DIFFERENTIATING BETWEEN HEPARIN-INDUCED THROMBOCYTOPENIA AND VACCINE-INDUCED THROMBOTIC THROMBOCYTOPENIA ANTIBODY EPITOPES ON PF4

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TITLE: Differentiating Between Heparin-induced Thrombocytopenia and Vaccine-induced

Thrombotic Thrombocytopenia Antibody epitopes on PF4

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ABSTRACT:
Introduction:
Heparin-induced thrombocytopenia (HIT) is a potentially life-threatening adverse event that occurs in approximately 5% of patients following heparin administration to prevent clotting during cardiac as well as orthopedic surgeries, kidney dialyses and blood transfusions. HIT is mediated by antibodies that bind complexes of platelet factor 4 (PF4 or CXCL4) and heparin that cross-link Fc receptors on platelets to cause platelet activation, thrombocytopenia with or without thrombosis, potentially leading to death. Recently, a new anti-PF4 disorder has emerged in response to COVID-19 adenoviral-vector based vaccines against SARS-CoV-2 known as vaccine-induced immune thrombotic thrombocytopenia (VITT). VITT is a rare but serious adverse syndrome reported 5-30 days after administration of a COVID-19 adenoviral vector vaccine (Oxford-AstraZeneca ChAdOx1 nCoV-19 (AZ) and Johnson & Johnson/Janssen (J&J/Janssen)) and is characterized by thrombocytopenia and thrombosis in unusual locations, including cerebral sinus venous thrombosis (CVST). HIT and VITT are characterized by platelet-activating antibodies that bind to PF4 and often lead to thrombocytopenia and/or thrombosis. There are two types of platelet-activating antibodies that lead to HIT, antibodies that activate platelets only in the presence of heparin (heparin-dependent (HD)) and antibodies that can activate platelets in the absence of heparin (heparin-independent (HI)). In VITT, the two types of platelet-activating antibodies are ones that only activate platelets in the presence of PF4 (PF4-dependent) and antibodies that can activate platelets in the absence of PF4 (PF4-independent). We hypothesize that the binding sites of HIT and VITT antibodies on PF4 differ and that identifying these sites could be relevant to differentiate between both anti-PF4 disorders.

Methods:

All 70 PF4 mutants produced by alanine-scanning mutagenesis (ASM) were expressed and purified from *E. coli* BL21 (DE3) cells. ASM was performed using HIT (n=10) and VITT (n=14) patient samples to determine the binding sites of HIT and VITT antibodies on PF4. Clinical outcomes of CVST, DVT, PE incidences in VITT patients with PF4-dependent and PF4-independent antibodies were also analyzed. In addition, ASM was performed on the HIT-mimicking monoclonal antibodies (1E12, 1C12, 2E1, P4B1, P3D4, P3E10) to confirm the pathogenic antibody binding sites of HIT antibodies on PF4. Finally, all 70 PF4 mutants were screened against HIT (n=22), VITT (n=9) and non-HIT (n=12) patient samples and we identified 10 PF4 mutants that differentiate between HIT and VITT using an EIA.

Results:

We confirmed all 70 PF4 mutants were free of contaminants compared to bacterial lysate by visualizing the purity using gel electrophoresis. Using a cut-off of 50% or greater reduction in antibody binding to PF4 mutants compared to wild-type PF4, we determined that most monoclonal antibodies that mimic platelet-activating HIT antibodies, as well as antibodies purified from HIT patients bind to a site on PF4 like the KKO site, a known monoclonal antibody against PF4/heparin complexes that mimics platelet-activating HIT antibodies. While most VITT antibodies recognize a site on PF4 overlapping the heparin-binding site, we found a subset of VITT patients have antibodies that recognize an additional site on PF4 that is like the KKO site. By observing the incidences of clinical thrombotic characteristics in VITT patients, it was determined that CVST (6/14, 42.9%), DVT (4/14, 28.6%), and PE (4/14, 28.6%) were the most common types of thrombosis. This unusually high incidence of VITT is not typically seen in HIT. Information on the binding characteristics of HIT and VITT anti-PF4 antibodies have sparked interest on whether there are characteristics that can help differentiate between HIT and VITT. After screening the 70

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PF4 mutants against HIT and VITT patient samples, we then identified 10 PF4 mutants (H23A, R20A, T25A, E28A, K46A, R49A, K50A, K62A, K65A, K66A) that help differentiate between HIT and VITT in an EIA. We also screened the 70 PF4 mutants against non-HIT patients, identified as individuals that have non-platelet activating anti-PF4/heparin antibodies. We found that 5 PF4 mutants (K14A, H23A, V29A, T38A, K50A, K66A) help differentiate HIT from non-HIT in an EIA.

Conclusion:
Purifying PF4 mutants reduced background reactivity likely caused by contaminants in assays using bacterial lysate. ASM using PF4 mutants revealed that there are 2 distinct binding sites on PF4 that are recognized by either HI and HD HIT or anti-PF4 VITT antibodies. We confirmed that the molecular characteristics of antibodies from VITT and HIT patients dictate the platelet activation profiles in these disorders and can be used to separate VITT from HIT antibodies in the EIA.

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LIST OF ABBREVIATIONS:

Amp	Ampicillin
Anti-PF4/heparin	Antigenic PF4/heparin
ASM	Alanine scanning mutagenesis
ChAdOx1 nCoV-19	Astra Zeneca
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
°C	Celsius
CVST	Cerebral venous sinus thrombosis
CHL	Chloramphenicol
Da	Daltons
DNA	Deoxyribonucleic acid DOC Deoxycholic acid
DVT	Deep vein thrombosis
EIA	Enzyme immunoassay
E. coli	Escherichia coli
GFP	Green fluorescent protein
GT	Glanzmann's thrombasthenia
HD	Heparin dependent
HI	Heparin independent
HIT	Heparin-induced thrombocytopenia
Hep Wash	Heparin Wash
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-10	Interleukin 10
IVIg	Intravenous immune globulin
IPTG	Isopropyl -D-1-thiogalactopyranoside
Ad26.COV2.S	Johnson and Johnson, J&J
kDa	Kilodaltons
L	Litre
LB	Luria-Bertani
LMWH	Low molecular weight heparin
mAb	Monoclonal antibody
MW	Molecular weight
NETs	Neutrophil extracellular traps
PDB	Protein data bank
PEA	P-selection expression assay
PF4	Platelet factor 4
PF4-SRA	PF4-enhanced 14C serotonin release assay
PNPP	P-nitrophenylphosphate
PE	Pulmonary embolism
rhPF4	Recombinant human platelet factor 4
RT	Room Temperature
NaCl	Sodium Chloride
SN	Supernatant
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SRA	14C-serotonin release assay

SD	Standard Deviation
TGF-β1	Transforming growth factor beta 1
UFH	Unfractionated heparin

DECLARATION OF ACADEMIC ACHIEVEMENT

Mercy Daka performed all the experiments described in this thesis under the supervision of Dr. Ishac Nazy and Dr. Donald M. Arnold with the exception of the following:

- James W. Smith and Mercy Daka ran the SDS-PAGE gels to visualize the purity of PF4 mutants

- Rumi Clare and Mercy Daka biotinylated PF4 for use in binding assays against anti-PF4 antibodies and for affinity purification.

- Taylor D. Elliot and Mercy Daka expressed and purified PF4 mutants

1 INTRODUCTION
1.1 Platelets
1.1.1 Production and Function

Platelet biology is central to the understanding of thrombosis and thrombocytopenia. Platelets are small anucleate blood cells derived from megakaryocytes.[1] Platelets become activated when damage is instilled upon the endothelial cell layer, making them primarily involved in hemostasis, a process that refers to the initiation of blood coagulation. [1,2] Upon activation, platelets change their shape from a distinct discoid shape and acquire a morphology with multiple filopodial projections.[11] At sites of endothelial injury, glycoprotein (GP) Ibα receptors on the membrane of platelets interact with von Willebrand factor (VWF) to aggregate and form a fibrin network in order to prevent blood loss at the site of injury. [1-3] Platelets contain dense-granules that release high concentrations of cations (K+, Mg2+) and nucleotides, and they also contain α-granules that secrete procoagulant factors such as thromboxane A2, ADP and platelet factor 4 (PF4), which serve as strong stimulants that further promote platelet activation.[2,4-6,7] The normal platelet count range in healthy individuals is between 150×10^9 to 400×10^9 platelets per liter (L) of blood.[2,4,5] Platelet activation must be tightly regulated, since too much can lead to clots and strokes that affect approximately 62,000 Canadians yearly, while too little can result in uncontrolled bleeding like Glanzmann's thrombasthenia (GT),[8,9] a bleeding disorder that is characterized by a severe mucocutaneous bleeding due to defects in the platelet receptors for fibrinogen GP II/IIIba and GPIbIX.[10] Another example is seen in heparin-induced thrombocytopenia (HIT), where the platelets of patients with HIT become activated in response to being administered the anticoagulant, heparin, this results in a paradoxical response caused by a drop in the platelet count which can cause a series of severe thromboembolic complications to arise.[11-13]

1.2 Heparin-induced thrombocytopenia

1.2.1 Definition and Prevalence of HIT

HIT is an immune-mediated, prothrombotic, and potentially life-threatening disorder initiated by platelet-activating immunoglobin G (IgG) antibodies directed against PF4 or PF4/heparin. [14-16] HIT occurs in 1-5% of patients that are exposed to intravenous heparin.[17] Since its introduction in the 1920's as an effective anticoagulant for major surgeries, the clinical usage of heparin continued to increase until patients began to form severe complications post-surgery due to the formation and prevalence of pathogenic antibodies in HIT that were triggered by the use of heparin.[11,12] The risk of thromboembolic events is greater than 50%, of which the mortality rate is 20%, and of those, 10% of patients suffer from major morbidities.[15] The immune response in HIT is unique and unlike like the characteristic of a primary immune response, where typically immunoglobulin M (IgM) antibodies are predominant approximately 5-7 days after symptom onset, followed by a weaker response of IgG antibodies.[18,19] HIT occurs 2-5 days following heparin treatment and is characterized by the rapid formation of immunoglobulin G without a preceding IgM.[16,20] The predominant response of IgG antibodies that bind to PF4/heparin complexes in HIT can be detected as early as 4 days after drug exposure.[21,22] Compared to other populations with persistent memory B cells, the memory B cells for anti-PF4/heparin IgG are lacking and become undetectable even after three to four months.[23,41] Therefore, re-exposure to heparin does not guarantee the re-occurrence of HIT in patients. Once HIT is suspected, heparin treatment is stopped immediately and alternative anticoagulants that do not cross-react with HIT antibodies like lepirudin and argatroban are administered where available.[23] Using alternative anticoagulants may still increase bleeding risks and there is also increasing evidence of a subset of patients with platelet-activating anti-PF4/heparin antibodies that are autoantibody-like in that they recognize PF4 bound to platelets in the absence of heparin.[24] Therefore additional therapies like intravenous immune globulin (IVIg)

become necessary to treat patients with HIT. [25] IVIg is an alternative therapy that inhibits HIT antibody-mediated platelet activation by blocking platelet Fc receptors and is especially useful in treating patients with severe HIT that becomes complicated by thrombosis and thrombocytopenia.[25]

1.2.2   Platelet factor 4 (PF4)

The pathophysiology of HIT involves an immune response triggered by antibodies that recognize platelet factor 4 (PF4), also known as chemokine ligand 4 (CXCL4). [26,27] PF4 is a cationic chemokine that is 7.8 kilodaltons (kDa) in its monomeric form and 32.0 kDa in its active, tetrameric form. [26,27 28] PF4 is one of the most abundant proteins stored in platelet α-granules and plays a dual role in inflammation and wound healing.[3] These activities are mediated by its interactions with glycosaminoglycans, (GAG's), like heparin, that are negatively charged and lie on the platelet surface.[3,29] PF4 is synthesized in megakaryocytes and makes up approximately 2% of the total protein in platelet α-granules.[26] PF4 is made of 70 amino acid residues and in its native, monomeric form, consists of a hydrophobic anti-parallel beta sheet domain. The C-terminal domain is amphipathic alpha helix containing four lysine residues and the N-terminus is divided into four domains where the first and fourth domains are located on the outer surface of PF4, while the second and third domains are located on the outer and inner surface of PF4.[30-32] Tetrameric PF4 exposes a ring of positive charges that is primarily attributable to the lysine residues and contributes to its attraction to negatively charged molecules that lie on the platelet surface. [33] PF4 is also suspected of its role as a danger signal for the presence of pathogens due to its cationic charge with a high affinity for negatively charged polyanions that are expressed on the cell walls of gram-negative and gram-positive bacteria.[24,34] High levels of PF4 are released into circulation upon platelet activation with 0.4-2μM found present in serum and as high as 280μM of PF4

tetramers being found within a clot.[26,35-37] Mouse models have also investigated the role of PF4 in inflammation and found that a deficiency in PF4 protects mice from local tissue damage, suggesting that PF4 is an important mediator of local and remote tissue damage.[27] Following its local release into circulation, PF4 also plays an important role in serving as a chemoattractant for leukocytes like neutrophils. Neutrophils are innate immune cells that play a role in protection against microbial infection by trapping many microbes through DNA fibers known as neutrophil extracellular traps (NETs) in a process known as NETosis.[38,39] During infection or inflammation, PF4 has shown to bind to NETs causing them to form neutrophil-platelet aggregates that can contribute to clot formation.[40] Of the various interactions that PF4 forms to contribute to clot formation, its interaction with blood thinners like heparin have proven to be life-threatening. Heparin is a polymer made up of linear disaccharide chains of glucosamine and uronic acid.[41] There are two types of heparin derivatives that exist for clinical usage and also bind to PF4; these include unfractionated heparin (UFH) which ranges 3 to 30 kDa and low molecular weight heparin (LMWH) which ranges from 4.5 to 5 kDa.[42] Many chemokines bind heparin and other glycosaminoglycans, however, PF4 is the most abundant and binds heparin with 10^2-10^3 fold higher affinity than other chemokines.[33] Heparin interacts with PF4 by binding among PF4 monomers and also by bridging PF4 tetramers to further stabilize them, this step is fundamental to change the conformation of PF4 and reveal the neoantigen in a disease phenomenon known as heparin-induced thrombocytopenia (HIT).[33,43]

