CHARACTERIZATION OF ANTI-PF4 ANTIBODIES IN VITT

CHARACTERIZATION OF ANTI-PLATELET FACTOR 4 ANTIBODIES AND THEIR PERSISTENCE IN VACCINE-INDUCED IMMUNE THROMBOTIC THROMBOCYTOPENIA

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TITLE: Characterization of anti-platelet factor 4 antibodies and their persistence in vaccine-induced immune thrombotic thrombocytopenia

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LAY ABSTRACT

Vaccine-induced immune thrombotic thrombocytopenia (VITT) is caused by antibodies that bind to platelet factor 4 (PF4), reducing the number of platelets (thrombocytopenia) and forming deadly blood clots (thrombosis) within the body. Not much is known about the characteristics of VITT antibodies, so this study aims to improve understanding of VITT and anti-PF4 disorders.

We found that 2 main types of antibodies were produced in VITT, but their roles remain unknown. We also monitored patients over a median of 715 days and found that some patients continued to produce VITT antibodies that can activate platelets, but we were unable to detect the cells that produce these antibodies long-term. These patients have not had recurrent thrombocytopenia or thrombosis possibly because they remain on treatment. Further monitoring and research are needed to improve our understanding of anti-PF4 disorders and provide better care for VITT patients.

ABSTRACT

During the COVID-19 pandemic, highly efficacious vaccines were developed to help stop the spread of SARS-CoV-2. The adenoviral vector-based vaccines have caused a rare but serious adverse side effect known as vaccine-induced immune thrombotic thrombocytopenia (VITT). VITT occurs 5 to 30 days after vaccine administration and is characterized by thrombocytopenia and thrombosis. VITT is caused by antibodies against platelet factor 4 (PF4), forming immune complexes that cause platelet activation. This study aims to deepen our understanding of VITT antibody characteristics by investigating anti-PF4 antibody class and subclass distribution, anti-PF4 antibody persistence, and anti-PF4 memory B cell presence in VITT patients. This will improve our understanding of VITT, and disorders caused by anti-PF4 antibodies (anti-PF4 disorders) overall. We hypothesize that anti-PF4 memory B cells cause the persistence and circulation of pathogenic anti-PF4 autoantibodies, but these antibodies change over time, losing their ability to activate platelets and cause thrombosis in VITT patients.

We found that IgG is the main antibody class and IgG1 and IgG2 are the main antibody subclasses in VITT, but more work is needed to understand the roles of these antibodies. We also observe that VITT patients continue to produce platelet-activating anti-PF4 antibodies years after VITT diagnosis. These patients have not experienced any recurrent thrombosis or thrombocytopenia, possibly because they have remained on treatment and physicians remain unsure what would happen should they be taken off treatment. We could not definitively detect circulating

anti-PF4 memory B cells in VITT patients, so it remains unknown whether immune memory cells play a role in keeping these antibodies around. There is a need to continue to monitor these patients to understand the long-term impacts of this disorder. This work will improve the treatment of patients suffering from anti-PF4 disorders and improve future vaccine safety and efficacy.

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LIST OF ABBREVIATIONS

VITT	Vaccine-induced immune thrombotic thrombocytopenia
PF4	Platelet factor 4
COVID-19	Coronavirus disease 2019
USA	United States of America
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
CVST	Cerebral venous sinus thrombosis
SVT	Splanchnic vein thrombosis
PE	Pulmonary embolism
DVT	Deep vein thrombosis
TTS	Thrombosis with thrombocytopenia syndrome
Fab	Antigen binding fragment
Fc	Crystallizable fragment
vWF	Von Willibrand Factor
ASH	American Society of Hematology
EIA	Enzyme immunoassay
PF4-SRA	PF4 serotonin release assay
OD	Optical density
IVIg	Intravenous immunoglobulin
HIT	Heparin-induced thrombocytopenia
SRA	Serotonin release assay
MGTS	Monoclonal gammopathy of thrombotic/thrombocytopenic significance
BCR	B cell receptor
TTP	Thrombotic thrombocytopenic purpura
PBMC	Peripheral blood mononuclear cells
ELISpot	Enzyme-linked immunosorbent spot
°C	Degrees Celsius
PEA	P-selectin expression assay
PBS	Phosphate-buffered saline
BSA	Bovine serum albumin
AP	Alkaline phosphatase
PNPP	p-Nitrophenyl phosphate
DEA	Diethanolamine buffer
SD	Standard deviations
FBS	Fetal bovine serum
ACK	Ammonium-chloride-potassium
TMB	Tetramethylbenzidine
APS	Anti-phospholipid syndrome

DECLARATION OF ACADEMIC ACHIEVEMENT

All the experiments detailed in this thesis were conducted by Michael Hack under the supervision of Rumi Clare and Dr. Ishac Nazy.

1.0 INTRODUCTION

1.1 COVID-19 pandemic and vaccination program

On March 11, 2020, the World Health Organization declared coronavirus disease 2019 (COVID-19) a pandemic [1]. As of April 2024, there have been 775 million confirmed cases of COVID-19 and 7 million deaths globally [1]. Case counts of COVID-19 were as high as 800,000 per day in the United States of America (USA) [2]. A strategy employed to curb infection and lower cases was vaccination mandates. These mandates aimed to limit the person-to-person spread and reduce mortality of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Vaccines were produced on shorter timelines than the typical 10 – 15 years, ranging from the more traditional adenoviral vector-based vaccines ChADOx1 nCoV-19 (Oxford-AstraZeneca) and Ad26.COV2.S (Johnson & Johnson/Janssen) to the new mRNA-based vaccines Comirnaty (Pfizer-BioNTech) and Spikevax (Moderna) [3]. As of April 2024, there have been 13.59 billion vaccines administered globally [1].

1.2 Rare side effect following adenoviral vector-based vaccination

Although these vaccines proved effective in minimizing death and hospitalization, some individuals developed thrombocytopenia and thrombosis after receiving adenoviral vector-based SARS-CoV-2 vaccination [2]. The first cases of this rare, but severe side effect were described by Greinacher *et al.*, Scully *et al.*, and Schultz *et al.* in April 2021 [4-6]. Greinacher *et al.* described the

clinical and laboratory features of 11 German and Austrian patients [4]. The median age of these 11 patients was 36 years old (range: 22 to 49) and 9 were women [4]. All patients displayed thrombocytopenia and thrombotic complications developed 5-16 days post-vaccination [4]. Of the 11 patients, 9 developed cerebral venous sinus thrombosis (CVST), 3 developed splanchnic vein thrombosis (SVT), 3 developed pulmonary embolism (PE), 4 developed other types of thrombosis, 5 developed disseminated intravascular coagulation, and 1 developed fatal intracranial hemorrhage [4]. Unfortunately, 6 patients passed away [4, 7]. Schultz *et al.* described the clinical presentations of 5 Norwegian healthcare workers following their first dose of ChADOx1 nCoV-19 vaccine [5]. The median age of these 5 healthcare workers was 39 years old (range: 32 - 54) and 4 were female [5]. Patients reported symptoms such as headaches 7 – 10 days post-vaccination [5]. Severe thrombocytopenia was noted in all patients, with 4 patients developing CVST and 1 developing SVT [5, 7]. Scully *et al.* published a study on 23 patients following their first dose of ChADOx1 nCoV-19 vaccine [6]. The median age of these 23 patients was 46 (range: 21 – 77) and 14 (61%) were female [6]. Symptoms were reported after a median of 12 days (range: 6 – 24) with all patients experiencing thrombocytopenia [6]. CVST was the most common site of thrombosis reported in 13 patients, followed by PE in 4 patients, SVT in 2 patients, and deep vein thrombosis (DVT) in 2 patients [6]. Unfortunately, 7 (30%) patients passed away [6, 7].

1.3 Vaccine-induced immune thrombotic thrombocytopenia

This side effect following administration of a SARS-CoV-2 adenoviral vector vaccine has now been termed vaccine-induced immune thrombotic thrombocytopenia (VITT). It is a rare but life-threatening syndrome reported 4-42 days post-vaccination [4, 5, 8, 9]. VITT is characterized by moderate to severe thrombocytopenia and venous or arterial thrombosis in unusual locations [6, 10]. CVST and SVT are more common in VITT when compared to other disorders characterized by both thrombocytopenia and thrombosis, with CVST being the most common site of thrombosis in VITT patients [4, 10, 11].

CVST is a rare type of stroke (0.5% - 0.7% of all strokes) caused by clot formation in the venous sinuses of the brain [12]. Acute severe headaches are one of the most frequent symptoms of CVST [13]. A study by Payne et al. utilized data from 3 large administrative data systems and analyzed the incidence, demographic patterns, comorbidities, and clinical severity of CVST and CVST with thrombocytopenia in the USA from 2018 to 2019 [12]. They found that the incidence rate of CVST was between 2.45 – 3.16 per 100,000 individuals [12]. The incidence rate of CVST with thrombocytopenia was lower at 0.16 – 0.22 per 100,000 individuals [12]. They also found that patients with CVST and thrombocytopenia had worse clinical outcomes such as longer hospital stays (10.5 days on average) and higher mortality rates (10.2% compared to 4.4%) when compared to patients with CVST alone [12]. Despite the rarity of CVST in the general population, the prevalence of CVST with thrombocytopenia in VITT

patients has been reported as 37% - 82% with a 2.7-fold increase in mortality, highlighting how life-threatening this disorder is [4, 10, 11].

1.4 Incidence and mortality of VITT

It has been estimated that VITT has a general incidence of 1 case per 100,000 doses of SARS-CoV-2 adenoviral vector-based vaccines, but the incidence varies geographically and is dependent on the vaccine used [11, 14, 15]. A study by Soboleva *et al.* found that thrombosis with thrombocytopenia syndrome (TTS) cases per million doses of ChAdOx1 nCoV-19 was highest in Nordic countries at 17.6 cases per million doses and the lowest in Asian countries and Brazil at 0.2 cases per million doses [14]. Some potential factors that could influence the observed geographical differences include reporting systems, vaccination strategies, and environmental/genetic factors [14]. The WHO and the Strategic Advisory Group of Experts on Immunization published an interim recommendations document that contained the TTS cases per million doses of Ad26.COV2.S and found that it was highest in the USA at 3.7 cases per million doses and the lowest in South Africa at 0.2 cases per million doses [15].

Improved recognition of VITT has been imperative, not only for improving data reporting and calculating incidence rates, but also for reducing mortality [16]. A study conducted by Munckhof *et al.* examined mortality rates in VITT patients with CVST before and after the first scientific publication of VITT and found a reduction in mortality from 47% to 22% [16]. This highlights the importance of

early recognition, prompt treatment, and improving our knowledge of VITT and the antibodies that cause this disorder.

1.5 Mechanism of VITT

Antibodies are an important component of the adaptive immune system. They are a Y-shaped glycoprotein comprised of 2 heavy chains and 2 light chains [17-21]. Antibodies have 2 distinct regions, one being the variable region which is contained within the antigen binding fragment (Fab) which forms the binding interface between the antibody (paratope) and the target foreign molecule or antigen (epitope) [17-21]. The other region is the constant region, which contains the crystallizable fragment (Fc) and is important for protein assembly and effector functions such as binding to Fc receptors, which are found on cells that play a role in host defense [17-21]. Once an antibody binds to its antigen, it labels it for destruction. This can be done through neutralization, opsonization, antibody-dependent cellular cytotoxicity, and complement-mediated lysis of pathogens or infected cells [17-21]. While most antibodies protect us from foreign pathogens, some antibodies react to endogenous proteins and are called autoimmune antibodies or autoantibodies [22, 23]. Typically, these antibodies are eliminated or inactivated, but when tolerance breaks down, these autoantibodies can escape destruction and be produced, which can lead to autoimmune diseases [22, 23].

VITT is caused by autoantibodies directed against platelet factor 4 (PF4, CXCL4), a 70 amino acid cationic chemokine contained in the α-granules of platelets [24]. This tetrameric chemokine is released at high local concentrations

upon platelet activation and binds to negatively charged molecules such as glycosaminoglycans. Its exact role remains unknown as PF4 demonstrates dual procoagulant and anticoagulant roles depending on the local concentration.

Platelets are produced in the bone marrow and are the smallest of the major types of blood cells (20% the diameter of a red blood cell) [25, 26]. Typical platelet counts in the human body range from 150 - 400 x 10^9 platelets/L [25, 26]. These cells roll along blood vessel walls as they do not adhere to endothelial cell surfaces [25, 27]. At sites of vascular injury, platelets will stick to proteins such as von Willibrand Factor (VWF) and swell and change shape, extending long filaments that allow them to stick blood vessel walls as well as other platelets forming an unstable platelet plug to seal the broken blood vessel [25, 28]. The coagulation cascade will then occur as part of secondary hemostasis to stabilize this plug [29]. Hemostasis is the delicate balance with platelets where too few (thrombocytopenia) can lead to bleeding disorders and excessive platelet activation can lead to the formation of blood clots within a blood vessel (thrombosis) impeding blood flow in a vessel or to tissues, leading to ischemia or tissue death [25, 29].

High titre anti-PF4 IgG antibodies are central to VITT pathogenesis [30]. Upon adenoviral vector-based vaccine administration, PF4 may interact with some constituent within the vaccine, which triggers anti-PF4 antibody production [30]. While it was initially thought that the hexon of the adenoviral vector could be the trigger, a recent study has shown this interaction is unlikely and PF4 itself could be the antigen, so it remains unknown what specifically within the vaccine

triggers this immune response [31, 32]. In VITT, anti-PF4 IgG antibodies bind to PF4, leading to the formation of immunogenic complexes. These complexes then crosslink low-affinity FcγRIIa on platelets leading to the activation and consumption of platelets. This causes the release of more PF4, serotonin, and procoagulant factors, leading to further platelet activation and ultimately thrombosis [33, 34]. These immunogenic complexes also cause neutrophil activation, further exacerbating thrombosis [30, 34]. Prompt diagnosis and treatment are important to managing symptoms and mitigating mortality.

1.6 Diagnostic testing and treatment of VITT

Early recognition and specialized laboratory tests (PF4-enhanced platelet activation test) are critical for diagnosis and treatment [4]. According to the American Society of Hematology guidelines (ASH), patients with severe or persistent symptoms such as intense headache, abdominal pain, back pain, nausea and vomiting, vision changes, change in mental status, shortness of breath, leg pain and swelling, and bleeding/petechiae from 4 to 42 days following COVID-19 vaccination should be considered for VITT diagnosis [9]. The criteria for VITT diagnosis include: 1) COVID-19 vaccination within 4–42 days prior to symptom onset, 2) evidence of venous or arterial thrombosis, 3) thrombocytopenia (platelet count < 150 x 10^9/L), 4) positive anti-PF4 enzyme immunoassay (EIA) test, and 5) markedly elevated D-dimer levels (>4x the upper limit of normal) [9].

