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BRIEF REPORT

Fibrinogenolysis and fibrinolysis in vaccine-induced immune thrombocytopenia and thrombosis

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Abstract

Background: Vaccine-induced immune thrombocytopenia and thrombosis (VITT) is a rare syndrome associated with adenoviral vector vaccines for COVID-19. The syndrome is characterized by thrombosis, anti-platelet factor 4 (PF4) antibodies, thrombocytopenia, high D-dimer, and hypofibrinogenemia.

Objectives: To investigate abnormalities in fibrinolysis that contribute to the clinical features of VITT.

Methods: Plasma samples from 18 suspected VITT cases were tested for anti-PF4 by ELISA and characterized as meeting criteria for VITT (11/18) or deemed unlikely (7/18; non-VITT). Antigen levels of PAI-1, factor XIII (FXIII), plasmin-$\alpha_2$antiplasmin (PAP), and inflammatory markers were quantified. Plasmin generation was quantified by chromogenic substrate. Western blotting was performed with antibodies to fibrinogen, FXIII-A, and plasminogen.

Results: VITT patients 10/11 had scores indicative of overt disseminated intravascular coagulation, while 0/7 non-VITT patients met the criteria. VITT patients had significantly higher levels of inflammatory markers, IL-1β, IL-6, IL-8, TNFα, and C-reactive protein. In VITT patients, both fibrinogen and FXIII levels were significantly lower, while PAP and tPA-mediated plasmin generation were higher compared to non-VITT patients. Evidence of fibrinogenolysis was observed in 9/11 VITT patients but not in non-VITT patients or healthy controls. Fibrinogen degradation products were apparent, with obvious cleavage of the fibrinogen α-chain. PAP complex was evident in those VITT patients with fibrinogenolysis, but not in non-VITT patients or healthy donors.

Conclusion: VITT patients show evidence of overt disseminated intravascular coagulation and fibrinogenolysis, mediated by dysregulated plasmin generation, as evidenced

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by increased PAP and plasmin generation. These observations are consistent with the clinical presentation of both thrombosis and bleeding in VITT.

KEYWORDS
fibrinogenolysis, fibrinolysis, plasmin, thrombosis, vaccine

1 | INTRODUCTION

Vaccine-induced immune thrombocytopenia and thrombosis (VITT) is a rare syndrome first identified in March 2021 by groups in the United Kingdom, Norway, and Germany [1–3]. Clinical features include the development of catastrophic thrombosis accompanied by thrombocytopenia in previously healthy individuals following vaccination with the Astra Zeneca (AZ) ChAdOx1 nCoV-19 vaccine [1–3]. Subsequently, cases were also reported with the Johnson & Johnson adenoviral vector-based vaccine [4]. Thrombosis may present at any site, however, the majority of VITT cases presented with thrombosis in unusual sites, such as cerebral sinus vein thrombosis (CVST) and splenic vein thrombosis. In addition to thrombosis and thrombocytopenia, patients presented with high D-dimer levels and hypofibrinogenemia [1–3,5]. Pathological antibodies to platelet factor 4 (PF4) were identified in early reports [1] and a positive ELISA for these antibodies later formed part of the case definition criteria for VITT [5,6].

The clinical presentation of thrombocytopenia and thrombosis combined with the presence of circulating anti-PF4 antibodies in VITT led to early parallels being drawn with heparin-induced thrombocytopenia (HIT) [1,3,5–11]. HIT is mediated by antibodies directed against heparin–PF4 complexes which subsequently crosslink the FcγRIIa receptors on platelets inducing activation, degranulation, and aggregation. In VITT, the pathogenic antibodies of IgG class bind to the heparin-binding site on PF4, forming IgG–PF4 immune complexes that trigger platelet activation via the platelet FcγIIa receptors [7]. In addition, they trigger Fcγ receptors on other immune cells, including neutrophils, stimulating NETosis and the formation of platelet-neutrophil aggregates, as well as downstream thrombotic complications [12–14].

