Increased Platelet Activation demonstrated by Elevated CD36 and P-Selectin Expression in 1-Year Post-Recovered COVID-19 Patients

Samuel Sherng Young Wang, MD[1] Keefe Chee, PhD[2] Shiun Woei Wong, MRCP[3,4,5] Guat Bee Tan, MSc[6,7] Hong Ang, BSc[6] Bernard PuiLam Leung, PhD[4,8,9] Chuen Wen Tan, FRCPath[10] Kollengode Ramanathan, FCCP[4,11] Rinkoo Dalan, FRCP[3,4,12] Christine Cheung, PhD[3,13] David Chien Lye, FRCP[3,4,14,15] Barnaby Edward Young, MRCP[3,4,14,15] Eng Soo Yap, FRCPath[4,16] Yew Woon Chia, FRCP[3,4,5] Bingwen Eugene Fan, MRCP[1,2,3,4] and The COVID-19 Clotting and Bleeding Investigators

[1] Department of Haematology, Tan Tock Seng Hospital, Singapore, Singapore
[2] Becton Dickinson, Singapore, Singapore
[3] Lee Kong Chian School of Medicine, Nanyang Technological University, Singapore, Singapore
[4] Yong Loo Lin School of Medicine, National University of Singapore, Singapore, Singapore
[5] Department of Cardiology, Tan Tock Seng Hospital, Singapore, Singapore
[6] Department of Laboratory Medicine, Tan Tock Seng Hospital, Singapore, Singapore
[7] Clinical Research and Innovation Office, Tan Tock Seng Hospital, Singapore, Singapore
[8] Health and Social Sciences, Singapore Institute of Technology, Singapore, Singapore
[9] Department of Rheumatology, Allergy and Immunology, Tan Tock Seng Hospital, Singapore, Singapore
[10] Department of Haematology, Singapore General Hospital, Singapore, Singapore
[11] Department of Cardiothoracic and Vascular Surgery, National University Heart Centre, Singapore, Singapore
[12] Department of Endocrinology, Tan Tock Seng Hospital, Singapore, Singapore
[13] Institute of Molecular and Cell Biology, A*STAR, Singapore, Singapore
[14] Department of Infectious Diseases, Tan Tock Seng Hospital, Singapore, Singapore
[15] National Centre for Infectious Diseases, Singapore, Singapore
[16] Department of Haematology-Oncology, National University Health System, Singapore, Singapore
[17] Department of Laboratory Medicine, Khoo Teck Puat Hospital, Singapore, Singapore

Address for correspondence Bingwen Eugene Fan, MRCP, Department of Haematology, Tan Tock Seng Hospital, Singapore, Singapore (e-mail: Bingwen_Eugene_Fan@ttsh.com.sg).

Semin Thromb Hemost

To the Editor:

Coronavirus disease 2019 (COVID-19)-associated hypercoagulability and platelet hyperactivity are characteristic of COVID-19 infection and contribute to an increased thrombotic burden. This clinical phenomenon is observed during acute illness and the immediate post-recovery period. In addition to findings of hypercoagulability, endotheliopathy, and inflammation from our previous study of 39 COVID-19 survivors at a time point approximating 1 year after their recovery from COVID-19,[1] we evaluated the platelet activation profile of a subset of 36 patients, demonstrating increased platelet activation with elevated CD36 and P-selectin expression compared with healthy individuals without COVID-19 infection.

As per the National Institutes of Health guidelines, our patients were classified to asymptomatic ($n = 9$), mild ($n = 19$), moderate (4 L oxygen supplementation) ($n = 2$), and severe (admitted to intensive care unit) ($n = 6$). We combined asymptomatic and mild patients as a first group ($n = 28$), and moderate or severe patients as a second group ($n = 8$). The median age of the patients was 43 years (range = 25–75) with no significant age differences between the asymptomatic/mild patients (median 41 years, range 27–75) and moderate/severe patients (median 43, range 25–60). The demographic profile was: sex: male 25 (69%), ethnicity: Chinese 12 (33%), Indian: 15 (42%), Malays, or others 9 (25%). Seven (16%) patients had a history of diabetes, 4 (11%) had hypertension and 7 (16%) had hyperlipidemia. Three patients had both hypertension and hyperlipidemia. There were no significant under- or overrepresentation of comorbidities in any group.