As Canada's national centre for VITT diagnostic testing, we utilize a commercial anti-PF4 EIA to detect antibody presence and the PF4 serotonin

release assay (PF4-SRA) to assess the ability of these antibodies to activate platelets. An optical density (OD) of ≥ 0.4 in the commercial anti-PF4 EIA indicates a patient possesses anti-PF4 antibodies. The PF4-SRA is a functional assay that tests the capabilities of anti-PF4 antibodies to activate platelets. Briefly, healthy donor platelets are incubated with radioactive serotonin [35, 36]. VITT patient serum and increasing concentrations of PF4 (0 – 50 μg/mL) are added and platelet activation is measured by serotonin release [35, 36]. A serotonin release ≥ 20% indicates that a patient possesses platelet-activating anti-PF4 antibodies [35, 36]. An anti-human CD32 Fc receptor-blocking monoclonal antibody (IV.3) is used in the PF4-SRA to confirm the involvement of FcγRIIa in platelet activation [35, 36].

Initial steps for management and treatment involve performing a complete blood count, imaging for suspected thrombosis, testing for D-dimer levels and anti-PF4 antibodies, and avoiding heparin-based anti-coagulants [9]. The recommended treatment involves a 1g/kg dosage of Intravenous Immunoglobulin (IVIg) for 2 days to suppress immune activation through FcγRIIa and to avoid heparin-based anticoagulants to manage the thrombotic risk in affected patients [4, 9]. Plasma exchange or complement inhibition may be used in refractory cases [9].

1.7 Heparin-induced thrombocytopenia

Clinical features of VITT resemble the immune-mediated drug reaction heparin-induced thrombocytopenia (HIT), which is a life-threatening and

prothrombotic reaction to the anticoagulant heparin [37-39]. Heparin is a large anionic polysaccharide commonly given to patients intra- and post-operatively due to its anticoagulant properties [40, 41]. HIT occurs 5 – 10 days post-heparin exposure and is characterized by thrombocytopenia and thrombosis in 30% - 60% of cases, with the most common sites of thrombosis being DVT and PE [40, 42-45]. In the USA, 1 in 1500 hospitalization admissions are due to HIT [40, 46]. The incidence of HIT varies depending on the type of surgery and the type of heparin. The highest incidence of HIT is observed post-cardiac surgery and with unfractionated heparin when compared to low molecular weight heparin, especially in postsurgical settings [40, 47-49]. Amputation occurs in 3% - 8% of HIT cases and the mortality rate varies from 6% - 50% [40, 50]. VITT most closely resembles the rare form of HIT that has been described without proximate heparin administration (spontaneous HIT) [51, 52].

1.8 Mechanism, diagnostic testing, and treatment of HIT

While HIT shares pathophysiological mechanisms with VITT, the target antigens differ – HIT antibodies recognize PF4/heparin complexes, while VITT antibodies recognize PF4 alone [4, 11, 53, 54]. Polyanionic heparin binds to cationic PF4 tetramers, exposing neoepitopes that HIT antibodies target [50]. Similar to VITT, this leads to the formation of immunogenic complexes and activation of platelets via the crosslinking of low-affinity FcγRIIa. This causes the release of procoagulant factors, leading to further platelet activation and ultimately causing thrombosis [33, 34, 40].

Clinical HIT diagnosis begins with a pre-screen called the 4Ts score [40, 55, 56]. This screen uses a point system and assesses thrombocytopenia, timing of platelet count fall, thrombosis, and other causes of thrombocytopenia [40, 55, 56]. Patients with an intermediate (4 – 5) or high (6+) score are sent for laboratory testing [40, 55, 56]. As we are a national centre for HIT diagnostic testing also, we utilize a commercial anti-PF4 EIA to detect antibody presence and the serotonin release assay (SRA) to assess the ability of these antibodies to activate platelets. An OD of ≥ 0.4 in the commercial anti-PF4 EIA indicates a patient possesses anti-PF4 antibodies. The SRA is the gold standard for HIT testing and is a functional assay that tests the capabilities of HIT antibodies to activate platelets. Similar to the PF4-SRA, healthy donor platelets are incubated with radioactive serotonin. HIT patient serum and different concentrations of heparin are added (0, 0.1, and 0.3 U/mL) and platelet activation is measured by serotonin release. The reaction is inhibited when high heparin concentrations (100 U/mL) and IV.3 are added [40, 57, 58]. Treatment involves stopping heparin administration and using non-heparin anticoagulants until platelet counts have recovered or thrombosis is no longer a concern. IVIg is also used in cases of severe thrombocytopenia and thrombosis in HIT [40].

1.9 Anti-PF4 disorders

Research in VITT has improved our understanding and awareness of anti-PF4 disorders. Other anti-PF4 syndromes have now been identified and described; our group and others have recently identified anti-PF4 immunothrombosis due to proven adenoviral infection without heparin and adenoviral vector-based vaccine

exposure [59-61]. A study by Jing Jing *et al.* used mass spectrometry to sequence the antibody from these patients after adenoviral infection (n = 5) and found that the antibodies produced are extremely similar to VITT [62]. Additionally, recurrent thrombosis and thrombocytopenia due to a premalignant clonal plasma cell disorder that causes persistent anti-PF4 monoclonal antibody production has been reported and termed monoclonal gammopathy with thrombotic/thrombocytopenic significance (MGTS) [63-66]. With the increased prevalence of anti-PF4 disorders, the need for further research to improve our understanding of these disorders and the antibodies causing them has been made more apparent.

1.10 Antibody classes and subclasses

Much remains unknown about the antibodies produced in VITT. All antibodies are produced by B cells, which begin their maturation process in the bone marrow [17, 67-69]. As a B cell transitions from the pro B cell stage to the naïve B cell stage, The B cell undergoes many rounds of selection and recombination to produce a unique IgM molecule that is displayed on the cell surface as a B cell receptor (BCR) [17, 67-69]. Once a B cell matures, it produces both IgM and IgD as BCRs and is ready to interact with an antigen [17, 67-69]. Once a mature B cell interacts with its antigen, it differentiates into an antibody-secreting cell called a plasma cell and begins to secrete its BCR as an antibody [17, 67-69]. Antibody secretion will remain as IgM until the plasma cell encounters specific signaling molecules from helper T cells or cytokines, which will initiate class switch recombination [17, 67-69]. During this process, the antibody heavy chain constant

region is replaced, which results in the antibody changing to a different class of antibody, affecting the Fc receptors an antibody can interact with [17, 67-69]. The variable region remains the same, so antigen specificity remains unchanged [17, 67-69].

Class switching follows a stepwise procedure that is dictated by gene order, where once a heavy chain gene is spliced out, that removal is permanent. The heavy chain gene order is μ, δ, γ3, γ1, α1, γ2, γ4, ε, and α2 [17]. The different classes of antibodies (IgM, IgD, IgG, IgA, and IgE) vary in their properties, locations, and roles in the immune system [19, 20]. IgM comprises the first antibody response in an immune reaction and forms a pentameric structure [19, 20]. IgG is the most abundant antibody produced (10% – 20% of plasma protein) [19, 20]. IgA is the primary antibody at mucosal surfaces and in secretions [19, 20]. IgG has different subclasses (IgG1, IgG2, IgG3, and IgG4) which have different functions and affinities for Fcγ receptors despite sharing more than 90% sequence homology [17]. IgG1 and IgG3 are defined as the complement-binding subclasses and IgG2 and IgG4 both have shorter hinge regions and are defined as noncomplement-binding subclasses [17]. Since antibody classes and subclasses differ in their biological functions, understanding the types of antibodies produced in a disorder can provide valuable information about the pathophysiology of disorder. This has been shown in other autoantibody-mediated thrombotic disorders such as acquired thrombotic thrombocytopenic purpura (TTP), which is characterized by antibodies against ADAMTS-13, an enzyme that regulates VWF [70]. Ferrari *et al.* found that TTP patients had high levels of IgG4 autoantibodies,

followed by IgG1 and that their levels in patients were inversely correlated [70]. Patients with high IgG1 and potentially IgA, but low IgG4 had a higher mortality rate during their first TTP event [70]. Conversely, patients with high IgG4, but low IgG1 had higher rates of TTP relapse [70]. IgG1 can have higher pathogenic potential than IgG4 due to the ability of IgG1 to activate complement and interact with many Fcγ receptors [17, 70]. IgG4 is typically considered immune protective as it has a lower affinity for Fcγ receptors and is seen after chronic antigen stimulation [17, 70]. This study highlights the importance of studying antibody types in a disorder as this can provide valuable information to physicians and guide patient treatment.

In VITT, several studies have determined that the anti-PF4 antibodies produced are of limited clonality. One study by Kanack *et al.* utilized mass spectrometry to show that the antibodies produced in VITT are monoclonal or oligoclonal, while HIT antibodies are more polyclonal [71]. Several other studies in VITT have also shown that a predominant singular antibody is being produced in different VITT patients globally [72-74]. In addition to the clonality of the antibodies, they can further be distinguished by their ability to activate platelets in the presence or absence of exogenous PF4. A study by Huynh *et al.* showed some VITT patients have antibodies that need additional PF4 to activate platelets (PF4-dependent) and other VITT patients have antibodies that can activate platelets without adding PF4 (PF4-independent antibodies) [75]. Of the 39 VITT patients examined, 17 (46.3%) possessed PF4-dependent antibodies, and 22 (56.4%) possessed PF4-independent antibodies [75]. Utilizing our epitope mapping

platform, we found that PF4-dependent antibodies bound to the heparin-binding site alone, while PF4-independent antibodies bound to both the heparin-binding site and the HIT-binding site [75]. These antibody profiles impact disease progression in VITT patients [75]. Worse clinical outcomes are more associated with PF4-independent antibodies, as CVST occurred almost exclusively in this group [75]. How these antibodies differ and why they lead to different clinical manifestations are poorly understood.

A deeper understanding of the types of anti-PF4 antibodies produced during VITT could explain these differences. Previous research in HIT by our lab and others has revealed both the classes and subclasses of antibodies produced in HIT [76-80]. This information remains unknown in VITT and could explain differences in antibody epitope profiles and clinical manifestations. Comparing knowledge gained in VITT with what is known in HIT can identify key differences caused by changes in disease origin, improving our understanding of anti-PF4 disorders.

1.11 Antibody persistence

It remains unclear how long VITT antibodies persist in patients. While it varies per subclass, IgG antibodies typically have a half-life of about 3 weeks in the body [81, 82]. This half-life can be extended due to factors such as repeated antigen stimulation and neonatal FcγR [82, 83]. Tracking antibody titre after vaccination has shown that antibodies can vary in how long they circulate, as some will remain detectable for life while others require booster vaccines to

continue circulating and provide protection [84, 85]. A study by Iyer *et al.* found that IgM and IgA against the receptor-binding domain of the spike protein of SARS-CoV-2 decayed after a median of 49 and 71 days, respectively, but anti-RBD IgG continued to persist, decaying slowly over 90 days [86]. Similarly, a study by Irrgang *et al.* found that anti-Spike IgG antibodies remain detectable 210 days after vaccination, indicating that these antibodies continue to provide protection long after vaccination [87].

In the case of autoimmune antibodies, antibody persistence is a problem for patients. If pathogenic antibodies remain in circulation, patients need continued treatment and monitoring as they will remain at risk for their autoimmune disorder [63-66]. In HIT, one study by Warkentin and Kelton examined the temporal aspects of anti-PF4/heparin antibodies. They examined 243 HIT patients and found that anti-PF4/heparin antibodies lost their ability to activate platelets after a median of 50 days and became undetectable in serum after a median of 85 days [41]. These antibodies are more transient which means that there is no long-term clinical concern for HIT patients.

Currently, there is limited data on the durability of VITT antibodies. Studies have shown that VITT antibodies appear to remain in circulation for longer than HIT antibodies, but there is conflicting data on how persistent these antibodies are [88-91]. One study by Schönborn and Greinacher examined 65 longitudinal VITT patients and found that none possessed platelet-activating antibodies after 9 months; however, the authors still reported on several cases of patients displaying recurrent symptoms of thrombocytopenia and thrombosis despite ongoing

treatment [89, 92, 93]. This would indicate that VITT antibodies persist in some patients, but it remains unknown how long they last and what the long-term complications of this disorder are. Current treatment options involve continued anticoagulation, but the risks of using these medications over a long period and whether other treatments are required remain uncertain [91]. Further studies and ongoing monitoring are needed to understand the complete time course of anti-PF4 antibody persistence and develop effective long-term management strategies for affected patients.

1.12 Memory B cells

The reason these antibodies are persisting in some VITT patients also remains unknown. In response to antigenic stimulation, specific B lymphocytes undergo clonal expansion and class switching, with some differentiating into plasma cells that secrete antibodies at a high rate and persist in niches in the bone marrow, while others become memory B cells [94, 95]. Memory B cells provide long-term immunity by producing antibodies and facilitating rapid response upon reinfection [23]. These B cells persist for decades and therefore carry the “history” of an individual’s immune response by maintaining the population of plasma cells, thereby maintaining serum antibody levels [96]. There is also increasing evidence that memory B cells are responsible for plasma cells that produce pathogenic autoimmune antibodies [23]. This is a problem because these cells can remain within individuals for life and memory B cells shorten the time needed to produce robust antibody responses from weeks to days [23, 84, 97, 98].

While there is no evidence of immune memory in HIT, one study by Warkentin *et al.* examined previous HIT patients who had been re-exposed to heparin and found that these patients produced platelet-activating anti-PF4/heparin antibodies upon heparin re-exposure at a higher rate than expected when compared to the general population, but only 1 patient redeveloped HIT [99]. Other studies have also found that heparin re-exposure in previous HIT patients has not led to a reoccurrence of HIT, highlighting the importance of immune memory not being present in a life-threatening disorder such as HIT [100].

This contrasts with what is seen in SARS-CoV-2 infected and vaccinated individuals as our group and others have found that anti-Spike and anti-RBD antibodies appear persistent [87]. Irrgang et *al.* found that while there is a lasting antibody response and memory B cells in in SARS-CoV-2 vaccinated individuals, there is a shift toward IgG4 production IgG4 memory B cells perhaps due to repeated antigen exposure [87]. Detection of memory B cells in SARS-CoV-2 infected and vaccinated individuals indicates that individuals have long-term protection from this virus, but a shift toward IgG4 can be detrimental as IgG4 was shown to be less effective at offering antiviral immunity when compared to IgG1 [87].

