

Platelets are Hyperactivated but Show Reduced Glycoprotein VI Reactivity in COVID-19 Patients

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Coronavirus disease-2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), has rapidly spread worldwide since December 2019. Disease severity is strongly associated with abnormal routine coagulation tests (particularly increased D-dimer levels) and thrombocytopenia.¹ These alterations are accompanied by a high prevalence of deep venous thrombosis, pulmonary embolism, and arterial thrombotic events at uncommon sites.² Platelets are at the crossroad of thrombosis and immunity and contribute to thrombus formation and disease severity during bacterial sepsis^{3,4} and viral infections.^{5,6} We here investigated platelet activation and responsiveness to relevant stimuli in hospitalized COVID-19 patients.

Patients were included in the ELDER-BIOME protocol (clinicaltrials.gov identifier NCT02928367). We prospectively enrolled suspected COVID-19 patients admitted to the ward, in two hospitals in The Netherlands (Academic Medical Center in Amsterdam and Flevo Hospital in Almere). COVID-19 infection was confirmed by polymerase chain reaction (PCR) of material obtained by nasopharyngeal swab. Controls were hospital employees ($n=8$) or patients visiting the outpatient clinics without infectious disease symptoms ($n=18$); the results obtained in these control subgroups did not differ and were combined. Informed consent was obtained from all participants. Citrated peripheral blood was collected within 48 hours after admission and processed within 4 hours. Platelet-rich plasma (PRP) was obtained by centrifugation ($180 \times g$,

15 minutes, 21°C), mixed with a 1:5 ratio of acid citrate dextrose and subsequently centrifuged ($200 \times g$, 2 minutes) to remove remaining erythrocytes and leukocytes. Platelets in buffer-diluted PRP were stimulated for 30 minutes with cross-linked collagen-related peptide (CRP-XL, CambCol-Laboratory, Cambridge, United Kingdom; 0.1 or 1 µg/mL) or thrombin receptor activator peptide-6 (TRAP-6, Bachem, Bubendorf, Switzerland; 15 µM). Platelet activation was assessed by flow cytometry using anti-CD61-AlexaFluor700 (clone VI-PL2), anti-CD62P-PerCP-Cy5.5 (clone AK4), anti-CD63-PE-Cy7 (clone H5C6), and anti-glycoprotein (GP) IIb/IIIa-AlexaFluor647 (clone PAC-1) (Biolegend, San Diego, California, United States). Analysis was performed on FACSCanto II until 10,000 CD61+ events were recorded. Data were analyzed using FlowJo v10 (BD Biosciences, San Jose, California, United States). Platelet-neutrophil complexes were measured in whole blood (without ex vivo stimulation) as previously described,⁷ using anti-CD66b-FITC, anti-CD11b-PE, anti-CD61-AlexaFluor700 (Biolegend), and Viability Dye-LFluor780 (eBioscience, San Diego, California, United States). Platelet numbers in PRP were established by flow cytometry, using Precision Count Beads (BD Bioscience). Soluble GPVI was measured in citrated plasma ($1,500 \times g$, 15 minutes) by enzyme-linked immunosorbent assay (ELISA) (Invitrogen, Waltham, Massachusetts, United States). Soluble P-selectin GP ligand-1 (PSGL-1), CD40L, and D-dimer were measured using LEGENDplex Human Thrombosis Panel

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(Biologend). Soluble P-selectin and platelet factor (PF) 4 were measured by ELISA (R&D Duoset, Minneapolis, Minnesota, United States). All analyses were done using GraphPad Prism v8.0 (San Diego, California, United States). Results were compared using paired Wilcoxon test or unpaired Mann–Whitney *U* tests. We applied linear regression to assess the correlation between neutrophil counts and soluble PSGL-1 levels. *p*-Values < 0.05 defined statistical significance.

We enrolled 35 patients and 26 controls between April 10 and May 15, 2020. Five patients had a negative SARS-CoV-2 PCR and were excluded from analyses. Baseline characteristics of patients and controls were comparable, except for body mass index and ethnicity (►Table 1). Neutrophil counts were higher in COVID-19 patients compared with controls, whereas platelet counts were unaltered. Three patients (10.0%) were subsequently admitted to the intensive care unit and four (13.3%) died during hospital stay. Two patients (6.6%) were diagnosed with pulmonary embolism.

