+$) platelets ($n = 12$), PMAs and PGAs ($n = 13$) (A); Platelet δ-granules content: ATP, ADP, ATP/ADP ratio and 5-HT ($n = 12$) (B). All parameters were measured in 13 patients at least 1 month after clinical recovery: all patients had no persistent signs and symptoms of COVID-19 at the time of blood sampling. The dotted lines represent 90th or 10th centiles, evaluated in healthy controls and analysed by Wilcoxon matched pairs or matched paired *t*-test as appropriate. HC, healthy control; mild-C, mild COVID-19; PGAs, platelet-granulocyte aggregates; PMAs, platelet-monocyte aggregates; severe-C, severe COVID-19.

DISCUSSION

Platelet activation is a known feature of severe COVID-19.[10–14,21] In our study, we detected abnormalities in platelet parameters not only in severe-C patients, confirming the results of previous reports,[10–14,21] but also in mild-C patients, although there was a relationship between the severity of the disease and the severity of abnormalities in platelet parameters. Moreover, among the limited cohort of 13 patients who achieved complete clinical recovery, we found that platelet parameters normalized in 11 individuals, including both retested severe-C and mild-C patients. It is noteworthy that we studied COVID-19 patients from 2021 onwards, in whom the clinical manifestations of the disease were milder than in the initial phase of the pandemic,[22] as a consequence of improved management and prophylaxis by vaccines. Two deaths occurred among the 100 enrolled patients.

In line with previous studies,[10–14,21] we describe the presence of activated platelets in the circulation of COVID-19 patients. This is highlighted by (i) the increased expression of P-selectin on the platelet plasma membrane in 43% of severe-C patients and 29% of mild-C patients, and (ii) the increased percentages of PMAs and PGAs both in severe-C and mild-C patients.

In our study, degranulated circulating platelets were present both in severe-C patients (43%) and, albeit to a lesser extent, in mild-C patients (16%). It is plausible that, during the acute phase of the disease, platelets are activated in the circulation by cytokines and immune complexes, leading to degranulation and depletion of platelet granules. This hypothesis is supported by our finding of an inverse correlation between the positivity of markers of platelet activation and the platelet ADP content. It has been demonstrated that degranulated platelets survive normally in the circulation[23,24] and are functional, although their aggregatory response to low concentrations of secretion-inducing agonists is impaired.[24] Taken together, these findings are compatible with acquired δ-Storage Pool Deficiency (δ-SPD).[25,26] In vivo platelet degranulation, which is the most prevalent determinant of acquired δ-SPD,[25,26] is likely responsible for the already described defective platelet secretion in COVID-19 patients[14,27] and for our findings of impaired collagen-induced PA and platelet thrombus formation on collagen-coated surfaces due to the diminished amplifying effect on platelet function of δ-granule contents. However, it is important to acknowledge that additional factors may also play a role in the observed impaired collagen-induced PA. We consider it highly unlikely that treatment with corticosteroids or prophylactic doses of LMWH could have affected the results of platelet thrombus formation because corticosteroids do not appreciably affect platelet function[28,29] and the mild anticoagulant effect of low doses of LMWH was outweighed by that of the high concentration of desirudin used to anticoagulate blood samples in vitro. Consistent with the rather mild bleeding diathesis of most patients with δ-SPD,[25] the clinical consequences of the acquired δ-SPD in our patients were negligible, as only two patients with low δ-granule content developed clinically relevant bleeding events.

Platelet activation might play a role in COVID-19 progression. This is the reason why some investigators have proposed aspirin as a strategy to prevent COVID-19-related

thrombotic complications and organ failure. However, the use of aspirin in COVID-19 is still controversial due to conflicting results in published data.[30,31] Our findings of impaired PA and thrombus formation highlight the potential harm of further decreasing platelet function with aspirin in these patients. An early use of non-steroidal anti-inflammatory agents,[30] including aspirin, could be considered with the aim of preventing both severe inflammation and platelet hyper-activation during SARS-CoV-2 infection.

An important finding of our study is that, following disease remission, platelets from most patients who accepted to be re-studied after recovery resumed a physiological phenotype, with a reduction of markers of activation and a parallel increase of the δ-granule content. None of the re-tested patients had persisting signs and symptoms supporting a diagnosis of long-term COVID-19. Isolated anomalies were observed in three patients: high PMAs in two patients (one was patient 4, who had polycythaemia vera, which could be responsible for the abnormality[32]), and a low ADP platelet content in a third patient. The persistence of activated platelets was recently described by Martins-Goncalves et al in a higher percentage of survivors of COVID-19 pneumonia compared to our cohort.[33] This difference could be ascribed to the fact that, in contrast to our patients, who were all asymptomatic, all patients described by Martins-Goncalves et al. still reported at least one persisting symptom, with 73% of them presenting persisting respiratory symptoms.[33]

Our findings should be read in light of the limitations of our study: (1) we carried out patient enrolment during 2021–2022; at this time, several milder variants of the ancestral strain of SARS-CoV-2 were circulating but were not systematically identified in our patients' cohorts; (2) we cannot exclude an underreporting of minor bleeding events in our patient population, as we relied on a retrospective review of clinical charts to capture patients' bleeding tendency; (3) the sample size of the group of COVID-19 patients who were re-tested after recovery is small, only 13 patients, due to low adhesion of the patients; (4) there is no relationship in this study of the laboratory findings and the clinical findings.

