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BRIEF REPORT

Platelet-activating functional assay resolution in vaccine-induced immune thrombotic thrombocytopenia: differential alignment to PF4 ELISA platforms

Christine S. M. Lee BSc (Hons), PhD[1] | Lisa J. Clarke MBBS[2,3] | Geoffrey W. Kershaw BSc[4] | Ibrahim Tohidi-Esfahani BMed, PhD[1,2] | Timothy A. Brighton MBBS, MD[5] | Sanjeev Chunilal MBChB[6] | Emmanuel J. Favaloro PhD[7,8,9] | Huyen Tran MBBS[6,10,11] | Vivien M. Chen MBBS, PhD[1,2,12]

[1]ANZAC Research Institute, University of Sydney, Sydney, New South Wales, Australia

[2]Department of Haematology, Concord Repatriation General Hospital, and NSW Health Pathology, Sydney, New South Wales, Australia

[3]Australian Red Cross Lifeblood, Sydney, New South Wales, Australia

[4]Institute of Haematology, Royal Prince Alfred Hospital, Sydney, New South Wales, Australia

[5]Department of Haematology, New South Wales Health Pathology, Prince of Wales Hospital, Randwick, Sydney, New South Wales, Australia

[6]Department of Health Sciences, Monash University, Melbourne, Victoria, Australia

[7]Department of Haematology, Institute of Clinical Pathology and Medical Research (ICPMR), Sydney Centres for Thrombosis and Haemostasis, NSW Health Pathology, Westmead Hospital, Westmead, New South Wales, Australia

[8]School of Medical Sciences, Faculty of Medicine and Health, University of Sydney, Westmead Hospital, Westmead, New South Wales, Australia

[9]Faculty of Science and Health, Charles Sturt University, Wagga Wagga, New South Wales, Australia

[10]Clinical Haematology Department, The Alfred Hospital, Melbourne, Victoria, Australia

[11]Australian Centre for Blood Diseases, Monash University, Victoria, Australia

Abstract

Background: Anti-platelet factor 4 (PF4) antibodies in vaccine-induced immune thrombotic thrombocytopenia (VITT) appear to be transient, with discrepant persistence depending on the platform used for detection.

Objectives: We aimed to report a longitudinal study of antibody persistence using 2 ELISA platforms and 2 platelet-activating functional assays in a clinical cohort of patients with VITT referred for follow-up testing.

Methods: In total, 32 Australian patients with VITT or pre-VITT, confirmed by expert adjudication, with samples referred for clinical follow-up were included. Clinical follow-up assays, including Stago and Hyphen ELISAs, procoagulant platelet flow cytometry, and modified PF4-serotonin-release assay, were performed according to the pattern of reactivity for that patient at diagnosis.

Results: The median follow-up was 24 weeks after diagnosis. A general decline in anti-PF4 antibody levels and platelet-activating capacity over time was observed with a more rapid median time to resolution of 16 weeks by functional assay vs 24 weeks by Stago ELISA. Decline in platelet-activating antibody levels detected by functional assays mirrored Stago ELISA titer but not Hyphen. However, 87% of patients received a documented second vaccination and 74% received an mRNA booster with no reported adverse events.

Conclusion: Anti-PF4 antibodies persist longer than functional platelet-activating antibodies in VITT but do not warrant avoidance of subsequent vaccinations. Persistence detection is assay-dependent. Stago ELISA may be a surrogate where functional assays are unavailable for follow-up testing of confirmed patients with VITT.

[12]Sydney Medical School, University of Sydney, New South Wales, Australia

Correspondence
Vivien Chen, ANZAC Research Institute, Building 27, Gate 3 Hospital Road, Concord, NSW 2139, Australia.
Email: vivien.chen@sydney.edu.au

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KEYWORDS
COVID -19 vaccine, enzyme-linked immunosorbent assay, platelet activation, platelet factor 4, vaccine-induced immune thrombotic thrombocytopenia

Essentials

- Antibodies in vaccine-induced immune thrombotic thrombocytopenia (VITT) decline over time.
- Serial testing of patients with VITT is advised to guide treatment duration.
- Platelet-activating antibodies resolved earlier than those detected by Stago or Hyphen ELISAs.
- No adverse events were reported following subsequent vaccinations of patients after VITT diagnosis.