1.2.3   Pathophysiology of HIT

Heparin helps to form large tetrameric complexes of PF4 through electrostatic attraction.[14] This interaction leads to a conformational change that exposes neoepitopes on the surfaces of these PF4 complexes to which anti-PF4/heparin antibodies bind.[43] Prothrombotic mechanisms of HIT

antibodies depend on Fc receptor (FcγRIIa) cell-mediated activation, where these antibodies bind to PF4/heparin (PF4/H) complexes to form immune complexes that then bind to and activate Fc receptors by crosslinking them. This results in platelet activation and leads to the release of more PF4 and enhanced platelet recruitment to the prothrombotic process.[21,44]

1.2.4 Clinical Identification of HIT

Clinical features of HIT include individuals whose platelet counts decline by about 50% from their normal range within 5-10 days following heparin exposure.[23] In this thesis, we refer to non-HIT patients, as those suspected of having HIT based on their clinical symptoms and develop anti-PF4/heparin antibodies, yet test negative for platelet-activating antibodies in functional assays. Symptoms that diagnose HIT and can also distinguish between non-HIT patients and patients with HIT can be classified using the 4Ts perspective, a scaled clinical scoring system for HIT that is based on 4 typical features of HIT; these include the magnitude of thrombocytopenia, timing of thrombocytopenia, the occurrence of thrombosis, and the likelihood of other causes of thrombocytopenia seen in HIT-mimicking syndromes like cancer-associated disseminated intravascular coagulation (DIC). [45,46] Once a patient is suspected of having HIT according to the 4Ts perspective, laboratory diagnostic assays are then implemented. The 4Ts clinical scoring system is useful at ruling out a diagnosis of HIT since a low 4Ts score represents a low probability (<2%) of the patients testing positive for platelet activation antibodies which is indicated by a sensitivity of 97.7%. By contrast, a high 4Ts score has a low probability of being of confirming HIT by a positive platelet activation test due to a low specificity of 32.9%.[47]

1.2.5 Laboratory Diagnosis of HIT

Enzyme immunoassays (EIAs) are the first tools involved in the initial diagnosis of HIT. EIAs capture and detect antigenic PF4/heparin (anti-PF4/heparin) antibodies in patients suspected of HIT.[48,49] EIAs are limited with a low specificity of approximately 50% because, many patients

treated with heparin produce antibodies against PF4/heparin but never get the disease. Therefore, the EIAs cannot differentiate between anti-PF4/heparin antibodies that are platelet-activating and cause disease from those that are non-platelet activating antibodies, which leads to false-positive results and disease overcall despite the patients not having HIT. [48,50,51] This is because antibodies produced by HIT patients are poly-specific and have been shown to bind multiple sites on PF4, many of which are non-pathogenic and do not cause platelet activation or HIT.[52] This limitation presents a challenge in using EIAs as a conclusive diagnostic tool since up to ~50% of patients receiving heparin risk developing anti-PF4/heparin antibodies but only 1-5% of these patients develop platelet-activating antibodies that lead to clinical HIT. [48,49] However, given the high mortality rates affecting 5-10% HIT patients, HIT should be suspected in any patient that receives heparin and develops thrombocytopenia.[49] Functional assays which include the heparin-induced platelet activation assay (HIPA) that visually assesses platelet aggregation at five minute intervals using an indirect light source,[53] p-selectin expression assay (PEA) that uses flow cytometry to measure the expression of P-selectin (CD62) on the surface of activated platelets[54] and ^{14}C-serotonin release assay (SRA), which measures the release of serotonin from the dense granules of platelets once they are activated are necessary to confirm the platelet activating ability of antibodies in HIT diagnosis. [55] The ^{14}C-serotonin release assay (SRA) is considered the gold standard laboratory test for HIT. [55] The SRA reports a diagnostic sensitivity of 95% and specificity of 95%, making it an accurate diagnostic assay for HIT. However, the SRA is limited by lack of availability since it is technically demanding and requires radioactive isotopes and specific platelet donors in addition to its high cost and long turnaround time. [55]

1.2.6 Pathogenic and non-pathogenic HIT antibodies

HIT is characterized by pathogenic and non-pathogenic antibodies that bind to PF4 and heparin complexes, yet only pathogenic antibodies trigger platelet activation and increases the risk of thrombotic complications.[56,57] Although HIT only occurs in 5% of heparin treated patients, up to 70% of patients treated with heparin can produce anti-PF4/heparin antibodies that are non-pathogenic and as a result, this can complicate diagnosis and lead to disease overcall.[58-60] An important characteristic that helps distinguish pathogenic HIT antibodies from non-pathogenic HIT antibodies is that pathogenic HIT antibodies strongly activate platelets at detectable levels in functional assays like the heparin-induced platelet activation assay (HIPA), p-selection expression assay (PEA) and ^{14}C-serotonin release assay (SRA), while non-pathogenic antibodies do not cause platelet-activation.[58] Although there is evidence of pathogenic antibodies that weakly activate platelets at levels that are insufficient to cause HIT,[50,58] here we refer to pathogenic HIT antibodies as a subset of strong platelet-activating, anti-PF4/heparin IgG that are likely to cause HIT.[47] A study on the HIT antibody characteristics determined that the pathogenic antibodies in HIT are effective at triggering normal platelets to produce procoagulant microparticles at levels that are comparable with other standard platelet agonists like thrombin and collagen, both of which also trigger platelet activation.[61] These results could offer explanations on how pathogenic HIT antibodies lead to thrombotic complications observed in some HIT patients.[61,62] Implications on why exposure to heparin causes the production of antibodies against PF4 have been attributed to immune dysregulation, a phenomenon that is evidenced by different levels of regulatory cytokines including interleukin-10 (IL-10) and transforming growth factor beta 1 (TGF-β1), both of which have been observed at low levels in HIT patients compared to healthy controls.[63] A subset of autoantibodies in patients with clinical and laboratory features of HIT have since been recognized;[24,64] however, these patients have anti-PF4/heparin antibodies that can strongly activate

platelets independent of heparin *in vitro*.[24,64] Plausible explanations for this immune response in HIT that manifests in the absence of heparin can be attributed to glycosaminoglycans that are produced following orthopedic surgery or bacterial antigens that precede infection,[34,65] both of which have been reported to bind to PF4 and are associated with anti-PF4/heparin antibody formation.[33,66] This suggests that patients with HIT can have HD and HI antibodies against PF4.

1.2.7   Heparin independent and Heparin dependent antibodies that cause HIT

The antibodies in HIT patients are polyclonal and polyspecific in nature, this makes it difficult to determine whether there are any common epitopes on PF4 that contribute to the pathogenicity seen in HIT.[32,67] A previous study by Greinacher *et al* recognized that there are two types of antibodies that cause platelet activation in HIT.[24] The first subset of HIT antibodies are defined as HD antibodies because, these antibodies bind to PF4 only as it is complexed with heparin to trigger platelet activation. Meanwhile, HI antibodies define those that can trigger platelet activation by binding to PF4 even in the absence of heparin. Previous work screening peptides of PF4 with patient sera showed that most HD HIT antibodies recognize a non-contiguous conformational epitope on PF4 when it binds to heparin.[68] Since then, HIT researchers have engineered murine-derived monoclonal antibodies like KKO, that display HIT-like activity.[43,69] Like pathogenic HIT antibodies, KKO recognizes PF4/heparin complexes and promotes HD platelet-activation in vitro, therefore, as a monoclonal antibody, KKO serves as the standard to studying polyclonal human HIT antibodies.[43,69] The use of KKO and alanine-scanning mutagenesis (ASM) have both proven to be useful tools in identifying the amino acids that comprise important binding sites on PF4.[52,69] ASM is a form of site-directed mutagenesis that substitutes an original amino acid in a protein sequence to alanine, a non-bulky and neutral residue, to help determine the stability or function of a protein. Alanine residues in the original protein sequence are mutated to

valine.[70] Our previous work using the ASM method used 70 PF4 mutants to show that most HD and HI HIT antibodies recognize similar amino acids on PF4 that are found in the KKO site, although HI HIT antibodies recognized additional amino acids that are found in the VITT site on PF4.[71] Studies on the different binding sites are important as they have led to the generation of more monoclonal antibodies that mimic those that cause HIT;[72] however, further characterization of these antibodies is needed. A study by Vayne *et al* predicted the binding sites of the three murine monoclonal antibodies 1E12, 1C12, and 2E1 that resemble HI HIT antibodies in activating platelets and found that these sites differed but were close to the KKO binding site.[72] The methods implemented in this study were done in silico (MAbTope method) through an online 3D modelling system that captured the docking of each monoclonal antibody to PF4. [72] Although such studies on monoclonal antibodies using online tools are useful models for studying the pathogenesis of HIT, these monoclonal antibodies need to be characterized further, hence the reason why we found it important that to use ASM with purified PF4 mutants as an important tool in identifying the binding sites of these monoclonal antibodies on PF4.

1.3 Vaccine-induced immune thrombotic thrombocytopenia

Vaccine-induced immune thrombotic thrombocytopenia (VITT) is a rare, yet serious prothrombotic syndrome that is linked to vaccines developed against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), including the ChAdOx1 nCoV-19 (Oxford-AstraZeneca; AZ) and the Ad26.COV2.S vaccine (Johnson & Johnson; J&J).[73-75] The frequencies of VITT are reportedly 1 per 90,000[76] to 1 per 260,000[77] doses of an AZ or J&J vaccine, respectively. Clinical features of VITT begin 5-10 days following primary vaccination, meanwhile clinical suspicion of VITT is largely based on commonly identified symptoms like cerebral venous and splanchnic vein thromboses.[75] Pathologically, the immunological mechanism of VITT resembles spontaneous

HIT.[78,79] Spontaneous HIT was first described in 2008, in which the same adverse drug reaction in HIT was observed in patients despite the lack of previous heparin therapy.[80] It was recently confirmed that the presence of highly reactive anti-PF4 antibodies in patients with VITT are like anti-PF4 antibodies in spontaneous HIT,[52] in that they are oligoclonal and likely recognize conserved sites on PF4 to cause platelet activation and thrombosis.[71,79] Although VITT and HIT antibodies share a common antigen in PF4,[81] we recently published evidence that the standard SRA used to diagnoses HIT with a high sensitivity of 100% had a poor sensitivity of 46.2% for VITT.[81] This suggests that the functional assays used to diagnose HIT are insufficient in diagnosing VITT, owing to differences in the antibodies found in both disorders.

1.3.1   Anti-PF4 antibodies in VITT

The immune response in VITT has interesting features concerning the persistence of anti-PF4 VITT antibodies.[82] For example, a recent study found that 72% of VITT patients remained positive for PF4 antibodies following a median of 105 days; in addition, patients relapsed at a rate of 12.6% and remained positive for anti-PF4 antibodies and low platelet counts.[83] The pathophysiological mechanism in VITT is currently unknown, however the proposed mechanism strongly resembles HIT in that VITT too, could also represent a misdirected antimicrobial response and immune dysregulation that could be contributing to HIT.[33,63,84,80] It is well reported that administering heparin is a trigger of a misdirected immune response involving anti-PF4/heparin antibodies.[33,63,84,80] In addition, surgeries, infection, or trauma can also stimulate the formation of anti-PF4/polyanion antibodies.[85,86] VITT is proposed to develop when immune complexes of anti-PF4 IgG antibodies and PF4 are formed. [16,44] PF4 then interacts with platelet surface FcγRIIa receptors, triggering platelet activation.[87] Studies that have looked at characterizing VITT antibodies proposed that the presence of monoclonal and oligoclonal anti-PF4 antibodies mediate

VITT and that these antibodies are variably inhibited by heparin from binding to PF4 in an EIA.[79] Furthermore, platelet-activating anti-PF4 antibodies in VITT are reported as high titer and clonally restricted.[78,79] In order to further characterize the VITT antibodies, our group used ASM to identify amino acids on PF4 that are critical for VITT anti-PF4 antibody binding. [71] We determined that anti-PF4 antibodies in VITT recognize amino acids that are also relevant for heparin binding to PF4.[71] Such studies have major implications in treating VITT; since heparin often inhibits the binding of VITT antibodies, it could serve as an antidote in blocking the pathogenicity caused by antigen binding.[78] Anti-PF4 antibodies have been proposed to cause VITT without the presence of heparin by binding to PF4 and crosslinking PF4 tetramers on their own, thereby changing the conformation of PF4 to reveal neoepitopes for other anti-PF4 antibodies to bind and activate platelets. Further work used biolayer interferometry (BLI) to support that VITT anti-PF4 antibodies have sufficient binding strength to form immune complexes and trigger platelet activation.[88,89]

1.3.2   Laboratory Diagnosis of VITT

Official recommendations for the laboratory diagnosis of VITT involve assays that are also currently used to diagnose HIT because of their similar pathophysiologies.[27] The EIA has a low specificity for antibodies in HIT patients however, we recently reported three diagnostic immunoassays with high specificities in detecting VITT antibodies including a commercial anti-PF4/heparin IgG/A/M enzyme immunoassay (EIA, PF4 Enhanced; Immucor; specificity=95.6%), in-house   IgG-specific   anti-PF4   (specificity=96.5%)   and   anti-PF4/heparin-EIAs (specificity=97.4%).[81] Initial evidence using the standard SRA that is used for HIT for VITT diagnosis showed that VITT antibody platelet activation was inhibited in the presence of heparin but became positive with the addition of PF4.[28] Therefore, the PF4-enhanced SRA is currently the

gold standard diagnostic test for VITT and is performed similarly to the standard SRA but uses exogenous PF4 rather than heparin.[15] Deciphering the anti-PF4 antibodies that cause VITT has important advantages in studies involving similar diseases like spontaneous HIT since anti-PF4 antibodies can vary in binding locations as seen in studies on VITT.[71,88]

2 RESEARCH OUTLINE

2.1.1 Overall Objective
The main objective of this study was to better understand the molecular role of antibodies in HIT and VITT and to develop a tool that could accurately diagnose and differentiate between anti-PF4 disorders.

