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ORIGINAL RESEARCH - CLINICAL

Low-Grade Inflammation in Long COVID Syndrome Sustains a Persistent Platelet Activation Associated With Lung Impairment

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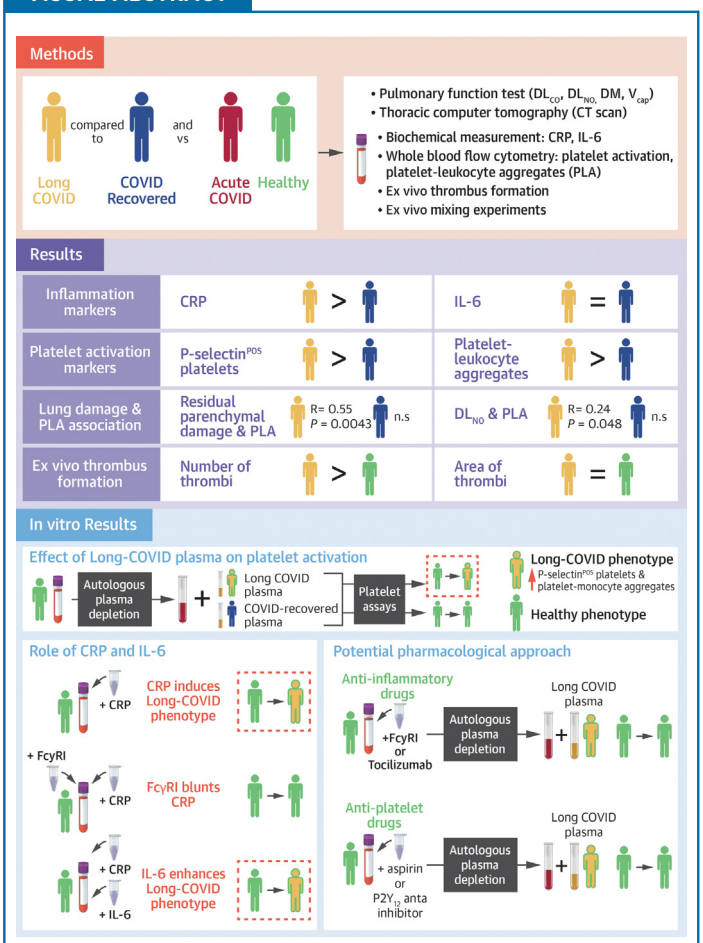
VISUAL ABSTRACT



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HIGHLIGHTS

- **Long COVID patients, compared to COVID-recovered asymptomatic subjects, present a platelet activation phenotype characterized mainly by a high number of circulating platelet-leukocyte aggregates.**
- **The number of platelet-leukocyte aggregates is significantly associated with residual lung damage that sustains the most frequently referred symptoms, such as dyspnea, chest pain, fatigue at rest and after exertion.**
- **Low-grade inflammation, sustained mainly by CRP but also by IL-6, is present in long COVID patients and correlates with the degree of platelet activation.**
- **In ex vivo mixing experiments, plasma of long COVID patients mixed with plasma-depleted blood of healthy subjects reproduces the platelet activation observed in vivo through a mechanism that is blunted by Fcγ-receptor inhibitor and tocilizumab, highlighting the role of CRP and IL-6, and also by antiplatelet drugs.**

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SUMMARY

In the present study, we provide evidence on the potential mechanisms involved in the residual pulmonary impairment described in long COVID syndrome. Data highlight that lung damage is significantly associated with a proinflammatory platelet phenotype, characterized mainly by the formation of platelet-leukocyte aggregates. In ex vivo experiments, long COVID plasma reproduces the platelet activation observed in vivo and highlights low-grade inflammation as a potential underpinning mechanism, exploiting a synergistic activity between C-reactive protein and subthreshold concentrations of interleukin-6. The platelet-activated phenotype is blunted by anti-inflammatory and antiplatelet drugs, suggesting a potential therapeutic option in this clinical setting. (JACC Basic Transl Sci. 2025;10:20–39)

ABBREVIATIONS AND ACRONYMS

CT = computed tomography
CRP = C-reactive protein
DL_{CO} = carbon monoxide lung diffusion
DL_{NO} = nitric oxide lung diffusion
DM = membrane diffusion
FU = follow-up
HAA = high attenuation area
HS = healthy subject
IL = interleukin
MV = microvesicle
PGA = platelet-granulocyte aggregates
PLA = platelet-leukocyte aggregates
PMA = platelet-monocyte aggregates
RT = room temperature
TF = tissue factor
VA = alveolar volume
V_{cap} = capillary volume
WB = whole blood

Although the COVID-19 emergency is over and the trend of new infections is currently downward, physicians still have to deal with long-term postinfective sequelae, which have been shown to affect the quality of life of a high percentage of patients who have experienced the disease.[1]

Long COVID is defined as a syndrome characterized by the persistence of COVID-19 symptoms over 4 weeks from the onset of the disease and cannot be explained by an alternative diagnosis. Long COVID patients have a wide range of fluctuating symptoms that can be present at different levels.[2] In this broad scenario, the most concerning symptoms are those resulting from lung parenchyma involvement (dyspnea, chest pain, fatigue at rest and after exertion)—evidenced by abnormal findings at follow-up computed tomography (CT) scan up to 12 months after the acute infection—mainly represented by ground glass opacity and fibrotic changes.[3] Another compelling aspect is the significant correlation between the percentage of residual lung damage on CT scan and the total lung capacity—the diffusing capacity of the lungs for carbon monoxide (DL_{CO}) and nitric oxide (DL_{NO})—over a period longer than 1 year.[4]

A full understanding of the pathophysiology of these alterations and persisting symptoms has not yet been achieved. Many investigators argue that the long-lasting lung impairment may be caused by prolonged endothelial dysfunction and immune cell activation, with the resulting cytokine production and ongoing inflammatory insult.[5,6] Chronic low-grade inflammation may indeed underlie the typical symptoms of long COVID, among which are neuroinflammation, intermittent fatigue, postexertional malaise, arthralgias, and mild dyspnea.[7] Studies have shown that C-reactive protein (CRP) levels in the range of chronic inflammation, in particular, are significantly associated with the presence of long COVID symptoms.[8]

This persistent inflammation, triggered by the initial COVID-19 infection, could also sustain an activated platelet phenotype, characterized by a trend toward increased P-selectin/aGPIIbIIIa expression and platelet clump formation, in individuals who have survived severe COVID-19.[9,10] The cytokine storm has been extensively shown to be the driving force of COVID-19 tissue injuries.[11] We have also provided compelling evidence that in SARS-CoV-2–infected patients during the first pandemic, the cytokine storm (mainly driven by interleukin [IL]-6) deeply affects endothelial functions, leading to a generalized cell-based tissue factor (TF)–mediated activation of blood coagulation, the release of procoagulant microvesicles (MVs), and a massive platelet activation testified by a strong up-regulation of

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The authors attest they are in compliance with human studies committees and animal welfare regulations of the authors' institutions and Food and Drug Administration guidelines, including patient consent where appropriate. For more information, visit the Author Center.

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platelet P-selectin (10-fold higher than in healthy subjects [HSs]) and TF expression.[12] These findings correlate with the disease severity in that the observed cell activation is greater in COVID-19 patients requiring mechanical ventilation. Moreover, we have also shown that the formation of platelet-leukocyte aggregates (PLA) is another hallmark of COVID-19; PLA could actively participate in the formation of the pulmonary microthrombi found in autopsies of acute COVID-19 patients.[12] Whether this phenotype may foster the pulmonary impairment observed in long COVID patients is currently unknown. We hypothesized that a chronic, low-grade inflammation underlies a persistent platelet activation that might sustain the lung impairment observed in patients with long COVID syndrome. Thus, we investigated the persistence of the platelet activation—observed during the acute phase—in patients recovered from COVID-19 by 6 months and, in those still symptomatic, its possible relationship with the pulmonary function.

METHODS

SUBJECT ENROLLMENT. We prospectively enrolled, between July and October 2020 at Centro Cardiologico Monzino IRCCS and Istituto Auxologico Italiano IRCCS in Milan, 204 subjects (Follow-up-COVID patients [COVID-FU patients]) who recovered from SARS-CoV-2 infection by 6 ± 1 months. Exclusion criteria were presence of a severe disease before COVID-19 infection (including anemia, infection, stroke, venous thromboembolism, cancer, collagen disease, and thyrotoxicosis), chronic atrial fibrillation, myocardial infarction within the last 30 days, and heparin or oral anticoagulant treatment. The subject evaluation included clinical information and routine laboratory tests. A subgroup of 34 COVID-FU patients still having symptoms (long COVID population) was compared with 34 consecutively enrolled asymptomatic subjects (COVID-recovered) and with 34 HSs, concomitantly recruited at Centro Cardiologico Monzino IRCCS, in terms of platelet activation and platelet procoagulant profile. HSs were defined as subjects who had never been affected by COVID-19, chronic respiratory disease, or cardiovascular disease. Data on platelet activation and platelet procoagulant profile of acute COVID-19 patients (n = 46) enrolled in a previous study[12] were also used for comparison. In the symptomatic group, a lung CT scan and pulmonary function tests were performed as per internal clinical protocol; for asymptomatic subjects, no further diagnostics over the follow-up period were recommended by physicians. The study was approved by the ethical committee of the institution (number CCM1293), and informed consent was obtained from all participants according to the principles of the Declaration of Helsinki.

BLOOD COLLECTION AND PLASMA PREPARATION. Whole blood (WB) was drawn 6 ± 1 months after the acute illness, under fasting conditions, with a 19-gauge needle without venous stasis into citrate (1/10 volume of 0.129 mol/L sodium citrate) or D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK) (Merk Millipore) (75 mmol/L) containing tubes and processed within 15 minutes. For platelet-rich plasma preparation, WB was centrifuged at 100*g* for 10 minutes and braked off at room temperature (RT).