1 | INTRODUCTION

The putative mechanism for vaccine-induced immune thrombotic thrombocytopenia (VITT) following ChAdOx1 nCoV-19 (Vaxzevria, AstraZeneca) vaccination involves generation of pathological anti-platelet factor 4 (PF4) platelet-activating antibodies. Anti-PF4 antibodies in VITT appear to be transient [1–4] but more persistent than those in heparin-induced thrombocytopenia (HIT) [5]. We recently reported the Australian multi-center study of immunological anti-PF4 antibody studies for VITT, highlighting the differences in sensitivity and specificity of both ELISA and functional assays at diagnosis [6], consistent with a previous report [7]. The optimal duration of anticoagulation remains unclear, and the Thrombosis and Haemostasis Society of Australia and New Zealand (THANZ) guidance recommends testing of serologically confirmed VITT at 3, 6, and 12 months to guide anticoagulation duration [8]. Five recent publications have separately documented the rate of decline in PF4 antibodies. Craven et al. demonstrated discrepant persistence between antibodies measured by Immucor and Stago ELISAs in 34 and 33 patients, respectively [2], whereas Schönborn et al. demonstrated more rapid resolution of platelet-activating antibodies by functional testing than antibodies detected by their in-house ELISA in 65 patients [1]. In smaller cohorts of 7 and 9 follow-up cases, similar patterns of decline in PF4 antibodies measured by Immucor ELISA or Hyphen ELISA and platelet-activating assays were reported [3,4,9]. Here, we report a longitudinal study of antibody persistence using 2 ELISA platforms and 2 platelet-activation assays in a subset of patients from the Australian VITT cohort who were referred for clinical follow-up, which suggests that 1 ELISA platform more closely aligns with functional assay decline than another.

2 | METHODS

2.1 | Study participants

Patients with samples were referred for clinical follow-up VITT testing as per THANZ guidance between July 2021 and April 2022 with a positive diagnostic functional assay, regardless of the diagnostic ELISA test result [8,10].

2.2 | Anti-PF4 antibody testing

Blood collection from healthy volunteers and comparison of assays were approved by the Sydney Local Health District Human Research Ethics Committees (HREC/18/CRGH/294, X21-0160, 2021/ETH00945, and 2021/ETH11929). Healthy blood donors for functional assays gave written informed consent and were screened for FcγRIIa responsiveness as described [11,12]. Anti-PF4 IgG antibodies were assessed prospectively using (a) Asserachrom HPIA IgG (Diagnostica Stago) as per manufacturer's instructions, (b) whole blood flow cytometry procoagulant platelet assay, and/or (c) serotonin-release assay (SRA) modified for VITT diagnosis were performed as described [12]. VITT or "Pre-VITT" [13] was confirmed by expert adjudication based on THANZ clinicopathologic criteria [8]. Samples were also retrospectively tested by Zymutest HIA IgG (Hyphen Biomed) as per manufacturer's instructions. Data analysis was performed using GraphPad Prism 9.2 (GraphPad Software).

3 | RESULTS AND DISCUSSION

Thirty-two patients were included (Table) and processed as per the THANZ clinical pathway with follow-up assays performed according to the pattern of reactivity for that individual at diagnosis (eg, patients positive for ELISA and PF4 flow cytometry at diagnosis and had follow-up with the same assays). Median follow-up was 24 weeks (range 8-36) after diagnosis. In total, 31 patients were followed at least 12 weeks and 4 patients for 36 weeks. Follow-up results on Stago ELISA, Hyphen ELISA, flow cytometry, and SRA were available for 24, 23, 30, and 19 patients, respectively. Consistent with our published diagnostic cohort, only 75% of patients were positive on the Stago ELISA immunoassay at diagnosis [6].

TABLE Laboratory and clinical characteristics of confirmed patients with VITT referred for follow-up VITT testing.