Whether immune memory is present in VITT remains unanswered. Studies have shown that VITT patients who receive a subsequent dose of mRNA-based SARS-CoV-2 vaccination have experienced no increase in anti-PF4 antibody titre or experienced no thrombotic or thrombocytopenic complications [89, 101]. While the incidence of VITT drops to 1 in 518,181 after a second dose of

adenoviral vaccination, the reoccurrence of VITT has been shown to occur in previous patients [102, 103]. Immune memory could play a role in VITT, similar to the role it plays in SARS-CoV-2 vaccinated and infected individuals. Repeated antigen exposure from the endogenous protein PF4 could stimulate memory B cells to continue producing pathogenic anti-PF4 antibodies for long durations, potentially even life. This information is crucial for the long-term management of VITT patients as patients with anti-PF4 memory B cells would need continuous monitoring and treatment to prevent recurrent life-threatening symptoms from arising again.

2.0 RESEARCH OUTLINE

2.1 Project goal

Investigating anti-PF4 antibody class/subclass distributions, persistence, and the presence of anti-PF4 memory B cells in VITT patients.

The knowledge garnered from research in HIT has aided our understanding of VITT, but some differences between these disorders and within VITT itself are poorly understood. Not much is known about the characteristics of VITT antibodies and the immune pathology unique to this disorder. While the duration and types of HIT antibodies are known, this information remains unknown in VITT and requires further research. This study aims to deepen our understanding of the characteristics of VITT antibodies by investigating anti-PF4 antibody class and subclass distribution, anti-PF4 antibody persistence, and anti-PF4 memory B cell presence in VITT patients. This will improve our understanding of VITT, but

also provide valuable insights into anti-PF4 antibody development in all anti-PF4 disorders and impact future vaccine development.

2.2 Project aims

This work aims to identify humoral characteristics of the anti-PF4 antibodies produced in VITT: 1) the class and subclass of anti-PF4 antibodies produced and whether different antibody profiles play a role in disease severity; 2) the persistence of anti-PF4 antibodies in circulation and investigate whether initial disease severity plays a role in antibody persistence patterns; and 3) the detection of anti-PF4 memory B cells in the circulation of VITT patients with persistent anti-PF4 antibodies.

2.3 Hypothesis

Anti-PF4 memory B cells cause the persistence and circulation of pathogenic anti-PF4 autoantibodies, but these antibodies change over time, losing their ability to activate platelets and cause thrombosis in VITT patients.

2.4 Project objectives

1) VITT anti-PF4 antibody class and subclass examination

We will test a cohort of acute phase VITT patients from Canada in a novel in-house PF4 EIA to characterize the antibody classes and IgG subclass distributions and compare these findings with previously published reports in HIT. As previously published data has shown that VITT antibodies are of limited clonality and that different antibody epitopes in VITT lead to more severe clinical

outcomes [71-75], we will investigate whether different anti-PF4 antibody class and subclass distribution patterns among patients correlate with more severe clinical outcomes in VITT.

2) VITT follow-up sample collection and longitudinal characterization

We will interview and collect whole blood samples from VITT patients longitudinally with their informed consent. From these samples, we will isolate peripheral blood mononuclear cells (PBMCs), serum, and plasma for each sample. These samples will be tested longitudinally for the presence of anti-PF4 antibodies utilizing a commercial diagnostic EIA and whether these anti-PF4 antibodies are platelet-activating utilizing the PF4-SRA. We will also monitor the longitudinal clinical picture of VITT patients to improve our understanding of the lasting impacts of VITT. We will then investigate whether clinical information from VITT patients plays a role in anti-PF4 antibody persistence.

3) VITT memory B cell investigation

Measuring serum antibody levels can be misleading as it excludes the detection of the memory B-cell pool. Therefore, we will elucidate whether memory B cells are playing a role in antibody persistence in VITT by examining VITT PBMCs for anti-PF4 memory B cells utilizing the enzyme-linked immunosorbent spot (ELISpot) assay. The ELISpot assay is an extremely sensitive assay that can measure the presence and frequency of PF4-specific memory B cells following polyclonal stimulation by causing memory B cells to differentiate into plasma cells and capturing the secreted antibody in the vicinity of each B cell to form a spot [104]. The supernatant from the incubated cells in

the ELISpot assay will also be collected and run in both commercial and in-house EIAs to detect the presence of secreted antibodies after stimulation.

3.0 MATERIALS AND METHODS

3.1 Study population

The McMaster Platelet Immunology Laboratory received serum and plasma samples used for this study based on clinical suspicion of VITT from across Canada between March and July 2021 [11]. All suspected VITT patients (n = 233) received one dose of the following vaccinations against COVID-19: ChADOx1 nCoV-19, Ad26.COV2.S, Comirnaty, or Spikevax. Therefore, patients were classified as VITT-positive based on the following clinical and laboratory criteria: 1) recent SARS-CoV-2 adenoviral vector vaccination without heparin administration; 2) the presence of thrombocytopenia and thrombosis; 3) the presence of anti-PF4 IgG/A/M antibodies in an enzyme immunoassay (EIA; $OD_{405nm} \geq 0.4$); and 4) a positive PF4-serotonin release assay result (^{14}C-serotonin release ≥ 20%) [11, 35, 36]. Patient samples were drawn during the acute stage of VITT and clinical data such as the time of symptom onset, platelet counts, and thrombosis was provided by referring physicians. The Hamilton Integrated Research Ethics Board (HiREB) approved this study and all participants provided written informed consent.

3.2 VITT Follow-up Study

The Michael DeGroote Centre for Transfusion Research recruited participants including patients with confirmed VITT diagnosis based on clinical testing (confirmed VITT), individuals who received at least one adenoviral SARS-COV2 vaccination but did not develop VITT (healthy controls), and individuals who were suspected of VITT but did not have VITT based on clinical testing (non-VITT). Whole blood was collected by venipuncture. Serum, plasma, and PBMCs were collected and cryopreserved in -80°C liquid nitrogen until use. Follow-up clinical data were obtained from patients via telephone and in-person interviews. This study was approved by the Hamilton Integrated Research Ethics Board (HiREB; study 10889) and written informed consent was obtained from all participants.

3.3 Diagnostic testing for anti-PF4 VITT antibodies

All suspected VITT patients were tested for IgG/A/M anti-PF4 antibodies using a commercially available enzyme immunoassay (EIA; LIFECODES PF4 Enhanced assay; Immucor, Peachtree Corners, GA, USA [positive OD 405nm $\geq$ 0.4]) as per manufacturer's instructions; suspected samples were also tested for platelet activation using the PF4-SRA with increasing concentrations of exogenous recombinant human PF4 (0, 10, 25, and 50 μg/mL) [35, 36]. To confirm that FcγRIIa is involved in platelet activation, IV.3 was also used in the PF4-SRA [35]. The P-selectin Expression Assay (PEA) was also utilized for longitudinal

analysis with different concentrations of exogenous recombinant human PF4 (0 and 37.5 μg/mL) [35, 36, 105].

3.4 Laboratory testing for anti-PF4 Ig classes in VITT patients

An in-house EIA was developed to detect anti-PF4 IgG, IgA, and IgM levels. Briefly,

96-well Nunc MaxiSorp (ThermoFisher Scientific, Waltham, MA, USA) plates were coated with 30 μg/mL recombinant human PF4 diluted in bicarbonate buffer pH 9.6 overnight at 4°C. On the next day, plates were washed twice with phosphate-buffered saline (PBS) with 0.05% Tween 20 and thrice with PBS alone, then blocked with PBS with 3% bovine serum albumin (BSA; MilliporeSigma, St. Louis, MO, USA) for 2 hours at room temperature. Plates were then washed, and patient samples were diluted 1:50 using PBS with 1% BSA and added in duplicate wells for 1 hour at room temperature. Plates were once again washed, and goat anti-human IgG, A, or M antibodies conjugated with alkaline phosphatase (AP; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) were diluted using PBS with 1% BSA 1:3333, 1:1000 and 1:1000, respectively, and added for 1 hour at room temperature. Next, plates were washed, and p-Nitrophenyl Phosphate (PNPP; MilliporeSigma) dissolved in diethanolamine buffer (DEA; MilliporeSigma) was added. ODs were kinetically read on a BioTek Synergy HTX plate reader (Agilent Technologies, Winooski, VT, USA) every two minutes for 30 minutes at 405 nm with a reference OD of 490 nm. A positive cut-off of 0.45, 0.29 and 0.51 was established using the mean and 2 standard deviations (SD) above a healthy control population for anti-PF4 IgG, IgA, and IgM, respectively.

3.5 Laboratory testing for anti-PF4 IgG subclasses in VITT patients

An in-house EIA for detecting anti-PF4 IgG subclass antibodies was also developed based on the procedure above with the following changes: mouse anti-human IgG1, 2, 3, or 4 conjugated with AP (SouthernBiotech, Birmingham, AL, USA) were diluted 1:200 using PBS with 1% BSA then added to each well. A positive cut-off of 0.15, 0.14, 0.17, and 0.10 was established using the mean and 2 SD above a healthy control population for IgG1, IgG2, IgG3, and IgG4, respectively.

3.6 PBMC isolations and cryopreservation

Whole blood collected in acid citrate dextrose tubes was diluted in PBS, layered over Histopaque-1077 (Sigma-Aldrich, St Louis, MO, USA), and separated by density gradient centrifugation at 400 x g for 30 min with no brakes. The plasma was collected, and the buffy coat layer was removed and washed using PBS with 10% heat-inactivated fetal bovine serum (FBS, Life Technologies-Gibco, Waltham, MA, USA) and heparin, spun at 510 x g for 10 mins with maximum brake. Residual red blood cells were removed by treatment with ammonium-chloride-potassium (ACK) lysing buffer (Life Technologies-Gibco, Waltham, MA, USA) for 10 minutes at room temperature in the dark and then washed again with PBS supplemented with 10% FBS, spun at 510 x g for 10 mins with max brakes. Next, PBMCs were resuspended in AIM-V media with 10% FBS, 2 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin (Life Technologies-Gibco, Waltham, MA, USA) then quantified using a Bio-Rad TC20 automated cell counter with trypan blue dye exclusion (Bio-Rad, Hercules, CA,

USA). PBMCs were spun down at 510 x g for 10 mins and resuspended in AIM-V media with 10% FBS and 20% DMSO (Sigma-Aldrich, St Louis, MO, USA) to protect cells against osmotic lysis. Finally, PBMCs were frozen in aliquots of $1x10^7$/mL per cryovial, placed in a -1°C/minute rate freezing container (Mr. Frosty, Thermo-Fisher Scientific, Waltham, MA, USA) with isopropanol in -80°C, and then moved to long-term storage in liquid nitrogen vapour phase for cryopreservation.

3.7 PBMC thawing

20 million PBMCs were thawed in a 37°C water bath and added dropwise to pre-warmed serum-free AIM-V media (Life Technologies-Gibco, Waltham, MA, USA) with 10% FBS, 2 mM L-glutamine, 100 units/mL penicillin, 100 µg/mL streptomycin, and 1 unit/mL heparin (Pfizer, New York, NY, USA). Thawed PBMCs were spun at 350 x g for 10 minutes and resuspended in 5 mL of the same pre-warmed AIM-V media. PBMCs were quantified using a Bio-Rad TC20 automated cell counter with trypan blue dye exclusion, spun at 350 x g for 10 minutes, and resuspended at $2x10^6$/mL using AIM-V media with 2 mM L-glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin.

3.8 ELISpot for the detection of VITT memory B cells

Thawed PBMCs from VITT patient PBMCs (n = 18) were cultured to assess for anti-PF4 specific memory B cells. PBMCs from COVID-19-infected individuals (n = 5) were used as controls. 500 µL of thawed PBMCs resuspended at $3x10^6$/mL

in AIM-V (with 2 mM L-glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin) were stimulated with an equal volume of 4 µg/mL (2 µg/mL final) R848 (Resiquimod; Mabtech, Stockholm, Sweden) with 20 ng/mL (10 ng/mL final) IL-2 (Mabtech, Stockholm, Sweden). Unstimulated PBMCs were cultured in 500 µL of AIM-V media alone in 24-well tissue culture plates (Corning, New York, NY, USA). PBMCs were incubated at 37°C, 5% CO_2 for 5 days. After 5 days, stimulated and unstimulated PBMCs were collected and pelleted at 350 x g for 10 minutes. Supernatants containing secreted antibodies were collected and frozen at -80°C. The pelleted PBMCs were then resuspended and washed three times with AIM-V serum-free media with 2 mM L-glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin. Finally, the stimulated and unstimulated PBMCs were quantified using a Bio-Rad TC20 automated cell counter with trypan blue dye exclusion. On day 4, a Millipore Multiscreen PVDF ELISpot plate (Mabtech, Stockholm, Sweden) was hydrated with 50 µL of 70% ethanol for less than 2 minutes to activate the membrane and then washed 3 times with sterile distilled water. The plate was coated with 100 µL/well of 10 µg/mL of mouse anti-human IgG, IgM or IgA (Mabtech) and incubated overnight at 4°C. On day 5, the plate was washed three times with PBS + 0.05% Tween20 and twice with PBS (250 µL/well) and blocked with PBS with 3% BSA for 2 hours at room temperature. Unstimulated and stimulated PBMCs were then added to the conditioned plate at 200,000 cells/well in duplicate to assess anti-PF4 antibody secretion and 10,000 cells/well of stimulated cells in duplicate to assess total antibody secretion. The plate was incubated at 37°C, 5% CO_2 overnight. On day

6, the cells were decanted and the plate was washed three times with PBS + 0.05% Tween20 and twice with PBS (200 µL/well). Secreted anti-PF4 specific IgG, IgM or IgA were detected with a 50% bioPF4 solution made by mixing 25 µg/mL recombinant PF4 with 25 µg/mL biotinylated recombinant PF4 in PBS + 0.05% BSA which was then added to unstimulated and stimulated specific wells (100 µL/well). For assessment of total IgG, IgM and IgA, biotinylated mouse anti-human IgG, IgM or IgA (Mabtech) at a concentration of 1 µg/mL in PBS + 0.05% BSA was added (100 µL/well) and the ELISPOT plate was incubated for 2 hours at 37°C. The plates were then washed 4 times with PBS + 0.05% Tween20 and twice with PBS and a 1/1000 dilution of streptavidin conjugated with horse radish peroxidase (Mabtech) in PBS + 0.05% BSA was added to each well and incubated for 1 hour at room temperature. The backing of the ELISPOT plate was removed and the front and back of the plate were washed four times with PBS + 0.05% Tween20, then twice with PBS and 100 µL of tetramethylbenzidine (TMB) substrate (Mabtech) was added to each well. The plates were incubated in the dark for 30 minutes at room temperature and then washed five times with distilled water. The plates were left to dry in the dark overnight and then read using a CTL Immunospot Analyzer (CTL, Shaker Heights, OH, USA) and frequencies of specific memory B cells were calculated from the ratio of cells secreting PF4 specific IgG/A/M antibodies versus total IgG/A/M secreting cells.