2.1.2 Rationale
The main challenge in HIT stems from pathogenic antibodies that cause HIT as well as non-pathogenic antibodies that do not cause HIT, yet test as false-positive in screening diagnostic assays.[49,58] Furthermore, these antibodies are polyclonal making it difficult to distinguish them by their affinity for PF4.[24,71] Currently, the only measurable difference between pathogenic and non-pathogenic antibodies in HIT is the diagnostic ability that pathogenic antibodies have, in that they recognize PF4/heparin complexes to activate platelets, while non-pathogenic antibodies also recognize PF4/heparin complexes but do not activate platelets.[90,91] Therefore, there is a pressing need to characterize the specific properties of pathogenic antibodies that mediate HIT.[24,49,58,71] Previous studies have shown that there are two types of pathogenic HIT antibodies whose characteristics are important in the development of HIT,[92] these include HD antibodies that recognize PF4 in the presence of heparin and HI antibodies that recognize PF4 independent of heparin.[92,93]

Studies on HIT in the last 60 years have provided insights into a newly discovered disorder termed vaccine-induced thrombotic thrombocytopenia (VITT).[73-75,94] The anti-PF4 antibodies in VITT are like HI HIT antibodies in that they can activate platelets independent of heparin to lead to blood clots and other life-threatening thrombotic symptoms.[64,79,80] HD and HI HIT antibodies and anti-PF4 VITT antibodies that activate platelets then lead to thrombosis/thrombocytopenia are central in HIT and VITT and the antibodies in both disorders display different platelet activation profiles

that are dictated by their binding sites on PF4.[71] The platelet activation profile of VITT anti-PF4 antibodies display consistent platelet activation in the absence of heparin. Huynh *et al* previously studied the binding characteristics of pathogenic antibodies in HIT and another platelet-activating monoclonal antibody called KKO using the bacterial lysate of 70 PF4 mutants that were produced by performing ASM.[52] The authors identified amino acids that were likely important for binding to KKO and HIT antibodies, however, there was a limitation on the number of HIT patients that could not be tested in the binding assays due to their background reactivity to bacterial lysate.[52] To improve on this limitation, my aim was to modify the previous method that used bacterial lysate by purifying the 70 PF4 mutants. By testing the purified PF4 mutants in binding studies, we found that the binding sites of VITT antibodies on PF4 were restricted to eight surface amino acids, all of which are located within the heparin binding site, and that the binding of VITT antibodies was inhibited by heparin.[71] I then used the PF4 mutants to determine the epitopes of six monoclonal antibodies to confirm that the differences in the epitopes dictate their platelet activation profiles. Furthermore, I purified HD antibodies and HI antibodies directly from HIT patient samples for the purpose of identifying their specific binding sites on PF4 to show that the previously identified epitopes correspond with the platelet activation profiles in HIT patients. By determining the most common PF4 mutants that affect the binding of HIT and VITT antibodies, I was then able to use ten PF4 mutants to develop an assay that can differentiate between HIT and VITT in an easy-to-use and efficient assay. This work aims to improve diagnostic strategies to provide a better understanding on the clinical course of HIT and VITT based on the molecular characteristics identified and thereby allows us to develop a new diagnostic tool that could help differentiate between both anti-PF4 disorders. A good example of this assays' potential utility is the fact there are already preliminary indications that heparin may be a safe and valid treatment option for VITT

patients, whereas heparin can be fatal in HIT. Another advantage of using PF4 mutants to differentiate between platelet-activating anti-PF4 disorders in an EIA would mean shorter turnaround times and cost-efficiency, which would allow us to provide clinicians with faster and more reliable results for the appropriate treatment to be administered.

Hypothesis:
We hypothesize that the molecular characteristics in VITT and HIT antibodies dictate their platelet activation profiles and can be used to diagnose and differentiate between both anti-PF4 disorders.

2.1.3 Specific Objectives
1. Identify the PF4 epitopes of the following monoclonal antibodies with HIT-like reactivity; 1E12, 1C12, 2E1, P4B1, P3D4, and P3E10

2. Map the binding sites of HIT antibodies on PF4 and compare the similarities and/or differences with the epitopes of the monoclonal antibodies

3. Map the epitopes of VITT anti-PF4 antibodies on PF4 and compare their similarities and/or differences from the HIT site and the epitopes of monoclonal antibodies on PF4 using patient samples.

4. Develop an assay that separates HIT from VITT using purified PF4 mutants.

3   MATERIALS AND METHODS

3.1   Patient demographics for VITT and HIT testing

Blood samples used for these studies were obtained from suspected VITT and HIT patients

who were referred for diagnostic testing at the McMaster Platelet Immunology Laboratory. VITT

diagnosis was confirmed based on a positive result (OD405-490nm ≥ 0.45) in the commercially

available EIA that detects antibodies of IgG/A/M classes [LIFECODES PF4 Enhanced assay;

Immucor GTI Diagnostics][15], positive in the PF4-enhanced SRA (positive PF4-SRA, ≥ 20% [14]C-

serotonin release), and no previous exposure to heparin[19]. HIT diagnosis was confirmed based on

a positive commercially available PF4-enhanced heparin dependent IgG/A/M-specific EIA and a

positive SRA result (≥ 20% ^{14}C-serotonin release). [95]

3.2   Immunoassays for the Detection of anti-PF4 Antibodies and anti-PF4/heparin Antibodies

Testing for IgG/A/M anti-PF4/heparin antibodies was performed using a commercially

available EIA (LIFECODES PF4 Enhanced assay; Immucor GTI Diagnostics, Waukesha, WI,

USA [positive optical density $(OD)_{405nm} \geq 0.4$]) as per the manufacturer's instructions.[48] Human

recombinant PF4 used in our immunoassays, as well as our platelet functional assay, were purified

in-house as previously described.[52] Subsequently, an in-house IgG-specific anti-PF4-EIA and anti-

PF4/heparin-EIA were performed on all samples as previously described.[48] Briefly, patient sera

were incubated in 96-well Maxisorp plates (Thermo Fisher Scientific, Waltham, MA, USA) coated

with 30 μg/mL recombinant PF4 (or with 30 μg/mL recombinant PF4 and 0.5 U/mL heparin

[Pfizer, New York, NY, USA]) in bicarbonate buffer pH 9.6 overnight at 4°C. Plates were then

washed twice with phosphate buffered saline (PBS) with 0.05% Tween-20 then PBS alone and

blocked in 3% bovine serum albumin (BSA; MilliporeSigma, St Louis, MO, USA) at room

temperature for two hours. Plates were washed again followed by the addition of 1/50 dilutions of

patient sera in PBS supplemented with 1% BSA in duplicate for one hour at room temperature. Bound human IgG antibodies were detected with alkaline phosphatase–conjugated goat anti-human IgG (Fc specific, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) diluted 1/3000 in PBS supplemented with 1% BSA. After washing, 1 mg/mL *p*-nitrophenylphosphate (*p*NPP; MilliporeSigma, St Louis, MO, USA) substrate was added and ODs were measured using a BioTek 800TS microplate reader at 405 nm to determine the presence of anti-PF4 or anti-PF4/heparin antibodies in patient sera (positive $OD_{405nm} \geq 0.45$). Results were reported as a percentage of binding relative to wild-type PF4 binding using the following formula:

$$\% \ binding \ relative \ to \ WT = \frac{(Abs405_{mutant} - Abs490_{mutant})}{(Abs_{wildtype \ PF4})} \times 100$$

3.3 Functional Platelet Activation Assays

All VITT patient samples were tested for platelet activation using the PF4-SRA which follows the method of the SRA but with exogenous PF4 added at increasing concentrations of 0, 25, 50, 100 µg/mL (positive $\geq$ 20% ^{14}C-serotonin release).[50,96] All HIT patient samples were tested for platelet activation in the standard SRA in the presence of therapeutic (0.1-0.3 U/mL) and high (100 U/mL) doses of unfractionated heparin (Pfizer, New York, NY, USA) without exogenous PF4, as previously described.[97] Platelet activation was also assessed in a PF4/heparin-SRA, which tests blood samples in the presence of 0.5 U/mL unfractionated heparin and 10 µg/mL recombinant PF4.[51] Each assay was performed with an anti-human CD32 Fc receptor-blocking monoclonal antibody (IV.3) to confirm FcγRIIa involvement in platelet activation.

3.4 Epitope mapping VITT and HIT antibodies binding to PF4

The binding of HIT Antibodies to wildtype PF4 and PF4 mutants was measured using a modified PF4/heparin IgG-specific EIA. [48]Streptavidin (10 µg/mL) was immobilized on a 384-

well microtiter plate (Immulon, Thermo Scientific Inc., Burlington, ON, CAN) overnight at 4

Celsius (°C). 1 IU/mL Biotinylated unfractionated heparin (UFH) was incubated with

streptavidin for 1 hr. 5 μg/mL of previously expressed library of 70 mutant PF4 proteins was

added to each well of the plate to form mutant PF4/complexes. Either VITT or HIT sera at 1:50

dilution was then added to bind PF4/mutant complexes on the plate for 1 hr at RT. Following

washing with PBS, the samples were incubated with alkaline phosphatase conjugated anti-mouse

IgG or anti-human IgG for 1 hr at RT. The absorbance at a wavelength of 405 nm with a

reference wavelength of 490nm was measured for 1 hour using a TECAN Sunrise plate reader

(Tecan Group Ltd., Mannedorf, Switzerland) to assess binding of antibodies to wild-type PF4

and PF4 mutants.

3.5   Vector construct of recombinant PF4 and cell growth

Recombinant human PF4 (rh-PF4) was previously cloned into a pET22b expression vector.[14]

The pET22b-rhPF4 construct was also previously transformed into *E. coli* BL21(DE3) cells and a

subset of these cells were stored in 50% glycerol at -80°C for long-term usage. [14] Overnight

cultures of BL21(DE3) cells were prepared by growing cells from the glycerol stock in 25mL of

Lysogeny Broth (LB) media supplemented with the antibiotics 100 μg mL^{-1} of ampicillin (AMP)

and 100 μg mL^{-1} chloramphenicol (CHL) diluted 1:1000 in LB media and grown overnight at 37°C

while shaking at 225 rpm.

3.6   Expression of wildtype PF4 and recombinant PF4 mutants

A large-scale expression was prepared by aliquoting a 1:50 dilution of the previously

described 25mL overnight culture in 1 Litre flask of fresh LB media also supplemented with

ampicillin (100 μg mL^{-1}) and CHL (100 μg mL^{-1}). The large-scale culture was incubated at 37°C

with shaking at 225rpm for about 2 hours until the mid-exponential phase at OD$_{600mn}$ reached 0.6

and then protein production was induced with 0.5M Isopropyl -D-1-thiogalactopyranoside

(IPTG). Following induction by IPTG, the culture was grown for 3 more hours and then harvested by centrifugation at 4000 x g for 20 minutes; the cell pellets were resuspended in 20mL phosphate buffered saline (PBS) to repeat centrifugation and then frozen at -80°C.

3.7   Purification of wildtype PF4

Wildtype recombinant human PF4 (WT-rhPF4) was previously produced using the pET22b expression vector and then introduced into BL21 cell strain of *Escherichia coli (E. coli)*.[98] An overnight culture of wildtype PF4 was grown in ~50mL of LB media supplemented with 100ug/mL of ampicillin. The overnight culture for WT-rhPF4 was diluted 1:50 in 1L of LB media and grown at a larger scale as previously described. Each *E. coli* cell pellet for WT-rhPF4 was lysed in 20mM sodium phosphate buffer (pH 7.2), 400 mM sodium chloride, 1.4 mM β-mercaptoethanol, 5% (v/v) glycerol, 1% (v/v) Triton X-100 (Thermo Fisher Scientific), and 0.5% (w/v) sodium deoxycholate (MilliporeSigma) with 2 mM $MgCl_2$, 10 μg ml–1 DNaseI (MilliporeSigma) and supplemented with an EDTA-free protease inhibitor cocktail (Roche). The supernatant was then obtained by centrifugation at 40,000 x g for 40min at 4 °C before being filtered with a 0.2 μm filter (Pall Acrodisc, St. Laurent, QC, CAN) and applied onto a HiTrap Q HP column (Cytiva Life Sciences) that had been equilibrated with 20mM sodium phosphate, pH 7.2, 400 mM sodium chloride, 1.4 mM β-mercaptoethanol and 5% (v/v) glycerol at 4 °C. The flow-through from the Q HP column was then stored at 4 °C, overnight. The next day, the flow-through was diluted 1:2 to yield a sodium chloride concentration of 200 mM with 20 mM sodium phosphate, pH 7.2, 1.4 mM β-mercaptoethanol and 5% (v/v) glycerol, syringe-filtered with a 0.2-μm filter (Acrodisc) and then the diluted supernatant was loaded onto a HiTrap Heparin HP column (Cytiva Life Sciences). 0.5M sodium chloride was then run through the column to elute any contaminants. WT-rhPF4 was then eluted with a linear gradient from 0.7 to 2M sodium

chloride using the ÄKTA purification system (Marshall Scientific LLC). Multiple eluted

fractions containing WT-rhPF4 were then pooled and concentrated using centrifugal filters (10K

Amicon Ultra-15; Sigma). The concentration of PF4 was determined using a bicinchoninic acid

(BCA) assay (Thermo Fisher Scientific). Protein expression and purity was assessed for purified

WT-rhPF4 using 18% SDS–PAGE.

