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RESEARCH ARTICLE

Persistence of Ad26.COV2.S-associated vaccine-induced immune thrombotic thrombocytopenia (VITT) and specific detection of VITT antibodies

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Abstract

Rare cases of COVID-19 vaccinated individuals develop anti-platelet factor 4 (PF4) antibodies that cause thrombocytopenia and thrombotic complications, a syndrome referred to as vaccine-induced immune thrombotic thrombocytopenia (VITT). Currently, information on the characteristics and persistence of anti-PF4 antibodies that cause VITT after Ad26.COV2.S vaccination is limited, and available diagnostic assays fail to differentiate Ad26.COV2.S and ChAdOx1 nCoV-19-associated VITT from similar clinical disorders, namely heparin-induced thrombocytopenia (HIT) and spontaneous HIT. Here we demonstrate that while Ad26.COV2.S-associated VITT patients are uniformly strongly positive in PF4-polyanion enzyme-linked immunosorbent assays (ELISAs); they are frequently negative in the serotonin release assay (SRA). The PF4-dependent p-selectin expression assay (PEA) that uses platelets treated with PF4 rather than heparin consistently diagnosed Ad26.COV2.S-associated VITT. Most Ad26.COV2.S-associated VITT antibodies persisted for >5 months in PF4-polyanion ELISAs, while the PEA became negative earlier. Two patients had otherwise unexplained mild persistent thrombocytopenia

(140-150 x 10^3/μL) 6 months after acute presentation. From an epidemiological perspective, differentiating VITT from spontaneous HIT, another entity that develops in the absence of proximate heparin exposure, and HIT is important, but currently available PF4-polyanion ELISAs and functional assay are non-specific and detect all three conditions. Here, we report that a novel un-complexed PF4 ELISA specifically differentiates VITT, secondary to both Ad26.COV2.S and ChAdOx1 nCoV-19, from both spontaneous HIT, HIT and commonly-encountered HIT-suspected patients who are PF4/polyanion ELISA-positive but negative in functional assays. In summary, Ad26.COV2.S-associated VITT antibodies are persistent, and the un-complexed PF4 ELISA appears to be both sensitive and specific for VITT diagnosis.

1 | INTRODUCTION

Recipients of the Ad26.COV2.S (Janssen, Johnson & Johnson),[1–7] and ChAdOx1 nCoV-19 (AstraZeneca)[8–11] have both been described to develop a rare and potentially life-threatening syndrome, characterized by thrombosis and thrombocytopenia caused by platelet-activating anti-PF4 antibodies. The clinical syndrome appears similar following both vaccines and is recognized as vaccine-induced immune thrombotic thrombocytopenia (VITT). However, whether differences may exist in the nature of the antibodies or clinical manifestations following each individual type of vaccination is unclear. Here use the terms Ad26.COV2.S-associated VITT and ChAdOx1 nCoV-19-associated VITT to distinguish between the two.

Studies on ChAdOx1 nCoV-19-associated VITT patients have demonstrated that the causative antibodies strongly recognize PF4-polyanion complexes[8–10] and require PF4-treated platelets (as opposed to heparin-treated platelets) for consistent detection by functional assays.[11] Much less is known about the characteristics of Ad26.COV2.S-associated VITT antibodies, including the duration of antibody persistence, information that would help define clinical and laboratory monitoring protocols in these patients. AD26.COV2.S-associated VITT is rare, with an estimated incidence of 3.55 individuals per million vaccinated,[6] and is difficult to distinguish from another rare anti-PF4 antibody-mediated syndrome, spontaneous HIT. Spontaneous HIT, like VITT, is seen in the absence of proximate heparin exposure and has been referred to as the "background rate" of anti-PF4 antibody-mediated thrombotic thrombocytopenia in recent publications from the CDC.[6] Currently-available diagnostic assays fail to distinguish between VITT and spontaneous HIT (and "classical" HIT), as patients with these disorders have antibodies detectable by both HIT ELISAs (that have PF4-polyanion targets) and functional assays.[8,12,13] Furthermore, VITT and spontaneous HIT are clinically similar, characterized by severe thrombocytopenia and thrombosis, including in uncommon locations such as the cerebral venous sinus and splanchnic vasculature.[14,15] In this study, we sought to assess VITT antibody persistence using currently available functional and antigen-based assays and to develop new laboratory tools for the specific detection of VITT that would allow its differentiation from classical and spontaneous HIT.