The levels of D-dimer seen in patients with VITT are elevated well beyond those observed in isolated venous thromboembolism. The presence of D-dimer serves as a marker of both thrombin and plasmin activity, as it reflects degradation of cross-linked fibrin. It was noted early in the history of VITT that many patients developed massive intracranial hemorrhage, particularly in the context of CVST, which was associated with increased mortality [5–7]. Given these 2 lines of evidence, we hypothesized that VITT is also associated with activation and/or dysregulation of the fibrinolytic system. Our data show for the first time that patients with VITT exhibit excessive plasmin generation, evidenced by plasmin-$\alpha_2$antiplasmin (PAP) complex. This results in fibrinogenolysis and loss of factor XIII (FXIII), which circulates in complex with fibrinogen [15] and is also a substrate for plasmin [16]. These data may help explain the clinical presentation of both thrombosis and bleeding in VITT patients.

Essentials

- Vaccine-induced immune thrombocytopenia and thrombosis (VITT) is a complex syndrome.
- This study investigates whether abnormalities in fibrinolysis contribute to the features of VITT.
- VITT is associated with disseminated intravascular coagulation, fibrinogenolysis, and elevated plasmin.
- These observations are consistent with the presentation of both thrombosis and bleeding in VITT.

2 | METHODS

The cohort consisted of patients in Scotland suspected of having VITT between April and June 2021. All patients in whom this clinical suspicion arose had routine hemostatic measurements taken at presentation, and samples were sent to Aberdeen Royal Infirmary to test for anti-PF4 antibodies. PF4 testing was performed at initial clinical presentation and further analysis carried out retrospectively, having obtained Caldicott approval and ethical approval from NHS Health Research Authority London-Stanmore Research Ethics Committee (IRAS 300610). Based on the PF4 results, platelet count, D-dimer level, vaccination schedule, and the presence of clinical thrombosis, patients were categorized as having VITT or non-VITT, as detailed in Pavord et al. [5]. In addition, cohort members were assessed for disseminated intravascular coagulation (DIC) according to the International Society on Thrombosis and Haemostasis guidelines [17]. Healthy donor plasma samples were obtained with ethical approval obtained from the University of Aberdeen College Ethical Review Board (CERB/2017/9/1411).

2.1 | Protein quantification

IL-1β, IL-6, IL-8, TNFα, C-reactive protein (CRP), total plasminogen activator inhibitor-1 (PAI-1), FXIII, and PAP complex levels were measured in plasma using the Simple Plex ELLA automated system or commercially available ELISA as per the manufacturer's instructions (Bio-Techne, Abcam, and Technoclone, respectively).

2.2 | Western blotting

Western blotting was performed on patient and healthy donor plasma as previously described [18] with purified fibrinogen and plasminogen

TABLE Clinical characteristics of VITT and non-VITT patients. Presenting clinical data from VITT and non-VITT patients was analyzed by Mann–Whitney *t*-test.

Clinical characteristics		VITT patients (*n* = 11)	Non-VITT patients (*n* = 7)
Age (y)	All	50 (43-56)	60 (51-68)
	Female	50 (48-54)	61 (35.25-66.5)
	Male	48.5 (39.5-62)	59 (51-87)
Days postvaccination to symptom onset		12 (9-15)	15.5 (6-37.25)[a]
Deaths		2/11 (18.2 %)	2/7 (28.6%)
Platelet count ($\times 10^9$/L)		41 (37-49)[b]	142 (99-312)
Fibrinogen (g/L) Clauss assay		1.7 (1.5-2)[b]	3.2 (2.2-3.7)
D-dimer (ng/mL)		18000 (6905-39578)[a,b]	1700 (551-6054)[a]
PT (s)		13 (12.5-14.5)[a]	12.1 (10-14)
APTT (s)		31 (25.5-33.5)[a]	30 (26–31)
Thrombosis sites		Acute aortic thrombosis (*n* = 1)	CVST (*n* = 1)
		Cortical vein thrombosis (*n* = 1)	DVT (*n* = 2)
		CVST (*n* = 3)	PE (*n* = 1)
		MCA stroke (*n* = 1)	
		MI and DVT (*n* = 1)	
		Multiple PEs, PVT, SMV thrombosis and splenic vein thrombosis, (*n* = 1)	
		PE (*n* = 1)	
		MI and PE (*n* = 1)	
		Superficial thrombophlebitis (*n* = 1)	

APTT, activated partial thromboplastin time; PT, prothrombin time. Thrombosis was detected at various sites, as detailed: CVST, cerebral venous sinus thrombosis; DVT, deep vein thrombosis; MCA, middle cerebral artery; MI, myocardial infarction; PE, pulmonary embolism; PVT, portal vein thrombosis; SMV, superior mesenteric vein.