3.9 Statistical analysis

All statistical analyses were performed using Python (version 3.12.0) with Jupyter lab (version 4.0.6). After determining the normality of the data with the Shapiro-

Wilk test, categorical variables were assessed using Fisher's exact test. For continuous variables, an independent two-sample t-test or the Mann-Whitney U test was used to assess normally or non-normally distributed data, respectively. *P*-values < 0.05 were considered statistically significant.

4.0 RESULTS

4.1 Antibody class and subclass distribution analysis of VITT patients

We tested 31 acute-phase VITT patient samples for anti-PF4 antibody classes and subclasses (Table 1). Single doses of ChAdOx1 nCoV-19 or Ad26.COV2.S were given to 29/31 (93.5%) and 2/31 (6.5%) patients, respectively. All patients initially presented with thrombocytopenia and 29/31 (93.5%) had thrombosis. All patients also tested positive for anti-PF4 antibodies (OD ≥ 0.4; mean OD = 2.27, range = 0.76 – 3.35) and for platelet activation (≥ 20% ^{14}C-serotonin release) in the PF4-SRA. We tested anti-PF4 IgG, IgA, and IgM antibody levels from 31 acute-phase unique VITT plasma samples (Figure 1A). All 31 patients had anti-PF4 IgG (mean OD = 2.50 ± 1.04, range = 0.55 – 3.75); 5 (16.1%) had IgA anti-PF4 (mean OD = 0.25 ± 0.32, range = 0.09 – 1.91); and 16 (51.6%) had IgM anti-PF4 (mean OD = 0.83 ± 0.90, range = 0.17 – 3.20). When analyzing the distribution of antibody class by patient, 14 (45.2%) VITT patients had IgG alone, 12 (38.7%) had IgG and IgM, 1 (3.2%) patient had IgG and IgA and 4 (12.9%) had IgG, IgA and IgM (Figure 1B).

Anti-PF4 IgG subclasses were also analyzed in the same 31 VITT patients (Figure 2A); 28 (90.3%) had IgG1 (mean OD = 1.47 ± 1.39, range = 0.08 – 3.74); 20

(64.5%) had IgG2 (mean OD = 0.25 ± 0.21, range = 0.07 – 0.96); 4 (12.9%) had IgG3 (mean OD = 0.25 ± 0.73, range = 0.03 – 3.90); and 1 (3.2%) had IgG4 (mean OD = 0.05 ± 0.02, range = 0.03 – 0.10). When analyzing the distribution of antibody subclass by patient, 11 (35.5%) patients had IgG1 only, 15 (48.4%) patients had IgG1 and IgG2, 1 (3.2%) patient had IgG2 alone, 2 (6.5%) patients had IgG2 and IgG3, 1 (3.2%) patient had IgG1, IgG2, and IgG3 and 1 (3.2%) patient had all 4 IgG subclasses (Figure 2B).

We assessed demographic and clinical data between different anti-PF4 antibody class and subclass groups but did not observe significant differences in age, disorder onset, diagnostic testing and thrombotic events (Table 2). We found no significant correlation between anti-PF4 IgG2 antibodies and more severe clinical outcomes, such as CVST or multiple thromboses (Table 2B).

4.2 Longitudinal analysis of VITT patients

As the reference laboratory for VITT testing in Canada, we received longitudinal samples from 30 VITT patients that were collected at various intervals (Table 3). Of the VITT follow-up patients, 15 (50%) were female, and the median age at the time of adenoviral-vector COVID-19 vaccination was 50 years (range: 38–72 years). At the time of VITT diagnosis, 29/30 (97 %) initially received the ChAdOx1 nCoV-19 (AstraZeneca) vaccine, and 1/30 (3%) received Ad26.COV2.S (Johnson & Johnson/Janssen). During the acute episode of VITT, the median platelet count was found to be 52 x 10^9/L (range 16–301 x 10^9/L), and 28/30 (93%) experienced at least one thrombotic event with 8/30 (29%) experiencing CVST. Initial VITT patient samples were collected over a median of

15 days (range 7–61 days) since vaccination and all were anti-PF4 EIA positive, with an average OD of 2.25, and PF4-SRA positive, with an average peak release of 87%. Longitudinal VITT patient samples were tested over time for anti-PF4 IgG/A/M antibodies presence and platelet activation with a median latest follow-up sample date of 715 days (range 126–1065 days) post-adenoviral SARS-CoV-2 vaccination (Figure 3A and B). As of their latest sample date, 22/30 (73.3%) VITT patients remained positive for anti-PF4 antibodies, and of those, 14/22 (63.6%) were found to be platelet-activating. We then used a Kaplan-Meier curve to more accurately track the total percentage of VITT patients who tested positive for anti-PF4 antibodies (blue) and platelet activation (red) over time (Figure 4). Points on the graph represent when patients became censored, meaning it was the last sample received from a patient that tested positive for anti-PF4 antibodies or platelet activation, which is accounted for in our probability. From this analysis, at our latest patient sample date of 1065 days post-adenoviral SARS-CoV-2 vaccination, 65.2% and 34.1% of VITT patients tested positive for anti-PF4 antibodies and platelet activation, respectively.

We then investigated for any correlations between initial clinical and demographic data and the longitudinal presence of anti-PF4 antibodies or platelet activation within VITT patients. We assessed initial demographic and clinical data between VITT patients with or without longitudinal anti-PF4 antibody presence and platelet activation (Table 4A and B). While no significant differences were observed, potentially due to our small sample size limiting our statistical power, there was a non-significant increase of CVST observed in the anti-PF4 EIA

positive (p = value = 0.07) and PF4-SRA positive groups (p-value = 0.10) and SVT observed in the anti-PF4 EIA negative group (p-value = 0.05).

Of the 30 VITT patients followed in this study, 19/30 (63.3%) patients were interviewed by telephone or recurrent symptomology and treatments from October 2023 – July 2024 (Table 5). Clinical follow-up data were available for 8/14 (57.1%) patients with persistent platelet-activating antibodies. Among these patients, 7/8 (87.5%) continue to receive anticoagulant therapy (Apixaban or Rivaroxaban; n = 5) or anti-platelet therapy (Aspirin or Jamp ASA EC; n = 2).

4.3 VITT memory B cell detection

As part of the VITT Follow-up Study and the COVID-19 PBMC study, longitudinal samples from VITT patients and SARS-CoV-2 infected individuals were collected and PBMCs were isolated. PBMC samples were drawn from both studies to investigate VITT memory B cell presence. Initial clinical presentation and demographic data were analyzed for the individuals chosen for this study (Table 6).

Memory B cells from VITT patients' PBMCs (n = 18) and SARS-CoV-2 infected participants' PBMCs (n = 5) were stimulated with R848 and IL-2 and the ELISpot assay was employed for the detection of VITT memory B cells and total memory B cells (Figure 5). PF4-specific memory B cells were enumerated relative to the total number of IgG, IgA, and IgM secreting memory B cells (Figure 6). VITT total IgG, IgA, and IgM spots were found to be a mean of 985.6 spots (range: 20 - 5040 spots), 468.4 spots (range: 30 - 2060 spots), and 3683.8 spots (range: 1420 - 9540 spots) per 200K PBMCs, respectively (Figure 6A). VITT anti-PF4 IgG, IgA,

and IgM spots were found to be a mean of 1.42 spots (range: 0 - 13 spots), 1 spot (range: 0 – 7.5 spots), and 2 spots (range: 0 - 23 spots) per 200K PBMCs, respectively. SARS-CoV-2 total IgG, IgA, and IgM spots were found to be a mean of 1180 spots (range: 580 - 1960 spots), 390 spots (range: 200 - 650 spots), and 4784 spots (range: 700 - 9010 spots) per 200K PBMCs, respectively (Figure 6B). SARS-CoV-2 anti-PF4 IgG, IgA, and IgM spots were found to be a mean of 0.1 spots (range: 0 – 0.5 spots), 0.8 spots (range: 0 – 3 spots), and 0.5 spots (range: 0 - 2 spots) per 200K PBMCs, respectively.

The stimulated and unstimulated VITT PBMC supernatants were examined for anti-Spike and anti-PF4 IgG, IgA, and IgM antibodies (Figure 7). First, the VITT supernatant was tested using an in-house anti-Spike EIA (Figure 7A). Stimulated supernatant had a mean OD of 1.31 (range: 0.13 – 3.18), 0.12 (range: 0.08 – 0.18), and 0.12 (range: 0.08 – 0.21) for IgG, IgA, and IgM, respectively. Unstimulated supernatant had a mean OD of 0.31 (range: 0.11 – 1.5), 0.12 (range: 0.09 – 0.15), and 0.09 (range: 0.07 – 0.13) for IgG, IgA, and IgM, respectively. Second, the VITT supernatant was tested using the Immucor GAM EIA kit (Figure 7B). Stimulated supernatant had a mean OD of 0.09 (range: 0.04 – 0.35) for anti-PF4 IgG/A/M. Unstimulated supernatant had a mean OD of 0.04 (range: 0.03 – 0.05) for anti-PF4 IgG/A/M. Third, the VITT supernatant was tested using an in-house anti-PF4 EIA (Figure 7C). Stimulated supernatant had a mean OD of 0.10 (range: 0.07 – 0.15), 0.12 (range: 0.08 – 0.16), and 0.80 (range: 0.21 – 3.15) for IgG, IgA, and IgM, respectively. Unstimulated supernatant had a mean OD of 0.10 (range: 0.07 – 0.13), 0.11 (range: 0.08 – 0.13), and 0.18 (range: 0.11 – 0.27) for IgG, IgA, and IgM, respectively.

As a control, the stimulated and unstimulated SARS-CoV-2 PBMC supernatants were also examined for anti-Spike and anti-PF4 IgG, IgA, and IgM antibodies (Figure 8). First, the SARS-CoV-2 supernatant was tested using an in-house anti-Spike EIA (Figure 8A). Stimulated supernatant had a mean OD of 2.05 (range: 0.22 – 3.11), 0.16 (range: 0.11 – 0.22), and 0.13 (range: 0.07 – 0.18) for IgG, IgA, and IgM, respectively. Unstimulated supernatant had a mean OD of 0.24 (range: 0.12 – 0.44), 0.15 (range: 0.14 – 0.17), and 0.08 (range: 0.08 – 0.09) for IgG, IgA, and IgM, respectively. Second, the SARS-CoV-2 supernatant was tested using the Immucor GAM EIA kit (Figure 8B). Stimulated supernatant had a mean OD of 0.13 (range: 0.04 – 0.26) for anti-PF4 IgG/A/M. Unstimulated supernatant had a mean OD of 0.04 (range: 0.03 – 0.05) for anti-PF4 IgG/A/M. Third, the SARS-CoV-2 supernatant was tested using an in-house anti-PF4 EIA (Figure 8C). Stimulated supernatant had a mean OD of 0.10 (range: 0.08 – 0.12), 0.14 (range: 0.12 – 0.17), and 1.55 (range: 0.18 – 3.07) for IgG, IgA, and IgM, respectively. Unstimulated supernatant had a mean OD of 0.08 (range: 0.06 – 0.10), 0.13 (range: 0.09 – 0.19), and 0.22 (range: 0.16 – 0.32) for IgG, IgA, and IgM, respectively.

5.0 DISCUSSION

5.1 Antibody class and subclass

Both HIT and VITT patients exhibited a predominant IgG anti-PF4 antibody response. VITT patients showed a similar frequency of IgM at 51.6% compared to 21% - 57%, as reported in HIT (Supplementary Table 2) [76-79]. However, only

16.1% of VITT samples had an anti-PF4 IgA antibody response compared to 29.0% - 57.7% previously reported in HIT, indicating the VITT immune response is likely more restricted towards generating anti-PF4 IgG class antibodies than IgA (Supplementary Table 2) [76-79]. The mechanisms controlling antibody class switch recombination are not fully understood, but perhaps differences in HIT and VITT pathogenesis promote distinct antibody class profiles. The invasive nature of surgery in HIT may contribute to increased IgA production, but this is not seen in VITT which occurs shortly after vaccination. Perhaps IgA production is lower in VITT due to the vaccination environment being less conducive to IgA production in general. The lack of IgA production and protective mucosal protection is an ongoing challenge for vaccine development; for instance, typical vaccine administration routes, namely subcutaneous and intramuscular, generally produce lower mucosal immune responses [106]. One study examined anti-Spike antibody classes following SARS-CoV-2 vaccines and showed that IgA titers were lower with adenoviral-based vaccines compared with mRNA-based vaccines [107, 108]. Thus, the adenoviral vector-based SARS-CoV-2 vaccine leads to less IgA production, which may also affect antibody production in VITT, as it occurs shortly after.

Antibody subclass switching is an irreversible stepwise procedure dictated by gene order, starting with IgG3 followed by IgG1, IgG2 then IgG4, with each IgG subclass having a different function despite 90% sequence homology amongst them [17]. The IgG subclass EIA proved to be quite challenging to develop. We started by utilizing a previously developed protocol that had been shown to work in HIT, but this proved ineffective at detecting subclasses in VITT [80]. This led us to try

and optimize the subclass EIA, creating a novel assay that provided more replicable results (Supplementary Table 1). After many rounds of optimization, we noticed an issue with our detection method for VITT. When testing known VITT patients that were positive for anti-PF4 IgG, we found that 100% (7/7) tested positive for anti-PF4 IgG when a directly conjugated detection antibody was used, but only 43% (3/7) tested positive for anti-PF4 IgG when 2 detection antibodies were used (Supplementary Figure 1). Our signal dropped when utilizing 2 detection antibodies and the reason why this occurs is unknown. This assay yielded good results in HIT, but not VITT, perhaps due to the more monoclonal nature or increased instability in the VITT antibody. Utilizing directly conjugated subclass antibodies gave us much better results. We found that VITT and HIT anti-PF4 antibodies have similar frequencies of IgG1 and IgG3, but VITT has a higher frequency of IgG2 (64.5%; Supplementary Table 3). Two previous studies reported lower anti-PF4 IgG2 frequencies in HIT, at 3.8% and 8.2%; however, one HIT study by Arepally *et al*. reported an IgG2 prevalence similar to what we observed for VITT anti-PF4 antibodies at 61.8% (Supplementary Table 3) [77, 79, 80]. Notably, their HIT cohort had an unusually high frequency of thrombosis (23/36; 63.9%) compared to the general HIT thrombosis rate of 10-20% [79]. While a shift toward IgG2 is considered immune-protective as it has limited complement-activation ability, perhaps IgG2 plays a role in thrombosis as it interacts primarily with a single Fc receptor, FcγRIIa, which is essential for platelet activation in VITT [7, 17, 18, 109]. This higher IgG2 prevalence may correlate with an increased risk of thrombosis, as seen in other highly thrombotic disorders such as anti-phospholipid syndrome (APS) [79, 110]. IgG1 and IgG2 could work synergistically by targeting different

epitopes, with IgG2 specifically activating cells with FcγRIIa (platelets and monocytes), causing increased complement activation, complex formation, and thrombosis [17, 79]. In our study, two VITT patients with high IgG3 levels did not have IgG1, indicating that IgG3 antibodies may serve the same function to activate complement and engage multiple Fc receptors [17].