2 | METHODS

2.1 | Patient samples and clinical course

Blood samples were obtained from nine patients with VITT after Ad26.COV2.S vaccination, ChAdOx1 nCoV-19-associated VITT ($n = 1$), classical HIT ($n = 8$), delayed-onset HIT ($n = 3$), spontaneous HIT ($n = 2$) and "false-positive" HIT (PF4-polyanion ELISA+/PEA-) patients ($n = 7$). One patient was from Germany, while the remainder were from the US. Clinical and laboratory information was extracted from patient medical records. Recurrence of thrombocytopenia was deemed to have occurred if the patient's platelet count decreased below 150 x 10^3/μL after having attained a prior count at or above that level. Recurrence of thrombosis was defined as new thrombosis that developed after the initial hospitalization episode. Research studies were approved by the Institutional Review Board of Mayo Clinic.

2.2 | ELISA testing

2.2.1 | Un-complexed PF4 ELISA

ELISA plates (Thermo Scientific) were incubated with recombinant PF4 (Protein Foundry; 10 μg/ml), and plates were washed three times with phosphate-buffered saline pH 7.4 (PBS)/0.1% Tween-20 and blocked with Superblock T20 (Thermo Scientific). Serum or plasma samples were incubated at 1:50 dilution for 60 min followed by four washes with PBS/0.1% Tween-20. After a 45-min incubation with 50 μl of alkaline phosphatase-conjugated goat anti-human IgG Fc antibody (Jackson Immunoresearch) at a dilution of 1:5000, four additional washes were performed using PBS/0.1% Tween-20. Colorimetric detection was performed using *p*-nitrophenyl phosphate (pNPP) substrate, and the optical density, OD (405 nm minus 492 nm) at 30 min, was recorded.

2.2.2 | PF4-Polyanion ELISA

A widely-used, FDA-approved in vitro diagnostic assay that uses PF4-polyvinyl sulfonate (PVS) targets, Lifecodes PF4 IgG (Immucor), was

TABLE 1 Demographic, laboratory, and clinical features of Ad26.COV2.S-associated VITT patients

Patient No.	Age (yrs)	Age (yrs)	Sex	Timing of Symptom onset after vaccination (days)	Platelets[a] (x 10^3/μL)	D-dimer (ng/ml FEU)	Thrombotic Features	Treatment (during acute episode)	Subsequent treatment	Outcome
1	46	46	M	13	54	28 980	PE and DVT	Bivalirudin, IVIg and prednisone	Apixaban	Alive
2[b]	40	40	F	6	20	27 150	PE and CVST	Bivalirudin, IVIg and prednisone	Rivaroxaban	Alive
3	34	34	F	14	126	39 930	CVST	Bivalirudin, IVIg and prednisone	Apixaban	Alive
4[c]	48	48	F	14	13	112 073	CVST and Splanchnic vein thrombosis	Argatroban, IVIg and prednisone	IVIg, Apixaban	Alive
5	28	28	M	10	66	22 546	PE, CVST and Splanchnic vein thrombosis	Argatroban and IVIg	Apixaban	Alive
6	51	51	F	7	55	>20 000	PE and CVST	Argatroban, IVIg and prednisone	Apixaban	Alive
7[d]	48	48	M	11	99	15 100[e]	PE and DVT	Argatroban, IVIg and prednisone	IVIg, Apixaban	Alive
8	33	33	M	8	31	>10 000[f]	Splanchnic vein thrombosis	Argatroban, IVIg and dexamethasone	Apixaban	Alive
9[g]	50	50	M	13	9	5270[e]	Splanchnic vein thrombosis	Argatroban, IVIg and prednisone	IVIg, Rituximab, TPE, Warfarin	Alive

Abbreviations: CVST, cerebral venous sinus thrombosis; DDU, D-dimer units; DVT, deep venous thrombosis; FEU, fibrinogen equivalent units; IVIg, intravenous immunoglobulin G; PE, pulmonary embolism; TPE, therapeutic plasma exchange.
[a]Lower limit of the platelet reference range was 150 x 10^3/μL for all laboratories.
[b]Patients previously reported in Reference 5.
[c]Patients previously reported in Reference 1.
[d]Patients previously reported in Reference 7.
[e]D-dimer reference range was <500 ng/ml FEU except for [e](<400 ng/ml FEU) and [f](<230 ng/ml DDU).
[g]Patients previously reported in Reference 3.

used according to manufacturer instructions (package insert on file). Briefly, similar to the un-complexed PF4 ELISA, patient serum/plasma incubation with PF4-PVS coated platelets was followed by stringent washing and incubation of an alkaline phosphatase labeled anti-human IgG antibody. pNPP substrate was then added, colorimetric detection was performed after 30 min of the reaction, and OD (405–492 nm) was recorded.