[a] Indicates missing data.

[b] Denotes significant differences between the VITT and non-VITT cohorts. Data presented as median ± interquartile range.

from Enzyme Research Laboratories, FXIII (Fibrogammin, CSL Behring) and Fragment D (Merck). Fibrinogen and plasminogen were detected using sheep and goat polyclonal peroxidase-conjugated antibodies (Enzyme Research Laboratories), respectively. Fibrinogen was analyzed under non-reducing and reducing conditions. Fibrinogen α-chain and FXIII-A subunit were detected using rabbit polyclonal antibodies (Abcam and Invitrogen respectively) and horseradish peroxidase-conjugated polyclonal swine anti-rabbit IgG (Dako).

2.3 | Activity measurements

Plasmin generation in plasma (10%) was measured using 0.5 mM S-2251 (Chromogenix) ± 10 nM tPA (Alteplase) and cyanogen bromide fibrinogen fragments (10 μg/ml; Technoclone). Absorbance readings at 405 nm were taken every 30 seconds for 8 hours at 37 °C on a Biotek Flx800 microplate reader. Neutrophil elastase activity was measured in plasma using a fluorometric assay as per manufacturer's instructions (Merck).

2.4 | Data analysis

Statistical analysis was completed using GraphPad Prism (version 9.4.1). Data was analyzed using either a Mann–Whitney *U*-test or a Kruskal–Wallis test with Dunn's multiple comparisons, and presented as median ± interquartile range. Plasmin generation rates were calculated using; Longstaff C, 2016, Shiny App for calculating zymogen activation rates, version 0.6, (https://drclongstaff.shinyapps.io/zymogenactnCL/) and analyzed by a Kruskal–Wallis test.

3 | RESULTS AND DISCUSSION

All 18 cohort members with a potential VITT diagnosis were screened on presentation for routine hematological parameters (Table) and were subsequently tested for anti-PF4 antibodies. Eleven patients from the suspected cases were anti-PF4 positive and classified as having VITT, according to the criteria described by Pavord et al. [5]. Seven patients, all anti-PF4 negative, were deemed unlikely and

