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Low α-Thrombin/GPIbα Interaction Is a Potential Contributor to Platelet Hyper-reactivity in COVID-19 Patients

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Several studies have demonstrated that platelets can interact with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), and consequently undergo programmed cell death, extracellular vesicle release, and increased platelet reactivity in coronavirus disease 2019 (COVID-19) patients in response to low doses of α-thrombin.[1–3] Such hyper-sensitivity could be partially due to increased mitogen-activated protein kinase (MAPK) signaling pathway activation, protein kinase C delta (PKCδ) phosphorylation, and thromboxane synthesis.[1,2] These posttranslational modifications are often triggered following activation of one or more receptors on the platelet surface.

GPIbα (CD42b) in the GPIb-IX-V receptor complex is the major binding site for α-thrombin associated with platelets, and through this function may support procoagulant activities and contribute to platelet activation and aggregation.[4] Importantly, blocking α-thrombin binding to the N-terminal region of GPIbα (His1-Glu282) can be achieved by a blocking antibody (SZ2) that selectively inhibits the α-thrombin binding site on GPIbα.[4] According to recent findings, GPIbα is the receptor through which SARS-CoV-2 spike protein binds to platelets as well as activates their increased expression of ligands.[5] However, the role of GPIbα in the α-thrombin-induced platelet hyper-responsiveness observed during SARS-CoV-2 infection is not well understood.

At the low enzyme concentration, we identified α-thrombin/GPIbα interaction as a novel mechanism triggering the hyper-reactivity observed in COVID-19 patients. Our findings showed that during SARS-CoV-2 infection, pretreatment of platelets with human anti-GPIbα antibody (SZ2) prevents platelet hyper-aggregation and degranulation. Interestingly, we found that platelets derived from patients with Bernard–Soulier syndrome (BSS) and who are infected with SARS-CoV-2 are not hyper-reactive. We thus have identified low α-thrombin–GPIbα interaction as a novel prothrombotic pathway which triggers the formation of procoagulant platelets in COVID-19 patients.

COVID-19 patients ($n = 10$) and COVID-19 patients with BSS ($n = 3$) who were admitted to the Cheikh Zaid Hospital (Rabat, Morocco) were included in this prospective, observational study conducted from December 7, 2020 to January 12, 2022. Patients with BSS have a homozygous mutation on GP9 which prevents GPIbα expression on platelets. All patients with COVID-19 were studied (on average) at

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5.8 ± 1.2 hours after receiving a nasopharyngeal swab that showed positivity for SARS-CoV-2. Sex- and age-matched (age: 47.5, interquartile range: 38–52.4, 50% female) healthy blood donors ($n = 10$) were used as controls. None of the patients or healthy volunteers were treated with antithrombotic drugs (either premorbidly or for the treatment of their COVID-19 infection), and that could affect platelet functions or coagulation. All human blood studies were approved by the Local Ethics Committee of Cheikh Zaid Hospital, Rabat, Morocco; Project: CEFCZ/PR/2020-PR04. Informed consent was obtained from each subject and all experiments were conducted according to the principles set out in the Declaration of Helsinki.

Washed platelets were prepared as previously described.[1] Markers of platelet activation, α-granule release (CD62P or P-selectin expression) (►**Fig. 1Ai, Aii**) and dense granule secretion, as assessed by ATP release (►**Fig. 1Aiii**) and loss of mepacrine fluorescence (►**Fig. 1Aiv**), were significantly enhanced in the presence of sub-threshold concentrations of α-thrombin in platelets from COVID-19 patients. We sought to investigate the contribution of GPIbα to the regulation of platelet hyper-activation. As shown in ►**Fig. 1A(i–iv)**, this