Recent studies have shown that VITT-like anti-PF4 antibodies can develop in patients with monoclonal gammopathy and after natural adenoviral infection without heparin administration or SARS-CoV-2 adenoviral vector-based vaccination [59-61, 63-65]. These observations and the rapid IgG immune response suggest that VITT is most consistent with a secondary immune response re-stimulated by adenoviral vector-based vaccination or other causes. The initial immune response could be anti-bacterial, as IgG responses to polysaccharide bacterial antigens are almost always IgG2, explaining the predominance of IgG2 in VITT and some HIT studies [18, 79, 111].

We then investigated whether different antibody profiles within VITT patients influenced clinical outcomes, focusing on IgG2, as we hypothesized it may correlate to an elevated severity of thrombosis. We assessed demographic and clinical data between different anti-PF4 antibody class and subclass groups but did not observe significant differences in age, disorder onset, diagnostic testing, and thrombotic events (Table 2). We found no correlation between anti-PF4 IgG2 antibodies and more severe clinical outcomes, such as CVST or multiple thromboses (Table 2B). This aligns with findings by Arepally *et al.*, who also found no correlation between anti-PF4 IgG2 and elevated thrombosis in HIT [79]. Despite observing no correlation between severe thrombosis and anti-PF4 IgG2, perhaps

elevated IgG2 is a unique characteristic of prothrombotic disorders in general, as seen in APS, VITT, and HIT [79, 110]. Additionally, we observed no correlation between VITT patients possessing both IgM and IgG2, as both are thymus-independent polysaccharide responses [17].

In summary, we characterized the antibody class and subclass distribution of anti-PF4 antibodies in VITT. Compared to previous studies in HIT, our findings revealed an increase in anti-PF4 IgG2 in VITT patients and a decrease in IgA. The specific roles of anti-PF4 IgG1 and IgG2 in the pathogenesis of VITT remain unclear, as we did not find a significant association between clinical characteristics at presentation and anti-PF4 antibody class or subclass. The role of anti-PF4 IgG1 and IgG2 in VITT is yet unclear, and further research will enhance our understanding of VITT pathology and the resulting clinical outcomes and aid in developing monoclonal antibodies to study anti-PF4 disorders further.

5.2 Longitudinal data

Our data on VITT follow-up samples revealed a heterogenous pattern with some patients remaining anti-PF4 positive with platelet-activating antibodies (n = 14), some remaining anti-PF4 positive but without platelet-activating antibodies (n = 8), and some eventually testing negative for anti-PF4 antibodies (n = 8). Samples were also tested for platelet activation using the PEA and the results aligned with those from the PF4-SRA (Supplementary Figure 2). Interviews were conducted with 8 longitudinal VITT patients who continued to test positive for platelet-activating anti-PF4 antibodies and 7/8 (87.5%) continued to receive anticoagulant therapy (Apixaban or Rivaroxaban; n = 5) or anti-platelet therapy (Aspirin or Jamp

ASA EC; n = 2). This continued treatment could explain why recurrent thrombosis and thrombocytopenia are not observed.

While no significant differences were observed when we investigated for a correlation between more severe initial clinical presentation and longitudinal antibody persistence, we found a non-significant increase of CVST observed in the anti-PF4 EIA positive (p-value = 0.07) and PF4-SRA positive groups (p-value = 0.10) and SVT observed in the anti-PF4 EIA negative group (p-value = 0.05). This could suggest that the presence of more rare types of thrombosis could indicate which patients will have persistent antibodies, but further work would be needed to elucidate this. Clinical information in the acute phase of VITT was recorded by the attending physician on site and the individuals with the most devastating symptoms unfortunately passed away.

While some VITT patients have experienced a rapidly progressive illness that can be fatal, others have experienced a milder disease course [112]. In addition, VITT can also cause a protracted illness characterized by persistent thrombocytopenia and recurrent thrombosis [93]. A study by Schönborn *et al*. had previously shown that VITT antibodies are transient in most patients [113]. Another study by Craven *et al*. found persistently positive serology with 72% of VITT patients remaining positive for anti-PF4 antibodies after a median of 105 days; furthermore, the rate of relapse for thrombocytopenia in this study was 12.6% with one case of recurrent thrombosis; all relapses occurred within 90 days of initial presentation [114]. Thaler *et al*. found that VITT anti-PF4 antibody levels slowly decreased over several weeks and recommended continued anticoagulation until patients are negative for anti-PF4 antibodies or until they

lose their ability to activate platelets [115]. Finally, a study by Schönborn and Greinacher found that anti-PF4 antibodies persist in some VITT patients; however, all platelet-activating antibodies disappeared over a 9-month follow-up period [89]. In contrast, our findings showed 28/30 (93.3%) of VITT patients remained positive for the presence of anti-PF4 antibodies and 19/30 (63.0%) still had platelet-activating antibodies at the 9-month time-point of our VITT cohort. This discrepancy could be due to different laboratory assays for detecting anti-PF4 antibodies, Schönborn and Greinacher used an in-house anti-PF4/heparin EIA to detect VITT antibodies and the study by Craven *et al*. identified the differences in two commercial anti-PF4 EIA results [89, 114]. When investigating pedigree SRA blood donors in the PF4-SRA, preliminary analysis revealed some donors with lower testing results (Supplementary Figure 3). This could suggest the need to investigate and standardize both EIA and functional testing for VITT, but more work is necessary. Panagiota *et al.* recently reported that 2/5 (40.0%) VITT patients remained positive for anti-PF4 antibodies for a median of 300 days [90]. Due to the low prevalence of VITT, the strength of this study is the availability of VITT samples from across Canada that were tested using high sensitivity and specificity platelet activation assays such as the anti-PF4 EIA and the PF4-SRA. To our knowledge, this is the first study to describe VITT anti-PF4 antibodies for nearly three years. Limitations include a selection bias with follow-up samples since not all patients in our cohort could be studied longitudinally due to mortality in the acute phase, they were lost to follow-up, or due to incomplete or absent clinical data. Furthermore, follow-up patient data was self-reported since patients were no longer in a hospital setting.

Our study showed platelet-activating anti-PF4 antibodies can remain in circulation in VITT patients for prolonged periods in contrast to what has been shown with HIT anti-PF4 antibodies, which tend to be transient [100]. Specifically, HIT antibodies lose their ability to activate platelets after a median of 50 days and become undetectable after a median of 85 days [100]. While there is no evidence of immune memory in HIT, one study has shown the risk of recurrent HIT is low but possible with the presence of platelet-activating anti-PF4 antibodies [99]. With the half-life of IgG being about 21 days, we define antibody persistence for VITT beginning at 100 days [81, 82]. This is about 5 half-lives of IgG and beyond the time where HIT antibodies last. A transient de novo immune response should have faded by this time point and anything longer would indicate a more persistent immune response. The differences seen in anti-PF4 antibody persistence in VITT and HIT are likely due to the presence or absence of the antigen, respectively. It was first experimentally shown that adenoviral vectors bound to PF4 via an electrostatic mechanism [31]. However, a recent study investigated the direct interaction of PF4 with Ad26.COV2.S using dynamic light scattering, biolayer-interferometry, and surface plasmon resonance and found this interaction with PF4 to be unlikely, implying the VITT antigen may be PF4 itself. [32] Thus, the presence of PF4 might be responsible for the persistence of anti-PF4 antibodies in VITT, since it could provide continued stimulation for anti-PF4 B cells in VITT patients. Continued laboratory testing of VITT antibodies will provide a better understanding of the long-term implications of VITT, such as the risk of recurrent thrombosis, and improve patient care for those affected by this disorder.

5.3 VITT memory B cell investigation

Lastly, we wanted to investigate whether circulating anti-PF4 memory B cells played a role in antibody persistence. ELISpot assays and anti-PF4 EIAs were employed to investigate VITT memory B cell presence. We stimulated the PBMCs with R848 and IL-2 as this stimulation has been shown to be the best at causing the differentiation of memory B cells into ASCs [116]. This method has been shown to work utilizing samples from SARS-CoV-2 infected individuals. First, we utilized an in-house anti-Spike EIA and detected anti-Spike antibodies in follow-up serum samples from SARS-CoV-2 infected individuals. This means that circulating antibodies still exist and are persisting. We then utilized an anti-Spike ELISpot assay and detected an average of 173 (range: 5 – 639.5) anti-Spike IgG spots in R848-IL2 stimulated PBMCs from SARS-CoV-2 infected individuals. This means that circulating anti-Spike memory B cells exist and could play a role in the maintenance of the anti-Spike antibody response. Previous work from our lab has also shown that while we can accurately detect and count hybridomas that produce a monoclonal anti-PF4 antibody in our anti-PF4 ELISpot assay, we could not detect any anti-PF4 memory B cells in HIT patients.

Anti-PF4 antibodies were still detected in some follow-up serum samples from VITT patients tested using a commercial anti-PF4 EIA. This means that some VITT patients still possess circulating anti-PF4 antibodies. When these samples were tested in the anti-PF4 ELISpot assay, we found an average of 1.4 anti-PF4 IgG spots per 200K PBMCs were detected using longitudinal stimulated PBMCs from VITT patients compared to 0.1 anti-PF4 IgG spots per 200K PBMCs

detected using longitudinal stimulated PBMCs from SARS-CoV-2 infected individuals. Despite observing a higher average frequency in our VITT cohort, our spot counts are low. In comparison, a study examining memory B cells in patients with pemphigus vulgaris, characterized by IgG autoantibodies against desmoglein 3, found desmoglein 3 specific spot counts ranging from 6.3 – 84.0 per 100K PBMCs [117]. Anti-PF4 spots were not conclusively detected in stimulated PBMCs from VITT-positive patients in the anti-PF4 ELISpot assay. This means that perhaps there are no circulating anti-PF4 memory B cells, despite the presence of anti-PF4 antibodies remaining in circulation.

This indicates that another cell type might be responsible for this prolonged antibody response. It is thought that memory B cells could be a more direct precursor to plasma cells responsible for both pathology and antibody-mediated protection when compared to naive B cells. Memory B cells are larger in number than naive B cells in peripheral blood, more stable, and can be cross-reactive. There is also increased evidence pathogenic plasma cells stem from memory B cells [23, 84, 98, 118, 119]. In one study, individuals were immunized with the 2018–2019 influenza seasonal vaccine. Examination revealed that memory B cells formed from a different influenza strain contained cross-reactive clones with the new 2018–2019 strain. Some of these cross-reactive memory B cells became plasmablasts [120]. Perhaps a cross-reactive memory B cell created after a previous adenoviral infection leads to plasmablasts and long-lived plasma cells that produce anti-PF4 antibodies. Upon antigenic stimulation and germinal center formation, B cells can become plasma cells, long-lived plasma cells, or memory B cells. It is unknown what dictates the fate of a B cell, but it is theorized that

antigen affinity could play a role [23]. B cells with high antigen affinity favouring long-lived plasma cells and B-cells with low antigen affinity favouring memory B cells [23]. Studies in other autoimmune conditions such as ITP and MGTS show that long-lived plasma cells reside in niches in the bone marrow or spleen and could be playing roles in these disorders [63-66, 121].

We also collected the supernatant from the stimulated and unstimulated PBMCs and tested them in anti-Spike and anti-PF4 EIAs. Anti-Spike antibodies were detected in the supernatant from both SARS-CoV-2 infected individuals and VITT patients, which was expected as both cohorts have either had a SARS-CoV-2 infection or vaccination and immune memory has been shown to develop. The commercial anti-PF4 IgG/A/M EIA kit and in-house anti-PF4 IgG and IgA testing were negative for both cohorts, which aligns with our ELISpot results. Interestingly, stimulated supernatant samples in both cohorts had high ODs in the in-house anti-PF4 IgM EIA. While this appears to be PF4-specific as there was no increase in IgM seen in the anti-Spike EIA, this was not VITT-specific as both the VITT and SARS-CoV-2 cohort had high ODs. This was unexpected as the commercial EIA testing ODs for IgG/A/M were low and anti-PF4 antibodies in VITT are primarily IgG. While this could suggest the existence of a native IgM B cell in the normal population, more testing is necessary with HIT and healthy supernatants.

Some limitations of our study include that we could only investigate for anti-PF4 memory B cells that circulate in peripheral blood and that there were a limited number of cells in PBMCs to conduct experiments. Future work will require adjusting cell counts, more sensitive techniques, or different samples, such

as bone marrow, to conclusively determine whether anti-PF4 immune memory exists.

6.0 CONCLUSION

In conclusion, this project has elucidated the predominance of IgG as the main antibody class and IgG1 and IgG2 as the main IgG subclasses in VITT. Platelet-activating anti-PF4 antibodies can persist in VITT patients for prolonged periods, but these patients have not experienced any recurrent symptoms and circulating anti-PF4 memory B cells do not appear to play a role in this persistence. Much remains unknown about the persistence of these antibodies, and we will continue to monitor these VITT patients longitudinally to improve our understanding of the long-term impacts of this disorder and help guide treatment options for these patients. More work needs to be done to uncover the role these different types of antibodies play in VITT and to understand what is causing antibody persistence in VITT.

This project has profound implications for antibody persistence, long-term patient care, and future vaccine development. The findings on the persistence of anti-PF4 antibodies can significantly enhance our understanding of VITT and help devise effective long-term treatment options for patients, many of whom continue to take anticoagulants due to the fear of symptom relapse. VITT has increased our knowledge and awareness of anti-PF4 disorders, and we now recognize that anti-PF4 antibodies can also appear in patients with MGTS, after adenoviral infection, and after other vaccines such as HPV [7, 59-61, 63-65, 122, 123]. Further research into VITT can provide crucial insights into the acute and long-term management

of patients suffering from all anti-PF4 disorders, as well as improve the efficacy and safety of future vaccines.