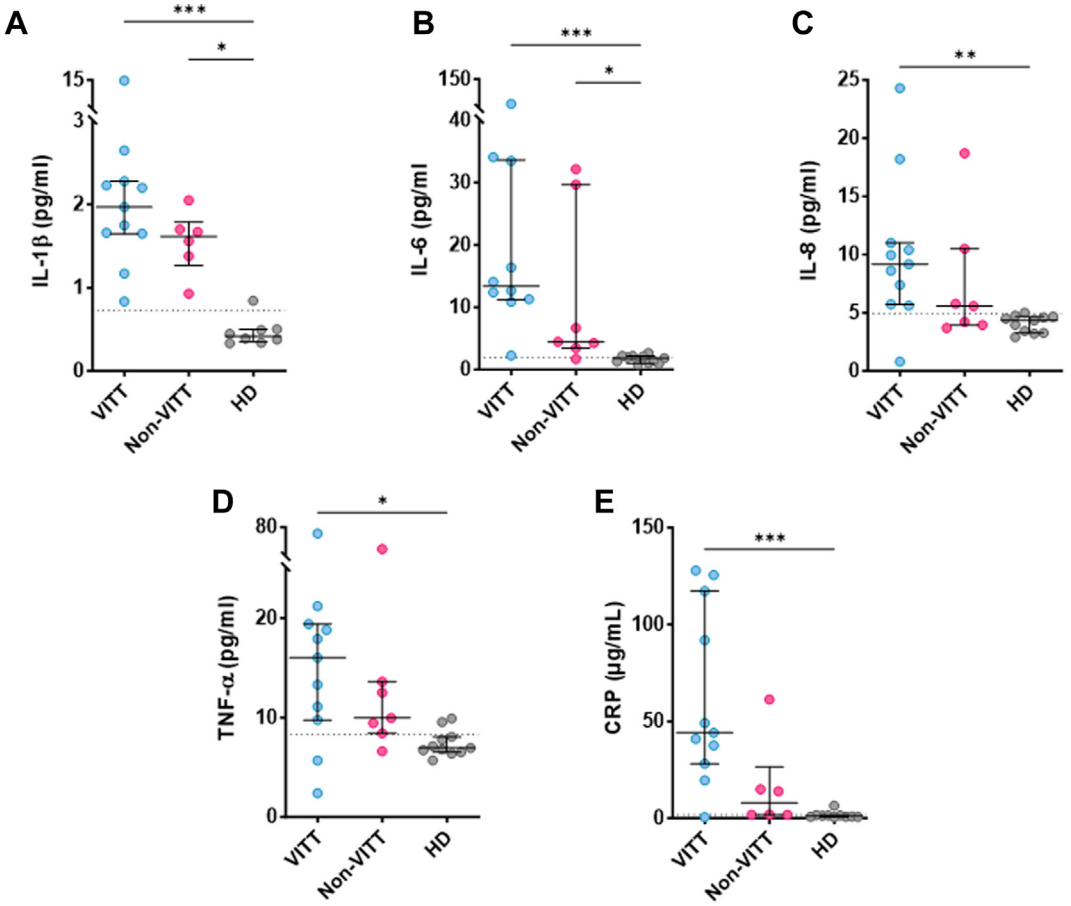

FIGURE 1 Inflammatory markers are elevated in VITT and non-VITT patients. Inflammatory markers were measured in VITT patient, non-VITT patient, and healthy donor (HD) plasma by Simple Plex ELLA automated assay system or ELISA. IL-1β (A), IL-6 (B), IL-8 (C), TNF-α (D), and C-reactive protein (E). Dotted lines indicate pooled normal plasma antigen concentrations. Data are shown as median ± interquartile range. * $P < .05$, ** $P < .01$, *** $P < .001$. VITT, vaccine-induced immune thrombocytopenia and thrombosis.

termed non-VITT (Table). The VITT patient cohort consisted of 7 females and 4 males, with a median age of 50 years. All patients presented 6 to 14 days post primary vaccination with the AZ ChAdOx1 nCov-19 vaccine. The nonVITT cohort consisted of 4 females and 3 males with a median age of 60 years who presented 6 to 59 days post vaccination with the AZ ChAdOx1 nCov-19 vaccine.

Patients with VITT presented with thrombocytopenia (<150 × 10^9/L platelets), while platelet counts in the non-VITT cohort were within the normal range ($P < .001$; Table). Elevated D-dimer levels were evident in both patient groups but were significantly higher in the VITT compared to the non-VITT cohort ($P = .01$; Table). Fibrinogen was significantly lower in patients with VITT than in the non-VITT patients ($P < .05$; Table). A range of sites of thrombosis, both arterial and venous were described in the VITT cohort (Table) while in the non-VITT cohort only 4 patients had a known thrombotic event. The majority of VITT patients (10/11) had scores indicative of overt DIC [17], which was not a feature of non-VITT patients (0/7).

In line with VITT being an inflammatory condition, plasma levels of IL-1β ($P < .001$), IL-6 ($P < .001$), IL-8 ($P < .01$), TNFα ($P < .05$), and CRP ($P < .001$) were significantly elevated in patients with VITT compared with healthy donors (Figure 1), as previously reported [7,12]. There are several reports of neutrophil activation in patients with VITT [12–14], but we were unable to detect active neutrophil elastase (HNE) in either VITT or non-VITT patient plasma.

The transglutaminase, FXIII, circulates in plasma in complex with fibrinogen [15]. Given the consumption of fibrinogen in the VITT cohort, we hypothesized that FXIII would also be reduced. Indeed, we found significantly decreased levels of FXIII in VITT patients compared with non-VITT patients ($P < .05$) and healthy donors ($P < .001$; Figure 2A). The serpin PAI-1, the principal inhibitor of tPA, is an acute phase protein that is frequently elevated in inflammatory states [19]. Surprisingly, despite the increased levels of proinflammatory cytokines (Figure 1), we found no significant difference in PAI-1 between the VITT and non-VITT groups (Figure 2B). We then looked for evidence of fibrinolysis by quantifying PAP complex and found it to be significantly elevated in VITT patients versus non-VITT patients ($P < .05$) and healthy donors ($P < .01$; Figure 2C). Analysis of tPA-mediated plasmin generation revealed higher levels of activity in VITT compared with healthy donors ($P < .05$, Figure 2D).