3.8   Purification of recombinant PF4 mutants

Recombinant PF4 mutants were previously designed by mutating non-alanine amino acids

in the wild-type PF4 sequence to alanine and cloned into pET22b expression vector and was

introduced to the BL21 (DE3) cell strain for expression and purification. An overnight culture

for each pET22b mutant PF4 construct was grown in ~50mL of Luria-Bertani (LB) media

supplemented with 100ug/mL of ampicillin and 100ug/mL of chloramphenicol (CHL). The

overnight culture for each recombinant mutant PF4 was diluted 1:50 in 1L of LB media and

grown at a larger scale as previously described. Each *E. coli* cell pellet for each PF4 mutant was

lysed in 20mM sodium phosphate buffer (pH 7.2), 400 mM sodium chloride, 1.4 mM β-

mercaptoethanol, 5% (v/v) glycerol, 1% (v/v) Triton X-100 (Thermo Fisher Scientific), and 0.5%

(w/v) sodium deoxycholate (MilliporeSigma) with 2 mM MgCl2, 10 μg ml−1 DNaseI

(MilliporeSigma) and supplemented with an EDTA-free protease inhibitor cocktail (Roche). The

supernatant was then obtained by centrifugation at 40,000g for 40min at 4 °C before being

applied onto a HiTrap Q HP column (Cytiva Life Sciences) that had been equilibrated with

20mM sodium phosphate, pH 7.2, 400 mM sodium chloride, 1.4 mM β-mercaptoethanol and 5%

(v/v) glycerol at 4 °C. The flow-through from the Q HP column was then stored at 4 °C,

overnight. The next day, the supernatant was diluted twofold to yield a sodium chloride

concentration of 200 mM with 20 mM sodium phosphate, pH 7.2, 1.4 mM β-mercaptoethanol

and 5% (v/v) glycerol, syringe-filtered with a 0.2-μm filter (Acrodisc) and then the diluted

supernatant was loaded onto a HiTrap Heparin HP column (Cytiva Life Sciences). 0.5M sodium chloride was then run through the column to elute any contaminants. PF4 was then eluted with a linear gradient from 0.5 to 2M sodium chloride using the ÄKTA purification system (Marshall Scientific LLC). Eluted fractions containing either pure wild-type PF4 or PF4 mutants were then pooled, concentrated using centrifugal filters (10K Amicon Ultra-15; Sigma). The concentration of PF4 was determined using a bicinchoninic acid (BCA) assay (Thermo Fisher Scientific). Protein expression and purity was assessed for each PF4 mutant using 4–18% denaturing SDS–PAGE.

3.9 Affinity purification of anti-PF4 antibodies from HIT and non-HIT patients

3.9.1 Patient demographics:
Samples of human HIT sera were obtained from patients referred for diagnostic testing at the McMaster Platelet Immunology Laboratory. Leftover patient sera that were referred for laboratory testing for HIT were then used for purification purposes, the samples selected had tested positive in the in-house PF4/heparin (PF4/H) IgG-specific EIA, [OD450 nm] $\geq$ 0.45 is positive and their platelet-activating functions were tested and confirmed as being positive for either platelet activating antibodies in i) the presence of heparin, making them heparin-dependent or ii) the Antibodiesence of heparin, making them heparin-independent antibodies in the serotonin-release assay (SRA). This study was approved by the Hamilton Integrated Research Ethics Board (HIREB).

3.9.2 Total IgG purification:
Protein G beads (GE healthcare, Mississauga, ON, CAN) were washed with PBS (1:10 dilution of beads in PBS) by spinning beads in PBS at 400 x g for 5 minutes and repeated for a total of 3 times. Patient samples confirmed to have either heparin-independent or heparin-dependent antibodies were diluted 1:3 with PBS. The diluted serum was then added to the

washed protein G beads and incubated at room temperature for 1 hour with shaking. The Protein

G beads were then spun down at 300 x g for 5 minutes to remove unbound antibodies in the

serum. The beads were then washed with PBS at a 5x volume and spun again at 300 x g for 5

minutes and repeated 3 times. Following washing, elution buffer (0.1M Glycine, pH 2.8) was

added to an Eppendorf containing neutralizing Tris buffer (1.5M, pH 8.8) and incubated at room

temperature for 15 minutes. Following incubation, the eluate was then spun down at 300 x g and

collected to obtain total IgG from the Protein G beads. This step was repeated 3 more times with

each elution collected into a new Eppendorf supplemented with neutralizing Tris buffer (1.5M,

pH 8.8). The absorbance was measured at OD_{280nm} using spectrophotometry (BioPhotometer

6131 Spectrophotometer, Eppendorf) and subsequently used to calculate the concentration. The

purity of the total IgG was confirmed using SDS-PAGE gel. Total IgG purified from patient

samples that were confirmed for heparin-independent (HI) antibodies was subsequently ran

through a PF4 column, meanwhile the total IgG from patient samples that were confirmed for

heparin-dependent (HD) antibodies was subsequently ran through a PF4/heparin column.

Biotinylating WT-rhPF4 was performed for its subsequent use in purifying heparin-

independent and heparin-dependent antibodies from the total IgG of the previously described

patients. WT-rhPF4 (12mg) was incubated with 2mL of Heparin Sepharose beads (GE

Healthcare) for 1 hr at room temperature (RT). EZ-Link Sulfo-NHS-LC-Biotin (Thermo Fisher

Scientific) was added to the WT-rhPF4 bound to heparin beads in 20 molar excess and the

reaction was incubated for 1 hour with shaking at RT. The biotinylated WT-rhPF4 was eluted

from the heparin beads using 2 M NaCl in PBS and the biotinylated WT-rhPF4 subsequently

diluted in PBS buffer to lower the concentration of NaCl to 0.5 M. The concentration of the

biotinylated WT-rhPF4 was determined using a spectrophotometer (OD280nm). Purity and

Biotinylating of WT-rhPF4 was confirmed using a streptavidin EIA.

3.9.3   PF4 affinity column to elute heparin-independent (HI) HIT antibodies:
Total IgG that was purified from samples confirmed to have had HI antibodies was run in

the PF4 column to purify HI antibodies. Biotinylated PF4 (bPF4) was incubated with total IgG

for 1 hour at RT and then transferred to streptavidin coated Sepharose beads (GE Healthcare)

overnight at 4°C. Following washing, bound HI Antibodies were then eluted using 10mL of

0.1M Glycine buffer, pH 2.7 and the solution was neutralized with 1mL of Tris buffer, pH 8.0.

The elution buffer containing HI Antibodies was immediately exchanged for PBS and the

concentrated through centrifugation.

3.9.4   PF4/heparin affinity column to elute heparin-dependent (HD) HIT antibodies:
Total IgG that was purified from samples confirmed to have had HD antibodies was run in

the PF4 column to purify HD antibodies. Complexes of 0.5IU/mL unfractionated heparin (UFH)

were incubated with a 1:4 mixture of biotinylated PF4 and PF4 and formed at RT for 1hr before

being suspended in streptavidin coated Sepharose beads and incubated overnight at 4°C. The

sample column was then washed, and the previously purified total IgG was transferred to the

PF4/H column. The sample column was then washed to remove any unbound antibodies, the HD

Antibodies were eluted from the protein G column with 0.1M Glycine buffer, pH 2.7 and

neutralized with Tris buffer, pH 8.0. The elution buffer containing total IgG was immediately

exchanged for PBS and concentrated through centrifugation.

3.10 Immunoassay to determine the binding of HIT and VITT antibodies against PF4 mutants
The binding of antibodies in VITT and HIT patient sera to wildtype PF4 and PF4 mutants

was measured using a modified PF4/heparin IgG-specific EIA. Streptavidin was immobilized on

a microtiter well plate (384 wells; Thermo Scientific Nunc) overnight at 4 °C. 1 IU/mL

Biotinylated UFH was incubated with streptavidin for 1 hr. 5 μg/mL of either purified

recombinant mutant or wildtype PF4 proteins were then added and incubated for 1 hour at

ambient temperature and then washed with PBS+0.05% Tween 20. 1:50 dilutions of the either

VITT or HIT patient samples were then added to the plate and incubated for 1 hr at RT.

Following washing, the alkaline phosphatase-conjugated anti-human or alkaline phosphatase-

conjugated CXCL4/PF4 Rabbit anti-Mouse Polyclonal Antibody (Jackson Immuno Research

Laboratories, Inc) was added and incubated for 1 hr at ambient temperature. 1 mg/mL p-

nitrophenylphosphate (PNPP, Sigma-Aldrich) substrate dissolved in 1 mol/L diethanolamine

buffer (pH 9.6) was then added for detection. Following a 30-minute incubation, the optical

density was measured at 405nm (OD405nm) using a TECAN Sunrise plate reader (Tecan Group

Ltd., Mannedorf, Switzerland) to assess binding of antibodies to wild-type PF4 and PF4 mutants.

Results were reported as a percentage of VITT and HIT antibody binding relative to wildtype

PF4 binding using the following

$$\% \ antibody \ binding = \frac{OD_{VITT \ or \ HIT \ patient \ against \ mutant \ PF4}}{OD_{VITT \ or \ HIT \ patient \ against \ wildtype \ PF4}} \ x \ 100$$

3.11 Data Acquisition, statistical analysis, and reproducibility

We visualized the number of hydrogen bonds that occur between the mutated amino acids

on PF4 that make up the VITT binding site and can also differentiate between HIT and VITT

antibodies on the X-ray crystal structure of PF4 using PyMOL. We analyzed the location of the

affected amino acids as being either on the alpha helix or beta sheet of PF4 using an online curated

database containing integrated protein information with cross-reference to multiple sources

(UniProtKB, entry: PLF4_Human). GraphPad Prism version 9.1.2 for Mac OS software (GraphPad Software, Inc) was used to create all graphs and perform statistical analysis. Performance characteristics, including sensitivity, specificity, and confidence intervals, were calculated using Microsoft Excel for Mac version 15.37. Differences between data sets were tested for statistical significance using an unpaired t-test or a testing for significant difference in medians, using R package *coin*. *P*-values are reported as two-tailed where $P < 0.05$ was considered statistically significant.

4   RESULTS

4.1.1   Expressing and purifying 70 PF4 mutants.

4.1.2   Expression and purification of PF4 mutants compared to bacterial lysate.

To purify each PF4 mutant was expressed by bacteria and visually confirmed using SDS-PAGE, wildtype recombinant human PF4 (WT-rhPF4) was used as a control to confirm protein expression (Figure 1). These results confirm that the induction of PF4 mutants at mid-exponential cell growth with IPTG produced the largest amounts of soluble mutant PF4. As an example, Figures 1a and 1b show the SDS-PAGE analysis of the cells of the PF4 mutants G7A-D8A and L9A-Q10A, respectively, before and after inducing PF4 expression with IPTG. In addition, Figure 2 shows the SDS-PAGE analysis of the cells of the PF4 mutants E2A-D6A and one wildtype PF4 sample used as a control after inducing PF4 expression with IPTG. Once protein expression of all 70 PF4 mutants was confirmed, we then purified them by way of affinity chromatography using a heparin column and visualized the purity of each sample by SDS-PAGE gel (Figure 3). Figure 3 shows an example of the multiple samples eluted using a salt gradient containing contaminants including supernatant (SN) and samples kept from washing the heparin column (Hep Wash) that

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were collected during the purification process. Finally, we obtained samples of PF4 mutants that were contaminant-free when compared to the previous protocol that used bacterial lysate as seen in Figures 4a and 4b. It was important to obtain samples of purified PF4 mutants that were contaminant-free to combat limitations that were faced in the previous study that used bacterial lysate, which included the high background reactivity that was observed when testing several HIT patient sera against bacterial lysate and consequently, this reduced the testing population and risked obtaining skewed data in the previous study.

4.1.3 Mapping the binding sites of HIT antibodies on PF4 using alanine scanning mutagenesis

4.1.4 Mapping the binding sites of pathogenic HIT monoclonal antibodies

4.1.5 Testing the activity of 1E12, 1C12, and 2E1 in the EIA

To optimize the detection of monoclonal antibodies in the EIA, we tested 1E12, 1C12, and 2E1 at varying concentrations of $1.6\ \mu g/mL$, $3.1 \mu g/mL$, $6.3\ \mu g/mL$, $12.5\ \mu g/mL$, $25.0\ \mu g/mL$ and $50.0\ \mu g/mL$ against wildtype-PF4/heparin as described in the methods section. A previous report of 1E12, 1C12, and 2E1 observed high OD values and maximal activity at $2\ \mu g/mL$ and positive OD values at concentrations as low as $0.5\ \mu g/mL$ in the EIA.[72] We too, observed positive OD values of 0.6, 0.7, and 0.5 for 1E12, 1C12, and 2E1 respectively, at antibody concentrations as low as $1.6\ \mu g/mL$ (Figure 7). Our results and Vayne *et al*'s results suggest a high affinity of 1E12, 1C12, and 2E1 for PF4/heparin complexes.