Variable	Confirmed VITT referred for follow-up testing
N	32
Female	18
Male	14
Age at presentation (years)	57.5 (27-80)
Days after vaccine	10 (4-43)
Platelet count at presentation ($\times 10^9$/L)	45 (7-477)[a]
D-dimer (fold-change over ULN) at diagnosis	40 (6.8-228)
Stago ELISA result at diagnosis	
Positive	25
Negative	7
Hyphen ELISA result at diagnosis	
Positive	23
Negative	9
Procoagulant platelet flow cytometry result at diagnosis	
Positive	31
Negative	1
Modified serotonin-release assay result at diagnosis	
Positive	27
Negative	4
Not available	1
Thrombosis	29
Cerebral venous sinus thrombosis	9
Splanchnic thrombosis	8
Pulmonary embolism	16
Deep vein thrombosis	5
Other	5
Follow-up period after diagnosis (weeks)	24 (8-36)

Continuous variables are expressed as median (range).

ULN, upper limit of normal.

[a]Patient with platelet count of 477 $\times$ 10^9/L had a platelet nadir of 52 $\times$ 10^9/L.

Consistent with published reports [1–4,9], a general decline was demonstrated in anti-PF4 antibody levels and platelet-activating capacity over time, with varying sensitivity between ELISA platforms [14]. Similar to Craven et al. [2], more rapid resolution of antibodies was detected using Stago ELISA compared with alternatives (Hyphen in our cohort and Immucor in Craven et al.), although all showed general declines in ODs over time. In total, 17 of 24 patients (71%) with positive Stago ELISA at diagnosis remained positive at 12 weeks and 11 remained positive by the end of follow-up. Hyphen ELISA detected anti-PF4 antibodies in 8 of 13 (62%) patients that returned a negative Stago ELISA at matching time points (Figure 1A and B). However, 22 of 23 (96%) patients remained Hyphen ELISA-positive 12 weeks after diagnosis, consistent with the report by Panagiota et al. [9], and 20 of 23 patients (87%) within the follow-up period (Figure 1A and B).

Because not all detectable anti-PF4 antibodies were platelet activating within the Australian cohort [6,12], only samples that tested positive by functional assays were included in this study. On follow-up functional assay testing using dichotomous positive or negative outcomes, 19 of 32 patients (59%) returned a negative result, whereas 13 remained positive throughout the follow-up period.

Using Stago ELISA, our median time to antibody resolution was longer than Craven et al., 24 weeks rather than 20 weeks [2], with only 7 patients returning a negative result within 12 weeks and 2 patients still positive at 36 weeks (Figure 1C). In contrast, retrospective retesting of the same 23 patients on Hyphen ELISA, which was more discriminatory at the diagnostic time point [6], 20 patients (87%) remained positive at the last follow-up, reflecting a similar discrepancy regarding persistence as previously shown between Stago and Immucor [2]. VITT antibody kinetics appear unpredictable. We found no correlation between the original antibody titer by ELISA and persistence rate—some patients with high initial OD values were not as persistent as those with lower values (Figure 1A and B).

The median time to resolution of platelet-activating antibodies detected by either flow cytometry or SRA was 16 weeks compared with 19 weeks in an Ad26.COV2.S-associated cohort [3] and 15.5 weeks in the cohort of Schönborn et al., noting that the median resolution of antibodies by in-house ELISA was not reached in the latter study [1]. Paired diagnostic and follow-up testing by flow cytometry demonstrated individual variation in the rate of decline of induced procoagulant platelet response, not predicted by procoagulant platelet fold-change at diagnosis (Figure 1D). Although patients with "severe" thrombosis (cerebral venous sinus, splanchnic, portal, arterial, central vessel, and multiple sites thromboses) appeared to have increased time to resolution in the functional assay compared with patients with "standard site" thrombosis (deep vein thrombosis or pulmonary embolism), we found no statistically significant difference between the groups by Kaplan–Meier analysis (P = .4542, Figure 1E) or by Fisher's exact test (P = .4495). Resolution of thrombocytopenia and normalization of D-dimer were seen in all patients in which data were available (Figure 1F and G). Analysis according to therapy was not helpful in this cohort as all patients received anticoagulation and >90% received intravenous immunoglobulin therapy.