BIOCHEMICAL MEASUREMENTS. High-sensitivity CRP and IL-6 levels were measured with Atellica Solution (Siemens) and D-dimer and fibrinogen with ACL-TOP5550 (Werfen).

FLOW CYTOMETRY. Platelet-associated TF expression and platelet activation markers were analyzed by conventional and imaging flow cytometry as previously described.[13,14] Briefly, WB (1×10^6 platelets) was labeled for 15 minutes at RT in the dark with saturating concentrations of centrifuged (17,000*g*, 30 minutes, 4 °C) αTF and αP-selectin monoclonal antibodies[14] together with αCD41 monoclonal antibody to identify platelets. The LA) formation was identified as double-positive events for platelet and leukocyte population markers ($CD41^{pos}/CD14^{pos}$ or $CD41^{pos}/CD66^{pos}$ for platelet-monocyte aggregates [PMA] and platelet-granulocyte aggregates [PGA], respectively). Fluorochrome-conjugated isotype controls were used to quantify the background labeling. A total of 10,000 $CD41^{pos}$ events and 3,000 $CD14^{pos}$ events per sample were acquired on a Gallios cytometer (Beckman Coulter). Imaging flow cytometry was performed with ImageStreamX Mk II (Amnis, Merck). Platelets were identified as $CD61^{pos}$ events and leukocytes as $CD45^{pos}$ events. Data were analyzed with Kaluza analysis software version 1.5 (Beckman Coulter) and reported as mean percentage ± SD of positive cells. Image analysis was performed using IDEAS software (Amnis, Millipore).

THROMBIN GENERATION. Platelets (40×10^6) isolated from long COVID patients (n = 32), COVID-recovered subjects (n = 21), and HSs (n = 21) were tested for their capacity to promote thrombin generation using the calibrated automated thrombogram assay as previously described.[15] Briefly, isolated cells were resuspended in an MV-free plasma pool

prepared from multidonor WB collected in corn trypsin inhibitor (50 μg/mL)—citrate buffer to prevent ex vivo contact pathway activation.

To make TF the only rate-limiting contributor of the thrombin generation assay, calibrated automated thrombogram experiments were performed by adding an excess of exogenous phospholipids (4 μmol/L, MP Reagent, STAGO). Corn trypsin inhibitor plasma samples, devoid of cells and MVs, were run in every assay to set the background level of thrombin generated by the assay. Thrombin generation was started by the addition of a $CaCl_2$/fluorogenic substrate mixture (FluCa Kit, STAGO), and fluorescence was read for 90 minutes in a Fluoroskan Ascent reader. Thrombin generation curves were analyzed by dedicated software (Thrombinoscope BV). Lag time (minutes), endogenous thrombin potential (nmol/L × minutes), and peak high (nmol/L thrombin) were used as main parameters describing thrombin generation.

EX VIVO PLATELET ADHESION AND AGGREGATE FORMATION UNDER FLOW CONDITIONS. Experiments of ex vivo platelet adhesion under flow conditions were performed as previously described[16] in a subgroup of long COVID patients (n = 14) and HSs (n = 14). Briefly, PPACK-anticoagulated WB was incubated with green fluorescent lipophilic dye 3,3′ dihexyloxacarbocyanine iodide (DiOC6, 1 μmol/L; Thermo Fisher Scientific). The microfluidic device was then placed on the stage of a fluorescence microscope (Axiovert A1 FL, Zeiss) equipped with a 16-bit camera, and WB was perfused in microchannels (1,000 μm wide, 100 μm high, and 3 cm long) coated with Horm fibrillar collagen type I (Mascia Brunelli) at a concentration of 100 μg/mL. Two flow rates, corresponding to 300/s and 1,600/s for 4 minutes, were used. Five images were acquired at the end of each experiment, and the average surface coverage and number of aggregates were calculated using a custom MATLAB script.[16]

EX VIVO STUDIES. To evaluate the effect of FU-COVID patients' plasma on platelet activation, blood from HSs who had not taken any antiplatelet medication in the previous 10 days (n = 3) was centrifuged at 1,000u for 10 minutes at RT, plasma-depleted, and replaced with frozen plasma pools (n = 3) from long COVID or COVID-recovered patients (n = 3 each) or with fresh autologous plasma as control. Ad hoc performed experiments comparing patients' fresh and frozen plasma showed no significant differences between the 2 types of samples. After 30 minutes of incubation, the blood thus reconstituted was labeled for P-selectin, CD41/CD14, or CD41/CD66 and analyzed by flow cytometry. To evaluate the impact of CRP and IL-6 on platelet P-selectin expression and platelet-leukocyte formation, HS blood was stimulated with CRP (10-100 μg/mL), IL-6 (1-100 pg/mL), or both (50 μg/mL CRP + 1-100 pg/mL IL-6) at RT for 30 minutes. To corroborate the in vitro findings, blood from HS was preincubated with Fcγ-receptor inhibitor (200 μg/mL) or tocilizumab (300 μg/mL) for 30 minutes at RT and then treated with plasma from the enrolled patient.

To test the effect of antiplatelet drugs on platelet activation, WB was preincubated with aspirin (8 μmol/L, 120 minutes) or with $P2Y_{12}$ antagonist AR-C69931MX (1 μmol/L, 30 minutes) at RT before the addition of COVID-FU patients' plasma.

PULMONARY FUNCTIONAL TESTS. DL_{CO} and DL_{NO} were simultaneously measured in the standard sitting position through the single-breath technique, with a breath-hold time of 4 seconds (MasterScreen-Pulmonary Function Testing analyzer, Jaeger Masterscreen). The membrane diffusion (DM) subcomponent was calculated by dividing DL_{NO} by 1.97, and capillary volume (Vcap) was estimated as equal to: $1/\theta_{CO} \times [1/(1/DL_{CO} - 1.97/DL_{NO})$, with $1/\theta_{CO} = (0.73 + 0.0058 \times$ alveolar peripheral oxygen saturation$) \times 14.6/$hemoglobin. We used as reference equations for DL_{CO}, DM, and V_{cap} those proposed in official European Respiratory Society (ERS) technical standards.[17] Alveolar volume (VA) was measured by helium decay slope.[18] DL_{CO} values (both measured and the percentage of predicted) were corrected for hemoglobin levels.

THORACIC CT. CT examinations were performed using a 256-slice high-resolution computed tomography scanner (Revolution CT, GE Healthcare). No contrast media was administered to the patients. The percentage of the extent of lung parenchyma affected by COVID-19 pneumonia was processed by a dedicated workstation (ADW4.6, GE Healthcare) using a specific reconstruction software (Thoracic-V-Car software, GE Healthcare). This quantitative approach enables an automated assessment of the pulmonary infection, depicting infection areas as high attenuation areas (HAAs) concerning a defined threshold value ranging from 650 to 3,071 HU. The number of infected lungs defined as the percentage of lung parenchyma above the predefined vendor-specific threshold of 650 HU (HAA% = HAA/total lung volume) was automatically calculated by the dedicated software for both lungs.[19]

STATISTICAL ANALYSIS. Continuous variables are expressed as the mean ± SD. The normality of the variable distributions was assessed by the use of the D'Agostino-Pearson omnibus K^2 test. Within-group

comparisons were made by Student's paired *t*-test or the Wilcoxon signed rank test, as appropriate, and between-group comparisons were made using an unpaired Student's *t*-test or Wilcoxon rank sum test, as appropriate. Categorical variables are presented using count (percentage) and compared using the chi-square or Fisher exact test. Spearman rank correlation coefficients (*r*) were computed to assess associations between 2 continuous variables. A *P* value of <0.05 was considered to be statistically significant, although some results should be interpreted with caution because adjustment for multiple post hoc tests was not used. Analyses were performed using Prism GraphPad version 9.0 (GraphPad Software) and SAS version 9.4 (SAS Institute).

RESULTS

CHARACTERISTICS OF THE ENROLLED SUBJECTS. In this study, 204 subjects with a previous COVID-19 infection (6 ± 1 months, between July and September 2021) were enrolled. Within the enrolled population, the prevalence of still-symptomatic subjects was about 16.7%, for a total of 34 subjects (long COVID). Table 1 shows the most frequent symptoms reported by long COVID patients. A group of 34 asymptomatic subjects (COVID-recovered) randomly selected within the enrolled subjects was used for comparison. Two subjects in the latter group withdrew informed consent and were excluded from the final analysis. The clinical characteristics, drug therapy, lung function, and structural parameters of the subjects who had experienced SARS-CoV-2 infection were all statistically comparable except hypertension, which was more prevalent in the COVID-recovered group (69% vs 26%; $P < 0.001$) (Table 2). In the long COVID group, the average time since acute infection was 5.0 ± 2.9 months; the mean age was 56 ± 12 years. Only 15% of the subjects had previous respiratory disease (12% had asthma, and 3% had chronic obstructive pulmonary disease), 6% were active smokers, 29% were past smokers, and 18% reported cardiovascular diseases. In the COVID-recovered group subjects, the average time since acute infection was 5.2 ± 1.2 months, they were older (age 62 ± 14 years), a higher proportion were male (75% vs 68%), and no one was an active smoker.

The values of DL_{CO}, DL_{NO}, DM, and V_{cap} were all significantly reduced in the long COVID group compared to HSs ($P < 0.001$). The average lung volume involved in structural changes within the long COVID group, as assessed by CT scan, was 7.6% ± 3.0% (Table 2).

TABLE 1 Long COVID Syndrome Symptoms (n = 34)

Memory loss	26 (76)
Fatigue	19 (59)
Dyspnea	15 (44)
Palpitations	10 (30)
Joint pain	8 (24)
Chest pain	8 (24)
Loss of taste (ageusia)	5 (15)
Loss of smell (anosmia)	3 (9)
Cough	1 (3)
Other[a]	16 (47)

Values are n (%). [a]Other symptoms include neuroinflammation, postexertional malaise, and arthralgias.