2.3 | Additional laboratory studies

The PEA was performed as previously described.[16] Serotonin release assay (SRA), platelet count, and D-dimer testing were performed at various reference/hospital laboratories. Normal ranges for the Lifecodes PF4 IgG assay, SRA, and PEA were < 0.4 OD, <20% serotonin release, and <19% P-selectin expression, respectively. D-dimer reference ranges varied by laboratory (Table 1).

2.4 | Statistics

One-way ANOVA was used to compare the three groups of VITT, HIT, and ELISA+/PEA- patient samples. One patient who had a negative PEA result followed by a positive result on longer follow-up had the earlier negative result excluded from the analysis. This patient also had two negative PF4-polyanion ELISA results (one from a post-therapeutic plasma exchange sample) followed by numerous samples with positive results. These two negative results were excluded for the purpose of the Kaplan–Meier curve calculation. Time to normalization of platelet count and D-dimer was calculated as the time to a normal result that was not followed by an abnormal result at a longer follow-up time point. Kaplan–Meier curves were calculated using Turnbull's method that accounts for both right and left censoring.

3 | RESULTS

3.1 | Presentation, clinical course, and management

The Ad26.COV2.S-associated VITT patient cohort included five males and four females with a median age of 46 years (range 28–51; Table 1). Patients were followed for a median of 190 days (range 166–202)—all patients presented with thrombocytopenia and thrombosis and the median time from Ad26.COV2.S vaccination to onset of