4.1.6 Pathogenic Binding Sites of monoclonal antibodies 1E12, 1C12, 2E1 on PF4

To confirm whether the previously predicted binding sites on PF4 that used polyclonal HIT sera were in fact pathogenic, we predicted the binding sites of 3 murine monoclonal anti-PF4 antibodies with a humanized Fc (1E12, 1C12, 2E1) that possessed pathogenic HIT antibody

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activity when tested against platelets (Figure 8a-c).[72] The results from mapping the binding sites of each monoclonal antibody are as follows:

1E12: the PF4 mutants R22A, H23A, L45A, K50A, K66A resulted in greater than 50% reduction when testing the binding of 1E12 against each corresponding mutant compared to wild-type PF4. Excluding L45, the amino acids that make up the 1E12 epitope on PF4 are part of the amino acids that make up the VITT binding site that is localized within the heparin binding region on PF4 (Figure 8a).[71]

1C12: the PF4 mutants E28A, I30A, N47A, G48A, R49A, K50A, I51A, C52A resulted in greater than 45% reduction when testing the binding of 1C12 against each corresponding mutant compared to wild-type PF4. The amino acids that make up the 1C12 epitope PF4 bind to the same region as VITT antibodies that is localized within the heparin binding region and the amino acids E28, N47, and K50 are part of the amino acids that make up the VITT binding site that is localized within the heparin binding region on PF4 (Figure 8b). [71]

2E1: The PF4 mutants C10A, T25A, L27A, T38A, L41A resulted in greater than 45% reduction when testing the binding of 2E1 against each corresponding mutant compared to wild-type PF4. The amino acids that made up the 2E1 epitope on PF4 are found in the KKO binding region on PF4 and the amino acid C10 is part of those that make up the KKO binding site on PF4 (Figure 8c). [52]

4.1.7   Testing activity of monoclonal antibodies P4B1, P3D4, and P3E10 in the EIA

To optimize the detection of monoclonal antibodies in the EIA, we tested P4B1, P3D4, and P3E10 at varying concentrations of 1.6 μg/mL, 3.3μg/mL, 6.3 μg/mL, 12.5 μg/mL, 25.0 μg/mL, 50.0 μg/mL and 75.0 μg/mL against wildtype-PF4/heparin as described in the methods section. We observed a positive OD value ($OD_{405nm} \geq 0.45$) of 1.3 at a concentration of P4B1 as low as 1.6

μg/mL, meanwhile positive OD values of 0.6 and 0.7 for P4B1 and P3D4, respectively (Figure 9). We too, observed that even a high concentration of P3D4 and P3E10 at 6.3 μg/mL was insufficient to distinguishing the platelet-activating (P3D4) from non-platelet activating (P3E10) antibody. However, we observed that at a concentration of 1.6 μg/mL, the OD values of P4B1 (1.3) can be distinguished from P3D4 (0.2) and P3E10 (0.2), a concentration at which both antibodies have negative OD values (Figure 9). P4B1 is a platelet activating antibody that displays heparin-independent activity by being able to activate platelets in the absence of heparin. We then performed a student's t-test to compare whether being able to distinguish P4B1 from P3D4 and P3E10 held any statistical relevance. We observed that at a concentration of 1.6 μg/mL, the differences in OD values for P4B1 vs. P3D4 (p-value <0.0001) and P4B1 vs. P3E10 (p-value <0.0002) were both statistically significant (Figure 10).

4.1.8 Pathogenic Binding sites of monoclonal antibodies P4B1, P3E10 and P3D4 on PF4

To determine whether the previously predicted binding sites on PF4 that used polyclonal HIT sera were in fact pathogenic, we predicted the binding sites of 3 human-derived HIT monoclonal antibodies (P4B1, P3E10 and P3D4) that possessed HIT-like reactivity when tested against platelets (Figure 11a-c).

P4B1: the PF4 mutants C12A, V13A, K50A, C52A, L53A resulted in greater than 20% reduction in binding of P4B1 against each corresponding mutant compared to wild-type PF4. The amino acids that make up the P4B1 binding site on PF4 are found in the KKO binding region on PF4 and the amino acids including C12 and C52 are part of those that make up the KKO binding site on PF4 (Figure 11a).[52]

P3D4: the PF4 mutants E1A, A2V, E3A, V13A, P37A, T38A resulted in greater than 20% reduction in binding of P3D4 against each corresponding mutant compared to wild-type PF4. The amino acids that made up the P3D4 binding site on PF4 are found in the KKO binding region on PF4 (Figure 11b). [52,71,88]

P3E10: the PF4 mutants K14A, T15A, L27A, C36A, A39V, Q40A resulted in greater than 20% reduction in binding of P3E10 against each corresponding mutant compared to wild-type PF4. The amino acids that made up the P3E10 binding site on PF4 are not located in any of the identified antibody binding regions on PF4, rather they are found outside the KKO binding site on PF4 (Figure 11c).[52]

4.1.9 Purifying total IgG antibodies from HIT sera:

To confirm the binding sites of HIT antibodies on PF4, we went on to purify HI and HD from HIT samples. To do this, we followed a method of sequential purification steps that first involves purifying the total amount of IgG class antibodies from HIT samples (Figure 12). We purified the total IgG antibodies from four HIT patient samples. Following purification of total IgG from the four HIT serum samples, we confirmed the purity of the IgG purified from HIT sera by SDS-PAGE gel analysis (Figure 13) and then tested the purified total IgG in a PF4/heparin EIA to confirm that antibody reactivity against the antigen was not lost. The ODs of the serum from patient 1, patient 2, patient 3, and patient 4 were 3.03, 2.87, 1.86, and 2.61, respectively and the ODs of the IgG purified from these patients were at values of 1.11, 1.95, 0.52, and 0.60, respectively and considered positive (OD405nm $\geq$ 0.45) with respect to the standard 0.45 cut-off used for serum samples against PF4/heparin in the EIA.

4.1.10 Purification and performance of HI and HD HIT antibodies

To determine whether HI and HD HIT antibodies from HIT patients bind to sites that dictate their platelet activation profiles and to identify amino acids that could be further used to separate them from VITT antibodies , I attempted to purify HI and HD HIT antibodies from four HIT patients in whom the anti-PF4/heparin antibodies are autoantibody-like, as they recognize PF4 bound to platelets even in the absence of heparin in the SRA.. Our method of affinity purification involved binding streptavidin coated beads to biotinylated PF4 to form a PF4 column to purify HI antibodies and another similar column, where heparin was added to form a PF4/heparin column to purify HD antibodies. Following affinity purification, we confirmed that the purified samples containing HI and HD antibodies from each column were free of contaminants using SDS-PAGE. The image of the SDS-PAGE in Figure 14 confirms the purity HI and HD samples with visible bands indicating the heavy chain at 50 kDa, and light chain at 25 kDa. We then tested the purified samples containing the HI antibodies for four HIT patients and HD antibodies for the same four HIT patients in an EIA. When tested in the PF4/heparin EIA, the mean OD_{405nm} of the purified HI antibodies from the four HIT patients was 0.51 (range 0.1-1.3) and the mean OD_{405nm} of the purified HD antibodies from the same four HIT patients was 0.55 (range 0.07-1.1) (Table 2).

4.1.11 Mapping the binding sites of purified HI HIT antibodies

To determine where the purified HI and HD antibodies bind to on PF4, we tested these antibodies against the 70 PF4 mutants in an EIA. We tested the 2 patients (patient 2 and patient 4) whose serum, HI and HD antibodies reacted positively in the EIA (OD405-490nm ≥ 0.45) (Table 2). We determined the binding sites of the 2 patients by mapping the purified HD and HI antibodies against the 70 PF4 mutants (Figure 15a, b). 7 PF4 mutants (L8A, C10A, T16A, R20A, C52A, K61A, and L67A (Figure 15b) resulted in ≥ 50% loss of binding of HI HIT antibodies and were of common importance between the 2 HIT patients. These 7 PF4 mutants identified represent

amino acids that are found in the binding sites of previously tested HIT-like monoclonal antibodies that display heparin-independent reactivity including 1C12 (C52A) and 2E1 (C10A and T16A), as well as those found in the previously published HI-HIT site by Huynh *et al.*[71] The binding sites of HI-HIT antibodies on PF4 were predicted using alanine-scanning mutagenesis, and changes to the antibody-binding ability were modelled using the crystal structure of the PF4 monomer (Figure 15b). The purified HD antibodies from the 2 HIT patients were also tested against the PF4 mutants. We found 6 PF4 mutants resulted in ≥ 50% loss of binding of HD HIT antibodies and were of common importance between the 2 HIT patients. These amino acids include K14A, H23A, V29A, T38A, K50A, K66A (Figure 15a). The 6 PF4 mutants identified represent amino acids that are found in the binding sites of previously tested HIT-like monoclonal antibodies that display heparin-dependent reactivity including P3D4 (T38A) and P4B1 (K50A).

4.1.12 Binding Sites of HIT antibodies on PF4

The binding of HIT antibodies to wildtype PF4 and PF4 mutants was measured using a streptavidin EIA as outlined in the methods section. Figure 5 outlines a schematic diagram of the streptavidin-based EIA used to detect HIT antibodies. A primary antibody like KKO was used as a HIT-like model to confirm the feasibility of the assay.[43,52,69] In alignment with previous results [43,52,69] we found that 9 PF4 mutants including C10A, V13A, A32V, C36A, C52A, L55A, Q56A, L67A, L68A resulted in greater than 50% reduction in binding of KKO against each corresponding mutant compared to wild-type PF4 (Figure 6). We then tested the 10 HIT patients in our binding assay and found that of the 70 PF4 mutants tested, 9 PF4 mutants: C10A, A32V, C36A, P37A, L41A, A43V, L53A, D54A, L67A were identified with a 50% or greater reduction in HIT patient antibody binding compared to wild-type PF4 (Figure 6a and Table 1). The PF4 mutants that affected HIT antibody binding are restricted to a similar region as KKO on PF4 and 4 out of 12 of the amino acids affected including C10A, A32V, C36A, and L55A overlap with the 9 amino acids

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that make up the KKO binding site on PF4. Although, these 9 amino acids in the KKO binding site confirm previous results from the study that used bacterial lysate,[52] using purified PF4 mutants allowed us to identify the 9 amino acids that comprise the KKO binding site on PF4 using 50% cut-off and no background interference.

4.1.13 Binding Sites of antibodies from HIT patients with non-HIT patients on PF4

We also selected 10 non-HIT patient samples to determine whether they bound to a restricted site on PF4. The non-HIT patient cohort includes patients that tested positive for antibodies against wild-type PF4 in our in-house IgG specific PF4/heparin EIA[48] ($OD_{405nm} \geq 0.45$) but subsequently tested negative for platelet activating antibodies in the SRA ($\geq 20\%$ ^{14}C-serotonin release). We determined that the PF4 mutants that affected the binding of non-HIT antibodies by greater than 15% in comparison to wildtype PF4 recognized arbitrary sites on PF4 and that the amino acids affected were not restricted to any identified region on PF4 (Figure 7b).

4.1.14 Binding sites of VITT antibodies on PF4

VITT patient samples were collected and tested against the PF4 mutants to confirm the recently identified VITT binding site on PF4 with a larger sample size than the 5 patients initially tested.[71] We selected a total of 14 diagnosed VITT patients to predict the binding sites of VITT antibodies on PF4. The binding sites were predicted once we made point mutations to each amino acid on PF4 to generate 70 recombinant PF4 mutants and with then we performed alanine-scanning mutagenesis. We tested each of the recombinant PF4 mutants against the 14 VITT positive patient samples and identified 9 PF4 mutants including L8A, R22A, H23A, E28A, K46A, N47A, K50A, K62A, K66A that affected the binding of VITT antibodies. The 9 PF4 mutants include those that resulted in greater than 50% reduction in binding of each corresponding mutant compared to wild-type PF4. The 9 affected mutants align with the 8 that are reported in literature[71] with the addition

of L8A (Table 3). These PF4 mutants represent 9 out of 70 (12.9%) amino acids found on the surface of PF4 that are critical for the binding of antibodies in VITT samples. (Figure 16a and b colored red).

4.1.15 Clinical description of PF4-dependent vs. PF4-independent VITT patient cohorts

Upon further assessing the laboratory and clinical characteristics of the 14 VITT patients we selected for the binding study: within the VITT cohort (n=14) we identified that 10 of these VITT patients are those whose antibodies display PF4-dependent reactivity, and the remaining 4 patients are VITT patients whose antibodies display PF4-independent reactivity. The laboratory characteristics of PF4-dependent and PF4 independent antibodies VITT patients are outlined as followed. PF4-dependent antibodies include VITT patient samples that display typical platelet activation profiles in the PF4-SRA, meaning that they are negative in buffer ($<20\%$ ^{14}C serotonin release) at 0 μg/mL of PF4 and positive ($\geq 20\%$ ^{14}C serotonin release) at either concentration of PF4 including 10 μg/mL, 25 μg/mL and 50 μg/mL and inhibited by IV.3 (Figure 16a). PF4 independent antibodies include a subset of VITT patients whose samples display unique platelet activation profiles in the PF4-SRA compared to the typical profiles outlined, meaning that PF4-independent antibodies react positively in buffer ($\geq 20\%$ ^{14}C serotonin release) in the absence of PF4 at 0 μg/mL and positive at either concentration of PF4 including 10 μg/mL, 25 μg/mL and 50 μg/mL and inhibited by IV.3 (Figure 16b). These patient samples were identified as PF4-independent since they can activate platelets in the absence of the supplemented antigen for VITT antibodies, PF4. Of the 14 VITT patients we selected, the antibodies in 10 patients displayed PF4-dependent platelet activity in the PF4-SRA. The 10 VITT patients with PF4-dependent antibodies were further assessed and their clinical characteristics are outlined as in Table 4 and as followed. 10 out of 10 (100%) of these patients had received at least one dose of the ChAdOx1 nCoV-19

(AstraZeneca) vaccine. The most common presentations of thrombosis in the PF4-dependent cohort were cerebral venous sinus thrombosis (CVST) (4 out of 10, 40%), deep vein thrombosis (DVT) (2 out of 10, 20%), and pulmonary embolisms (PE) (2 out of 10, 20%). Meanwhile, 4 patients with PF4-independent antibody reactivity in the PF4-SRA were screened further and their clinical characteristics are outlined in Table 2 and as followed: 3 out of 4 (75%) of these patients had received at least one dose of the ChAdOx1 nCoV-19 (AstraZeneca) vaccine and the 1 patient had received Ad26.COV2.S (Johnson and Johnson, J&J). The most common presentations of thrombosis in the PF4-dependent cohort were CVST (2 out 4, 50%), DVT (2 out of 4, 50%), and PE (2 out of 4, 50%). There was a significant difference (P < 0.001) in the platelet counts between the PF4-dependent (mean = 50 x10^9/L) and PF4-independent (mean = 171 x10^9/L) patient samples (Table 4). With differences observed in the clinical and laboratory characteristics between PF4-dependent and PF4-independent VITT patient samples, we went on to analyze whether there would also be differences observed in the binding characteristics to PF4 of the antibodies found in these patients.