Regarding potential relationships between immune and functional assays, we previously reported a moderate correlation between plasma-induced procoagulant platelet response and Stago OD at diagnosis (r^2 = 0.406) [12], and Craven et al. speculated that antibodies detected by Stago ELISA might align more with platelet-activation assays [2]. Our data support this concept, demonstrating a pattern of functional assay decline in individuals that mirrored the Stago titer but not Hyphen (Figure 2A-D). In contrast, Platton and

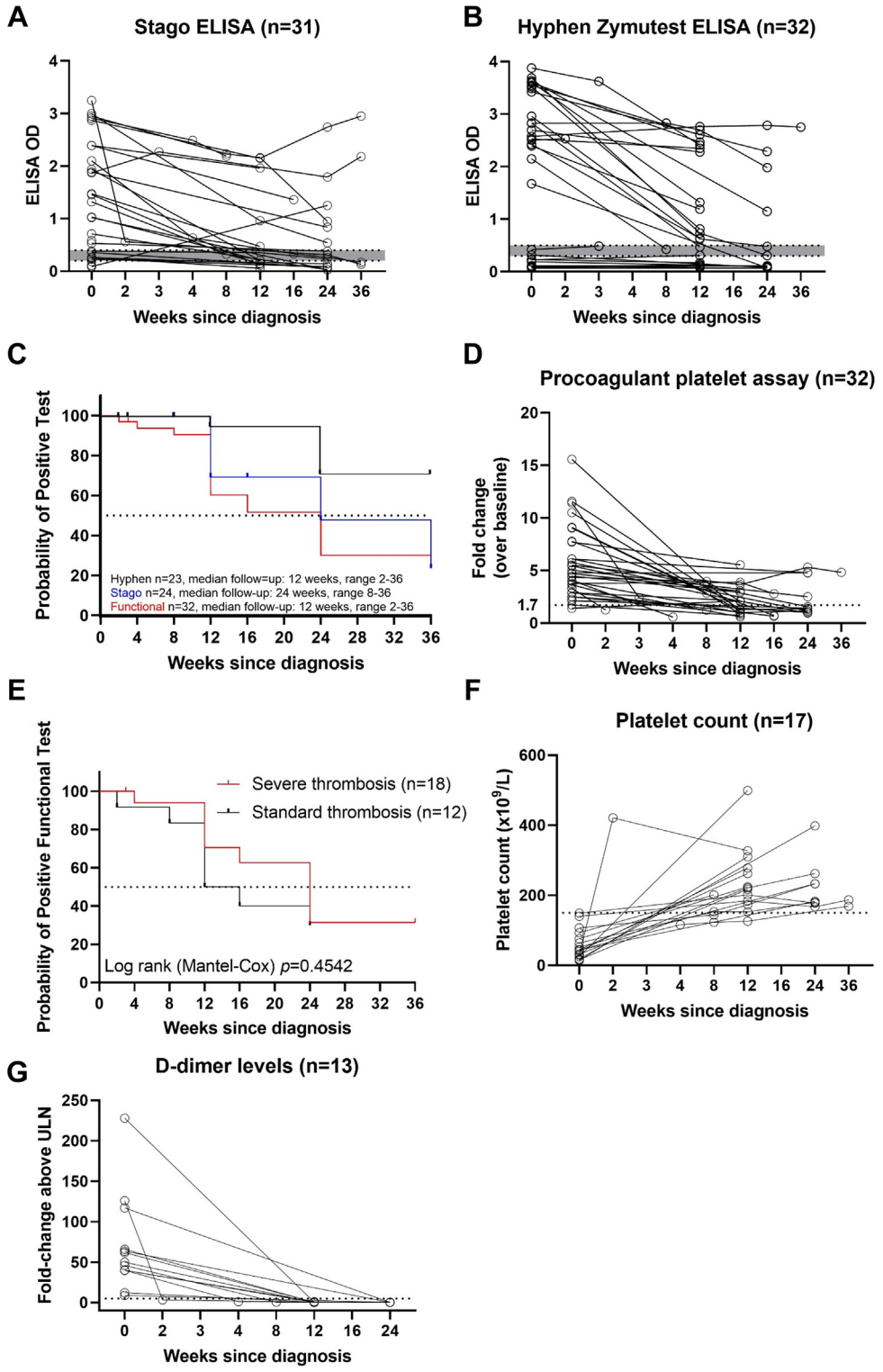

FIGURE 1 The rate of decline of anti-PF4 antibodies in 32 patients with VITT with follow-up samples depends on the testing platform used. Anti-PF4 antibodies in confirmed VITT patient plasma or serum were measured over time by (A) Asserachrom HPIA IgG (Diagnostica Stago) and (B) Zymutest HIA IgG (Hyphen Biomed). One Stago ELISA-negative VITT patient was excluded from panel A as their OD value was not available. VITT was confirmed by clinical adjudication by members of the THANZ VITT advisory group. Shaded region denotes manufacturer's cutoff values. (C) Kaplan–Meier curve showing the proportion of patients with VITT testing positive on Hyphen ELISA (black line), Stago ELISA (blue line), and either procoagulant platelet flow cytometry and/or serotonin-release functional assays (red line) over time. Horizontal dotted line shows median time to resolution. (D) Platelet-activating antibodies were detected by treating healthy donor whole blood with VITT patient plasma in the presence of 5 μM platelet agonist (SFLLRN) and assessed for procoagulant platelet formation. A greater than 1.7-fold increase in procoagulant response relative to no-plasma baseline control indicates a positive result. (E) Kaplan–Meier curve of proportion of patients with VITT with severe (cerebral venous sinus, splanchnic, portal, arterial, central vessel, and multiple sites thromboses) or standard thrombosis (deep vein thrombosis and pulmonary embolism) returning a positive functional test over time. Temporal profiles of (F) platelet count and (G) D-dimer levels (fold-change above upper limit of normal, ULN) in patients with VITT where follow-up data are available. Each line in panels A, B, D, F, and G represents a unique patient with VITT.