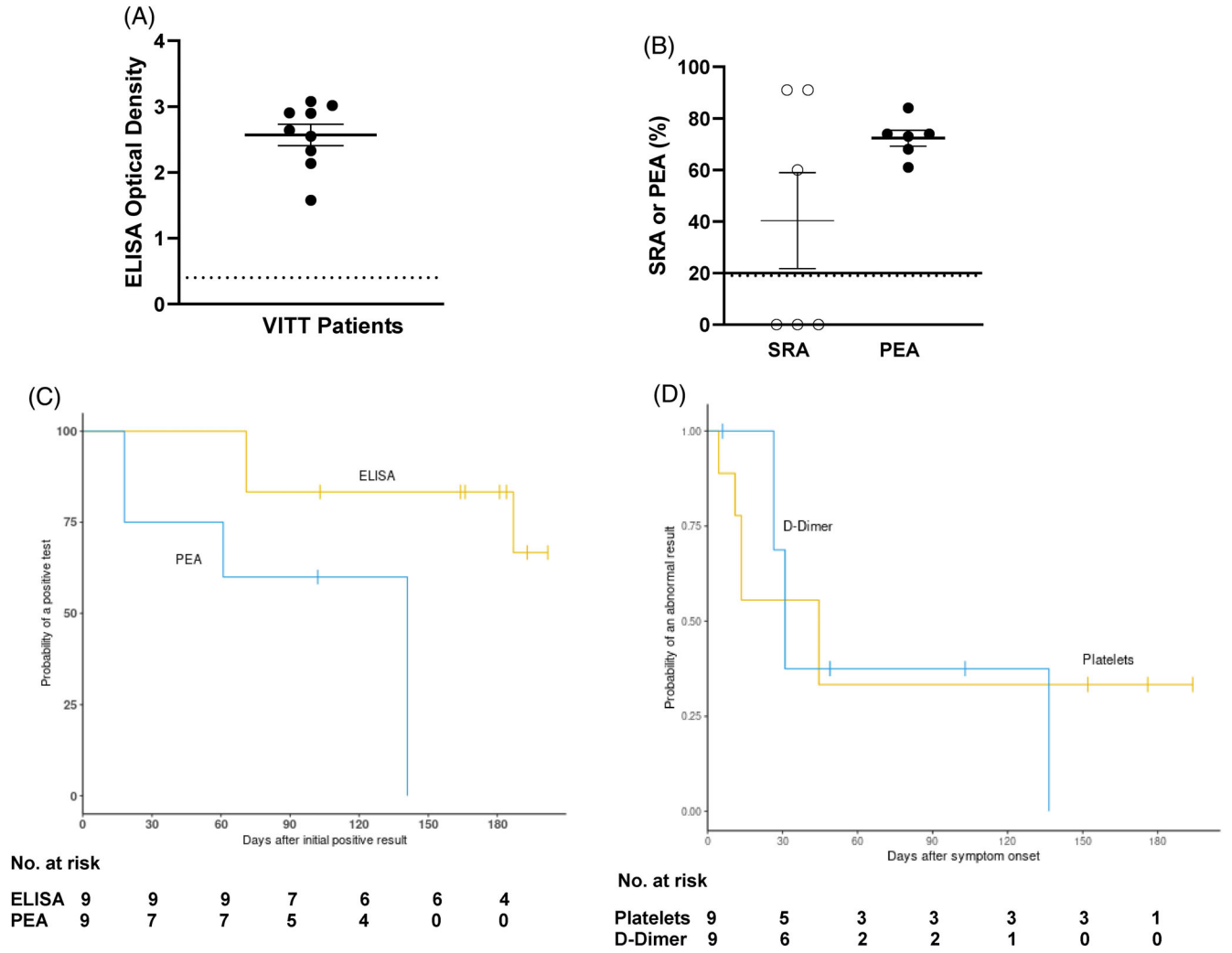

FIGURE 1 VITT antibodies are strongly ELISA positive, activate PF4-treated platelets, and are persistent. (A) PF4-Polyanion ELISA testing from nine patients upon initial presentation with VITT is presented. The Y-axis denotes Optical density, OD. Eight patients were tested in the LIFECODES PF4 IgG (IgG-specific), while one was tested in the LIFECODES PF4 Enhanced assay (detects IgG, IgA, and IgM), both FDA-approved in vitro diagnostic assays for HIT. Mean and standard error are shown. The dotted line represents the positive cut-off of the assay. (B) SRA and PEA results upon initial presentation of six VITT patients are presented. Open circles, SRA; Closed circles, PEA. Mean and standard error are shown. The solid and dotted line represents the positive cut-offs of the SRA and PEA, respectively. (C) Kaplan Meier curves for time to negative ELISA OD (<0.4 OD; yellow) and PEA (<19%; blue) are shown. Due to the small number of patients ($n = 9$), confidence intervals were wide (not shown). (D) Kaplan Meier curves for time to normalization of D-dimer (cut-off varied by testing laboratory, as shown in Table 1; blue) and platelets count (>150 x 10^3/μL; yellow) are presented. Due to the small number of patients ($n = 9$), confidence intervals were wide (not shown) [Color figure can be viewed at wileyonlinelibrary.com]

symptoms was 11 days (range 6–14 days). At presentation, the median platelet count was 54 x 10^3/μL (range, 9–126 x 10^3/μL), and D-dimer was markedly elevated in all patients (Table 1). Thrombosis at uncommon locations predominated, with cerebral venous sinus thrombosis (CVST) and splanchnic vein thrombosis seen in five and four patients, respectively (some patients had thrombosis at both sites ($n = 2$); Table 1). Five patients developed pulmonary embolism. Only two of the nine patients presented with non-CVST/splanchnic vein thromboses, with both developing pulmonary embolism and deep venous thrombosis. All patients were treated with high-dose intravenous immunoglobulin G (IVIg) and a direct thrombin inhibitor during the acute episode; eight of the nine patients also received steroids (Table 1). After recovery from the acute episode, patients received anticoagulation with direct oral anticoagulants (DOACs). Three patients experienced recurrent thrombocytopenia. Two received additional IVIg therapy–no platelet increment was seen in one patient, and the second required IVIg re-dosing for platelet normalization. In the third patient, thrombocytopenia resolved without additional intervention. Three additional patients had long-term persistence of thrombocytopenia, one of whom was treated with IVIg, rituximab, and therapeutic plasma exchange. At last follow-up, all but two patients were on DOACs (stopped at 6 and 7.5 months in patients off DOACs). No patient had a recurrence of thrombosis, and there were no fatalities.