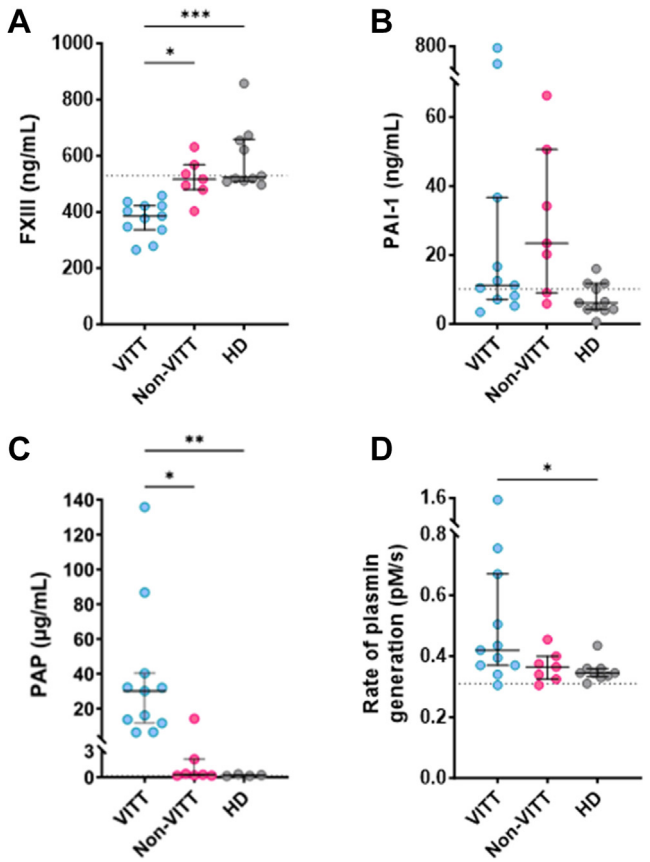

FIGURE 2 Patients with VITT exhibit reduced levels of factor XIII (FXIII) alongside increased levels of plasmin-α_2-antiplasmin and plasmin generation. Fibrinolytic parameters were quantified in VITT patient, non-VITT patient, and healthy donor (HD) plasma samples by Simple Plex ELLA automated assay system or ELISA; FXIII (A), plasminogen activator inhibitor-1 (PAI-1) (B), and plasmin-α2-antiplasmin (PAP) (C). Plasmin generation (D) was determined using a chromogenic substrate. Rates of plasmin generation (pM/s) were calculated and presented as median ± interquartile range. Dotted line indicates levels of antigen and rate of plasmin generation in pooled normal plasma. * $P < .05$, ** $P < .01$, *** $P < .001$. VITT, vaccine-induced immune thrombocytopenia and thrombosis.

Inflammatory stimuli can modulate the fibrinolytic response, but interestingly there was no association between fibrinolytic markers and CRP or TNFα in VITT patient plasma. However, we found that PAP and PAI-1 significantly correlate with IL-8 ($r = 0.78$, $P < .01$; $r = 0.7$, $P < .05$, respectively) and IL-1β ($r = 0.82$, $P < .01$; $r = 0.95$, $P < .001$, respectively). A correlation also existed between PAI-1 and IL-6 ($r = 0.7$, $P < .05$). No correlation existed with the levels of FXIII or fibrinogen with the inflammatory markers measured. Not surprisingly, a strong correlation existed between the levels of PAP and plasmin generation ($r = 0.89$, $P < .001$) in VITT patient plasma. A possible explanation for this increase in plasmin generation may relate to differences in systemic PAI-1. We were unable to detect this by quantifying total antigen and if sample permitted it would have been beneficial to measure PAI-1 activity. Further studies are required to explore the relationship between PAI-1 and the inflammatory state in VITT. Quantifying systemic levels of this serpin in VITT is confounded by the fact that the circulating platelet-derived pool will be diminished, due to the severe thrombocytopenia, but that levels of endothelial-derived PAI-1 are likely to be elevated due to the inflammatory response [19].