4.1.16 PF4-dependent antibody vs. PF4-independent antibody binding sites on PF4
We selected a total of 14 VITT patients and confirmed the amino acids that comprise the VITT binding site on PF4 as described in the results section above and in the recent study by Huynh et al.[71] Upon assessing the PF4-SRA results, we determined that 10 out of 14 (71.4%) of these patient samples displayed PF4-dependent reactivity profiles in the PF4-SRA meanwhile, the remaining 4 out of 14 (7.1%) patients displayed PF4-independent reactivity profiles. Nine amino acids (L8, R22, H23, E28, K46, N47, K50, K62, K66) were common among 10 of the 14 VITT patients with PF4-dependent reactivity profiles that resulted in greater than 50% reduction in binding (Figure 16a). These PF4 mutants are like the nine amino acids that make up the previously

identified VITT site. [71] The amino acids L8, E28, K46, K50 and N47 are located on the beta strand

of PF4 meanwhile, amino acids R22, H23, K62, K66 are located on the alpha helix of PF4. Eight

out of 9 (88.9%) of these PF4 mutants make up the amino acids that comprise the VITT binding

site as predicted by Huynh *et al*.[71] The binding sites of 4 patient samples with PF4-independent

reactivity profiles in the PF4-SRA were also mapped using the 70 PF4 mutants and outlined in

Figure 16b. Two different binding sites were identified in this patient group. All PF4 mutants

affected PF4-independent antibodies with greater than 50% reduction in binding. The first binding

site was like the published VITT binding site and the binding site we previously determined for

PF4-dependent antibodies and included the PF4 mutants L8A, R22A, H23A, E28A, K46A, N47A,

K62A, K65A, and K66A. Meanwhile, the second binding site recognized by PF4-independent

antibodies included 10 PF4 mutants namely, Q9A, C10A, C12A, T15A, A39V, Q40A, C52A,

L53A, D54A, and K61A and are outlined in Table 5. The amino acids Q9, C52, L53 are located

on the beta strand of PF4. C12 and K61 are located on the alpha helix of PF4 (Table 3). The 10

PF4 mutants that affected PF4-independent VITT antibodies bind to the region of the previously

identified KKO site on PF4 (Figure 16b).[52,71]

4.1.17 PF4 mutants that differentiate VITT from HIT

We selected the 10 PF4 mutants R20A, H23A, T25A, E28A, K46A, R49A, K50A, K62A,

K65A, K66, of which the corresponding amino acids are important either for VITT or heparin

binding to PF4 to determine whether they could differentiate between HIT and VITT. We found

that 5/10 (50%) of these mutants (H23A, K46A, K50A, K62A, and K66A) consistently showed

the largest decrease in VITT antibody binding to PF4 when tested against a larger subset of

samples in previous screens and the additional 5 PF4 mutants (R20A, T25A, E28A, R49A, and

K65A) resulted either in no change or an increase in HIT antibody binding when compared to their binding to wildtype PF4 in previous screens. These 10 PF4 mutants were then tested against larger subset of HIT (n=22) and VITT (n=9) patient samples. Patient samples were selected based on having a positive test result for antibodies against wild-type PF4 in our in-house IgG specific PF4/heparin EIA[48] ($OD_{405nm} \geq 0.45$) and subsequently tested positive for platelet activating antibodies in the SRA ($\geq 20\%$ ^{14}C-serotonin release). The average binding to each mutant was compared between the groups. All 10 selected mutants (R20A: 19.1% vs. 94.6%, $p < 0.05$, H23A: 17.4% vs. 97.0%, $p < 0.0001$, T25A: 24.5% vs. 146.1%, $p < 0.001$, E28A: 20.5% vs. 111.0%, $p < 0.0001$, K46A: 12.2% vs. 76.4%, $p < 0.001$, R49A: 17.8% vs 132.9%, $p < 0.01$, K50A: 13.4% vs. 79.0%, $p < 0.0001$, K62A: 19.6% vs. 94.6%, $p < 0.0001$, K65A: 20.1% vs. 124.5%, $p < 0.001$, K66A: 18.5% vs. 114.4%, $p < 0.0001$) were able to distinguish HIT from VITT antibodies with statistical significance (Figure 17 and Table 6). The mutants H23A, E28A, K50A and K66A were better at separating HIT from VITT antibodies when using cut-offs of 45.5%, 42.7%, 31.1%, and 41.8%, respectively and all had high sensitivities (100%) and specificities (100%). 6 out of 10 (60%) of these PF4 mutants (H23A, E28A, K46A, K50A, K62A, K66A) represent 6 amino acids that were identified as part of the 8 amino acids that make up the VITT antibody binding site on PF4 determined by Huynh *et al*.[71,88]

4.1.18 PF4 mutants that differentiate HIT from non-HIT

To differentiate between HIT and non-HIT, the serum samples of patients that presented with HIT-like symptoms but were negative for HIT were sent in for diagnostic testing. The patient samples from both HIT (n=22) and non-HIT (n=12) patient groups that tested positive for antibodies against wild-type PF4 in our in-house IgG specific PF4/heparin EIA[48] ($OD_{405nm} \geq 0.45$) and the streptavidin/biotinylated-heparin/PF4 EIA ($OD_{405nm} \geq 0.45$) were selected for further

analysis (Tables 9 and 10). These HIT and non-HIT patient cohorts were then tested against the

10 previously selected PF4 mutants in an EIA. The average binding to each mutant was compared

between the groups. Only 5 out of the 10 selected mutants (H23A: 96.9% vs. 50.0%, $p < 0.0001$,

T25A: 146.1% vs. 57.2%, $p < 0.01$, R49A: 132.9% vs. 63.9%, $p < 0.05$, K65A: 124.5% vs. 59.3%,

$p < 0.05$ and K66A: 114.4% vs. 74.4%, $p < 0.05$) were able to distinguish HIT from non-HIT

antibodies with statistical significance (Figure 18 and Table 8). The mutants H23A, T25A, and

K65A were better at separating HIT from VITT antibodies when using cut-offs of 49.8%, 58.9%,

and 52.4% respectively and all had low sensitivities (50%) and high specificities of 95.7%, 82.1%

and 91.3% in the EIA testing HIT and non-HIT samples against the PF4 mutants H23A, T25A and

K65A, respectively. These 5 amino acids are part of those that make up the previously defined

heparin binding site on PF4.[99]

5   DISCUSSION

Platelet-activating antibodies are a source of the pathogeneses in blood disorders

like HIT and VITT. The pathogenic antibodies in HIT are mostly specific for PF4/heparin

and can activate platelets to cause arterial and venous thrombosis with a mortality rate as high as

20% in the event of missed or delayed diagnoses.[100] EIAs alone are insufficient to diagnose HIT

since they have a low specificity of approximately 51%,[48,50,51] and thus are unable to rule out the

presence of non-pathogenic, non-platelet activating antibodies. By contrast, we recently showed

that the EIAs used for diagnosis in VITT have an improved specificity as high as 96%.[81,101,102]

Evidently, differences in the types of antibodies that activate platelets in VITT and HIT have been

of particular interest as they could provide a promising way of identifying the mechanism that

triggers VITT and offer more insight on the antibodies that lead to HIT. Therefore, the aim of my

project was to purify 70 PF4 mutants and to determine whether the molecular characteristics of HIT and VITT antibodies dictate their platelet activation profiles and from this, I developed an assay that can be used to differentiate between both anti-PF4 disorders. I did so by determining the binding sites where pathogenic antibodies in HIT bind to on PF4 using HIT-like monoclonal antibodies and HIT antibodies purified directly from patient samples. We recently identified 8 amino acids on PF4 that are important for VITT antibody binding.[71] Initial studies showed that VITT caused by the AstraZeneca vaccine had 1 antibody that recognizes an epitope in the heparin-binding site, meanwhile VITT anti-PF4 antibodies after the J&J vaccine had 2 antibodies, where the additional epitope recognizes the binding site of heparin-dependent antibodies and alsomodel HIT pathogenic monoclonal antibodies.[88] Based on such findings, another aim of my project was to perform ASM on more VITT samples for the purpose of identifying whether there is a second VITT antibody binding site on PF4. Lastly, there has been emerging interest on whether there are intrinsic characteristics that can help to differentiate HIT from VITT. [103-105] From this, I developed an assay that uses specific PF4 mutants to differentiate HIT antibodies from VITT antibodies in an easy-to-use and efficient assay. This work is relevant to improving diagnostic strategies and provide a better explanation on the triggers that cause HIT and VITT. The implication of these aims and results will be discussed below.

5.1.1   Modifying ASM using purified mutants compared to bacterial lysate

I purified 70 PF4 mutants for the purpose of determining whether the binding sites of HIT pathogenic monoclonal antibodies and VITT antibodies dictate their platelet activation profiles and whether these characteristics can be used to differentiate between both anti-PF4 disorders. To do this, I modified a previous assay by Huynh *et al* that used bacterial lysate of 70 PF4 mutants to

identify immunodominant sites in HIT.[52] The methods implemented in this study produced bacterial lysate of 70 PF4 mutants by way of ASM to first identify as a control, the binding site of the PF4 mutants to bind to the monoclonal antibody KKO, then to experimentally determine the binding sites of the pathogenic HIT antibodies from patient sera.[52] They found 13 amino acids on PF4 that were important for KKO binding and identified 10 amino acids that are recognized by most HIT antibodies, however, several HIT patient sera could not be tested in the binding assays due to the background reactivity to bacterial lysate. [52] While the assay I used involved purified 70 PF4 mutants, I too, found that some HIT patient samples reacted with high background OD values against the purified 70 PF4 mutants compared to wildtype PF4 in the EIA, making it difficult to identify important epitopes on PF4 that antibodies in those HIT patients bind to. However, I observed 9 amino acids that were important for KKO binding on PF4, which was less than the 13 amino acids identified when using bacterial lysate.[52] Using purified PF4 mutants was important since, the increased background signal in the previous assay could be explained by the reaction of anti-PF4/heparin antibodies found in HIT patient sera against bacterial lysate. Bacterial lysate in its soluble form has been shown to be a useful tool during structural and biochemical protein characterization in experiments like Native Mass spectrometry and SDS-PAGE. [3] However, the use of bacterial lysate in binding assays can leave contaminants like cell debris and furthermore, lead to the background expression of *E.coli* proteins that could compete with the activity of the protein of interest.[106] For example, a study by Didovyk *et al* looked at B-galactosidase (LacZ), an enzyme that cleaves lactose into glucose and galactose for the advancement of glycolysis.[107,108] LacZ was tagged with a green fluorescent protein (GFP) and similar to our protocol, was expressed in *E. coli* BL21 (DE3) strain for the purpose of analyzing the activity of the bacterial lysate produced, the reactions were monitored by GFP fluorescence and then measured at a readout of

OD 574 nm. They observed high OD readings in the LacZ deficient bacterial lysate, which the authors explained by possible background expression of *E. coli* genes as mentioned before. [106] For this reason purified proteins have been found to provide more extensive characterization of the physiochemical and functional aspects of a tetramer, including the effect of mutations on the structural properties of proteins.[109]

5.2 Optimizing identification of VITT and HIT antibody binding sites on PF4

We then used the purified mutants to confirm the HIT and VITT antibody binding sites as first described by Huynh *et al*. [71] By mapping the binding sites of antibodies in 10 HIT and 14 VITT patients, we were able to confirm that there are two distinct binding regions made up of 12 amino acids on PF4 for HIT antibodies and 9 amino acids for VITT antibodies to bind to on PF4. 12/12 (100%) of the amino acids that we found important for the HIT site were part of the same 10-13 amino acids identified for HIT antibody binding by Huynh *et al*.[52,71] This suggests that despite likely having polyclonal antibodies, there are dominant clones in HIT patients that recognize similar amino acids on PF4 and are likely to trigger platelet activation and disease.[71,79] Meanwhile, 8/9 (88.9%) of the amino acids that we found important for the binding of antibodies from VITT patients were part of the same 8 amino acids identified for VITT antibody binding by Huynh *et al* in 5 of VITT patients. [71] Based on suggestions that VITT antibodies have oligoclonal/monoclonal characteristics, this indicates that VITT patients likely recognize a distinct region on PF4 to trigger the pathogenesis of VITT. [71] The HIT site we identified and Huynh *et al* published is confirmed to be in a similar region on PF4 as the KKO site [52,69,71] and again similarly to Huynh *et al*., our findings revealed that the VITT site is in a similar region on PF4 as the heparin binding site on PF4. [71,99] The antibodies in both diseases bind to distinct sites from each other due to the way they recognize PF4 to elicit platelet activation, majority of HIT antibodies recognize

complexes of PF4/heparin since heparin causes a confirmation change to PF4 that reveals neoepitopes on PF4 for antibodies to bind and cause platelet activation.[110-112] Meanwhile, the antibodies in VITT patients recognize PF4 alone to cause VITT in patients that have received an adenoviral vector-based vaccine, suggesting that the heparin binding site allows for sufficiently large PF4-antibody complexes to form such that they can elicit an immune response in VITT patients.[73,88]