7.0 FIGURES

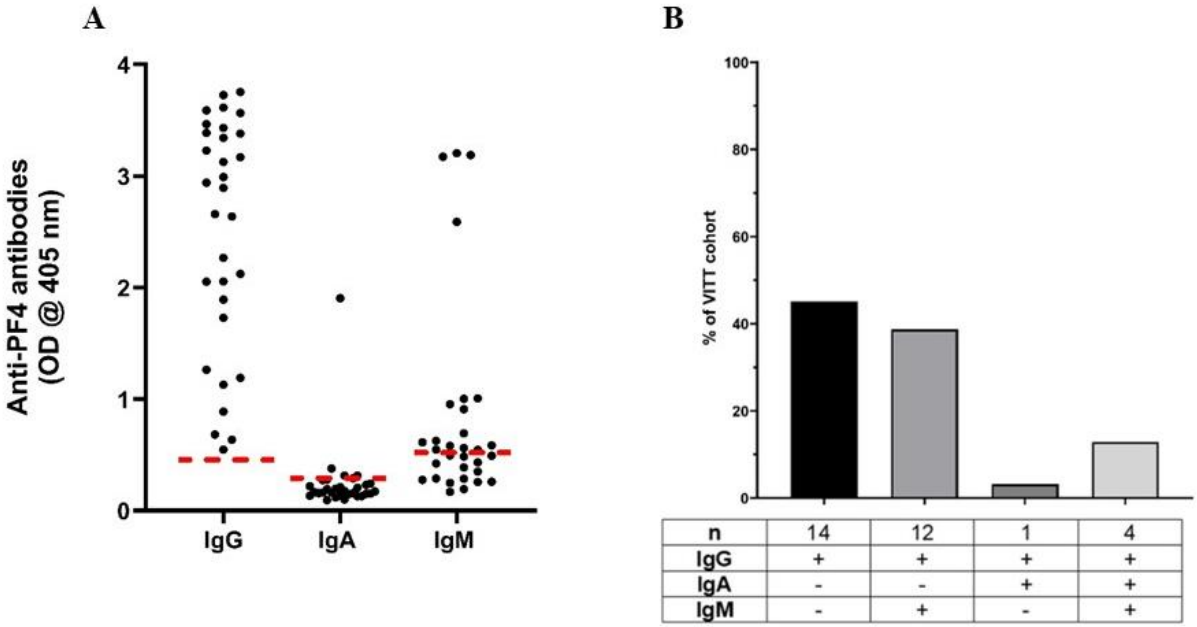


Figure 1: Overall Ig class distribution of anti-PF4 antibodies from clinically defined VITT patients. (A) Acute-phase VITT patients (n = 31) were screened for IgG, IgA, and IgM anti-PF4 antibodies. All 31 (100.0%) tested positive for IgG antibodies with a mean optical density (OD) of 2.50 ± 1.04; 5 (16.1%) tested positive for IgA antibodies with a mean OD of 0.25 ± 0.32; and 16 (51.6%) tested positive for IgM antibodies with a mean OD of 0.83 ± 0.90. A cut-off of 0.45, 0.29, and 0.51 (dotted line) was established using healthy controls for IgG, IgA, and IgM, respectively. **(B)** The anti-PF4 IgA, IgM, and IgG antibody profiles for VITT patients are shown; 14 patients (45.2%) had IgG alone; 12 patients (38.7%) had IgG and IgM; 1 patient (3.2%) had IgG and IgA; and 4 patients (12.9%) had IgG, IgA and IgM.

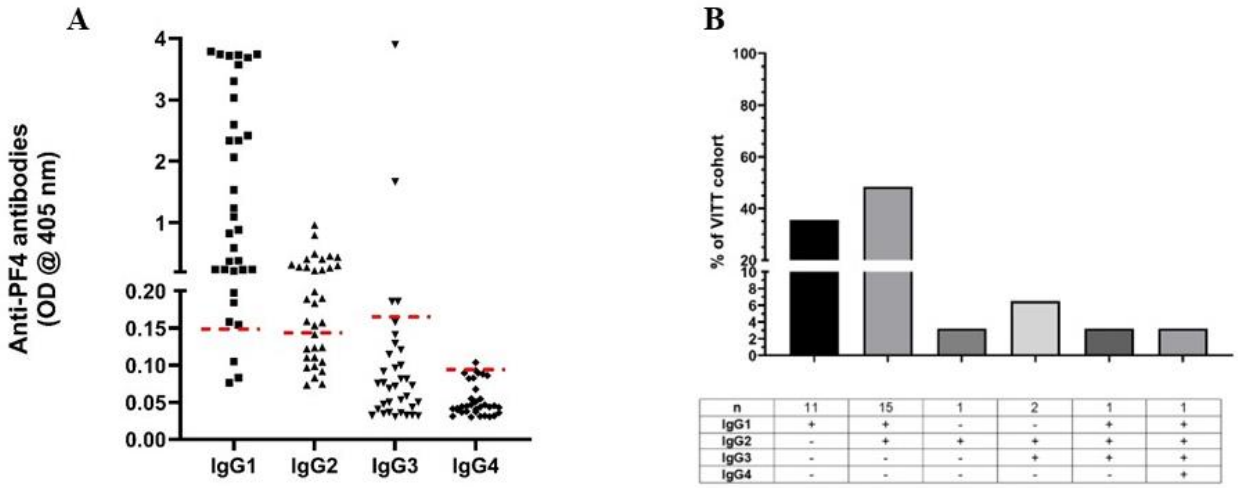


Figure 2: Overall IgG subclass distribution of anti-PF4 antibodies from clinically defined VITT patients. (A) Acute-phase VITT patients (n = 31) were screened for anti-PF4 IgG1, IgG2, IgG3 and IgG4 subclass antibodies; 28 (90.3%) tested positive for IgG1 antibodies with a mean OD of 1.47 ± 1.39; 20 (64.5%) tested positive for IgG2 antibodies with a mean OD of 0.25 ± 0.21; 4 (12.9%) tested positive for IgG3 antibodies with a mean OD of 0.25 ± 0.73; and 1 (3.2%) tested positive for IgG4 antibodies with a mean OD of 0.05 ± 0.02. A positive cut-off of 0.149, 0.144, 0.165, and 0.095 (dotted line) was established using healthy controls for IgG1, IgG2, IgG3 and IgG4, respectively. **(B)** The IgG1, IgG2, IgG3 and IgG4 antibody profiles for the VITT patients are shown; 11 patients (35.5%) had IgG1 alone, 15 patients (48.4%) had IgG1 and IgG2, 1 patient (3.2%) had IgG2 alone, 2 patients (6.5%) had IgG2 and IgG3, 1 patient (3.2%) had IgG1, IgG2 and IgG3; and 1 patient (3.2%) had all Ig subclasses.

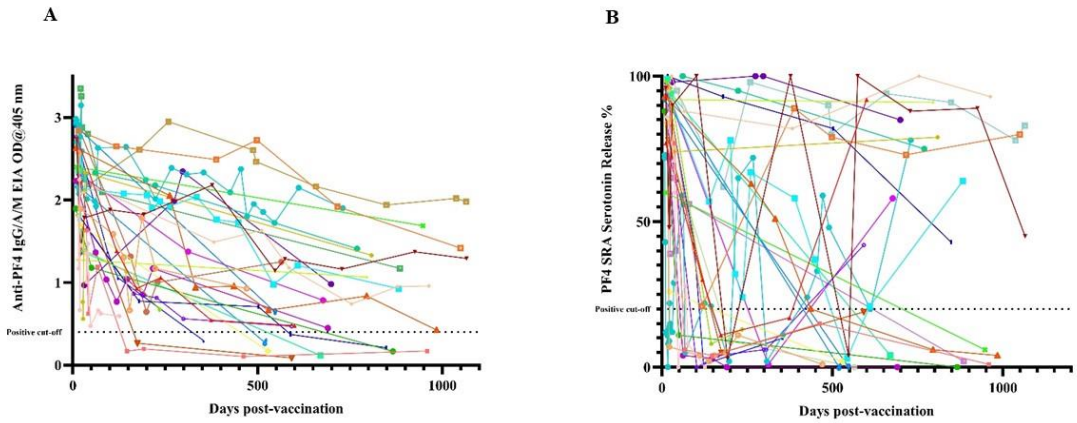


Figure 3: Longitudinal samples were collected on VITT patients (n = 30) with a median latest follow-up sample date of 715 days (range 126-1065 days) post-vaccination with an adenoviral vector-based SARS-CoV-2 vaccine. Each sample was tested for anti-PF4 antibodies and platelet activation. (A) Anti-PF4 IgG/A/M antibodies were detected using the commercial Immucor anti-PF4 EIA. Binding was expressed as ODs (positive $OD_{405nm} \geq 0.4$) and plotted for each patient over time. At their last follow-up, 22 (73.3%) patients were positive for anti-PF4 antibodies. **(B)** The ability of anti-PF4 antibodies to activate platelets was detected using the PF4-SRA and expressed as ^{14}C-serotonin percent release (positive ≥ 20% ^{14}C-serotonin release). The highest serotonin release percentage was plotted for each patient over time. At their last follow-up, 14 (46.7%) patients were positive for platelet-activating anti-PF4 antibodies.

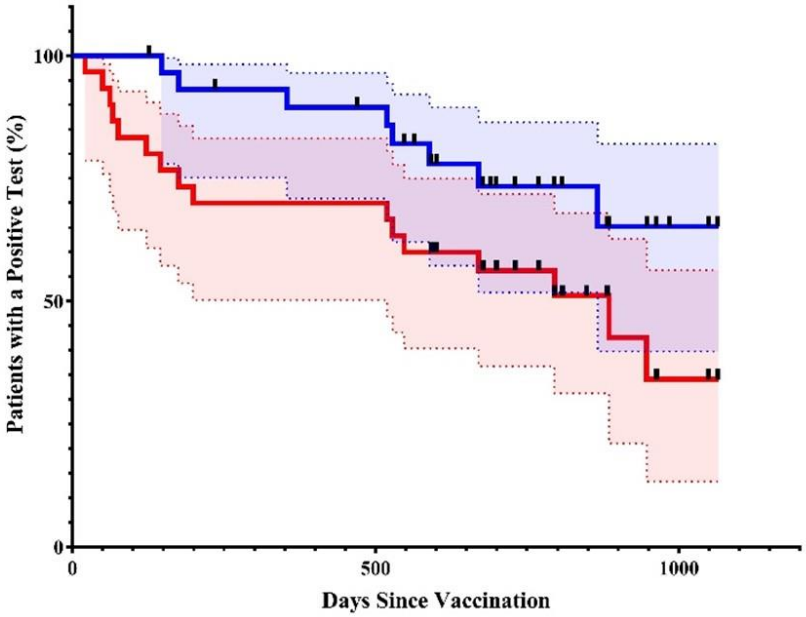


Figure 4: Kaplan-Meier analysis of anti-PF4 antibodies and platelet activation. Longitudinal VITT patients (n = 30) were assessed for presence of anti-PF4 antibodies (blue) and platelet activation (red) while accounting for differences in the latest follow-up date. Initially, 100% of VITT patients tested positive for anti-PF4 antibodies and platelet activation. Points on the graph represent censored patients, which is accounted for in our analysis. As of day 1065, 65.2% and 34.1% of VITT patients tested positive for anti-PF4 antibodies and platelet activation. Shading represents the 95% confidence interval.

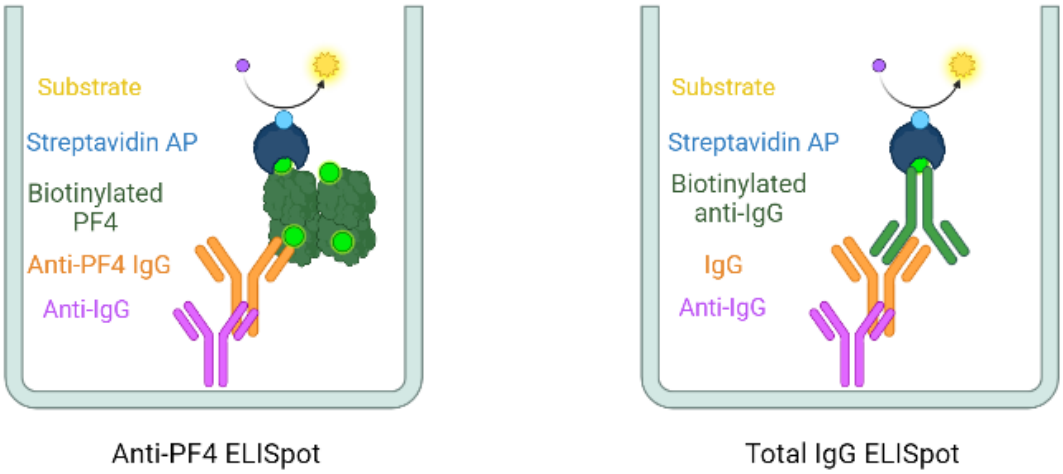


Figure 5: A schematic demonstrating the methodology behind the VITT ELISpot assay. The left well shows the detection of anti-PF4 IgG and the right well shows the detection of total IgG.

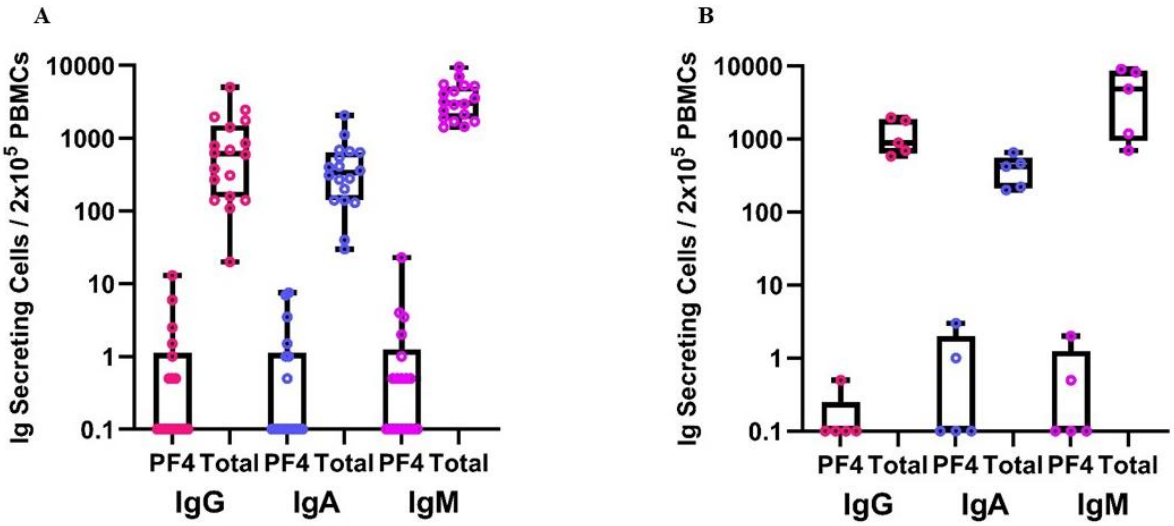


Figure 6: Anti-PF4 and total antibody-secreting cell counts per 2 x 10^5 PBMCs from VITT patients and SARS-CoV-2-infected individuals. A) Longitudinal VITT patients' PBMCs (n = 18) were stimulated with R848 and IL-2 for 5 days and tested for IgG, IgA, and IgM anti-PF4 specific and total ASCs. Cell counts per 2 x 10^5 PBMCs were shown in a box and whisker plot. **B)** Longitudinal SARS-CoV-2-infected individuals' PBMCs (n = 5) were stimulated with R848 and IL-2 for 5 days and tested for IgG, IgA, and IgM anti-PF4 specific and total ASCs. Cell counts per 2 x 10^5 PBMCs were shown in a box and whisker plot.