The analysis of the inflammatory profile indicated that all of the assessed parameters were significantly lower in COVID-FU patients compared to those measured during the acute phase of the disease and, on average, within the reference range of HSs (Figure 1). Long COVID patients, however, had markedly higher CRP levels (7-fold; $P < 0.001$) than COVID-recovered subjects. Specifically, when analyzed for the presence of low-grade inflammation, defined as CRP levels between 0.3 and 1 mg/dL, 18 of 30 (60%) symptomatic patients had CRP levels above 0.3 mg/dL, with 16 of these 18 patients having a markedly elevated concentration (>1 mg/dL) (Figure 1A). Of note, IL-6 levels, which were similar on average in both groups of COVID-FU patients, exceeded the reference range of HSs in 30% and 35% of long COVID and COVID-recovered patients, respectively (Figure 1B).

FEATURES OF PLATELET ACTIVATION PERSISTING AFTER COVID REMISSION. Long COVID patients showed marked platelet activation compared to COVID-recovered subjects. Indeed, the percentage of circulating P-selectin^{pos} platelets was significantly greater in symptomatic than in the asymptomatic subjects (2.5-fold; $P < 0.001$) and HSs (7-fold; $P < 0.001$), and it was still comparable to values found in acute-phase patients[12] (Figure 2A). As a result, the percentages of PGA and PMA aggregates were found to be significantly higher in long COVID patients than in COVID-recovered subjects (1.5-fold; $P = 0.006$ and 1.3-fold; $P = 0.027$, respectively) (Figures 2B and 2C) and in HSs (1.5-fold; $P = 0.001$ and 2-fold; $P < 0.001$, respectively). Nevertheless, it is worth mentioning that COVID-recovered subjects showed a still greater percentage of P-selectin^{pos} platelets (3-fold; $P < 0.001$) and of PMA (1.5-fold; $P < 0.001$) compared to HSs (Figures 2A to 2C). Of note, in the overall enrolled cohort, CRP levels of ≥0.3 mg/dL significantly correlated with the number of P-selectin^{pos}

TABLE 2 Clinical Characteristics of the Enrolled Patients

	Long COVID Patients (n = 34)	COVID-Recovered Patients (n = 32)	*P* Value	COVID-Follow-Up Patients (n = 204)	Healthy Patients (n = 34)	*P* Value[a]
Months from acute infection	5.0 ± 2.9	5.2 ± 1.2	0.99	6.0 ± 1.0	–	–
Men	23 (68)	24 (75)	0.5096	115 (56)	18 (53)	0.07
Age, y	56 ± 12	62 ± 14	0.0879	56 ± 14	34 ± 02	<0.001
Risk factors						
Cardiovascular disease	6 (18)	4 (12)	0.5599	47 (29)	0	–
COPD	1 (3)	1 (3)	0.9652	9 (4)	0	–
Asthma	4 (12)	3 (9)	0.7526	14 (7)	0	–
Hypertension	9 (26)	22 (69)	0.0005	66 (32)	0	–
Diabetes	0	2 (6)	–	15 (7)	0	–
Dyslipidemia	5 (15)	9 (28)	0.1826	41 (20)	0	–
Active smokers	2 (6)	0	–	11 (6)	16 (47)	<0.001
Past smokers	10 (29)	9 (28)	0.9081	25 (12)	2 (6)	0.009
Pharmacologic treatments						
ASA	4 (12)	5 (15)	0.6478	41 (20)	0	–
$P2Y_{12}$ inhibitors	1 (3)	3 (9)	0.2736	15 (7)	0	–
ACE inhibitors	3 (9)	5 (15)	0.3974	19 (9)	0	–
β-Blockers	10 (29)	13 (41)	0.3393	49 (24)	0	–
Diuretics	4 (12)	7 (22)	0.2707	23 (11)	0	–
Insulin/antidiabetic agents	1 (3)	2 (6)	0.4864	14 (7)	0	–
Pulmonary function						
DL_{CO}, mL/min/mmHb	21.19 ± 7.20	–	–	–	29.35 ± 5.33	<0.001
Predicted DL_{CO}, %	76 ± 22	–	–	–	95 ± 9	<0.001
DL_{NO}, mL/min/mmHb	91 ± 30.45	–	–	–	121.95 ± 20.23	<0.001
DM, mL/min/mmHb	46.39 ± 14.84	–	–	–	62 ± 10.30	<0.001
Vcap, mL	62.44 ± 20.43	–	–	–	86 ± 18.55	<0.001
Pulmonary structure/anatomy						
Residual parenchymal damage at CT, %	7.62 ± 3.03	–	–	–	–	–

Values are n (%) or mean ± SD. [a]Long COVID patients vs healthy subjects.

ACE = angiotensin-converting enzyme; ASA = acetylsalicylic acid; COPD = chronic obstructive pulmonary disease; CT = thoracic computed tomography; DL_{CO} = diffusing capacity of the lungs for carbon monoxide (data corrected for hemoglobin); DL_{NO} = diffusing capacity of the lungs for nitric oxide; DM = membrane diffusion; V_{cap} = capillary volume.

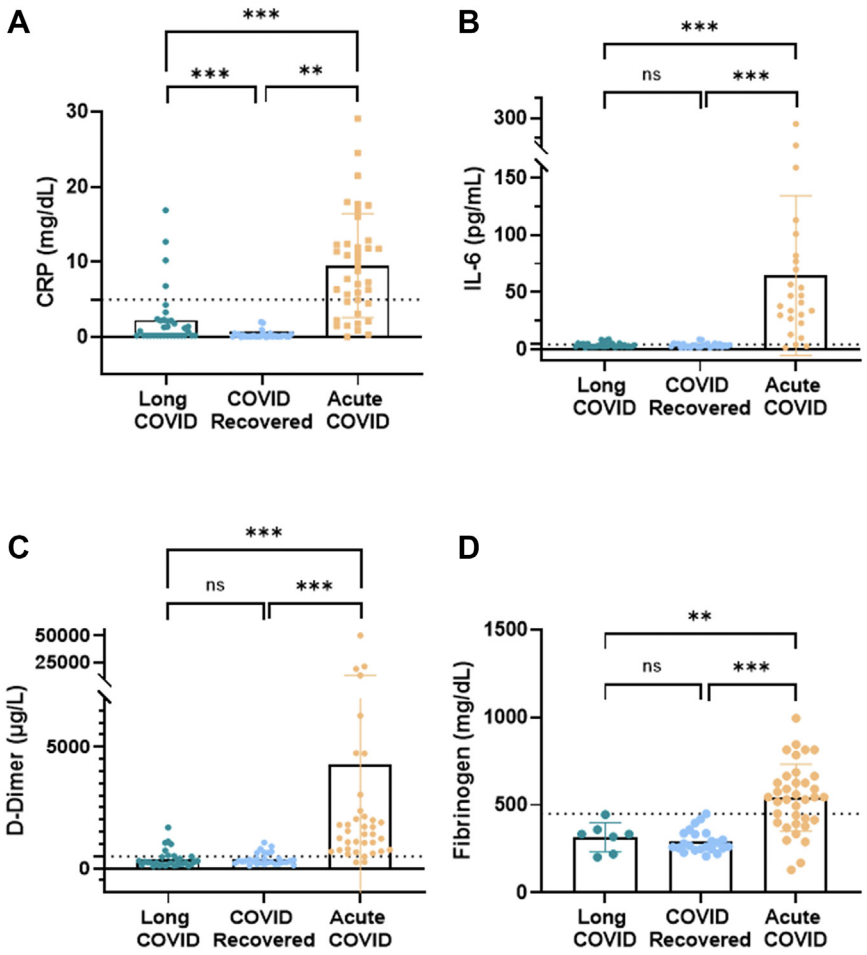
platelets as well as with the percentages of PGA and PMA (Figures 2D to 2F).

LUNG IMPAIRMENT IN LONG COVID PATIENTS IS ASSOCIATED WITH PERSISTENT PLATELET ACTIVATION. Interestingly, the percentages of PGA and PMA found in long COVID patients were significantly associated with residual parenchymal damage assessed by CT scan ($r = 0.55$; $P = 0.004$ and $r = 0.54$; $P = 0.006$, respectively) (Figures 2D and 2E). Among the variables describing the pulmonary functional tests, PGA and PMA were also associated with DL_{NO} values ($r = 0.16$; $P = 0.036$ and $r = 0.24$; $P = 0.049$, respectively) (Figures 3A and 3F). No association was found with V_{cap} (Figures 3B and 3G), DM (Figures 3C and 3H), or DL_{CO} value (absolute and percentage of predicted) (Figures 3D, 3E, 3I, and 3J).

PLATELET-ASSOCIATED TF EXPRESSION AND ACTIVITY REVERTED TO LEVELS OF HSs 6 MONTHS AFTER COVID-19 REMISSION. Six months after SARS-CoV-2 infection remission, the percentage of TF^{pos} platelets (surface expression) in long COVID patients, although still 1.5-fold greater than in COVID-recovered subjects and HSs ($P = 0.025$ and $P = 0.050$, respectively) (Figure 4A), was significantly lower than that observed during the acute phase ($P = 0.014$).[12]

When the overall procoagulant potential of platelets was tested in a thrombin generation assay, both endogenous thrombin potential and the maximum amount of generated thrombin (peak) were comparable among long COVID patients, COVID-recovered subjects, and HSs and significantly lower compared to the acute COVID patients (Figures 4B and 4C). Furthermore, the time required to generate detectable amounts of thrombin—that is, the lag time (Figure 4D)—was significantly prolonged in long COVID patients (28.6 ± 7.8 minutes) compared to that measured in patients during the acute phase (18 ± 2.7 minutes; $P < 0.001$) (Figure 4D), similar to that measured in HSs (27.9 ± 9.3 minutes; $P = 0.30$) and