The proposed mechanism for VITT is currently under investigation, however, VITT antibodies bind to PF4 efficiently enough to activate platelets without prior heparin treatment, suggesting that the conformation of PF4 might not need to be modified to reveal neoepitopes for platelet activation in VITT.[113] Unlike in HIT where majority of the antibodies bind to different epitopes within a region on PF4, anti-PF4 VITT antibodies bind to a distinct site on PF4 that is made up of 8-9 common amino acids, giving anti-PF4 VITT antibodies monoclonal/oligoclonal characteristics. [71,79] Anti-PF4 VITT antibodies display these monoclonal/oligoclonal characteristics since they have a distinct preference for the heparin binding site on PF4 and by their ability to be variably inhibited by heparin. [79] A recent study by Singh *et al* presented a patient diagnosed with ChAdOx1 nCoV-19-associated (AstraZeneca) VITT that recovered after being treated with heparin, evidently, this is a phenomenon that provides insight on the distinguishable mechanisms that trigger VITT from HIT since heparin is possibly fatal to HIT patients.[79]

Non-pathogenic antibodies have not been detected in VITT patients, whereas in HIT, non-pathogenic antibodies make up the majority of the antibodies in patients suspected of HIT and are the reason for the low specificity of HIT EIAs.[114] For this reason, we also screened 10 patients with suspected HIT that were positive in the EIA for antibodies that were PF4/heparin specific but non-pathogenic because, they did not activate platelets in the SRA. We found that the antibodies in

non-HIT patients did not recognize distinct sites on PF4 and had no distinguishable binding region identified like in HIT and VITT. This was needed to confirm whether the site that majority of the antibodies in HIT patient sera is in fact the site where pathogenic antibodies bind to on PF4 to cause HIT.

I went on to determine whether the region on PF4 where HIT antibodies bind to is pathogenic by mapping the sites of 1E12 and 1C12, 2 mouse-derived monoclonal antibodies with a human Fc-fragment that activate platelets in functional assays. When testing these antibodies against the 70 PF4 mutants, I found that 1E12 and 1C12 bound to a region on PF4 that was like the VITT site on PF4. These results coincide with previous functional assay results on 1E12 and 1C12 by Vayne *et al* , by predicting antibody binding sites *in silico* they found that both 1E12 and 1C12 bind to sites outside of the KKO site on PF4, furthermore with the SRA they found that similar to VITT, both monoclonal antibodies can activate platelets in the absence of heparin and are inhibited at high concentrations of UFH (10 IU/mL).[72] Determining the binding sites of HIT-like monoclonal antibodies provides important insight on spontaneous HIT which is a type of HIT that affects a small subset of HIT patients. Patients with spontaneous HIT are unique in that they have antibodies like 1E12 and 1C12 that can cause platelet activation even in the absence of prior heparin exposure, suggesting a mechanism of PF4 recognition that can occur in the absence of heparin, to change in the conformation of PF4 and reveal neoepitopes for pathogenic antibodies to bind and cause platelet activation. For this reason, the proposed mechanism of PF4 recognition in spontaneous HIT has been attributed to Gram-negative bacteria found on endothelial cells, which could behave similarly to heparin by binding to and altering PF4 conformation and lead to thrombosis.[24] Future experiments could involve purifying the antibodies from clinically diagnosed patients with spontaneous HIT and mapping them against the 70 PF4 mutants to confirm whether

they share similar binding sites to 1E12 and 1C12 on PF4. This is important because spontaneous HIT is unlike typical HIT that affects most patients since typical HIT manifests because of heparin exposure, suggesting that there could possibly be differences in the mechanisms that lead to the pathogenesis of HIT caused by heparin exposure and HIT observed without heparin exposure.

We also mapped the binding site of 2E1, another mouse-derived monoclonal antibody that activates platelets in functional assays. We observed that 2E1 bound to a site like the KKO site on PF4. Our results differed from findings by Vayne *et al* , they determined that 2E1 recognizes a site on PF4 different but close to the KKO binding site, furthermore, 2E1 exhibited unique bivalent binding mechanism to PF4 through its antigen recognition site and charge-related interactions with heparin.[72] It binds to two threonine and the two leucine residues, both of which are hydrophobic amino acids and there has been an observed bias toward hydrophobic amino acids in immunogenic epitopes. [115,116]

Next, we mapped the binding sites of the 3 human-derived monoclonal antibodies P4B1, P3D4, and P3E10 that display HIT-like reactivity in functional assays and activate platelets in the presence of heparin. P4B1 and P3D4 are EIA positive antibodies that activate platelets and P3E10 is an EIA positive antibody that does not activate platelets. Our results suggest that P4B1 has a high affinity for PF4/heparin complexes while P3D4 and P3E10 have a lower affinity for PF4/heparin complexes. We found that that P4B1 and P3D4 recognized similar sites on PF4 as the KKO site. These antibodies recognize a site on PF4 like the recently published heparin-dependent HIT site by Huynh *et al*.[71] As expected by an antibody derived from a non-HIT patient, P3E10 recognized a site different from and outside both the KKO and VITT binding regions on PF4. This was consistent with the binding characteristics we found from screening the 10 non-HIT patients above, where we reported that the antibodies in non-HIT patients bind to indistinct and

indistinguishable sites on PF4, unlike most antibodies in HIT and VITT that are restricted to 2 distinct binding sites on PF4. This portion of our study is the first reported attempt at mapping the binding sites of 3 human-derived monoclonal antibodies directly from HIT patients where ours and previous studies have looked at mouse-derived monoclonal antibodies. We were limited in the amount of sample available to map each monoclonal antibody at higher concentrations, therefore, future studies may look to obtaining more of each sample to show repeatability in being able to map P4B1, P3D4 and P3E10 at varying concentrations against PF4 mutants.

Once we were able to confirm the binding sites of HIT antibodies through an optimized method that uses purified PF4 mutants where bacterial lysate was once used, we then used this method to confirm the binding sites of VITT antibodies on PF4. In doing so, we found that majority of the antibodies in VITT patients bound to the previously defined VITT site published by Huynh *et al* .[71] Furthermore, we identified two cohorts of antibodies in VITT patients, those typically observed with PF4-dependent reactivity and those less observed with PF4-independent reactivity in the SRA. The difference between these antibodies is the ability of PF4-independent the antibodies to activate platelets in the absence of PF4 (0 μg/mL PF4) while PF4-dependent antibodies display typical platelet activation profiles in the presence of PF4. The reason why some patients had platelet-activating PF4-independent VITT antibodies could be due to the presence of the antigen PF4 in the peripheral blood.[36] PF4 is released from stimulated platelets following injury and can be found at minimum detectable levels ranging from 4 ng/mL to 24 ng/mL in the plasma of healthy patients[37] to levels as high as 19.24 mg/mL to 24.1 mg/mL in patients with inflammatory diseases or patients that have undergone cardiopulmonary bypass surgery. [117,118] From mapping the binding sites of VITT patient samples that displayed PF4-independent reactivity profiles, we found that the antibodies in these patients recognized both the KKO site and the VITT site. We suspect

that the reason for evidence of additional amino acids within the KKO region recognized outside the VITT site is due to the suggested mechanism for VITT by anti-PF4 antibodies; like in HIT where heparin causes neoepitopes to form on PF4, VITT antibodies are suspected of binding and orienting PF4 in a similar manner as heparin does to trigger platelet activation like HD antibodies in HIT.[52,71,78]

Knowledge on the pathogenic binding sites in HIT and VITT allowed us to select appropriate PF4 mutants to differentiate VITT from HIT in an EIA. We selected mutants that affected the binding of most HIT and VITT samples. We discovered 10 PF4 mutants that can differentiate VITT from HIT in an EIA and we also discovered 6 PF4 mutants that can differentiate HIT from non-HIT. We found that all 10 PF4 mutants selected were able to differentiate VITT from HIT. We found that although the binding ability of the antibodies in about half of the HIT samples against the 10 PF4 mutants was affected while the other half did not lose their binding ability against the PF4 mutants. The difference was not similarly evident as seen with VITT antibodies against the 10 PF4 mutants where all the VITT patient samples lost more than 50% reactivity against each corresponding PF4 mutant when compared to wildtype (Figure 3). This could be explained by the effect that antibody clonality in HIT and VITT have on binding. HIT antibodies are likely polyclonal in nature, constituting their ability to bind to multiple amino acids on PF4 to trigger an immune response whereas VITT antibodies are defined by their monoclonal/oligoclonal characteristics and high affinity for 8 specific amino acids on PF4.

This relevance of these 10 PF4 mutants has important laboratory and clinical implications concerning both diseases. There is an unmet need for the development of antigen tests that would reliably be able to differentiate between VITT and HIT antibodies. From a clinical perspective, although reported incidences of VITT remain low, VITT can be debilitating or even fatal if left

untreated.[1] The current screening test for VITT is an EIA that is similarly used to help diagnose patients with HIT, these EIAs are positive in patients with VITT with a specificity as high as 96%.[81] Currently, the only way to distinguish a confirmed diagnosis of VITT from that of HIT, is through a modified functional PF4-enhanced SRA, however treatment has been recommended to start before diagnosis has been confirmed, likely due to the longer turnaround time of 1-3 days for the SRA as opposed to the shorter turnaround time and cost-efficiency that EIAs provide.[1, 2] As of now, any presumptive diagnosis of VITT by clinicians is promptly initiated with VITT-appropriate treatment, however presumptive clinical diagnoses made by clinicians lead to false-positives and unnecessary treatment.[2] Based on our analyses of suspected VITT samples sent in based on presumptive diagnoses by physicians, we have found that very few "suspected VITT" patients are positive for VITT once confirmed diagnostically.[81] Therefore, increased reporting will help us to provide clinicians with faster and more reliable results for the appropriate treatment to be administered. Given that we do not know the trigger for anti-PF4 antibody development in diseases like HIT, spontaneous HIT and VITT, it is important to identify and distinguish those that are pathogenic and seen in VITT from those that are non-pathogenic and seen in non-pathogenic HIT. This is because such studies have potential when screening for future VITT-like responses that may arise from adenoviral infections.

Being able to differentiate between HIT and VITT is important from a vaccine epidemiology perspective as this would inform strategies for risks associated with platelet-activating immune complexes[119] and risks regarding any future vaccinations that COVID-19 patients may need to receive.[71,82,88] An easily accessible assay that differentiates VITT from HIT would allow us to narrow the focus on VITT alone to discover the origin and risks associated with this immune response and give better insight on the best way to treat the disease.[82] A good example

of this assays' potential utility is the fact there are already preliminary indications that heparin may be a safe and valid treatment option for VITT patients since IVIG itself is potentially prothrombotic,[120] whereas heparin can be fatal in HIT. The lack of heparin-enhanced platelet activating properties permits treatment with heparin in VITT patients, of whom many experience CVST and secondary hemorrhages.[121,122] Separation between the two disorders could yield important insights regarding PF4 mediated antibody pathologies and lead to better and more optimized treatment options. Furthermore, there is relevance in identifying PF4 mutants that differentiate between two disorders using samples that are positive for platelet-activating antibodies. Such an assay could also be useful in determining whether there are PF4 mutants that can distinguish platelet-activating antibodies from non-platelet activating antibodies found in HIT patients.[50] The rationale behind this idea lies in the fact that clinicians rely on presumptive diagnoses to begin treatment for VITT before receiving laboratory diagnostic confirmation, which can lead to many false-positive results and unnecessary treatment.[81] A clinical rationale for an assay that can distinguish VITT from HIT is that the use of anticoagulants will be necessary to treat thrombosis, and while the use of heparin is contraindicated in treating HIT, the use of heparin-associated anticoagulants in VITT needs to be further studied as they have a potential clinical benefit for VITT patients.

5.2.1 Limitations while purifying and mapping heparin-independent and heparin-dependent antibodies from HIT patients

The premise behind affinity purifying HI and HD antibodies relies on previous studies that have identified pathogenic antibodies in HIT as having strong binding forces against PF4/heparin and PF4 complexes. [24,64] It has also been shown that there are similarities between the binding sites in HIT serum to HI and HD binding sites while using HIT serum and plasma samples and purified

total IgG from these samples.[1,2] We expected that the serum of a patient with autoimmune HIT would contain both HD and HI antibodies, and that the HI would recognize a site like the heparin binding site on PF4, meanwhile the HD antibodies would recognize a site like the KKO binding site on PF4.. However, the pathogenic antibodies specific for HIT only make up a small percentage of the total amount of antibodies found in a HIT sample, therefore, purifying the HI and HD antibodies using affinity purification allows us to better observe the different classes from each other. [24,52,71] With these caveats in consideration, my project also focused on using various methods of affinity purification to obtain HI and HD HIT antibodies from the samples of HIT patients. This was done in hopes of better identifying the pathogenic epitopes that the pathogenic antibodies that are specific for HIT bind to on PF4 to cause HIT. In attempt to map the epitopes of the purified HI and HD antibodies from two HIT patients, I found that some of these antibodies recognize additional amino acids on PF4, making it difficult to identify the specific amino acids that exclusively make up the restricted epitopes of the HI from the HD antibodies. Detection of purified HI and HD antibodies in the EIA was also limited since we had low concentrations of sample obtained following affinity purification. This might be explained by suggestions that pathogenic HIT antibodies are present at a low concentration in the serum of HIT patients [50,123]and the more purification steps we apply, the more likely that there will be a loss of total antibodies obtained following elution. We were also limited by the amount of each sample available to purify the antibodies and map them. These are some of the limitations involved in our study and should be considered when selecting patient samples for future experiments.