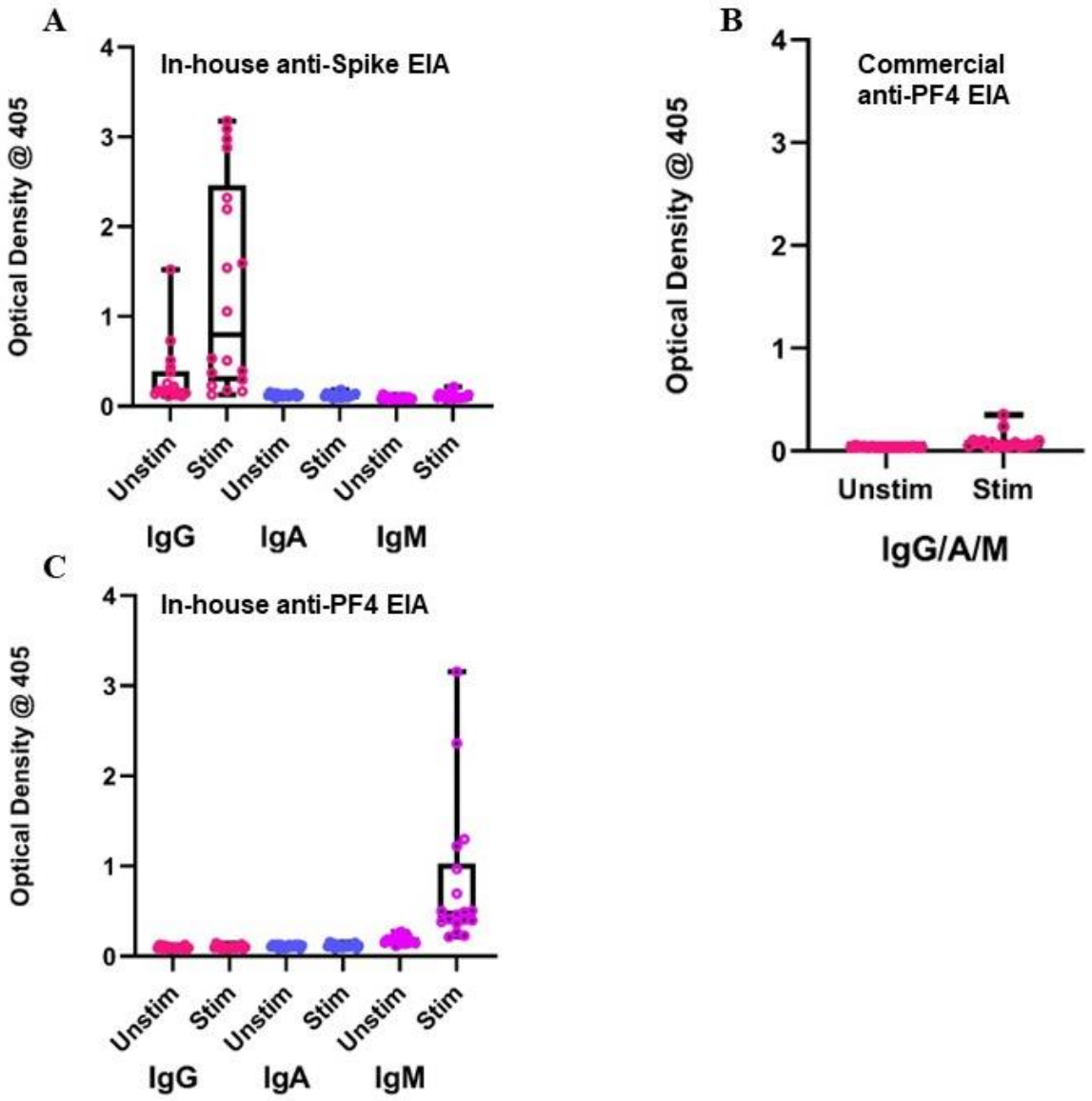


Figure 7: Anti-Spike and anti-PF4 antibody class distributions from supernatant collected from stimulated or unstimulated VITT patient PBMCs. **A)** Supernatant was collected from longitudinal VITT patients' PBMCs (n = 18) stimulated with R848 and IL-2 or unstimulated for 5 days. Unstimulated and stimulated supernatant was tested for IgG, IgA, and IgM anti-Spike using in-house anti-Spike EIAs and ODs were shown in a box and whisker plot. **B)** Supernatant was collected from longitudinal VITT patients' PBMCs (n = 18) stimulated with R848 and IL-2 or unstimulated for 5 days. Unstimulated and stimulated supernatant was tested for IgG/A/M anti-PF4 using the commercial Immucor anti-PF4 EIA and ODs were shown in a box and whisker plot. **C)** Supernatant was collected from longitudinal VITT patients' PBMCs (n = 18) stimulated with R848 and IL-2 or unstimulated for 5 days. Unstimulated and stimulated supernatant was tested for IgG, IgA, and IgM anti-PF4 using in-house anti-PF4 EIAs, and ODs were shown in a box and whisker plot.

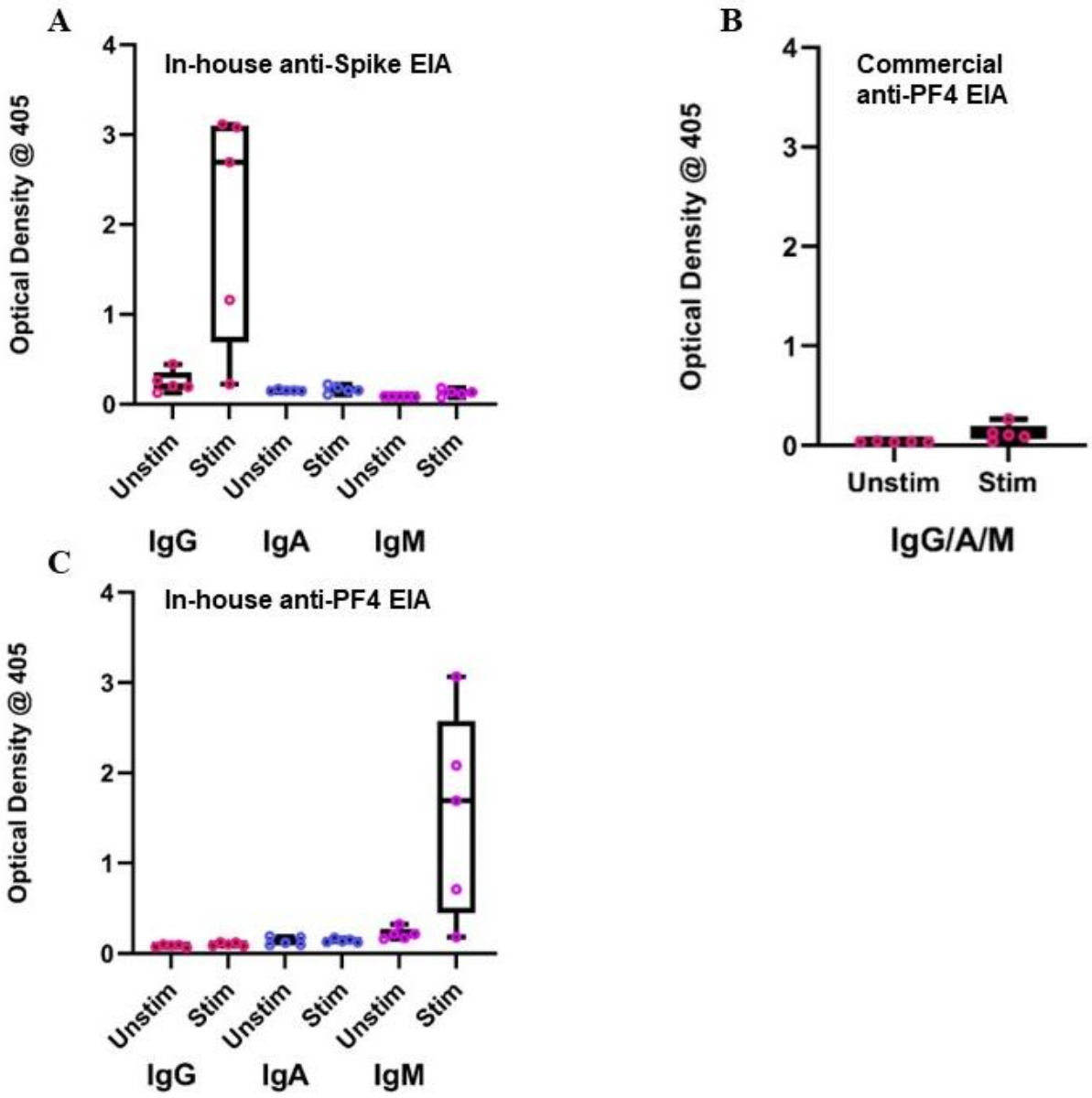


Figure 8: Anti-Spike and anti-PF4 antibody class distribution from supernatant collected from stimulated or unstimulated SARS-CoV-2-infected PBMCs. A) Supernatant was collected from longitudinal SARS-CoV-2-infected individuals' PBMCs (n = 5) stimulated with R848 and IL-2 or unstimulated for 5 days. Unstimulated and stimulated supernatant was tested for IgG, IgA, and IgM anti-Spike using in-house anti-Spike EIAs and ODs were shown in a box and whisker plot. **B)** Supernatant was collected from longitudinal SARS-CoV-2-infected individuals' PBMCs (n = 5) stimulated with R848 and IL-2 or unstimulated for 5 days. Unstimulated and stimulated supernatant was tested for IgG/A/M anti-PF4 using the commercial Immucor anti-PF4 EIA and ODs were shown in a box and whisker plot. **C)** Supernatant was collected from longitudinal SARS-CoV-2-infected individuals' PBMCs (n = 5) stimulated with R848 and IL-2

or unstimulated for 5 days. Unstimulated and stimulated supernatant was tested for IgG, IgA, and IgM anti-PF4 using in-house anti-PF4 EIAs, and ODs were shown in a box and whisker plot.

8.0 TABLES

Table 1: Demographic and clinical characteristics of VITT patients used to test anti-PF4 antibody classes and subclasses.

Demographic and Clinical Data	Patient Data (n = 31)
Demographics	
Sex, n (%)	
Male	13 (48.4)
Female	16 (51.6)
Median age (y, median [range])	52.8 (34.9 - 81.2)
ChAdOx1 nCoV-19 (Oxford-AZ), n (%)	29 (93.5)
Clinical	
Mean anti-PF4 IgG/A/M OD (OD_{405nm}, mean [range])	2.27 (0.76 - 3.35)
Positive in the PF4-SRA at 0 μg/mL PF4 added, n (%)	18 (58.1)
Time from vaccination to symptom onset (days, median [range])	16.4 (7 - 61)
Patients with platelet count available	
Median platelet count (10^9/L, median [range])	47.5 (15 - 301)
Clinical presentation, n (%)	
Thrombosis*	29 (93.5)
Multiple thrombotic sites	10 (34.5)
CVST	11 (37.9)
PE	11 (37.9)
DVT	8 (27.6)
SVT	4 (13.8)
Arterial clot	4 (13.8)

*Some patients experienced multiple thromboses.

Table 2: Demographic and clinical data analysis between patients with different anti-PF4 antibody classes and subclasses. (A) VITT patients with anti-PF4 IgG alone (n = 14) or anti-PF4 IgG with another antibody class (n = 17). **(B)** VITT patients with anti-PF4 IgG2 (n = 20) or without anti-PF4 IgG2 (n = 11).

A

All patients	IgG alone (n = 14)	IgG, M and/or A (n = 17)	P-value
Demographics			
Sex, n (%)			0.07
Male	4 (28.6)	11 (64.7)	
Female	10 (71.4)	6 (35.3)	
Median age (years, median [range])	52.9 (38.8 - 72.3)	47.1 (34.9 - 81.2)	0.56
Clinical			
Mean anti-PF4 IgG/A/M OD (OD_{405nm}, mean [range])	2.36 (0.76 - 3.35)	2.20 (1.27 - 3.15)	0.45
Positive in the PF4-SRA at 0 μg/mL PF4 added, n (%)	8 (57.1)	10 (58.8)	1.00
Time from vaccination to symptom onset (days, median [range])	15 (7 - 27)	14 (8 - 61)	1.00
Patients with platelet count available			
Median platelet count (10^9/L, median [range])	69 (15 - 301)	43 (17 - 111)	0.45
Clinical presentation, n (%)			
Thrombosis	13 (92.9)	16 (94.1)	
Multiple thrombotic sites	3 (23.1)	7 (43.8)	0.43
CVST	6 (46.2)	5 (31.3)	0.47
PE	3 (23.1)	8 (50.0)	0.25
DVT	2 (15.4)	6 (37.5)	0.24
SVT	2 (15.4)	2 (12.5)	1.00
Arterial clot	2 (15.4)	2 (12.5)	1.00

B

All patients	With IgG2 (n = 20)	Without IgG2 (n = 11)	P-value
Demographics			
Sex, n (%)			0.72
Male	9 (45.0)	6 (54.5)	
Female	11 (55.0)	5 (45.5)	
Median age (years, mean [range])	52.9 (34.9 - 81.2)	46.3 (37.7 - 64.5)	0.61
Clinical			
Mean anti-PF4 IgG/A/M OD (OD_{405nm}, mean [range])	2.39 (0.76 - 3.35)	2.06 (1.27 - 2.75)	0.12
Positive in the PF4-SRA at 0 μg/mL PF4 added, n (%)	10 (50.0)	8 (72.7)	0.28
Time from vaccination to symptom onset (days, median [range])	15 (7 - 33)	13 (10 - 61)	0.80
Patients with platelet count available			
Median platelet count (10^9/L, median [range])	48 (15 - 111)	47.5 (17 - 301)	0.79
Clinical presentation, n (%)			
Thrombosis	18 (90.0)	11 (100.0)	
Multiple thrombotic sites	5 (27.8)	5 (45.5)	0.43
CVST	8 (44.4)	3 (27.3)	0.45
PE	8 (44.4)	3 (27.3)	0.45
DVT	4 (22.2)	4 (36.4)	0.43
SVT	1 (5.6)	3 (27.3)	0.14
Arterial clot	3 (16.7)	1 (9.1)	1.00

Table 3: Demographic and clinical characteristics of VITT patients used in longitudinal anti-PF4 study.