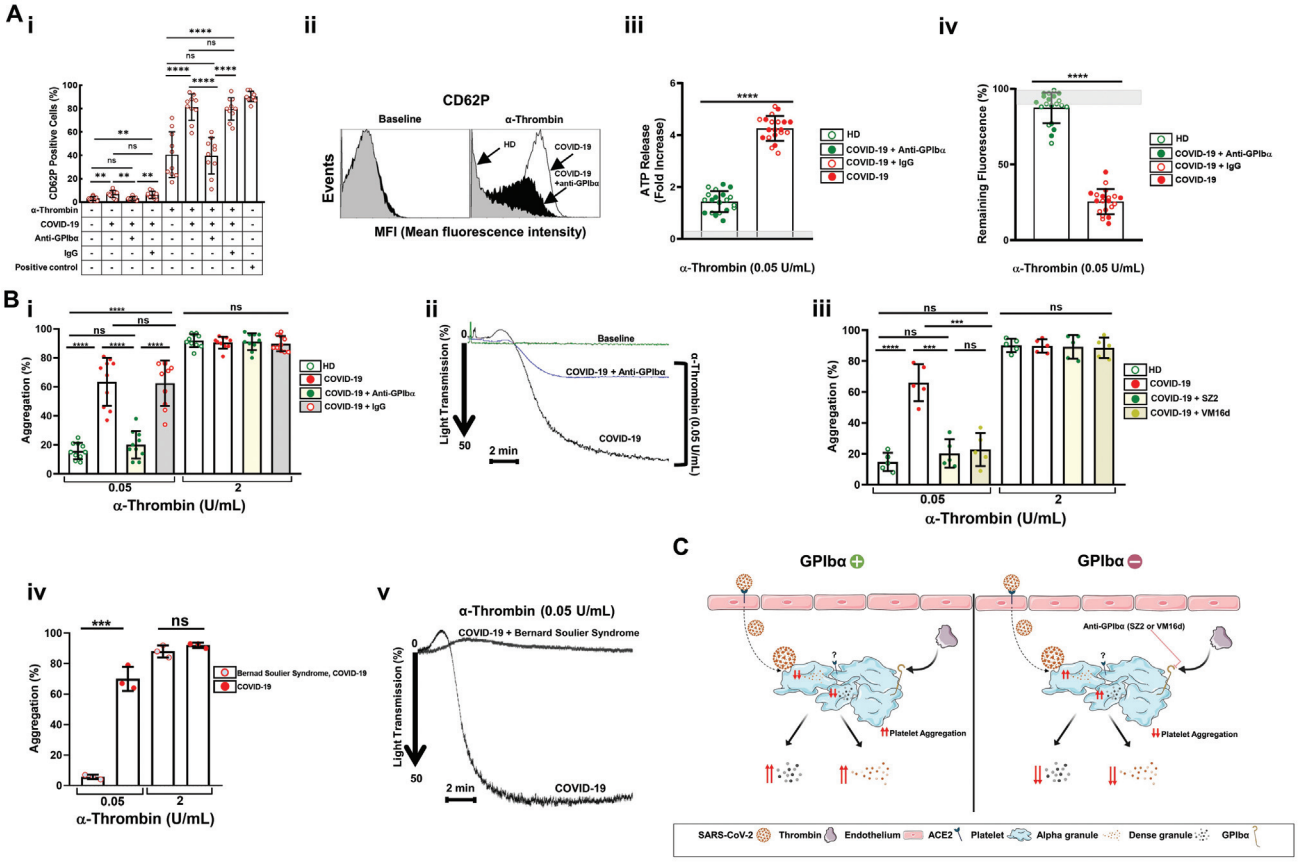

Fig. 1 (**A**) GPIbα positively regulates human platelet degranulation induced by low doses of α-thrombin in COVID-19 patients. (Ai) Platelet P-selectin expression was measured (percent of CD62P positive platelets) in washed human platelets by flow cytometry at baseline in 10 healthy donors and 10 COVID-19 patients. Platelets were pretreated with human anti-GPIbα antibody (SZ2, 20 μg/mL) or its isotype IgG control for 5 minutes at 37°C. Degranulation was then initiated by α-thrombin at 0, 0.05 (priming dose), and 0.2 U/mL (positive control). Histogram represents the mean of data ± standard deviation (SD) of plots for P-selectin (CD62P) expression ($n = 10$); $^{**}p < 0.01$; $^{****}p < 0.0001$. Statistical significance was analyzed using one-way analysis of variance (ANOVA) with subsequent Dunnett's *t*-test for comparison against a single group. (Aii) Effect of anti-GPIbα on P-selectin (CD62P) expression in COVID-19 patients, as assessed by flow cytometry. Left plots represent resting platelets. Right plots represent platelets in the presence of a priming concentration of α-thrombin (0.05 U/mL). (Aiii) ATP release was measured by a Lumi-Aggregometer (Luciferase assay; Chrono-Lume, Chrono-log). Results are expressed as a measure of increase in luminescence and (Aiv) dense granule secretion was evaluated by measuring the loss of mepacrine fluorescence following activation by α-thrombin (0.05 U/mL). Histogram represents the mean of data ± SD of (Aiii) ATP release (fold increase) and (Aiv) remaining mepacrine fluorescence ($n = 10$); $^{****}p < 0.0001$. The reference range (resting platelets) is represented by the shaded gray region. (**B**) Pretreatment with a human anti-GPIbα antibody prevents COVID-19-induced potentiation of platelet aggregation while Bernard–Soulier Syndrome (BSS) platelets are not hyper-reactive. (Bi) Platelets were pretreated with the human anti-GPIbα antibody (SZ2, 20 μg/mL) for 5 minutes at 37°C. Aggregation was then initiated by low concentrations of α-thrombin (0.05 U/mL). Histogram represents the mean of data ± SD of aggregation traces ($n = 10$); $^{****}p < 0.0001$. (Bii) Representative traces of platelet aggregation induced by a priming dose of α-thrombin (0.05 U/mL). (Biii) Specific blockade of the thrombin-binding