FIGURE 1 Circulating Levels of CRP, IL-6, D-Dimer, and Fibrinogen

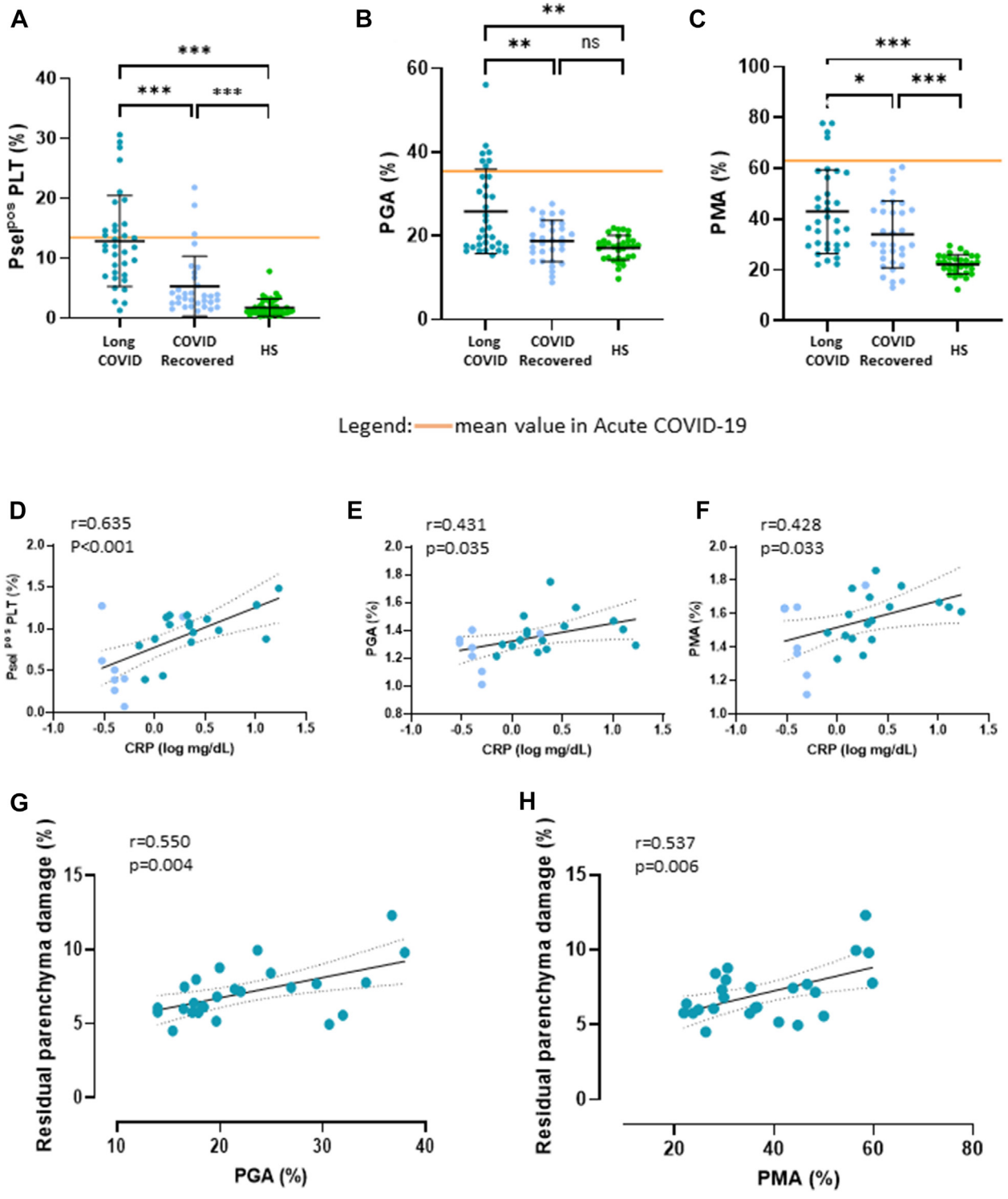


Concentrations of (A) CRP, (B) IL-6, (C) D-dimer, and (D) fibrinogen measured in plasma of long COVID, COVID-recovered, and acute COVID-19 patients.[12] Reference range of healthy subjects is shown as a dotted line. Data are reported as individual and mean ± SD. $^{**}P < 0.01$; $^{***}P < 0.001$. Data were compared by Student's *t*-test or Wilcoxon test, as appropriate. CRP = C-reactive protein; IL = interleukin; ns = not significant.

slightly prolonged compared to that of COVID-recovered subjects. TF^{pos} PGA and PMA values in long COVID patients were 2.5-fold lower ($P < 0.001$ for both) than in acute patients and 2- and 5.7-fold greater, respectively, than in HSs (Figure 4E and 4F). Interestingly, in long COVID patients, TF^{pos} PGA levels were considerably greater than those of COVID-recovered patients (2.3-fold; $P < 0.001$), whereas TF^{pos} PMA levels were comparable but significantly greater than in HSs ($P < 0.001$). Of note, imaging flow cytometry analysis clearly documented that TF expression in PLA was mainly associated with platelets (Figure 4G).

THE PLATELET-ACTIVATED PHENOTYPE OF LONG COVID PATIENTS SUSTAINS EX VIVO PLATELET ADHESION UNDER FLOW. Considering the activated phenotype of platelets from long COVID patients, we next assessed how prone they were to form ex vivo aggregates in a platelet adhesion assay. Perfusion of collagen (100 µg/mL)-coated surfaces with long COVID patient blood resulted in the formation of a significantly greater number of aggregates ($P = 0.022$ and $P = 0.016$ under 300/s and 1,600/s shear rate, respectively), although smaller in size—and, thus, not affecting the area of platelet adhesion—compared to perfusion with blood from HSs (Figure 5).

FIGURE 2 Assessment of Platelet Activation and Association Between Residual Parenchymal Damage at CT Scan With Platelet-Leukocyte Aggregate Formation



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IN VITRO MODEL OF PLATELET ACTIVATION INDUCED BY LONG COVID PLASMA. We then tested whether plasma from long COVID patients could reproduce in vitro, on cells from HSs, the platelet activation observed in vivo.

To this aim, blood from HSs was plasma depleted and reconstituted with plasma pools obtained from long COVID or COVID-recovered patients. The results indicate that plasma from long COVID patients, but not from COVID-recovered subjects, significantly raised the number of P-selectin^{pos} platelets (3-fold) (Figure 6A) promoting PLA formation (1.5-fold) (Figures 6B and 6C).

Thus, considering the low-grade inflammation still present in long COVID patients (characterized by CRP levels significantly higher compared to COVID-recovered subjects) (Figure 1), we analyzed whether CRP could reproduce in vitro, in HS blood, the effect observed with long COVID plasma. Interestingly, CRP, at levels comparable to those found in long COVID patients, concentration-dependently induced P-selectin expression and PLA formation (Figures 7A to 7C). This effect was blunted by preincubating platelets with an Fcγ-receptor inhibitor (Figures 7A to 7C). In contrast, IL-6, a cytokine strongly implicated in acute COVID complications and whose levels were above the reference range in about 30% of COVID-FU patients, did not induce per se cell activation, but it significantly enhanced the effect of CRP (Figures 7D to 7F), resulting in a cell stimulation that resembled that observed with long COVID plasma for all parameters measured.

Based on these findings, we next assessed the effect of blocking plasma CRP and IL-6 signaling. When platelets from HSs were treated with either Fcγ-receptor inhibitor or tocilizumab before exposure to long COVID patients' plasma, platelet P-selectin expression and PLA formation were significantly reduced (Figures 8A to 8C).

To further confirm the involvement of the CRP/IL-6 axis in platelet activation observed with long COVID plasma, experiments were performed to assess the impact of CRP (50 μg/mL) added to the COVID-recovered subjects' plasma and the effect of tocilizumab in this experimental setting. The addition of CRP increased platelet P-selectin expression and PLA formation. Interestingly, tocilizumab did not affect the expression of platelet activation markers on HS platelets when exposed to COVID-recovered subjects' plasma but significantly reduced platelet activation in the presence of increased levels of CRP (Figures 8D to 8F). These data therefore suggest that although IL-6 per se does not affect platelet activation, it contributes to cell activation in the presence of an inflammatory primer.

Finally, we evaluated the effect of commonly used antiplatelet drugs, such as aspirin and $P2Y_{12}$ inhibitors, in preventing the observed platelet activation. The data show that both aspirin and the $P2Y_{12}$ inhibitor AR-C69931MX significantly inhibited long COVID plasma–induced platelet activation to a similar degree (Figure 9), highlighting the potential benefit of antiplatelet agents in the modulation of platelet activation in still-symptomatic post-COVID patients.