To assess circulating platelet activation, we measured the expression of P-selectin, CD63 (reflecting α - and dense granules secretion, respectively), and the fibrinogen-binding

conformation of GPIIb/IIIa.⁸ Platelets from COVID-19 patients showed increased expression of P-selectin and active GPIIb/IIIa, while CD63 expression was comparable to that of control platelets (►Fig. 1A). The plasma levels of α -granule proteins PF4 and soluble CD40L were higher in COVID-19 patients, further indicating activated platelets in vivo (►Fig. 1B). While earlier studies also reported enhanced α granule release,^{9,10} our study adds to this that dense granules likely are not affected in COVID-19 patients not requiring intensive care.

Platelet-neutrophil complexes are linked to platelet hyperactivation in vivo.³ COVID-19 patients had more platelet-neutrophil complexes compared with controls (►Fig. 1C). Moreover, neutrophils from COVID-19 patients had higher CD11b expression, suggesting a higher activation status (►Fig. 1D). Interestingly, neutrophils that were in complex with platelets had the highest CD11b expression, implying that platelet activation and interaction with neutrophils is associated with increased neutrophil activation. Likely, the enhanced platelet P-selectin expression in COVID-19 patients contributed to the interaction with neutrophils.⁸ Plasma levels of soluble P-selectin and PSGL-1 (the neutrophil

Table 1 Baseline characteristics of patients and control subjects

	Controls <i>n</i> = 26	COVID-19 <i>n</i> = 30	<i>p</i> -Value
Age	56.5 [45.5–71.7]	61.5 [50.2–68.5]	0.53
Gender (male)	14 (53.8)	13 (43.3)	0.61
Ethnicity			
African	2 (7.2)	10 (33.3)	< 0.01
Caucasian	23 (88.5)	10 (33.3)	
Surinam	1 (3.8)	7 (23.3)	
Other	0 (0.0)	3 (10.0)	
Body mass index	25.9 [22.3–28.5]	29.5 [27.0–32.7]	< 0.01
Comorbidities			
Respiratory	4 (15.4)	3 (10.0)	0.45
Asthma	1 (3.8)	1 (3.3)	> 0.99
COPD	2 (7.7)	1 (3.3)	0.90
Cardiovascular	9 (34.6)	15 (50.0)	0.37
Hypertension	9 (34.6)	13 (43.3)	0.70
Ischemic cardiopathy	3 (11.5)	2 (6.7)	0.87
Peripheral ischemic disease	1 (3.8)	1 (3.3)	> 0.99
Congestive heart failure	3 (11.5)	1 (3.3)	0.50
Stroke	2 (7.7)	2 (6.7)	> 0.99
Diabetes	5 (19.2)	8 (26.7)	0.73
Malignancy	6 (23.1)	1 (3.3)	0.07
Chronic renal disease	0 (0.0)	2 (6.7)	0.54
Chronic medication			
Platelet antagonist	4 (15.4) ^a	5 (16.7) ^b	> 0.99
NSAID	2 (7.7) ^c	0 (0.0)	0.41

(Continued)

Table 1 (Continued)

	Controls	COVID-19	p-Value
	n = 26	n = 30	
Clinical characteristics			
Oxygen saturation	–	95.5 [90.5–97.8] ^d	
Temperature	–	37.5 [36.6–38.2]	
Systolic blood pressure	–	128 [119–141]	
Diastolic blood pressure	–	80 [73–89]	
qSOFA score 0	–	10 (33.3)	
1	–	19 (63.3)	
2	–	1 (3.3)	
Laboratory at admission			
Hemoglobin (g/L)	138.6 [125.7–151.5]	128.9 [117.6–133.7]	0.19
Platelets (10 ⁹ /L)	236 [180–272]	240 [194–344]	0.48
Leukocytes (10 ⁹ /L)	5.85 [5.08–7.17]	7.00 [5.82–9.07]	0.13
Neutrophils	3.71 [3.07–4.64]	5.00 [4.20–6.09]	0.01
Lymphocytes	1.37 [1.12–1.91]	1.05 [0.72–1.40]	0.04
Monocytes	0.49 [0.42–0.68]	0.38 [0.27–0.60]	0.07
D-dimer (mg/L)	0.031 [0.019 - 0.046]	0.045 [0.024–0.11]	0.10
Outcome			
Thrombotic event	–	2 (6.6) ^e	
ICU admission	–	3 (10.0)	
In-hospital death	–	4 (13.3)	

Abbreviations: COPD, chronic obstructive pulmonary disease; ICU, intensive care unit; IQR, interquartile range; NSAID, nonsteroidal anti-inflammatory drug; qSOFA, quick Sequential Organ Failure Assessment.