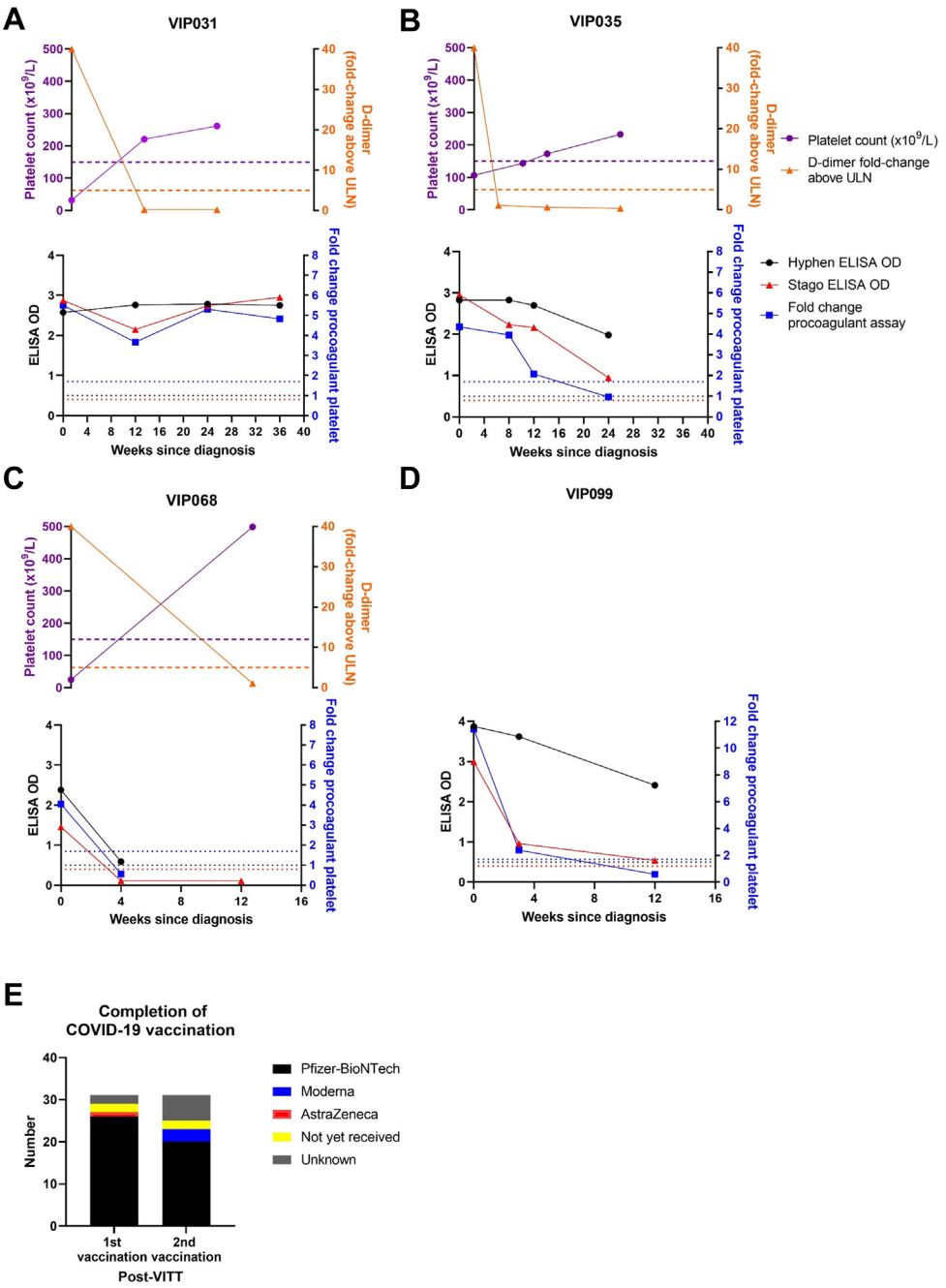

FIGURE 2 Temporal profiles of selected individual patients with VITT showing that Stago but not Hyphen ELISA OD closely aligning with functional platelet-activation assays. Platelet count (purple), fold-change of D-dimer levels over the upper limit of normal (orange), Hyphen ELISA OD (black), Stago ELISA OD (red), and procoagulant platelet response (blue) measured in patients with VITT over time. Horizontal dotted lines reflect the cutoff values for each measure. Follow-up platelet count and D-dimer levels were not available for patient VIP099 (panel D). (E) Breakdown of patients with VITT who had received their subsequent COVID-19 vaccination and the type of vaccine received after VITT diagnosis. OD, optical density.

colleagues reported an earlier resolution of Hyphen assays compared with a PF4-induced platelet aggregation functional assay in a smaller cohort of 6 patients [15].

The significance of antibody persistence by immunoassay vs functional assay remains unclear. In a longitudinal study of 243 patients with HIT, anti-PF4 antibodies persisted at a median of 12 weeks by immunoassays and 7 weeks by functional assays [5]. Similar to other reports, we found that VITT antibody detection persisted significantly longer than historical reports of HIT [1,5]. Two longitudinal studies on VITT demonstrated that the median time to antibody resolution was 15.5 weeks by washed platelet-activation assay [16] and was not reached at 20 weeks by Immucor ELISA [2]. The antibodies detected in our VITT cases were more persistent with a median of at least 24 weeks by ELISA, with some patients remaining positive

at 36 weeks. The median time to achieve normalization of a functional assay was 16 weeks, more than twice the duration in HIT. This may suggest differences in antibody clonality and/or antibody production between HIT and VITT [16,17].