3.2 | Presence and prevalence of anti-PF4 antibodies

All patients had strongly positive PF4-polyanion ELISA results (OD > 1.0), and eight patients demonstrated ODs > 2.0 (Figure 1A). Two patients were tested in an automated, latex enhanced immunoturbidimetric assay (HemosIL HIT-Ab[PF4-H], Instrumentation Laboratory; data not shown), and both were falsely-negative. Eight of the nine VITT patients were tested by conventional serotonin release assay (SRA) using heparin-treated platelets, with four testing positive. Available blood samples from six patients were tested in the PEA, a functional HIT assay that uses PF4 rather than heparin-treated platelets.[16] All six samples activated PF4-treated platelets in this assay, while only three were positive by the SRA (Figure 1B). Longitudinal samples from the nine VITT patients were tested in PF4-polyanion ELISA and PEA. At last follow-up, seven of the nine patients had positive ELISA results, all with OD > 1.0 (range 103–202 days; median 181 days). One of the two patients who became seronegative had received four doses of rituximab (at 375 mg/m^2) and therapeutic plasma exchange (TPE) treatment. The probability of a positive ELISA result was still high after 5 months of the acute event (Figure 1C). In contrast, eight of the nine patients were PEA-negative at their last follow-up with only one positive result (46%) 103 days after acute presentation. In contrast to ELISA, the probability of a positive PEA was very low 5 months after acute presentation (Figure 1C).

3.3 | Persistence of thrombocytopenia and D-dimer elevation

Platelets were mild-moderately decreased in three patients even after 5 months of follow-up (176, 194, and 152 days) at 141 x 10^3/μL, 149 x 10^3/μL, and 89 x 10^3/μL, respectively. One of the three patients (with a platelet count of 89 x 10^3/μL) had pre-existing thrombocytopenia secondary to hepatic cirrhosis. Thus, it was deemed unlikely that the persistence of thrombocytopenia was due to VITT antibodies. Most patients had normalized their D-dimer levels within 30 days of presentation (Figure 1D).

3.4 | Specific detection of VITT antibodies

To compare the current HIT ELISAs that employ PF4-polyanion targets and a novel un-complexed PF4 ELISA that uses PF4 in the absence of a polyanion for their abilities to distinguish between VITT, spontaneous HIT, and HIT, acute samples from six Ad26.COV2.S (5) and ChAdOx1 nCoV-19 (1)-associated VITT, two spontaneous HIT, eight "classical" HIT, and three delayed-onset HIT[17] patients were studied. Spontaneous HIT patients were heterogeneous in presentation, with one patient developing disease after knee arthroplasty and the other after several days of presumed infection characterized by nausea and fatigue. Both patients had severe outcomes due to thrombosis (above knee amputation and permanent neurological disability due to stroke, in the surgical and medical case, respectively). All three patients with delayed-onset HIT presented with heavy thrombotic burden and thrombocytopenia and needed readmission after being initially discharged after successful cardiac surgery. Table 2 presents platelet nadirs and the presence of thrombosis in these patients. The eight "classical" HIT patients had varied presentations with a platelet nadir range of 7–134 x 10^3/μL and five of the eight patients developed thrombosis. Thrombosis was seen in all five patients with spontaneous HIT/delayed-onset HIT. As shown in Figure 2A, the PF4-polyanion assay was highly non-specific for the detection of VITT antibodies, with samples from all groups (VITT, spontaneous HIT, delayed-onset HIT, and HIT) producing strong reactions in this test. An additional control group of seven ELISA+/PEA- samples demonstrated low but positive ODs in the PF4-polyanion ELISA, with some overlap with VITT samples. In contrast, when these cohorts were tested in an experimental ELISA assay using un-complexed rather than polyanion-complexed PF4 targets (Figure 2B), clear differentiation of VITT from all other groups was seen with anti-PF4 antibodies from VITT patients producing significantly higher optical densities relative to non-VITT groups. Of note, the sample from the ChAdOx1 nCoV-19-associated VITT patient[19,20] included in these studies yielded high OD in this assay despite being drawn >2 months after initial presentation (4.00, Figure 2B).