Given the reduced circulating levels of fibrinogen and increase in plasmin generation, we analyzed fibrin(ogen) in further detail by western blotting. Evidence of fibrinogenolysis was observed in 9/11 VITT patients but was not detected in the non-VITT, or healthy donor samples (Figure 3A–B). The size of one of the fibrinogen degradation products was consistent with fragment D (data not shown), indicative of plasmin-mediated cleavage of fibrinogen [5,20]. Western blotting for individual fibrinogen chains showed degradation of the α-chain in VITT patient plasma (Figure 3C), consistent with its strong susceptibility to cleavage by plasmin [7,21]. There was no obvious degradation of the fibrinogen β- and γ- chains (data not shown). In line with the ELISA data, there was a reduction in the FXIII-A subunit in VITT patient plasma compared with controls (Figure 3D). Evidence of PAP was found in the same 9/11 VITT patients with fibrinogenolysis (Figure 3E). The presence of PAP, as detected by both ELISA and western blotting, is evidence of excessive plasmin generation in VITT patients.

These results demonstrate that patients with VITT have severe DIC with prominent evidence of fibrinolysis and fibrinogenolysis. This pattern of findings in DIC is typically seen in association with high levels of PAP complexes, as evidenced herein [22]. Fibrinogenolysis is not typically a feature of sepsis; however, it is observed in acute promyelocytic leukemia and certain carcinomas, such as prostate cancer, where it is often associated with pathologic bleeding, including intracranial and interalveolar hemorrhage, in addition to thrombosis [23]. The early description of VITT in the United Kingdom described a high incidence of intracranial hemorrhage in VITT patients with CVST, which correlated with increased mortality [5]. Indeed, hemorrhage has been described in various cohorts of patients with VITT [1–3,24]. Soluble fibrin degradation products are able to stimulate tPA-mediated fibrinogenolysis [25], which is a complication associated with therapeutic thrombolysis [26]. In addition to the low platelet counts in VITT, excessive plasmin generation, due to the presence of circulating fibrin degradation products to support systemic tPA-mediated activation, as well as proteolysis of fibrinogen and FXIII may contribute to the clinical observations of bleeding.

Comparisons have been made between the pathogenesis of HIT and VITT for obvious reasons [3,5–10]. However, unlike HIT, VITT appears to be frequently complicated by DIC and additional hyperfibrinolytic features, as reported here, which give rise to bleeding complications including intracranial hemorrhage [1–3,5,24]. Interestingly, there have been a few reports suggesting that bleeding is more common in HIT then generally recognized [27,28]. Autoimmune HIT (aHIT) is a rare form of the syndrome that occurs in the absence of any heparin stimulus [29]. Sera levels of anti-PF4 antibodies in aHIT are extremely high, but platelet activation properties are heparin independent. Features of aHIT include significantly elevated D-dimer