Our study confirms that meaningful interpretation of follow-up requires comparison of results from the same ELISA platform at diagnosis. However, variability in the normalization rate between assay platforms raises the question of which assay is most relevant. Discrepancies between Stago and Hyphen ELISAs may be attributed to titer and assay-specific threshold, differences in dynamic range or technical differences between platforms. Hyphen ELISA includes platelet lysate as a reagent, which may provide uncomplexed PF4 antigen with different epitopes compared with immobilized PF4-polyanion in Stago. Although it is possible that activating the anti-PF4 antibody declines in Hyphen positive/Stago-negative follow-up samples, whereas persistent antibodies against a non-PF4 platelet lysate chemokine remain detectable in the Hyphen assay, this is unlikely to be the full explanation, given VITT antibodies, unlike HIT antibodies, appear to be monoclonal and stereotypic [16,18]. These questions can be addressed by isolating antibodies from patient samples; however, plasma dilution and antibody isolation were not performed due to limited sample availability. Of interest, although the increased sensitivity of Hyphen ELISA might be considered advantageous, if detectable platelet-activating antibodies are more relevant to disease and relapse risk during anticoagulation cessation, the Stago platform may be more relevant during the follow-up period. Stago ELISA demonstrated greater concordance with functional assays during follow-up and might be preferred when functional tests are unavailable. These results highlight the heterogeneity of VITT antibodies. We caution against calling patients "negative" based on a single platform noting that 6 patients in our report did not have anti-PF4 detectable by either ELISA at diagnosis but demonstrated PF4-dependent platelet activation on functional assays. We also previously reported that up to one-third of patients with VITT showing platelet activation may be ELISA negative [6,12].