TABLE 2 Platelet nadir and thrombosis of HIT cohort

N	Age	Sex	Platelet Nadir (x 10^3/μL)	Thrombosis
"Classical" HIT				
1	87	M	87	Y
2	57	M	39	N
3	76	M	134	Y
4	50	F	34	Y
5	48	M	7	Y
6	58	M	53	N
7	73	M	95	N
8	60	F	95	Y
Delayed-Onset HIT				
9	66	M	40	Y
10	75	M	37	Y
11[a]	47	M	8000	Y
Spontaneous HIT				
12[b]	30	M	41 000	Y
13	70	F	19 000	Y

Note: Y, Thrombosis noted; N, No thrombosis.
[a]Patients previously reported in Reference 18.
[b]Patients previously reported in Reference 12

4 | DISCUSSION

In this study, nine Ad26.COV2.S-associated VITT patients (~15% of 57 cases reported to the CDC to date) were followed over a median

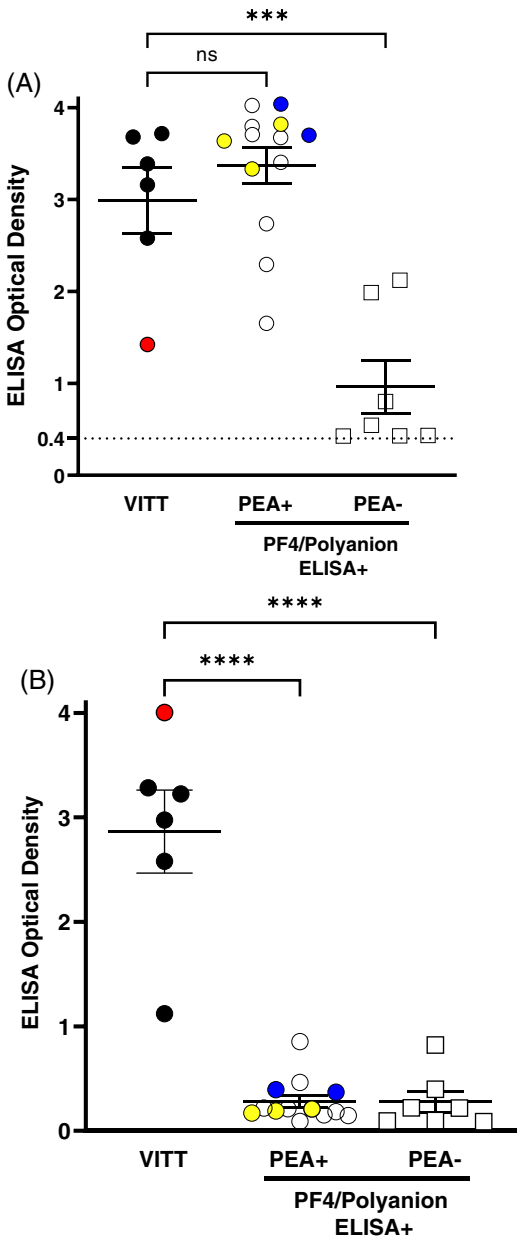

FIGURE 2 PF4/polyanion ELISAs are sensitive but highly non-specific, while an un-complexed PF4 ELISA is both sensitive and specific for the detection of VITT antibodies. Binding of antibodies from five Ad26.COV2.S-associated VITT (closed black circles), one ChAdOx1 nCoV-19-associated VITT (closed red circle), two spontaneous HIT (closed blue circles), three delayed-onset HIT (closed yellow circles), eight classical HIT (open circles), and seven ELISA +/PEA- samples (open squares) to immobilized PF4-polyanion complexes was evaluated using the LIFECODES PF4 IgG immunoassay (A) and the un-complexed PF4 ELISA (B). Groups were compared using one-way ANOVA. Mean and standard error are shown. ns, not significant ($p = .5192$); ***$p < .001$; ****$p < .0001$. Twenty-five healthy control sera had a mean OD of 0.130 (range 0.075–0.233) in the un-complexed PF4 ELISA (data not shown) [Color figure can be viewed at wileyonlinelibrary.com]

of 6 months and were demonstrated to have persistent anti-PF4 antibodies, as defined by PF4-polyanion ELISA positivity, for several months after acute presentation. We also present a new laboratory test for the sensitive and specific detection of VITT antibodies, using un-complexed PF4. The median age of our VITT cohort was 46 years, significantly lower than that seen with HIT (66 years[21]), but similar to the median of 48 years observed in ChAdOx1 nCoV-19-associated VITT.[22] While a female sex preponderance was not seen in this limited referred cohort, a recent analysis of Ad26.COV2.S-associated VITT in the United States shows that 70% were female.[6]