site on GPIbα, by either SZ2 or VM16d blocking monoclonal antibodies, prevented the potentiation of thrombin-induced platelet aggregation in COVID-19 patients. (Biv) Platelets isolated from COVID-19 patients with (or without) BSS (deficiency in GPIb-V-IX complex) were stimulated by low doses of α-thrombin (0.05 U/mL). (Bv) Representative traces of platelet aggregation (shown in Biv) induced by a priming dose of α-thrombin (0.05 U/mL). (**C**) Scheme of the potential role the α-thrombin/GPIbα axis plays in platelet hyper-reactivity in COVID-19 patients. During SARS-CoV-2 infection, α-thrombin stimulates its high-affinity receptor GPIbα leading to α- and dense granule release and platelet hyper-aggregation. Receptor blockade or deficiency prevents the hyper-coagulable state occurring in COVID-19 patients. IgG, immunoglobulin G.

process was prevented following pretreatment of platelets with a selective human anti-GPIbα antibody (SZ2), whereas a control immunoglobulin G (IgG) antibody had no effect. This suggests that SARS-CoV-2 triggered platelet degranulation in an α-thrombin- and GPIbα-dependent manner. This is consistent with a report that GPIbα is the major α-thrombin binding site on the platelet surface with evidence for a high affinity site within its extracellular domain adjacent to the von Willebrand factor (VWF) binding site.[6] Also, at a higher α-thrombin concentration, the protease-activated receptors (PAR1 and PAR4) likely become significantly engaged and further enhance degranulation and extent of platelet hyper-activation and aggregation.[7]

Similarly, we assessed whether the α-thrombin–GPIbα axis plays a role in the hyper-aggregation of platelets from COVID-19 patients. As anticipated, priming, but not high concentration of α-thrombin-induced aggregation, was significantly inhibited in platelets that were pretreated with a human anti-GPIb antibody (SZ2) (Beckman Coulter, sodium azide free), as compared with COVID-19 and to COVID-19 control IgG groups (►**Fig. 1Bi, Bii**). Thus, specific inhibition of the high-affinity binding site of α-thrombin on GPIbα was found to inhibit potentiated washed human platelet secretion and aggregation response, indicating that the α-thrombin/GPIbα axis can potentiate platelet function in the presence of suboptimal α-thrombin concentrations.