In total, 27 (87%) and 23 (74%) of 31 patients with VITT received their second and third COVID-19 vaccines, respectively. For second vaccination, 26 patients received messenger RNA (mRNA) vaccine BNT162b2 (Pfizer–BioNTech). One individual received ChAdOx1 nCoV-19 (Vaxzevria, AstraZeneca). This individual was already anticoagulated for stroke prevention with atrial fibrillation, demonstrated thrombocytopenia without thrombosis with the first dose and no adverse effects with the second dose (Figure 2E). For third vaccination, 20 patients received BNT162b2 and 3 received mRNA-1273 (Spikevax, Moderna). No adverse events to revaccination or episodes of relapse were reported despite the persistence of anti-PF4 antibodies in most patients (Figure 1C). Our observation is consistent with other reports on the safety of subsequent COVID-19 vaccination in confirmed patients with VITT [1,19,20].

This study was limited to samples from confirmed patients with VITT who had been referred for follow-up VITT testing between July 2021 and April 2022 and, therefore, did not capture the temporal profile of all confirmed patients with VITT in Australia. The data, thus, may be subjected to bias, such as severity of initial presentation and access to tertiary care follow-up. It is also possible that ELISA-negative individuals at diagnosis have an antibody directed against a non-PF4 chemokine. Ethnicity data were incomplete and was therefore not reported in this study.

In summary, within this subset of the Australian cohort, anti-PF4 antibodies persist longer than functional platelet-activating antibodies in VITT but did not appear to be associated with cerebral/splanchnic thrombosis at presentation, recurrent thrombocytopenia, or to warrant the avoidance of subsequent vaccinations. We confirm that detection of persistent antibody is assay-dependent, with Stago ELISA closely aligning with platelet activation. This is the first demonstration of a particular ELISA OD closely aligning with functional assay resolution. Thus, although Stago ELISA only detects 66% of platelet-activating antibodies at diagnosis [6], in cases that are initially positive, Stago ELISA may be a surrogate where functional assays are unavailable. Whether the decline in platelet-activating antibodies allows withdrawal of anticoagulation in these patients warrants further investigation but could be informative.

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ETHICS STATEMENT

Blood collection from healthy volunteers and comparison of assays were approved by the Sydney Local Health District Human Research Ethics Committees (HREC/18/CRGH/294, X21-0160, 2021/ETH00945, and 2021/ETH11929). Healthy blood donors for functional assays gave written informed consent.

AUTHOR CONTRIBUTIONS

C.S.M.L. designed and performed the experiments, analyzed the data, and wrote the manuscript. G.W.K. provided the ELISA results. T.B. provided the serotonin-release assay data. L.C., I.T-E., S.C., E.J.F., and H.T. collected clinical and ELISA information. V.M.C. designed and supervised the study, collected clinical information, analyzed data, and wrote the manuscript. All authors helped revise the manuscript and approved its submission.

RELATIONSHIP DISCLOSURE

V.M.C. holds a US patent "Selective targeting of procoagulant platelets" US15/521435. C.S.M.L. and V.M.C. hold International (PCT) Patent Application No. PCT/AU2021/051233 for "Identification of

prothrombotic conditions." All the authors declare no competing financial interests.

DATA AVAILABILITY

Data will be made available upon reasonable request.

TWITTER

Christine S.M. Lee @christine_lsm

Lisa J. Clarke @DrLisaJClarke

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