Note: Data are shown as median (%) or median [IQR].

^aAspirin (n = 2), clopidogrel (n = 1), aspirin + clopidogrel (n = 1).

^bAspirin (n = 3), clopidogrel (n = 1), aspirin + clopidogrel (n = 1).

^cDiclofenac (n = 1), naproxen (n = 1).

^dWith oxygen therapy (median 2 L/min).

^ePulmonary embolism (n = 2).

receptor mediating platelet-neutrophil complex formation) were not different between patients and controls (–**Fig. 1B**). Soluble PSGL-1 was positively correlated with neutrophil counts in COVID-19 patients ($r = 0.53$, $p = 0.0029$) but not in controls ($r = 0.35$, $p = 0.24$) (–**Fig. 1E**), suggesting that PSGL-1 shedding is associated with neutrophil activation and demargination in COVID-19 patients. Recent studies corroborate our findings of enhanced platelet P-selectin expression with normal plasma soluble P-selectin levels in COVID-19 patients not requiring intensive care.^{11,12} Together, these results suggest that circulating platelets are hyperactivated in COVID-19 patients, which is associated with enhanced neutrophil activation.

To assess platelet reactivity in COVID-19 patients, we stimulated platelets *ex vivo* with a GPVI agonist (CRP-XL) or protease-activated receptor-1 agonist (TRAP), mimicking collagen and thrombin stimulation, respectively. Remarkably, platelets from COVID-19 patients showed a decreased sensitivity to low dose CRP-XL as measured by P-selectin, CD63, and active GPIIb/IIIa surface expression; this hyporeactivity was also observed at a higher dose of CRP-XL for CD63 and

active GPIIb/IIIa expression (–**Fig. 1F**). In sharp contrast, platelet reactivity to TRAP was increased in COVID-19 patients when compared with controls with regard to P-selectin expression, in agreement with previous study,¹² while CD63 and active GPIIb/IIIa were not different. Our result of platelet hyporeactivity to GPVI stimulation in COVID-19 patients, confirmed with all measured membrane markers of α - and dense-granule release and GPIIb/IIIa activation, is corroborated by a recent study that reported unaltered P-Selectin expression but decreased GPIIb/IIIa activation in response to a high dose of CRP, another specific GPVI agonist.¹² Of note, earlier studies reporting increased platelet aggregation in COVID-19 patients used collagen to stimulate platelets,^{12,13} which unlike CRP-XL used here is not selective for GPVI.

GPVI is an essential platelet receptor that binds collagen and fibrin. We hypothesized that platelets desensitization of GPVI to CRP-XL in COVID-19 could be due to GPVI shedding, following *in vivo* transient interaction with its ligands such as collagen, fibrin, D-dimer, or fibrinogen (as previously reviewed by Nurden¹⁴). GPVI shedding,