DISCUSSION

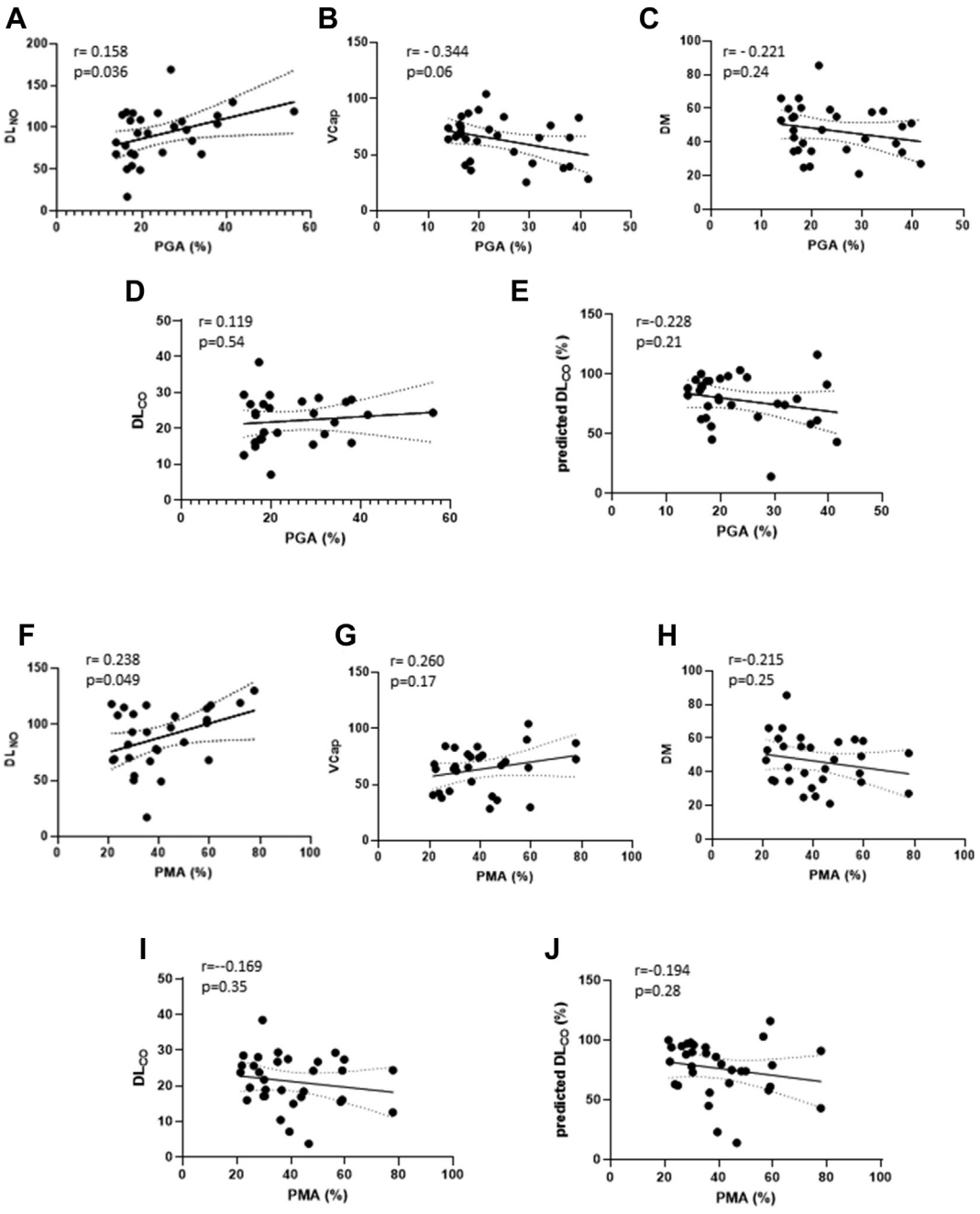
This study expands knowledge on the association between thromboinflammation and long COVID syndrome. Data showed that patients experiencing long COVID syndrome present a low-grade inflammation characterized by moderately increased levels of CRP. In these subjects, a significant association between the residual parenchymal damage assessed by CT scan and a persistent platelet activation, in terms of PGA and PLA, was documented. Interestingly, in ex vivo experiments, the blood of long COVID patients formed, on collagen-coated surfaces, a number of aggregates significantly greater than the blood of HSs. Furthermore, the plasma of long COVID patients mixed with the plasma-depleted blood of HSs closely reproduced the platelet activation observed in vivo through a mechanism mediated by CRP and IL-6 and blunted by anti-inflammatory and antiplatelet drugs.

In line with other reports, long COVID patients enrolled in this study showed several degrees of

FIGURE 2 Continued

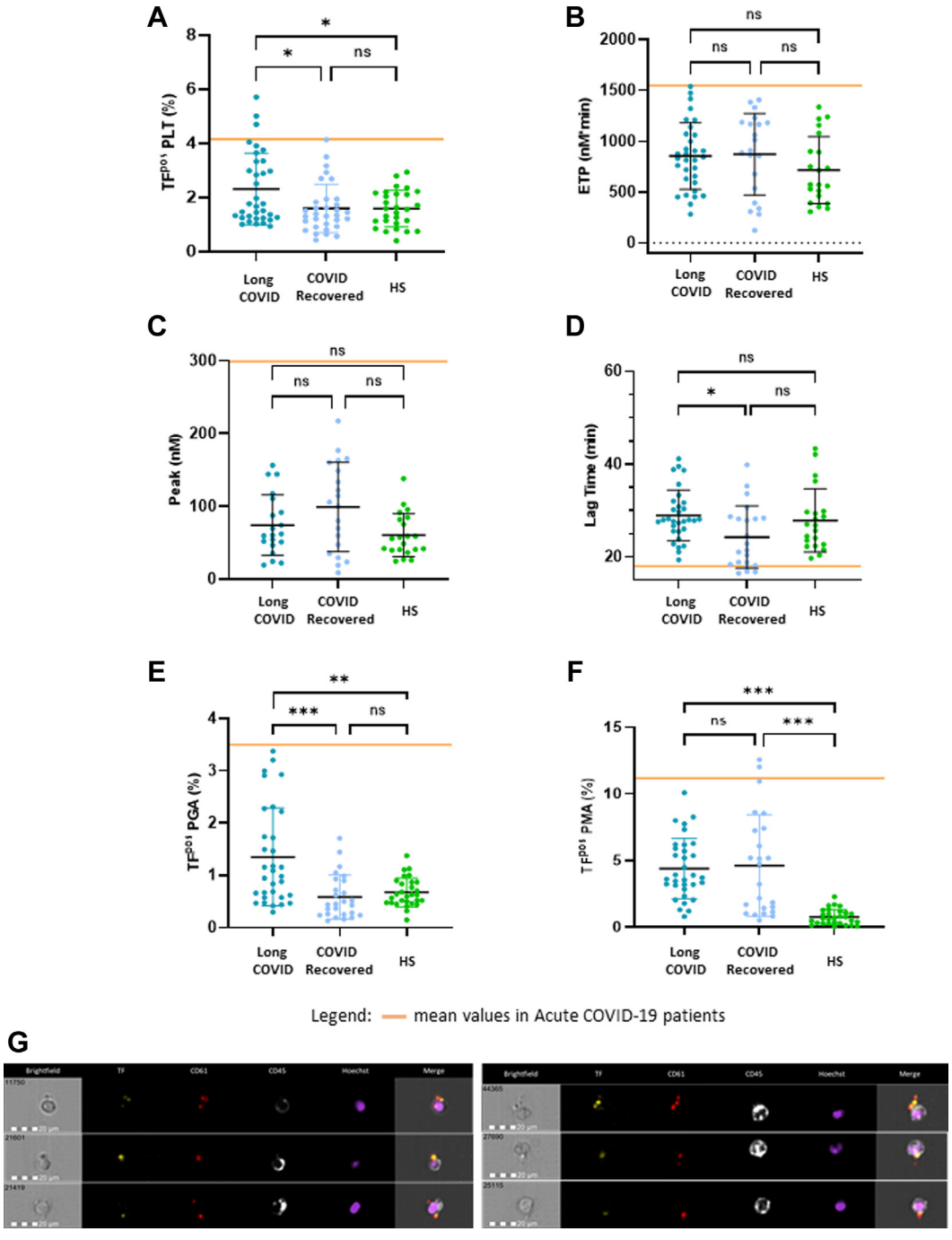
(A) Platelet-associated P-selectin expression as well as (B) PGA and (C) PMA aggregate formation, were evaluated by whole-blood flow cytometry in long COVID (teal dots) in COVID-recovered patients (6-month follow-up; light blue dots) and in the control group (HSs; green dots) and compared with those measured in acute phase patients,[12] indicated by the orange line. Data were analyzed by Student's *t*-test or the Wilcoxon test, as appropriate. Correlations between CRP levels of >0.3 mg/dL and the percentages of (D) P-selectin^{pos} platelets, (E) PGA, and (F) PMA are shown. Correlations between the number of (G) PGA and (H) PMA in long COVID patients with the percentage of residual parenchymal damage measured at CT scan are shown and analyzed by the Spearman correlation test. Data are reported as individual and mean value ± SD of positive cells. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. CT = computed tomography; HS = healthy subject; PGA = platelet-granulocyte aggregate; PLT = platelets; PMA = platelet-monocyte aggregate; pos = positive; Psel = P-selectin; other abbreviations as in Figure 1.

FIGURE 3 **Association Between Pulmonary Function Values and PLA Formation**



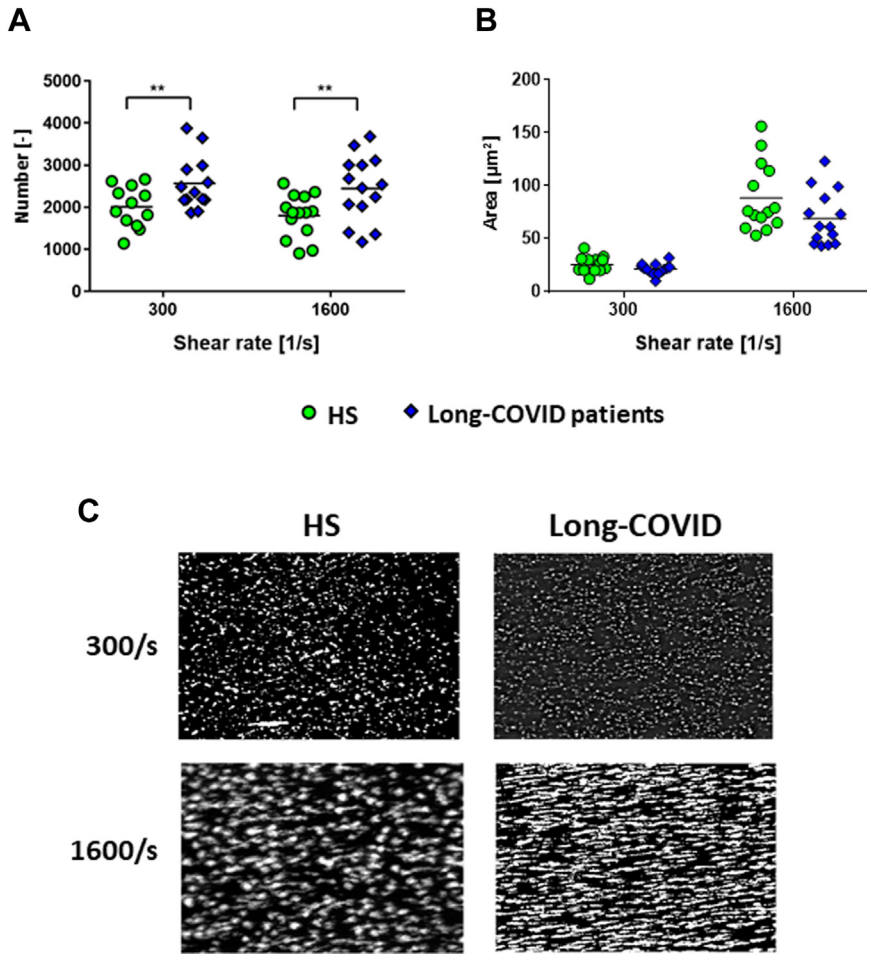
Correlation between the DL_{NO}, V_{cap}, DM, measured and percentage of predicted DL_{CO}—corrected for hemoglobin levels and assessed by pulmonary function tests—and the number of (A-E) PGA and (F-J) PMA aggregates, measured by whole-blood flow cytometry in the subgroup of long COVID patients. DL_{CO} = carbon monoxide lung diffusion; DL_{NO} = nitric oxide lung diffusion; DM = membrane diffusion; V_{cap} = capillary volume; other abbreviations as in Figure 2.