Issue Theme Editorial Compilation—Part XIII; Guest Editors: Emmanuel J. Favaloro, PhD, FFSc (RCPA); Leonardo Pasalic, FRCPA, FRACP, PhD; and Giuseppe Lippi, MD

Thieme Medical Publishers, Inc., 333 Seventh Avenue, 18th Floor, New York, NY 10001, USA

DOI https://doi.org/10.1055/s-0043-1762578.
ISSN 0094-6176.

Blood collection and sample preparation was performed as per standard flow cytometry guidelines.[2] Whole blood was collected aseptically by venepuncture into acid citrate dextrose/sodium citrate vacutainer tube using a 20-gauge needle. The first tube was discarded to avoid activated platelets and a second tube with released tourniquet was collected for flow cytometry staining. All samples were stained within 30 minutes from blood draw. Whole blood was also collected from 17 healthy donors who did not have prior history of COVID-19 to act as controls.

For the adenosine diphosphate (ADP) platelet function assay, each donor/patient blood sample was further divided into four samples with the following treatments: resting/untreated, 10 µM ADP, 20 µM ADP, and 40 µM ADP. Each of the four blood samples were incubated with their agonist agent for 2 minutes prior to incubation for staining.

A 10-color panel was designed with the following monoclonal antibodies: CD63 BV421 (H5C6 clone), CD61 BV510 (VI-PL2 clone), CD36 BV605 (CB38 clone), PAC-1 FITC (PAC-1 clone), GPVI PE (HY101 clone), CD42a PerCP (Beb1 clone), CD235a PE-Cy7 (GA-R2 clone), CD62P APC (AK-4 clone), CD42b R718 (HIP1 clone), and CD45 APC-H7 (2D1 clone) and the panel was prepared with Brilliant Stain Buffer (all Becton Dickinson Biosciences [BD], San Diego, CA). Whole blood was stained in room temperature in the dark for 10 minutes, diluted with Pharmingen Stain Buffer (BSA, BD), and acquired on a BD FACSCanto 10C immediately. An unstained set of blood samples were also used as a basis of comparison. Flow cytometry data was analyzed using FlowJo 10.8.1, with Pairwise Controlled Manifold Approximation (PaCMAP) v1.0. This was used as a plugin on FlowJo for unbiased data evaluation. Maps were created using PAC-1, GPVI, CD42a, CD42b, CD62P (P-selectin), CD63, CD36 (7 parameters), with 265,000 events that were concatenated with 5,000 events from 53 patients. The following parameters were used for PaCMAP visualization: nearest neighbors: 15; mid-near ratio: 0.5; FP ratio: 2. To layer clusters on the PaCMAP dimensions, Phenograph v3.0 was used with K 200. PaCMAP was chosen for its preservation of global structure and preservation of local structure.[3] Intergroup differences were then tested using analysis of variance with Tukey's multiple comparison method using GraphPad Prism 9.4, with $p < 0.05$ considered statistically significant.

PaCMAP analysis using only resting platelets without ADP stimulation demonstrated that prior COVID-19 infection generates a subset of activated platelets with a phenotypic profile of CD62P+ (surface P-selectin), PAC1+ (activated glycoprotein [GP] IIb/IIIa), and CD36+ (GP IV) that persists beyond the initial infective period. This platelet subset is observed in both groups of asymptomatic/mild and moderate/severe patients (►**Fig. 1A** and **B**). Furthermore, the size of the platelet subpopulation correlated with severity of patient symptoms. Further population gating confirmed the dual expression of CD62P+/PAC1+, with increased expression associated with worsening symptom severity (►**Fig. 1C**). However, additional analysis of CD62P+/PAC1+ populations showed nonsignificance between the healthy, asymptomatic/mild, and moderate/severe groups (►**Fig. 1D**), which could be secondary to the small sample size of the moderate/severe group. Another explanation might be the flow cytometry captured platelets in different stages of activation/exhaustion.[4] The standard deviation for CD62P+/PAC1+ was wider in the moderate/severe group compared with the asymptomatic/mild group (457.7 vs. 245.9). This larger standard deviation is likely to impact average mean fluorescence intensity of the moderate/severe patient group and