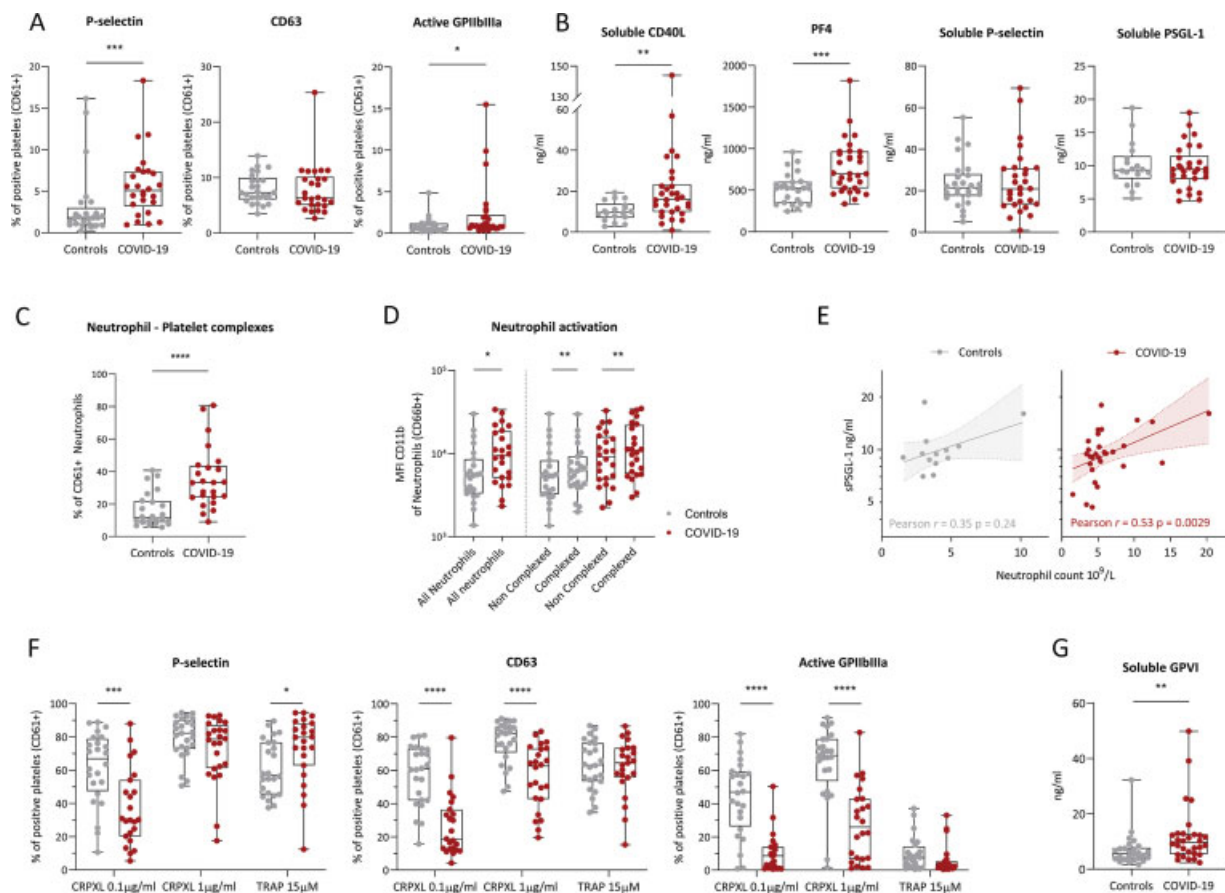


Fig. 1 Platelet activation and reactivity in COVID-19 patients and controls. (A) Platelet expression of P-selectin, CD63, and active glycoprotein (GP) IIb/IIIa measured at baseline by flow cytometry. (B) Plasma levels soluble CD40L, platelet factor (PF)4, soluble P-selectin, and soluble P-selectin GP ligand-1 (PSGL-1). (C) Platelet-neutrophil complexes (defined as CD61+ neutrophils) measured in whole blood by flow cytometry. (D) CD11b median fluorescence intensity (MFI) analyzed for all neutrophils (CD66b+), neutrophils complexed with platelets (CD66b+ CD61+), and non-complexed neutrophils (CD66b+ CD61-). (E) Correlation plot between soluble PSGL-1 and neutrophil counts. (F) Platelets were stimulated ex vivo with cross-linked collagen-related peptide (CRP-XL) 0.1 μ g/mL, CRP-XL 1 μ g/mL, or thrombin receptor activator peptide (TRAP) 15 μ M for 30 minutes; activation markers were studied by flow cytometry. (G) Plasma levels of soluble GPVI. Results are shown as dot plot with box and whiskers. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

estimated by measuring soluble GPVI in plasma, was higher in COVID-19 patients than in controls (\rightarrow Fig. 1G). Besides GPVI shedding, an additional mechanism of GPVI hyporesponsivity could be the disruption of GPVI signaling, for example, due to Fc γ RIIA irreversible proteolysis,¹⁵ in the context of a viral infection and circulating immune complexes.¹⁶

In conclusion, we report hyperactivated platelets in COVID-19 patients associated with increased activation of neutrophils and complex formation. Platelets from COVID-19 patients were desensitized to CRP-XL stimulation which was accompanied by increased shedding of the CRP-XL receptor GPVI. Although previous studies have implicated platelet GPVI in thromboembolism,¹⁴ our data argue against a role of platelet GPVI in COVID-19 hypercoagulability.

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Conflict of Interest
None declared.

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