All patients	Longitudinal testing (n = 30)
Demographics	
Sex, n (%)	
Male	15 (50)
Female	15 (50)
Median age (y, mean [range])	50 (38 - 72)
AstraZeneca, n (%)	29 (97)
Clinical	
Mean anti-PF4 IgG/A/M OD (OD_{405nm}, mean [range])	2.25 (0.76 - 3.35)
Positive at 0 µg/mL PF4 in PF4 SRA, n (%)	18 (60)
Median time to symptom onset (d, median [range])	15 (7 - 61)
Patients with platelet count available	
Median platelet count (10^9/L, median [range])	52 (16 - 301)
Clinical presentation, n (%)	
Thrombosis	28 (93)
CVST	8 (29)
PE	10 (36)
DVT	7 (25)
SVT	4 (14)
Arterial clot	6 (21)

Table 4: Demographic and clinical data analysis between patients with or without anti-PF4 antibodies and platelet activation. (A) VITT patients with anti-PF4 antibodies (n = 22) or without anti-PF4 IgG antibodies (n = 8). **(B)** VITT patients with platelet activation (n = 14) or without platelet activation (n = 16).

A

All patients	***EIA*+ at latest follow-up (n = 22)**	***EIA*- at latest follow-up (n = 8)**	**p-value**
Demographics			
Sex, n (%)			0.68
Male	12 (55)	3 (38)	
Female	10 (45)	5 (63)	
Median age (y, mean [range])	51 (39 - 72)	54 (38 - 64)	0.42
Clinical			
Median anti-PF4 IgG/A/M OD (OD_{405nm}, median [range])	2.31 (0.76 - 3.35)	2.06 (1.63 - 2.75)	0.22
Positive at 0 µg/mL PF4 in PF4 SRA, n (%)	12 (55)	6 (75)	0.42
Median time to symptom onset (d, median [range])	15 (7 - 61)	16 (8 - 22)	0.98
Patients with platelet count available			
Median platelet count (10^9/L, median [range])	64 (16 - 301)	39 (19 - 226)	0.15
Clinical presentation, n (%)			
Thrombosis	20 (91)	8 (100)	
CVST	8 (40)	0	0.07
PE	6 (30)	3 (38)	0.67
DVT	5 (25)	2 (25)	1.00
SVT	1 (5)	3 (38)	0.05
Arterial clot	5 (25)	1 (13)	1.00

B

All patients	*PF4 SRA*+ at latest follow-up (n = 14)	*PF4 SRA*- at latest follow-up (n = 16)	p-value
Demographics			
Sex, n (%)			1.00
Male	7 (50)	8 (50)	
Female	7 (50)	8 (50)	
Median age (y, mean [range])	49 (41 - 69)	48 (38 - 72)	0.27
Clinical			
Mean anti-PF4 IgG/A/M OD (OD_{405nm}, mean [range])	2.15 (0.76 - 2.98)	2.33 (1.63 - 3.35)	0.44
Positive at 0 µg/mL PF4 in PF4 SRA, n (%)	9 (64)	9 (56)	0.72
Median time to symptom onset (d, median [range])	17 (8 - 61)	14 (7 - 22)	0.19
Patients with platelet count available			
Median platelet count (10^9/L, median [range])	57 (37 - 301)	52 (16 - 226)	0.55
Clinical presentation, n (%)			
Thrombosis	14 (100)	14 (88)	
CVST	6 (43)	2 (14)	0.10
PE	6 (43)	3 (21)	0.24
DVT	5 (36)	2 (14)	0.20
SVT	1 (7)	3 (21)	0.60
Arterial clot	2 (14)	4 (29)	0.66

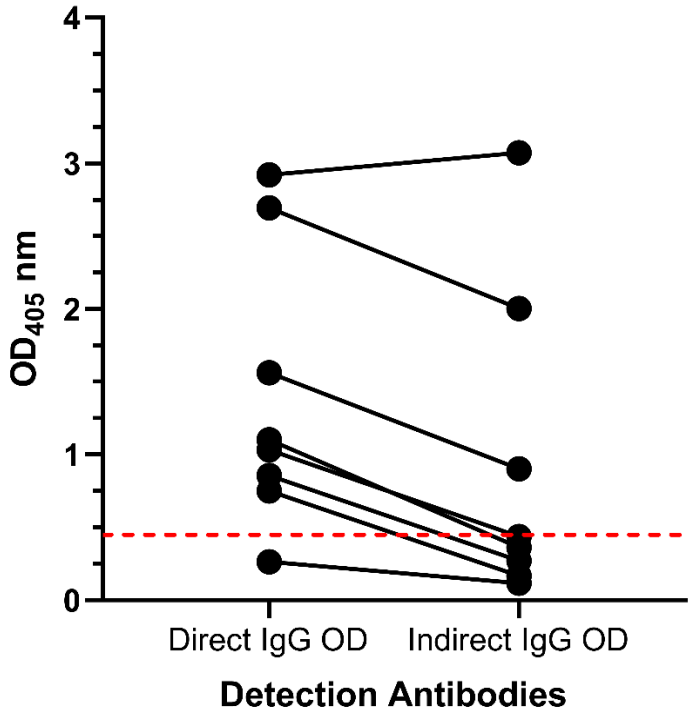
Table 5: Follow-up clinical interview data on VITT patients involved in longitudinal study.

Follow-up Clinical Data	Patient Data (n = 19)
Demographics	
Sex, n (%)	
Male	8 (42.1)
Female	11 (57.9)
Clinical	
Positive for platelet-activating anti-PF4 antibodies at last sample date, n (%)	8 (42.1)
Positive for platelet-activating anti-PF4 antibodies and currently on anticoagulant or anti-platelet treatment, n (%)	
Anticoagulant treatment (Apixaban, Rivaroxaban)	5 (62.5)
Anti-platelet treatment (Aspirin, Jamp ASA EC)	2 (25.0)
Clinical presentation, n (%)	
Recurrent thrombosis	0 (0.0)

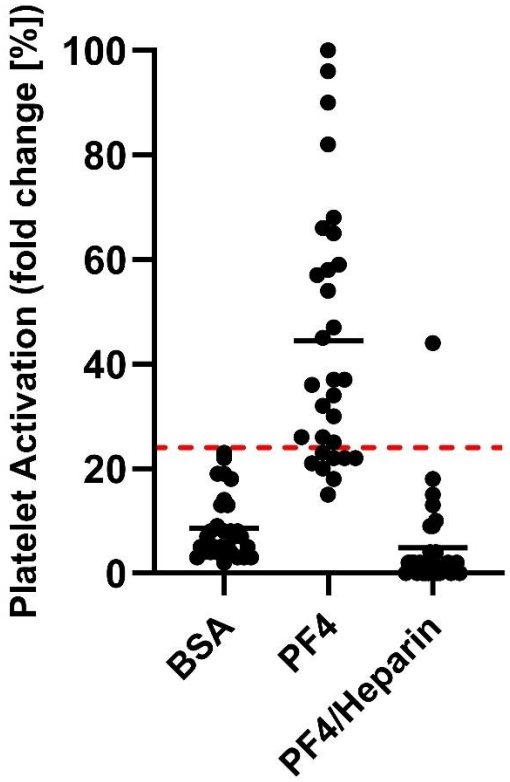
Table 6: Longitudinal clinical and demographic characteristics of VITT patients and SARS-CoV-2 infected individuals involved in anti-PF4 memory B cell testing.

All patients	VITT (n = 18)	COVID (n = 5)
Demographics		
Sex, n (%)		
Male	10 (56)	2 (40)
Female	8 (44)	3 (60)
Mean age at vaccination/infection (y, mean [range])	53.6 (37.7 - 72.3)	59.6 (48 - 80)
Clinical		
Positive for anti-PF4 IgG/A/M, n (%)	13 (72)	
Mean anti-PF4 IgG/A/M OD (OD_{405nm}, mean [range])	0.95 (0.09 - 2.72)	
Positive for platelet-activating anti-PF4 IgG/A/M, n (%)	7 (39)	
Mean serotonin release (%, mean [range])	32 (0 - 92)	
Mean anti-Spike IgG OD (OD_{405nm}, mean [range])		2.87 (2.46 - 3.09)
Mean anti-RBD IgG OD (OD_{405nm}, mean [range])		2.49 (1.47 - 3.11)
Mean time since vaccination/infection (d, mean [range])	648 (354 - 959)	504 (416 - 534)

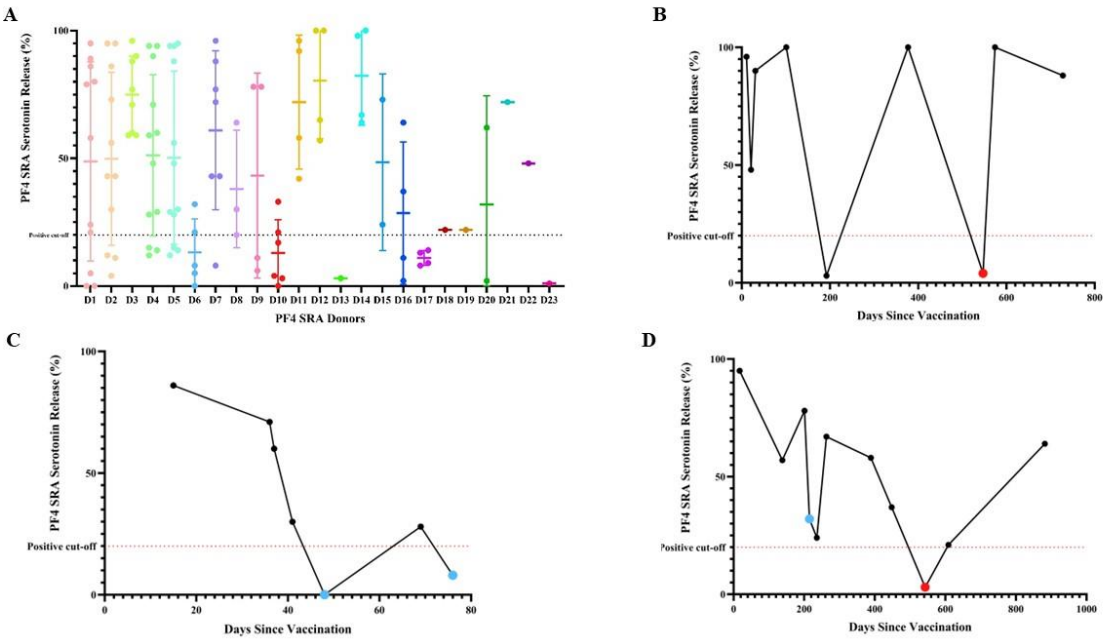
9.0 SUPPLEMENTARY FIGURES



Supplementary Figure 1: A scatter plot displaying in-house PF4 EIA ODs for total IgG detection in VITT patients (n = 7) and a normal (n = 1) through 1-step detection antibody or 2-step detection antibodies. Plates were run until control samples reached reference values and any $OD_{405nm} \geq 0.45$ was considered positive for total anti-PF4 IgG, indicated by the red dotted line. In summary, 2-step detection antibodies yield lower ODs.



Supplementary Figure 2: Longitudinal samples were collected on VITT patients (n = 30) with a median latest follow-up sample date of 715 days (range 126-1065 days) post-vaccination with an adenoviral vector-based SARS-CoV-2 vaccine. Each sample was tested for anti-PF4 platelet activation. The ability of anti-PF4 antibodies to activate platelets was detected using the PEA and expressed as P-selectin expression percentage (positive ≥ 25%). At their last follow-up, 22 (73.3%) patients were positive for platelet-activating anti-PF4 antibodies.



Supplementary Figure 3: Pedigree donors in the SRA were assessed for their ability to detect platelet activation in the PF4 SRA. A) Different pedigree SRA donors were utilized in the PF4 SRA and samples were grouped together based on the donor used. **B-D)** The PF4 SRA profiles for 3 different longitudinal VITT patients were plotted and donors that resulted in lower PF4 SRA results were highlighted.

10.0 SUPPLEMENTARY TABLES

Supplementary table 1: All optimization conditions tested in the in-house anti-PF4 ELISA for IgG1, IgG2, IgG3, and IgG4 antibody detection using VITT (PF4 antibodies) and HIT (anti-PF4/heparin antibodies) patient samples The left column highlights each variable that was manipulated and the right column shows the test conditions. Standardizing reagent and incubation temperatures, utilizing AP, diluting primary antibodies 1:1000, and diluting secondary antibodies 1:500 provided the best results.

Subclass EIA Variables	Conditions Tested					
Coating	PF4	PF4 + Heparin	PF4 + Polyvinly Sulfate	PF4 + Polyclonal anti-PF4		
Blocking Incubations Times	Overnight	2 Hours				
Sample Incubations Times	1 Hour	2 Hours				
PNPP	Tablets	Powder				
PNPP Incubation Times	5 Minutes	10 Minutes				
Incubation Temperatures	Room Temperature	37 ° Celcius				
Reagent Temperatures	Room Temperature	37 ° Celcius				
Primary Antibody Concentrations	1/4000	1/2000	1/1000	1/750	1/500	1/250
Primary Antibody Companies	Sigma-Aldrich	Millipore Sigma				
Primary Antibody Ages	Antibodies in Lab	New Antibodies				
Primary Antibody Binding Sites	CH2	Fc Fragment				
Primary Antibody Clones	8c/6-39	HP6002	HP-6050	HP6023	MK1A6	
Secondary Antibody Concentrations	1/4800	1/3400	1/2500	1/1000	1/500	
Secondary Antibody Enzymes	Alkaline Phosphatase	Horseradish Peroxidase				
Secondary Antibody Hosts	Rabbit	Goat				
Secondary Antibody Companies	Millipore Sigma	Invitrogen				

Supplementary table 2: Comparison of VITT findings to previously published data on anti-PF4 IgG, IgA, and IgM prevalence in HIT.

Reference	IgG	IgA	IgM
HIT Patients			
Warkentin *et al.*(2005) [76]	14/14 (100.0%)	6/14 (42.9%)	3/14 (21.4%)
Arepally *et al.* (1997) [79]	34/36 (94.4%)	15/36 (41.7%)	12/36 (33.3%)
Suh *et al.* (1997) [77]	26/26 (100.0%)	15/26 (57.7%)	11/26 (42.3%)
Greinacher *et al.* (2007) [78]	92/93 (98.9%)	27/93 (29.0%)	53/93 (57.0%)
VITT Patients			
Current Study	31/31 (100.0%)	1/31 (3.2%)	19/31 (61.3%)

Supplementary table 3: Comparison of VITT findings to previously published data on anti-PF4 IgG1, IgG2, IgG3, and IgG4 prevalence in HIT.

Reference	IgG1	IgG2	IgG3	IgG4
HIT Patients				
Denomme *et al.* (1997) [80]	55/61 (90.2%)	5/61 (8.2%)	8/61 (13.1%)	n/a
Arepally *et al.* (1997) [79]	30/34 (88.2%)	21/34 (61.8%)	5/34 (14.7%)	0/34 (0.0%)
Suh *et al.* (1997) [77]	24/26 (92.3%)	1/26 (3.8%)	9/26 (34.6%)	0/26 (0.0%)
VITT Patients				
Current Study	28/31 (90.3%)	20/31 (64.5%)	4/31 (12.9%)	1/31 (3.2%)

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