To further assert our finding based on the mouse-anti-human antibody, clone SZ2, that targets the anionic/sulfated tyrosine sequence 269 to 282 of GPIbα, we performed a similar experiment using a mouse anti-human antibody, clone VM16d (Abcam, dialyzed against phosphate-buffered saline in our laboratory to remove sodium azide), that maps the C-terminal flanking sequence 226 to 268 of GPIbα.[8] Our results show that specific blockade of the thrombin-binding site on GPIbα, by either SZ2 or VM16d (►**Fig. 1Biii**) blocking monoclonal antibodies, prevented the potentiation of thrombin-induced platelet aggregation in COVID-19 patients.

To confirm our pharmacological-based approach, the key role for GPIbα was assessed using platelets isolated from COVID-19 patients with BSS (deficiency in GPIb-V-IX complex causing an absence of GPIb expression). Platelets from these patients were found to have markedly diminished aggregation in response to low concentrations of α-thrombin compared with COVID-19 patients without BSS (►**Fig. 1Biii, Biv**). Indeed, SARS-CoV-2 infection failed to trigger platelet hyper-aggregation in patients with BSS suggesting that GPIbα positively regulates platelet aggregation downstream of α-thrombin in COVID-19 patients (►**Fig. 1C**).

BSS is characterized by prolonged bleeding time, thrombocytopenia,[9,10] and giant platelets lacking the surface membrane glycoprotein GPIb of the GPIb-IX-V complex.[10–12] The GPIb-IX-V complex is a platelet-specific adhesion-signaling complex, and it consists of GPIbα linked to GPIbβ via a disulfide bond and to GPIX and GPV non-covalently. Here, GPIbα is the major ligand-binding subunit and binds thrombin and the adhesive ligand VWF.[8] Given that platelets of BSS patients hardly express GPIb, BSS platelets lack GP1b-specific response to α-thrombin. Similarly, SZ2, an anti-GPIbα monoclonal antibody, is known to inhibit VWF binding to platelet and platelet aggregation.[13] Thus, both SZ2 antibody-treated platelets and platelets of BSS lack GP1bα function and GP1bα-driven response to α-thrombin. Other factors may contribute to the diminished response of BSS platelets to thrombin and SARS-CoV-2 infection, including the additional loss of GPV and GPIX. The loss of surface-membrane glycoprotein in BSS platelets has been closely linked to in vitro and in vivo functional impairment.[9,10]

Our finding was supported by a recent study[14] that worked on S100A8/A9, also known as "calprotectin" or "MRP8/14," an alarmin primarily secreted by activated myeloid cells with antimicrobial, proinflammatory, and prothrombotic properties. This research group identified the S100A8/A9-GPIbα axis as a novel targetable prothrombotic pathway inducing procoagulant platelets and fibrin formation, in particular in diseases associated with high levels of S100A8/A9, such as COVID-19.

Taken together, GPIbα interaction with low concentration α-thrombin is a potential contributor to the formation of procoagulant platelets in COVID-19 patients. Such interaction is probably only one aspect of many that likely regulate platelet activity in COVID-19 patients. These observations provide a valuable foundation for understanding the disease pathophysiology and to identify a new target for treatment options.

Data Availability Statement

The original contributions presented in the study are included in the article, and further inquiries can be directed to the corresponding author.

Ethical Approval Statement

Written informed consent was obtained from the individuals for the publication of any data included in this article.

Authors' Contribution

Y.Z., F.J., and F.G. designed the research. Y.Z., L.K., Y.T., N.Z., A.N., and M.O. performed the research and statistical analyses. Y.Z., L.K., KA., and F.G. analyzed the data. Y.Z., E.O.A., and F.G. contributed to discussion and revised the manuscript. Y.Z. and F.G. wrote the manuscript. All authors read and approved the paper.

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Conflict of Interest

None declared.

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