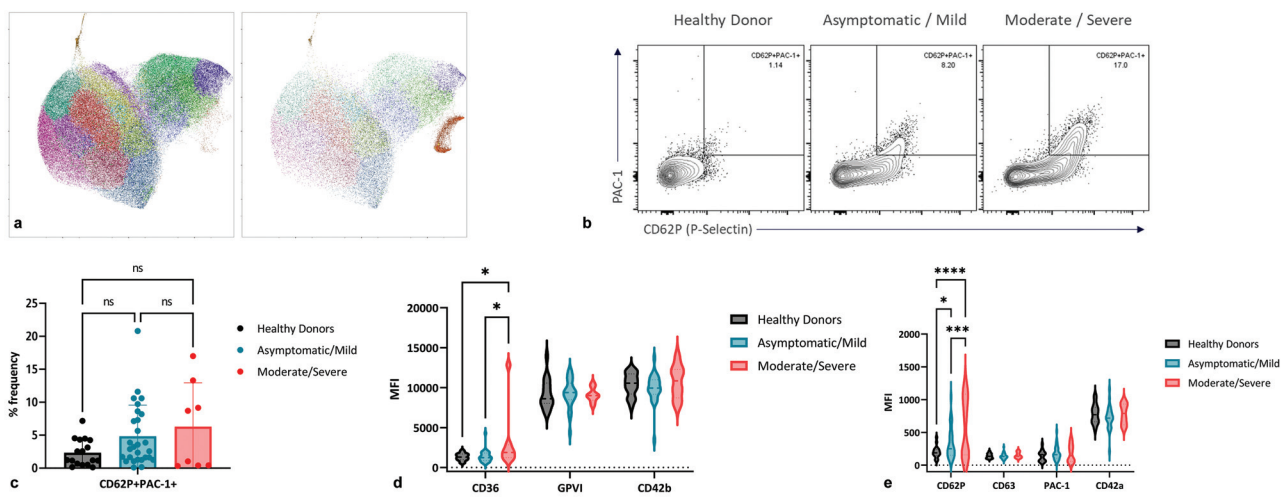

Fig. 1 (**A**) Pairwise Controlled Manifold Approximation (PaCMAP) analysis of patients with asymptomatic/mild symptoms. (**B**) PaCMAP analysis of patients with moderate/severe symptoms. A significant population of cells can be seen elevated in patients that experienced severe symptoms, gated within the black box. The phenotypic profile of the population is CD62+ (surface P-selectin), PAC1+ (activated glycoprotein [GP] IIb/IIIa), and CD36+ (data not shown). (**C**) Canonical gating of representative samples confirmed the dual expression of CD62P+ and PAC1+. (**D**) Statistical analysis of CD62+PAC1+ dual positive populations showed nonsignificance between the three patient groups using nonparametric Kruskal–Wallis test for pairwise comparisons. (**E**) Mean fluorescence intensity (MFI) for CD36, GPVI, CD42b, CD62P, CD63, PAC-1, and CD42a. Intergroup differences were tested using analysis of variance (ANOVA) with Tukey's multiple comparison test. *p*-Value of: $\leq 0.05 = ^{*}$; $\leq 0.01 = ^{**}$; $\leq 0.01 = ^{***}$; $\leq 0.0001 = ^{****}$.

consequently could mask any statistical difference between asymptomatic/mild and moderate/severe patient groups (►**Fig. 1D** and ►**Supplementary Fig. S1A**).

When comparing individual platelet activation markers between patient groups, CD36 levels were significantly higher in patients irrespective of their disease severity when compared with healthy volunteers. CD62P was significantly different between all three patient groups. However, there was no statistically significant difference for GPVI, CD42b, CD63, PAC-1, and CD42a (►**Fig. 1E**). This might imply a physiological saturation of CD36 activation with the COVID-19 infection irrespective of symptom severity.