FIGURE 4 Analysis of Platelet-Associated TF Expression



(A) Platelet surface TF expression was evaluated by whole-blood flow cytometry, and (B-D) platelet-associated TG was measured by calibrated automated thrombogram assay in long COVID and COVID-recovered patients (6-month follow-up) and in the control group (HS). (E, F) TF^{pos} PGA and PMA in long COVID and COVID-recovered patients and HSs. The mean value measured in COVID patients during the acute phase is displayed with an orange line.[12] Data are reported as individual and mean ± SD. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. (G) Analysis by imaging flow cytometry of TF expression in PLA of long COVID patients. Data were compared by Student's *t*-test or the Wilcoxon test, as appropriate. ETP = endogenous thrombin potential; TF = tissue factor; TG = thrombin generation; other abbreviations as in Figures 1 and 2.

FIGURE 5 Platelet Adhesion on a Collagen-Coated Microfluidic Surface

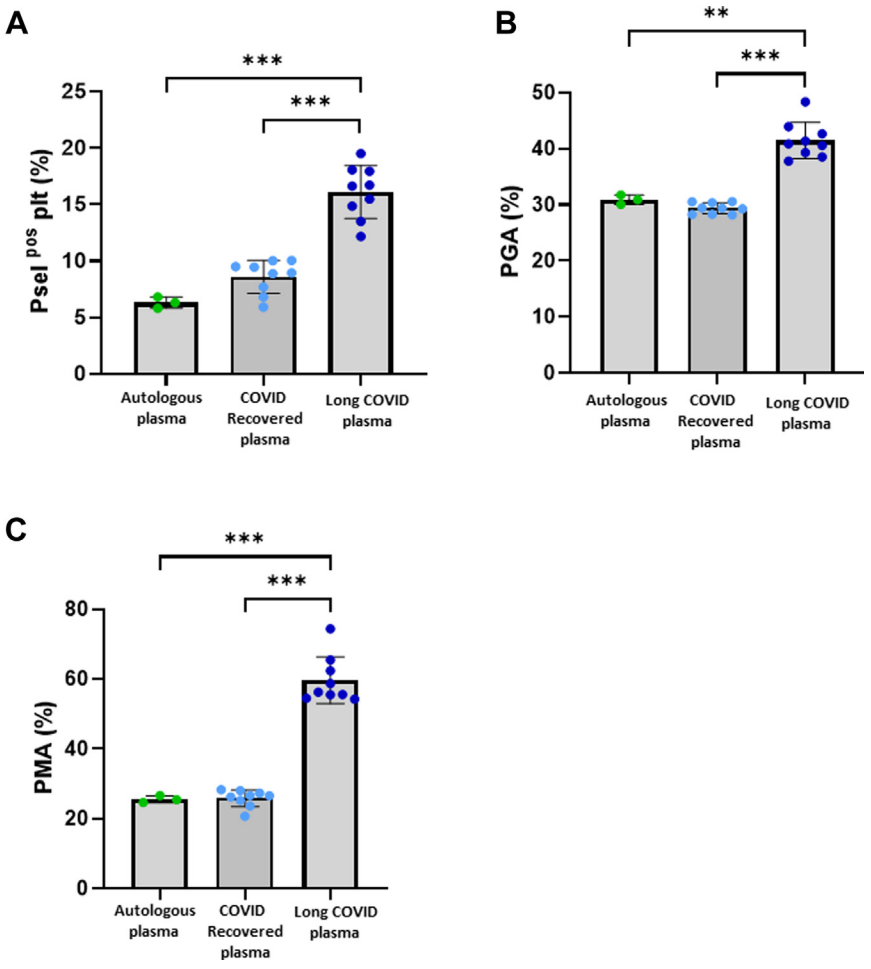


Ex vivo platelet adhesion was evaluated perfusing blood in collagen-coated microchannels. (A) Average number and (B) area of aggregates in long COVID patients (n = 14) HSs (n = 14) for shear rates of 300/s and 1,600/s were calculated. (C) Representative images (original magnification: 10×) of platelet adhesion over collagen-coated (100 mg/mL) microchannels at 300/s (top) and 1,600/s (bottom) for a patient (long COVID) and an HS. Flow is from left to right. Images were acquired after 4 minutes of perfusion. $^{**}P < 0.01$. Data were compared by Student's *t*-test or the Wilcoxon test, as appropriate. HS = health subject.

residual parenchymal damage on CT, reported as ground-glass opacity and reticular or fibrotic pulmonary areas.[20-24] Interestingly, these patients showed a percentage of circulating P-selectin^{pos} platelets greater than that of COVID-recovered subjects and HSs, thus sustaining the formation of heteroaggregates, a well-known marker of platelet activation that has also been documented in studies reporting an increase in soluble markers such as PF4 and vWF.[25-27] The persistence of high levels of PLA, although decreased compared to the acute phase, could reflect the presence of a healing reaction that translates into fibrotic tissue deposition. Indeed, it has been widely shown that PLA might be a trigger mechanism for alveolar and vascular injury culminating in fibrosis in idiopathic pulmonary fibrosis.[28] PLA increased in animal models of lung injury[29] during both bacterial and viral infection.[30] The heteroaggregates can occlude small pulmonary vessels and disrupt lung endothelial barrier function, causing local ischemia and perpetuating local inflammation as it happens during acute respiratory distress syndrome,[31,32] such as in the COVID-19 acute phase.

After the acute phase, platelets and leukocytes persist at the injured site to repair the inflammatory damage and express several mediators with

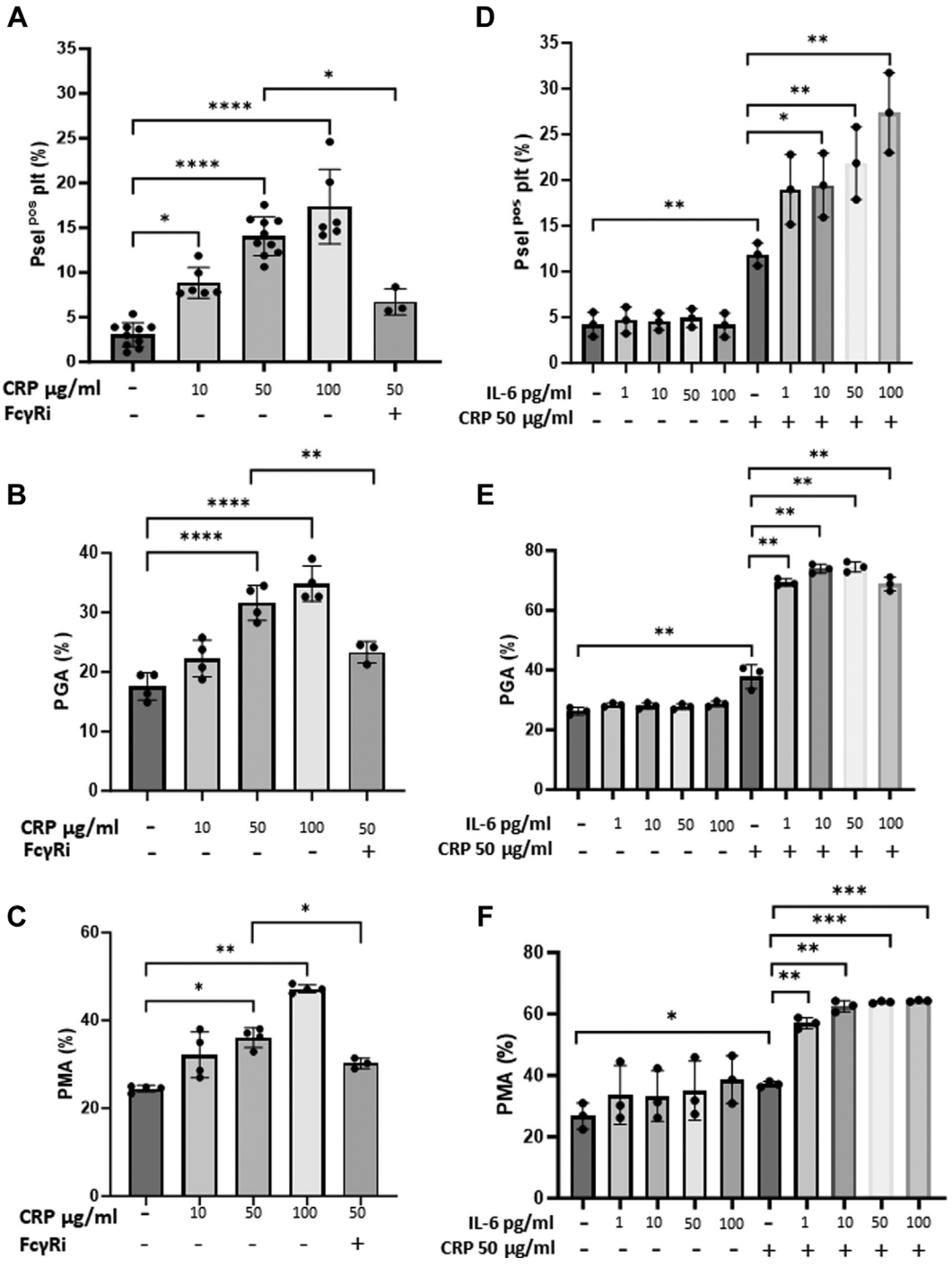
FIGURE 6 Ex Vivo Effect of Plasma From Long COVID and COVID-Recovered Patients on HS Platelets



Plasma-depleted blood from HSs (n = 3) reconstituted with autologous plasma (green dots) and plasma from long COVID patients (blue dots; n = 9) or from COVID-recovered subjects (light blue dots; n = 9). Percentages of (A) P-selectin^{pos} platelets, (B) PGA, and (C) PMA are shown. Data are reported as individual and mean value ± SD. $^{**}P < 0.01$; $^{***}P < 0.001$. Data were compared by Student's *t*-test or the Wilcoxon test, as appropriate. Abbreviations as in Figure 2.