The strong PF4-polyanion ELISA reactivity seen in our patients is consistent with recent findings in ChAdOx1 nCoV-19-associated VITT.[9] Like in ChAdOx1 nCoV-19-associated VITT,[8,11] this study emphasizes the criticality of testing samples in PF4-enhanced functional assays, as heparin-based assays like the SRA can be falsely negative. These results also show that automated (non-ELISA) HIT assays should be avoided for Ad26.COV2.S-associated VITT investigation due to poor sensitivity, as has been recommended for ChAdOx1 nCoV-19-associated VITT.[23] Ad26.COV2.S-associated VITT antibodies were similar to ChAdOx1 nCoV-19-associated antibodies,[24] wherein antibodies positive in the PF4-polyanion ELISA persisted much longer relative to platelet-activating antibodies in PF4-enhanced functional assays. During recovery, the seroconversion profile of a negative platelet activation assay followed by a negative ELISA was similar to HIT[25]; however, the duration of antibody positivity was significantly longer with VITT similarly, platelet count recovery occurs within 7 days in 90% of cases of HIT,[26] while it was significantly longer in our VITT cohort.

Two patients (excluding one with hepatic cirrhosis-related thrombocytopenia) continued to have mild thrombocytopenia (141 and 149 x $10^3/\mu L$) even after 6 months, but it is unknown whether persisting thrombocytopenia was due to strong PF4/polyanion ELISA + antibodies (1.55 and 2.03 OD, but both were PEA-negative) detected at the ~6-month time point in these two patients; however, no other obvious cause was apparent. D-dimers are known to be markedly elevated in VITT, but this study demonstrates that most patients have normalization of levels within a month after acute presentation, an indication that there was no ongoing thrombosis after IVIg therapy and anticoagulation in most patients.

ChAdOx1 nCoV-19-associated VITT antibodies bind the heparin-binding site of PF4,[27] although much less is known about PF4 epitopes recognized by Ad26.COV2.S-associated VITT antibodies. Recent work from multiple groups demonstrate that anti-PF4 antibodies from ChAdOx1 nCoV-19-associated VITT and Ad26.COV2.S-associated VITT patients recognize un-complexed PF4.[5,8,20] Here, we leveraged this information to show that an ELISA assay developed on the basis of this finding can differentiate VITT from another rare anti-PF4-mediated thrombotic thrombocytopenic disorder, spontaneous HIT, that like VITT, can develop in the absence of heparin exposure. Being able to differentiate between these entities is critical from a vaccine epidemiology perspective and to inform strategies for booster COVID-19 vaccinations that VITT patients may need to receive. In addition, this assay may be used to differentiate HIT, delayed-onset HIT, and non-pathogenic anti-PF4 antibodies from antibodies in VITT patients. The mechanistic basis of the differences we have noted in PF4 recognition by VITT versus HIT antibodies is still unclear, especially with spontaneous HIT antibodies, which like VITT antibodies, develop in the absence of heparin exposure.

In summary, patients with VITT demonstrate long-term ELISA-positivity, although functional assays become seronegative earlier during the course of recovery. The data presented here suggest that the un-complexed PF4 ELISA is sensitive and significantly more specific for the detection of VITT than currently available tests; however, given the condition's rarity and the small number of VITT and comparator HIT cohorts tested, future studies should further assess its diagnostic utility.

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CONFLICT OF INTEREST

Anand Padmanabhan reports pending/issued patents (Mayo Clinic, Retham Technologies and Versiti), equity ownership in Retham Technologies, and serving on the advisory board of Veralox Therapeutics. Geoffrey D. Wool receives honoraria from and serves on an advisory board for Diagnostica Stago. The remaining authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

Adam J. Kanack and Bandana Singh performed research testing. Gemlyn George, Krishna Gundabolu, Scott A. Koepsell, Mouhamed Yazan Abou-Ismail, Karen A. Moser, Kristi J. Smock, David Green, Ajay Major, Clarence W. Chan, Geoffrey D. Wool, Mark Reding, Aneel A. Ashrani, and Antonios Bayas provided critical clinical correlates and helpful feedback. Diane E. Grill performed the statistical analysis. Anand Padmanabhan conceived the study. Adam J. Kanack and Anand Padmanabhan wrote the first draft, and all authors provided input and approved the final version.

DATA AVAILABILITY STATEMENT

Data will be made available upon reasonable request to the corresponding author.

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