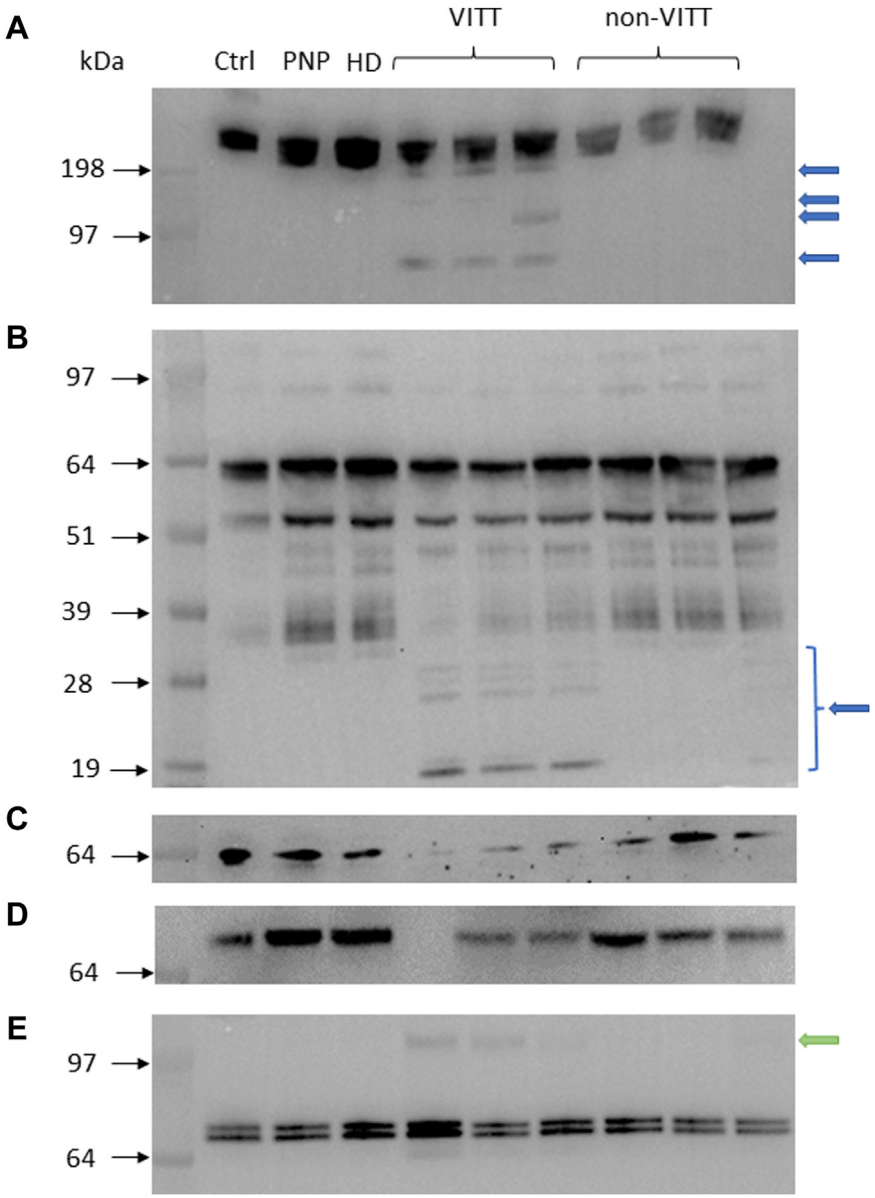

FIGURE 3 Patients with VITT show evidence of fibrinogenolysis and plasmin-α_2-antiplasmin complex. VITT, non-VITT, healthy donor (HD) plasma samples, pooled normal plasma (PNP), and control protein (ctrl) (fibrinogen, FXIII, or plasminogen) were separated and immunoblotted with appropriate antibodies. Representative blots of fibrinogen under non-reducing conditions (A) and reducing conditions (B) are shown. Samples were also analyzed for fibrinogen α-chain (C), FXIII-A subunit (D), and plasminogen (E). Blue arrows indicate fibrinogen degradation products, and the green arrow denotes plasmin-α2-antiplasmin complex. VITT, vaccine-induced immune thrombocytopenia and thrombosis.

levels and hypofibrinogenemia, along with evidence of overt DIC [29]. Interestingly, a recent report described a VITT-like disorder triggered by a symptomatic adenovirus infection [30]. This VITT-like condition also featured marked hypofibrinogenemia, greatly elevated D-dimer levels and evidence of overt DIC [30]. Clearly additional studies are required to define these complex syndromes; however, these data highlight striking similarities between aHIT, VITT, and adenoviral-induced disorder, suggesting that similar immunoregulatory mechanisms exist in these syndromes.

The presence of fibrinogenolysis and fibrinolysis combined with the critically low levels of platelets in VITT patients may contribute to the clinical observations of intracranial hemorrhage, which was associated with excess mortality in patients with CVST [5]. A criticism of the study would be the size of the cohort and the restricted sample availability limiting some tests. Nonetheless, these data clearly highlight that overt DIC, fibrinogenolysis and loss of FXIII are features of VITT that complicate this rare condition and likely contribute to disease progression. In conclusion, in addition to the thrombocytopenic and prothrombotic complications in VITT, we show for the first time that there is an associated hyperfibrinolytic state, driven by systemic activation of plasmin, and consumption of fibrinogen and FXIII.

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AUTHOR CONTRIBUTIONS

M.S. designed, executed, and analyzed the research, and wrote/edited the manuscript. A.N. and E.W. executed and analyzed the research and reviewed the manuscript. C.N.B. and A.R.P. collected samples and clinical data and reviewed the manuscript. J.M. analyzed the samples. H.G.W., C.S.W., and N.J.M. designed the research, analyzed clinical and laboratory data and wrote/edited the manuscript.

DECLARATION OF COMPETING INTERESTS

The authors have no relevant conflict of interests to declare.

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