A platelet functional assay using ADP as an agonist was performed, with CD62P and PAC1 levels being measured (►**Supplementary Fig. S2**). The data highlights no difference between asymptomatic/mild and moderate/severe groups despite progressive stimulation with ADP. CD62P and PAC1 activation also appears to be at near physiological maximum activation even at lowest dose of ADP, with further doubling/quadrupling of ADP at supraphysiological doses eliciting minimal further activation.

CD62P is a GP which rapidly translocates from platelet α-granules during platelet activation.[5] CD62P mediates platelet rolling on endothelial cells and platelet interactions with neutrophils and monocytes, and stabilizes platelet aggregates formed by GP IIb/IIIa-fibrinogen interactions. For platelets, CD36 functions as a receptor for cell-derived microparticles promoting platelet activation, aggregation, and secretion; thereby promoting thrombus formation in vascular injury and inflammation.[6] It has recently been reported in COVID-19 literature that higher levels of CD62P and CD36 were noted in patients with more severe disease who had a higher risk for arterial and venous thromboembolism.[7,8] The phenomenon of COVID-19 platelet activation is likely due to the association between inflammation/infection and platelet production. Platelet production and activation is driven by thrombopoietin,[9,10] which is upregulated by inflammatory cytokines activated in COVID-19 infection, such as interleukin-6. Platelets also possess cell surface receptors that contribute to immune cell interaction and function. The GPIb receptor, a key mediator of platelet adhesion, is also capable of binding to von Willebrand factor exposed on immune cells infected with bacterial pathogens. Autopsies in COVID-19 confirm the presence of microvascular thrombi comprising of neutrophil extracellular traps and platelets secondary to COVID-19-related inflammation and thrombosis.[11,12] This thromboinflammatory milieu and hyperactivated platelet phenotype is most prominent in critically ill COVID-19 patients.[11] The literature is still unclear regarding the exact mechanism for a hyperactivated platelet phenotype, one plausible explanation is the severe acute respiratory syndrome coronavirus 2 virus directly interacts and mediates platelet activation due to the presence of viral ribonucleic acid in platelets,[13] while another possible cause is the presence of soluble inflammatory mediators that may result in platelet activation as evidenced by healthy volunteer platelet activation with critically ill COVID-19 patient plasma.[14]

Post-COVID-19 persistent platelet activation may explain why high-risk patients such as intensive care unit patients could benefit from antiplatelet treatment regimens post-COVID-19 infection.[15] We postulate that persistent platelet activation may be due to immune dysregulation and persistent inflammation triggered by the initial COVID-19 infection that is still driving platelet activation (see ►**Supplementary Fig. S3**).[16] The limitation of our study is that our study population comprises of a relatively young and small cohort with a low prevalence of cardiovascular risk factors, where we recognize this might not be reflective of older survivors with cardiovascular comorbidities who may benefit most from antiplatelet treatments. Further comprehensive studies measuring other aspects of platelet function, such as platelet aggregometry and the inclusion of other physiological platelet agonists such as collagen and thrombin to assess platelet activation and platelet exhaustion in COVID-19 survivors are required. Our study provides possible early evidence for a hyperactivated platelet phenotype in our patients who have recovered (~1 year) from COVID-19, given their elevated CD62P and CD36 levels, with this phenotype manifesting more frequently in recovered survivors with severe infections.

The authors declare that they have no conflict of interest.

Ethics Approval

This study was approved by the National Healthcare Group Domain Specific Review Board (2020/01426): Cross-sectional study of long-term endothelial, hematological, and cardiovascular complications after recovery from COVID-19.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' Contributions

B.E.F. had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Concept and design: B.E.F., Y.W.C., E.S.Y., C.W.T., B.E.Y., D.C.L. Performing of laboratory tests: G.B.T., H.A. Statistical analysis: K.C. Drafting of the manuscript: S.S.Y.W., K.C., B.E.F. Acquisition, analysis, or interpretation of data and critical revision of manuscript for important intellectual content: All authors.

Funding

This work was supported by a National Healthcare Group-National Centre for Infectious Diseases (NHG-NCID) COVID-19 Centre Grant (COVID19 CG008).

Conflict of Interest

None declared.

Acknowledgments

The authors greatly appreciate the efforts of our fellow healthcare workers during this pandemic. Special thanks to Becton Dickinson for their technical support.

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