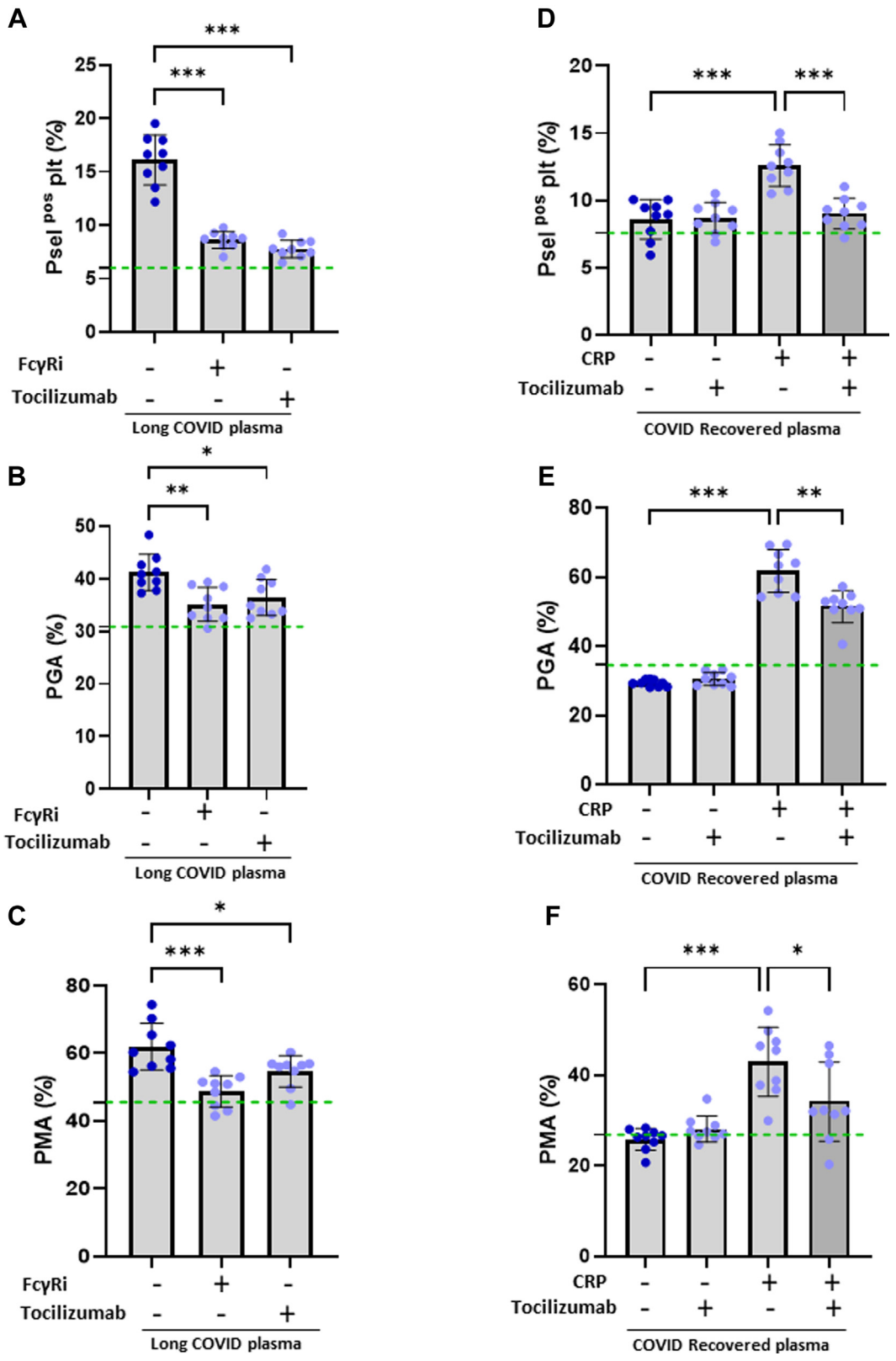
profibrotic activity,[33,34] such as metalloproteinases, vascular endothelial growth factor, cytokines, and TF,[35-37] that contribute to vascular, alveolar ,and endothelial/epithelial interface fibrotic injury progression. Our data show that long COVID patients still had a greater percentage of TF^{pos} platelets, TF^{pos} PGA, and CRP levels than asymptomatic COVID-recovered subjects. Some investigators have described the presence of TF and thrombin signaling pathway dysregulation in patients who experienced thrombosis during the COVID-19 convalescence phase.[6,26,38,39] No new thrombotic clinical events occurred 6 months after the acute infection in the subjects of this study. Notably, despite the greater percentage of TF^{pos} platelets, the overall platelet thrombin generation potential was comparable in symptomatic and asymptomatic subjects and HSs, highlighting the switch from the acute phase to the healing process. Furthermore, it is well known that TF is closely associated with fibrin deposits in the lungs of idiopathic pulmonary fibrosis and systemic sclerosis patients.[37] However, its contribution to fibrogenesis is mainly caused by its cytoplasmatic domain—notably not involved in coagulation—by inhibiting the PAR-2 downstream pathway,[40] and the kinetic requested to produce thrombin and fibrin is lower than in hemostatic challenges.

FIGURE 7 **Ex Vivo Effect of CRP and IL-6 on HS Platelet Activation**



Whole blood from HSs was incubated with CRP (10-100 µg/mL), IL-6 (1-100 pg/mL), or IL-6 (1-100 pg/mL) + CRP (50 µg/mL). Preincubation of platelets with FcγRi (200 ng/mL; 10 minutes) before CRP (50 µg/mL) stimulation confirmed the specificity of the effect. (A, D) P-selectin^{pos} platelets, (B, E) PGA, and (C, F) PMA were analyzed by flow cytometry, and the percentages of positive cells are reported. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.001$. Data were compared by Student's *t*-test or the Wilcoxon test, as appropriate. FcγRi = Fcγ-receptor inhibitor; other abbreviations as in Figures 1 and 2.

FIGURE 8 Ex Vivo Effect of Fcγ-Receptor Inhibitor and Tocilizumab on Platelet Activation Induced by Long COVID and COVID-Recovered Patients' Plasma



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In line with the relationship between PLA and anatomic abnormalities, we also found a correlation between the PLA and DL_{NO} in long COVID patients. Conversely, we reported no association between the amount of PLA with lung function in terms of DM, V_{cap}, and DL_{CO} (measured as the percentage of predicted value), although these lung parameters were significantly decreased compared to HSs, as recently reported.[41] It has been documented that COVID-19 survivors without lung function impairment, investigated with DL_{CO} or cardiopulmonary exercise test, complained of pulmonary symptoms,[42] indicating a lack of correlation between pulmonary symptoms and functional data. Indeed, the most frequently studied functional parameters hold a low sensitivity in screening this subtype of patients. It has been shown that the DL_{CO}, used to assess endothelial/alveolar DM, is not a reliable measure because of its many limitations and its close dependence on hematocrit in this type of patient.[43-45]

In our symptomatic cohort, the decreased V_{cap} values and preserved DM accounted for a minimally impaired DL_{CO}. Not surprisingly, it has been recently observed that DL_{CO} impairment was mainly associated with V_{cap} alteration in long COVID patients.[46] In line with this observation, a few reports suggest a major clinical role of the thromboinflammatory vasculopathy and hypoxemia-driven remodeling over the acute phase in the genesis of post-COVID syndrome.[38,43,47-50] Indeed, accumulating evidence has demonstrated that COVID-19-induced lung microangiopathy may be the cause of the subclinical gas exchange abnormalities[50] and inefficiency of ventilation[41] resulting in symptomatic alveolar-perfusion mismatch in long COVID patients.[51] Consistently, the findings reported in this report show an elevated susceptibility of long COVID patient blood to form a higher number of aggregates on collagen-coated surfaces, although smaller in size and thus not affecting the overall area of platelet adhesion.