In conclusion, our study shows that 67% (63/94) of our cohort of COVID-19 patients (61% of mild-C and 73% of severe-C) had at least one marker of circulating activated platelets or low δ-granule content, highlighting a platelet dysfunction even in mild-C patients, although to a lesser degree compared to severe-C. Such impairment in platelet function was mild and did not entail a clinically relevant bleeding tendency in the absence of other predisposing factors. After recovery, all parameters normalized spontaneously in 11 of 13 re-evaluated patients.

AUTHOR CONTRIBUTIONS

Mariangela Scavone, Claudia Ghali, Mariagrazia Calogiuri, Norma Maugeri and Antonella Fioretti contributed to the design of the study, performed laboratory analysis, analysed the data; Matteo Sala, Valeria Bono and Giulia Marchetti consulted on COVID-19 patients management and analysed the data; Elena Bossi performed laboratory analysis; Bianca Clerici and Simone Birocchi contributed on HCs enrolment and analysed the data; Tatiana Mencarini and Silvia Bozzi contributed to microfluidic device production and analysed thrombus formation data; Marco Cattaneo and Gian Marco Podda designed the study, coordinated the group, contributed to data analysis interpretation and wrote the manuscript. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

No conflicts of interest to disclose.

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REFERENCES

1. Guan WJ, Ni ZY, Hu Y, Liang WH, Ou CQ, He JX, et al. Clinical characteristics of coronavirus disease 2019 in China. N Engl J Med. 2020;382(18):1708–20.
2. Synowiec A, Szczepanski A, Barreto-Duran E, Lie LK, Pyrc K. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2): a systemic infection. Clin Microbiol Rev. 2021;34(2):e00133-20.
3. Lopez-Collazo E, Avendano-Ortiz J, Martin-Quiros A, Aguirre LA. Immune response and COVID-19: a mirror image of sepsis. Int J Biol Sci. 2020;16(14):2479–89.
4. Zheng J, Miao J, Guo R, Guo J, Fan Z, Kong X, et al. Mechanism of COVID-19 causing ARDS: exploring the possibility of preventing and treating SARS-CoV-2. Front Cell Infect Microbiol. 2022;12:931061.
5. de Andrade SA, de Souza DA, Torres AL, de Lima CFG, Ebram MC, Celano RMG, et al. Pathophysiology of COVID-19: critical role of hemostasis. Front Cell Infect Microbiol. 2022;12:896972.
6. Tincati C, Cannizzo ES, Giacomelli M, Badolato R, d'Arminio Monforte A, Marchetti G. Heightened circulating interferon-inducible chemokines, and activated pro-cytolytic Th1-cell phenotype features Covid-19 aggravation in the second week of illness. Front Immunol. 2020;11:580987.

7. Hsu RJ, Yu WC, Peng GR, Ye CH, Hu S, Chong PCT, et al. The role of cytokines and chemokines in severe acute respiratory syndrome coronavirus 2 infections. Front Immunol. 2022;13:832394.
8. Battistoni I, Francioni M, Morici N, Rubboli A, Podda GM, Pappalardo A, et al. Pre- and in-hospital anticoagulation therapy in coronavirus disease 2019 patients: a propensity-matched analysis of in-hospital outcomes. J Cardiovasc Med (Hagerstown). 2022;23(4):264–71.
9. Birocchi S, Manzoni M, Podda GM, Casazza G, Cattaneo M. High rates of pulmonary artery occlusions in COVID-19. A meta-analysis. Eur J Clin Investig. 2021;51(1):e13433.
10. Canzano P, Brambilla M, Porro B, Cosentino N, Tortorici E, Vicini S, et al. Platelet and endothelial activation as potential mechanisms behind the thrombotic complications of COVID-19 patients. JACC Basic Transl Sci. 2021;6(3):202–18.
11. Hottz ED, Azevedo-Quintanilha IG, Palhinha L, Teixeira L, Barreto EA, Pao CRR, et al. Platelet activation and platelet-monocyte aggregate formation trigger tissue factor expression in patients with severe COVID-19. Blood. 2020;136(11):1330–41.
12. Manne BK, Denorme F, Middleton EA, Portier I, Rowley JW, Stubben C, et al. Platelet gene expression and function in patients with COVID-19. Blood. 2020;136(11):1317–29.
13. Maugeri N, De Lorenzo R, Clementi N, Antonia Diotti R, Criscuolo E, Godino C, et al. Unconventional CD147-dependent platelet activation elicited by SARS-CoV-2 in COVID-19. J Thromb Haemost. 2022;20(2):434–48.
14. Garishah FM, Huskens D, Pramudo SG, Andriani D, Astrilia M, Sentosa RA, et al. Hyperresponsive platelets and a reduced platelet granule release capacity are associated with severity and mortality in COVID-19 patients. Thromb Haemost. 2022;122(12):2001–10.
15. COVID-19 Treatment Guidelines Panel. Coronavirus Disease 2019 (COVID-19) Treatment Guidelines. [Cited 2022 Oct 4]. Available from: https://www.covid19treatmentguidelines.nih.gov/
16. Scavone M, Femia EA, Caroppo V, Cattaneo M. Inhibition of the platelet P2Y12 receptor for adenosine diphosphate does not impair the capacity of platelet to synthesize thromboxane A2. Eur Heart J. 2016;37(44):3347–56.
17. Cattaneo M, Cerletti C, Harrison P, Hayward CP, Kenny D, Nugent D, et al. Recommendations for the standardization of light transmission aggregometry: a consensus of the working party from the platelet physiology subcommittee of SSC/ISTH. J Thromb Haemost. 2013;11:1183–9.
18. Dangelmaier CA, Holmsen H. Platelet dense granule and lysosome content. In: Harker LA, Zimmerman TS, editors. Methods in hematology: measurement of platelet function. Edinburgh, Scotland: Churchill Livingstone; 1983. p. 92–114.
19. Drummond AH, Gordon JL. Letter: rapid, sensitive microassay for platelet 5HT. Thromb Diath Haemorrh. 1974;31(2):366–7.
20. Scavone M, Bozzi S, Mencarini T, Podda G, Cattaneo M, Redaelli A. Platelet adhesion and thrombus formation in microchannels: the effect of assay-dependent variables. Int J Mol Sci. 2020;21(3):750.
21. Leopold V, Pereverzeva L, Schuurman AR, Reijnders TDY, Saris A, de Brabander J, et al. Platelets are hyperactivated but show reduced glycoprotein VI reactivity in COVID-19 patients. Thromb Haemost. 2021;121(9):1258–62.
22. Menni C, Valdes AM, Polidori L, Antonelli M, Penamakuri S, Nogal A, et al. Symptom prevalence, duration, and risk of hospital admission in individuals infected with SARS-CoV-2 during periods of omicron and delta variant dominance: a prospective observational study from the ZOE COVID study. Lancet. 2022;399(10335):1618–24.
23. Michelson AD, Barnard MR, Hechtman HB, MacGregor H, Connolly RJ, Loscalzo J, et al. In vivo tracking of platelets: circulating degranulated platelets rapidly lose surface P-selectin but continue to circulate and function. Proc Natl Acad Sci U S A. 1996;93(21):11877–82.
24. Reimers HJ, Packham MA, Kinlough-Rathbone RL, Mustard JF. Effect of repeated treatment of rabbit platelets with low concentrations of thrombin on their function, metabolism and survival. Br J Haematol. 1973;25(5):675–89.
25. Cattaneo M. Inherited disorders of platelet function. In: Michelson AD, Cattaneo M, Frelinger AL, Newman P, editors. Platelets. 4th ed. London, UK: Elsevier - Academic Press; 2018. p. 877–905.
26. Podda G, Femia EA, Pugliano M, Cattaneo M. Congenital defects of platelet function. Platelets. 2012;23(7):552–63.
27. Kalbhenn J, Pooth JS, Trummer G, Kranzhofer D, Schlagenhauf A, Zieger B. Pervasive platelet secretion defects in patients with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Cell. 2023;12(1):193.
28. Moraes LA, Paul-Clark MJ, Rickman A, Flower RJ, Goulding NJ, Perretti M. Ligand-specific glucocorticoid receptor activation in human platelets. Blood. 2005;106(13):4167–75.
29. Hutton RA, Mikhailidis DP, Georgiadis E, Hyden A, Ginsburg J. The effect of tetracosactrin and corticosteroids on platelet aggregation: an in vivo and in vitro study. Thromb Res. 1980;17(1–2):5–11.
30. Chow JH, Rahnavard A, Gomberg-Maitland M, Chatterjee R, Patodi P, Yamane DP, et al. Association of early aspirin use with in-hospital mortality in patients with moderate COVID-19. JAMA Netw Open. 2022;5(3):e223890.
31. RECOVERY Collaborative Group. Aspirin in patients admitted to hospital with COVID-19 (RECOVERY): a randomised, controlled, open-label, platform trial. Lancet. 2022;399(10320):143–51.
32. Maugeri N, Malato S, Femia EA, Pugliano M, Campana L, Lunghi F, et al. Clearance of circulating activated platelets in polycythemia vera and essential thrombocythemia. Blood. 2011;118(12):3359–66.
33. Martins-Goncalves R, Campos MM, Palhinha L, Azevedo-Quintanilha IG, Abud Mendes M, Ramos Temerozo J, et al. Persisting platelet activation and hyperactivity in COVID-19 survivors. Circ Res. 2022;131(11):944–7.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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