Thromboinflammation, driving a high prothrombotic state, is a major cause of acute COVID-19 syndrome.[52] The evidence linking thromboinflammation to long COVID has also been recently reviewed by Nicolai et al[53] Increased systemic inflammation has indeed also been suggested as a potential trigger for the heterogeneous symptoms of long COVID syndrome.[7,54] The mechanisms behind this ongoing inflammation are still unclear. Viral persistence in certain tissue reservoirs may maintain a prolonged inflammatory state by dysregulating the peripheral immune response.[55-57] Additionally, Ryu et al[58] recently provided the evidence of a causative role of fibrinogen in the COVID-19 immune response. Fibrin indeed is able to bind to the SARS-CoV-2 spike protein, forming proinfammatory blood clots that drive systemic thromboinflammation and neuropathology that can also persist in long COVID syndrome. The study, conducted by Iwasaki et al,[57] offers valuable insights into the immunologic mechanisms underlying the development of long-term effects of the SARS-CoV-2 infection. The investigators reported significant differences in circulating myeloid and lymphocyte populations compared to the matched controls as well as evidence of heightened humoral responses directed against SARS-CoV-2 among participants with long COVID. A machine learning model designed to accurately classify the long COVID and control populations demonstrated that the immunologic analyses and patient-reported outcomes were concordant in diagnosing long COVID syndrome.[53,57]

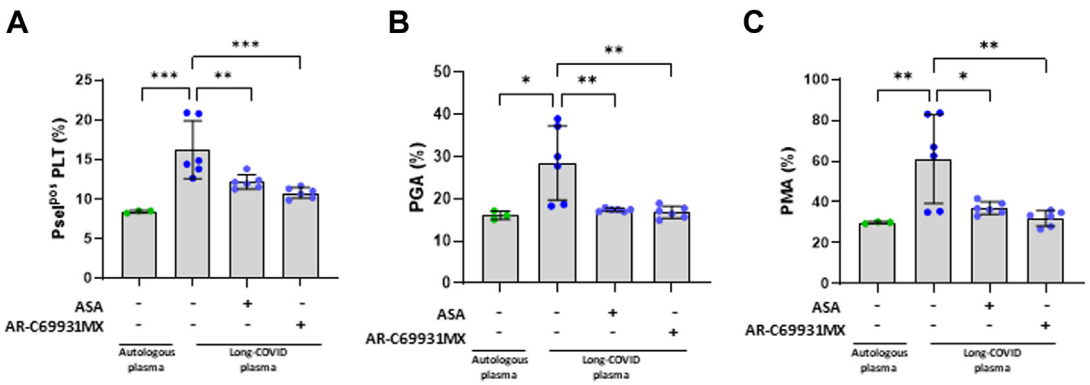
The downstream effects of the pathologic immune dysregulation may include platelet and endothelial activation, formation of PLA, and ultimately persistent cytokine production. Elevated levels of inflammatory cytokines, among which are IL-6, IL-1β, and tumor necrosis factor α, have been found up to 24 months after acute infection.[59]

Here, we report that a low-grade inflammation, sustained mainly by CRP but also by subthreshold levels of IL-6, is present in long COVID patients and mediates the peculiar platelet activation status described. The involvement of the CRP/IL-6 axis was confirmed by means of *in* vitro experiments showing that IL-6 per se has no effect on platelet activation, but it significantly synergizes with CRP, reproducing an activation pattern comparable to that observed in long COVID subjects. It is well known that plasma CRP levels correlate with the severity of inflammatory diseases.[60] CRP has also the capacity to enhance the

FIGURE 8 Continued

Whole blood from healthy subjects, preincubated with FcγRi (200 ng/mL, 10 minutes) or tocilizumab (300 μg/mL), was plasma depleted and reconstituted with (A to C) long COVID (n = 9) or (D-F) COVID-recovered patients' plasma. Percentages of (A, D) P-selectin^{pos} platelets, (B, E) PGA, and (C, F) PMA are shown. Data are reported as individual and mean ± SD. The mean values measured in the presence of autologous plasma are indicated by the dotted green line. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Data were compared by Student's *t*-test or the Wilcoxon test, as appropriate. Abbreviations as in **Figures 1, 2, and 7**.

FIGURE 9 Ex Vivo Effect of Aspirin and P2Y$_{12}$ Inhibitor on Platelet Activation Induced by Long COVID and COVID-Recovered Patient Plasma



Blood from HSs (n = 6), preincubated or not with aspirin (8 μmol/L) or with P2Y$_{12}$ inhibitor (AR-C69931MX; 1 μmol/L), was plasma-depleted and reconstituted with autologous plasma (green dots) or with plasma from long COVID patients (n = 6-12; blue dots). Percentages of (A) P-selectin^{pos} platelets as well as (B) PGA and (C) PMA formation were assessed by flow cytometry. Data are reported as mean value ± SD. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Data were compared by Student's *t*-test or the Wilcoxon test, as appropriate. ASA = acetylsalicylic acid; other abbreviations as in Figure 2.

inflammatory properties of IL-6. Indeed, CRP promotes the formation of the soluble interleukin-6 receptor (sIL-6R)/IL-6 complex by inducing sIL-6R release. The sIL-6R/IL-6 complex functions as an agonist, activating cells through the membrane-bound IL-6 receptor gp130 in a transsignaling mechanism.[61] The IL-6 transsignaling induces an autocrine activation loop (as measured by an increase in gp80 and gp130 content) and STAT3 phosphorylation that, in turn, drives the release of proinflammatory cytokines and chemokines.[62] It has been reported that, although IL-6 transsignaling has no effect on platelet aggregation, it is indeed crucial for the evolution of inflammation.[63] In mice, the specific IL-6 transsignaling inhibition reduced mortality and symptoms caused by SARS-CoV-2 infection, thus providing evidence of the beneficial effect of IL-6 transsignaling inhibition in the short- and long-term consequences of COVID-19 infection.[64]

We did not quantify the levels of sIL-6R that could sustain platelet transsignaling activation. However, the involvement of these mediators in sustaining the platelet phenotype observed in long COVID subjects was confirmed by means of in vitro mixing experiments. Indeed, the effect of long COVID patients' plasma on platelets of HSs, faithfully reproducing the platelet activation and PLA formation observed in vivo, was prevented by inhibiting CRP and IL-6 signaling, that is, by Fcγ-receptor inhibition and by tocilizumab.

Aspirin and P2Y$_{12}$ inhibitors have been previously shown to reduce PLA formation.[65] Accordingly, we assessed the effects of these drugs on the plasma-depleted blood from HSs treated with long COVID patients' plasma, providing evidence that aspirin and the P2Y$_{12}$ inhibitor AR-C69931MX significantly and similarly inhibited long COVID plasma-induced platelet activation and PLA formation. These results may have relevant clinical implications because, by providing insights into the pathophysiologic mechanism sustaining the platelet activation observed in long COVID syndrome, at least in the studied cohort, they suggest that the biomarkers be measured in order to fine-tuning the clinical status and, based on this, to tailor the drug therapy. To date, indeed, no pharmacologic treatment has proven to be clearly beneficial in the management of long COVID syndrome. Intervention trials are ongoing to evaluate the effect of direct oral anticoagulant agents and of the anti-inflammatory colchicine on the disease progression (IRSCTN10665760). Vaccination also seems to lower long COVID syndrome incidence.[66] No direct evidence to date has been provided on the effects of antiplatelet drugs in long COVID resolution. However, a secondary analysis of the REMAP-CAP trial showed the efficacy of in-hospital initial treatment with antiplatelet drugs or anti IL-6R antagonists in improving 180-day mortality compared with patients randomized to the control group.[67] These data, in line with the results provided in the present report, this

highlight the role of platelet activation as well as of inflammation in driving the postacute sequelae of COVID-19.

STUDY LIMITATIONS. This study presents some limitations. First, we lacked a detailed medical history of the enrolled subjects documenting the severity of the acute phase of COVID-19, so we could not correlate biochemical findings at follow-up with the need for intensive care unit care during the acute illness and compare follow-up radiologic abnormalities with previous patients' CT scan images. Nevertheless, because none of the enrolled subjects were on anticoagulant treatment at the time of recruitment, we can argue the absence of thromboembolic and major ischemic events during acute disease in this population. Moreover, because it has been demonstrated that ongoing hemostatic dysfunction is more common in patients who required intensive care support during acute SARS-CoV-2 infection,[40,42] long COVID patients with higher TF^{pos} platelet and CRP levels probably experienced a more severe acute illness.

Second, although it has been hypothesized that various SARS-CoV-2 variants may have distinct impacts on platelet behavior,[68] nevertheless, because of the limited enrollment timeframe, it is likely that both long COVID and COVID-recovered patients were infected with a unique original strain of the virus, and therefore the observed differences are not the result of a different viral variant.

Third, we did not assess monocytes and neutrophil activation markers or their enzyme plasma levels to prove their activation. However, because the formation of aggregates implies activation of the involved cells,[69,70] we did not consider it necessary to investigate this further. In addition, the antigenic phenotype of the aggregates generated in experiments under flow has not been characterized. Nevertheless, existing literature suggest that, within 5 minutes of perfusion, aggregates typically arise from leukocytes binding to platelets in the absence of fibrinogenesis.[71]

Fourth, data on lung CT and functional parameters of COVID-recovered subjects were not available because radiologic and functional assessment were not performed, as they reported no symptoms. Moreover, for pulmonary function tests, it was not possible to recruit an older healthy population, which would have allowed us to make a more accurate comparison with the long COVID patients. Fifth, we did not quantify the levels of sIL-6R that could sustain platelet transsignaling activation. Nevertheless, the role of IL-6 in maintaining the platelet phenotype observed in long COVID subjects was substantiated through ex vivo mixing experiments using appropriate pharmacologic controls.

CONCLUSIONS

The data presented in this study indicate that long COVID-related pulmonary symptoms, characterized by impaired diffusing capacity and consistent with radiologic interstitial change, are associated with a peculiar platelet activation process, featured mainly by PLA formation. Because a persistent low-grade inflammation seems to be the mechanism underpinning the platelet activation, this could be controlled, according to ex vivo data, by anti-inflammatory and antiplatelet drugs.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: Long COVID syndrome is characterized by a low-grade inflammation sustained by moderately increased levels of CRP and IL-6 that synergize, leading to persistent platelet activation in terms of PLA formation. This peculiar platelet activation correlates with residual parenchymal damage—that sustains the most frequently referred symptoms, such as dyspnea, chest pain, fatigue at rest and after exertion—and can be reduced by anti-inflammatory and antiplatelet drugs, suggesting a potential therapeutic approach in the treatment of long COVID symptoms.

TRANSLATIONAL OUTLOOK: The data reported here provide insights into the pathophysiologic mechanisms that sustain the observed platelet activation in long COVID syndrome. This suggests biomarkers that should be measured to fine-tune the clinical status and, based on this, tailor drug therapy for the management of long COVID syndrome.

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