6-Conclusion

This thesis focuses on differentiating between platelet factor 4 epitopes that bind heparin-induced thrombocytopenia and vaccine-induced thrombotic thrombocytopenia antibodies. Previously, the binding of antibodies from VITT patients identified was restricted to eight surface amino acids on PF4 that were all located within the heparin-binding site. These eight amino acids Suggesting a probable epitope for pathogenic VITT antibodies on PF4. Similarly, the binding of antibodies from HIT patients was restricted to two independent sites on PF4, suggesting the epitopes of pathogenic HIT antibodies are found within either of the two probable sites on PF4. This knowledge gave rise to questions like, "Do the VITT and HIT antibody epitopes on PF4 dictate their platelet activation profiles?" and despite similarities in their clinical manifestations and ability to recognize the same antigen (PF4), "How can we differentiate VITT from HIT for diagnosis?" The work described in this thesis addresses these questions and contributes to the growing knowledge on VITT, a rare blood disorder with a familiar clinical picture.

The experiments needed to address these research questions would require us to modify a previous assay by expressing and purifying 70 PF4 mutants produced by way of alanine-scanning mutagenesis. The previous assay used the bacterial lysate of the 70 PF4 mutants to map the epitopes of HIT antibodies. Although useful, this method reduced the testing population due to the high background reactivity between HIT antibodies and bacterial lysate. We used purified PF4 mutants to account for and omit the limitations involving background reactivity when using bacterial lysate. This body of work produced high yields of purified PF4 mutants for future experiments involving HIT and VITT.

With the 70 PF4 mutants purified, we were then able to identify the binding sites of HIT and VITT antibodies. Our work with alanine-scanning mutagenesis with PF4 mutants has helped to narrow the epitopes of pathogenic HIT and VITT antibodies to specific amino acids within restricted regions on PF4. Screening HIT patients samples and HIT-like monoclonal antibodies confirmed that although there is no single common epitope among pathogenic HIT antibodies because of their polyclonal nature, however, the epitopes that pathogenic antibodies bind to on PF4 are found within either of two different and distinguishable sites on PF4. The first site is similar to where KKO, a monoclonal antibody against PF4/heparin complexes binds to and the second site is found within the heparin binding site on PF4. Additional screening with VITT samples revealed that an additional 8 amino acids are part of a second VITT site for a small subset VITT patients on PF4. Following the identification of the sites for pathogenic antibodies in both HIT and VITT, we were able to identify 10 PF4 mutants that help to differentiate between HIT and VITT antibodies, as well as 6 PF4 mutants that help to differentiate between HIT and non-HIT antibodies in an EIA.

The importance of developing an assay that differentiates between HIT and VITT and an assay that differentiates between HIT and non-HIT patients provides implications on how we can better understand the pathogenesis of VITT and modify the biochemical structure of PF4 to increase the specificity of the EIA on a diagnostic level. Currently, the functional assays that can differentiate between these disorders are costly, cumbersome, and not widely available. EIAs on the other hand are easy-to-use and commercially available, making them ideal for diagnostic laboratories where functional assay testing for patient samples is unavailable. Ultimately, this would increase the turnaround time for disease diagnosis and improve patient treatment. The long-term aim for this project is to definitively state the molecular attribution of PF4 to the pathogenesis

of HIT and VITT, for instance, further research is needed to fully describe the conformational change of PF4 as it either binds to heparin to cause HIT and its possible association with adenoviral vector-based vaccines to cause VITT. To understand the role that vaccines play in the pathogenesis of VITT, the amino acids involved in the interaction between VITT antibodies and PF4 should be analyzed in the presence of adenoviral vector-based vaccines. This aim will inform key concepts in the mechanism of VITT antibodies and any vaccine-associated blood disorders that may arise in the future.

6   FIGURES
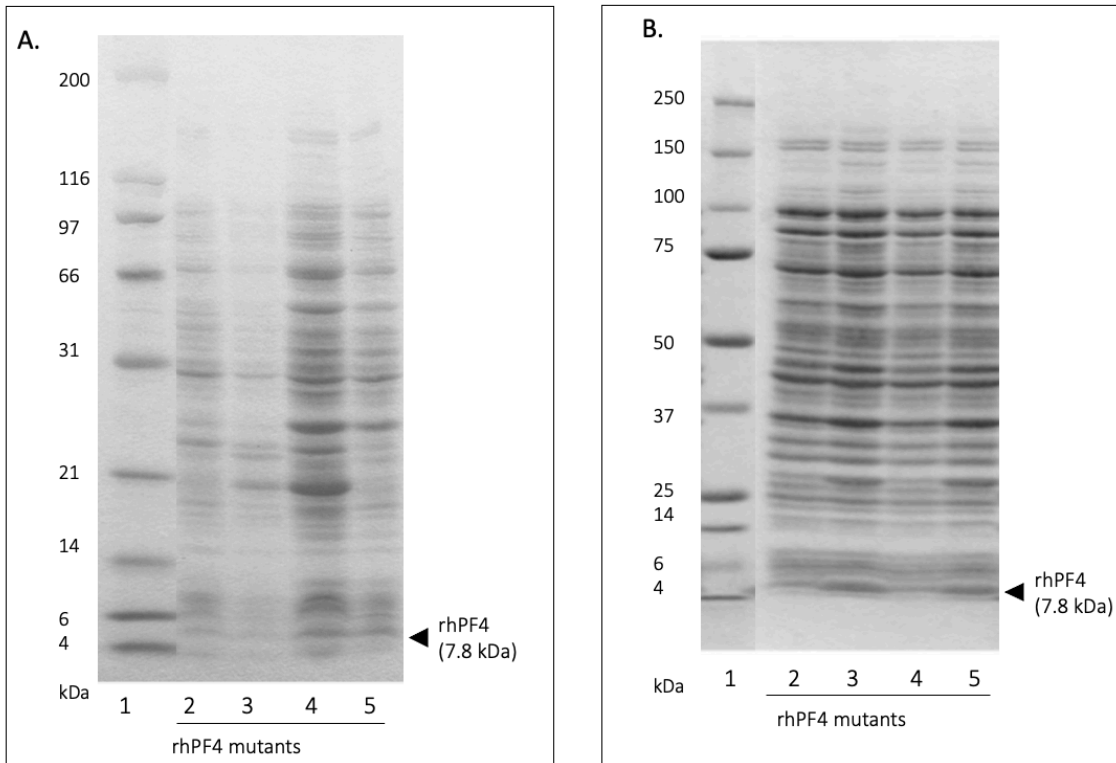
Figure 1. SDS-PAGE showing the expression of PF4 mutants following induction with IPTG. Purity was confirmed on Coomassie stained 18% SDS-polyacrylamide gel. Both gels show the expression of PF4 mutants: G7A-D8A in A and L9A-Q10A in B that were grown in BL21 (DE3) at 37°C for 3 hours before being induced by the addition of 0.5 mM IPTG. Lane 1 in both A,B) represents protein ladder standards. Lanes 2 and 3 in A and lanes 2 and 4 in B represent the bacterial culture of PF4 before expression while lanes 4 and 5 in figure A and lanes 3 and 5 in figure B represent the bands of each PF4 mutant following expression.
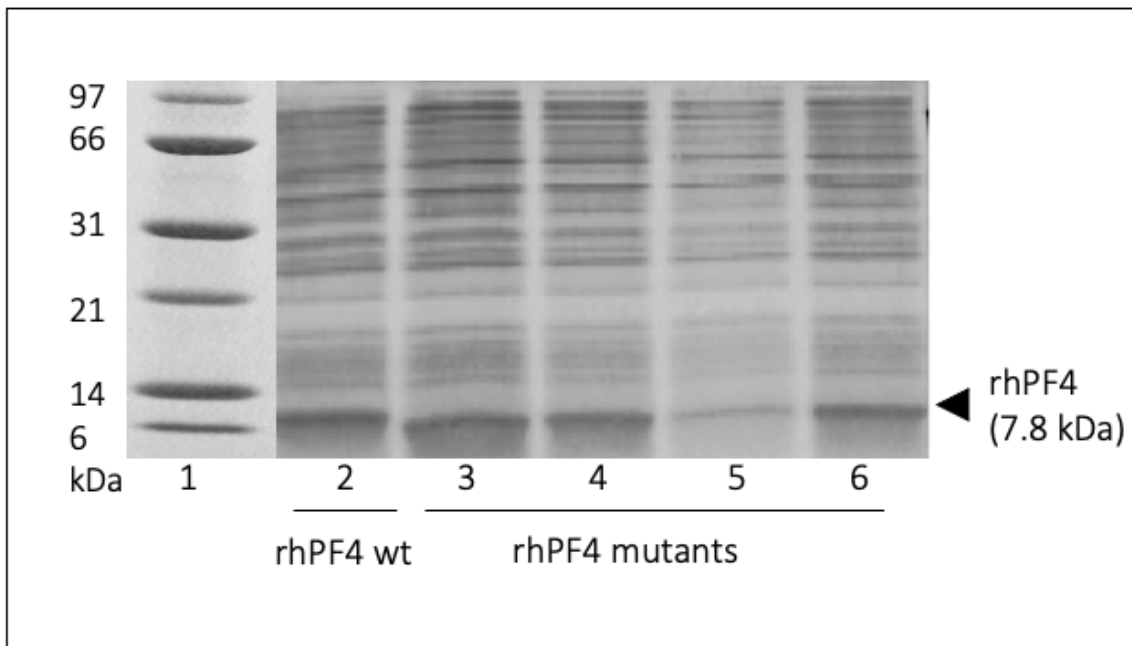
Figure 2. SDS-PAGE showing the expression of wildtype PF4 and PF4 mutants following induction with IPTG. Lane 1 represents protein ladder standards. Lanes 2-6 show cultures of (Lane 2) Wildtype PF4 Lanes 3-6) and PF4 mutants E2A-E5A were grown in BL21 (DE3) at 37°C for 3 hours before being induced by the addition of 0.5 mM IPTG.
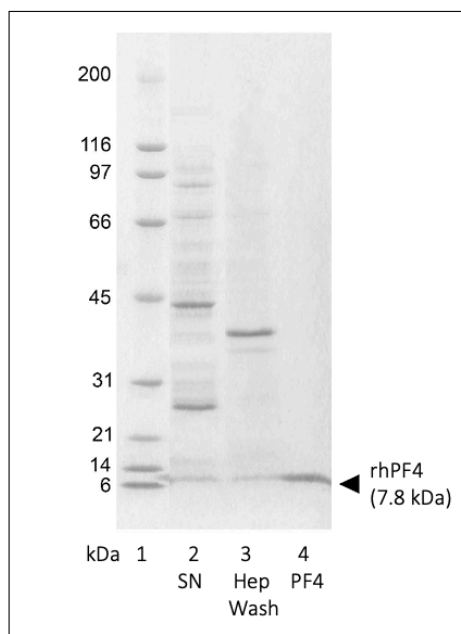
Figure 3. SDS-PAGE following purification of rhPF4 from *E. coli* using affinity chromatography. shows the corresponding for the heparin column purification of recombinant PF4. MW represents protein ladder standards. Lane 2 represents supernatant (SN) containing miscellaneous proteins and contaminants separated from the rhPF4 that was in the supernatant. Lane 3 represents Hep Wash containing the proteins removed from the column at 0.5M NaCl. Lane 4 represents samples containing purified fractions of PF4 that were contaminant free. The arrowhead indicates protein band that corresponds to rhPF4 (approx. 7.8kDa). All protein samples were run on a denaturing 18% SDS-polyacrylamide gel.
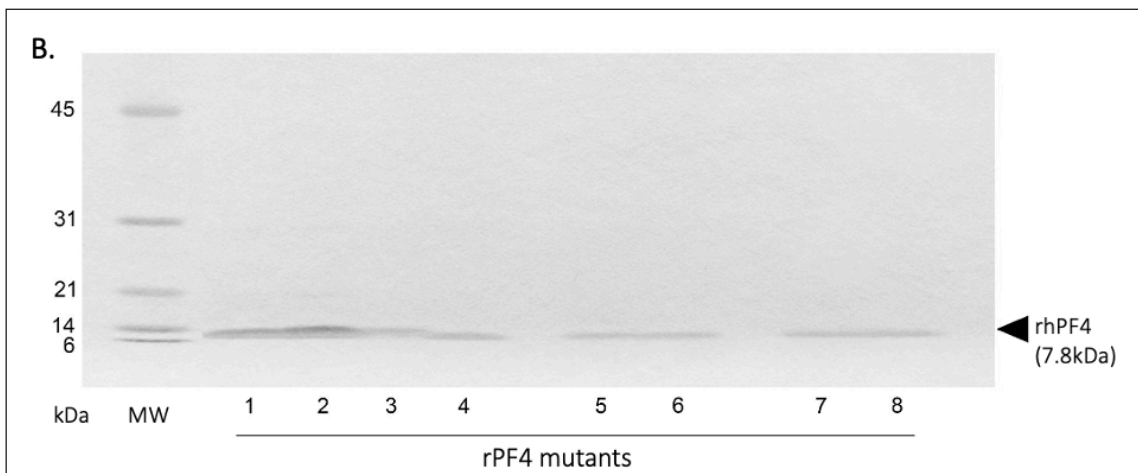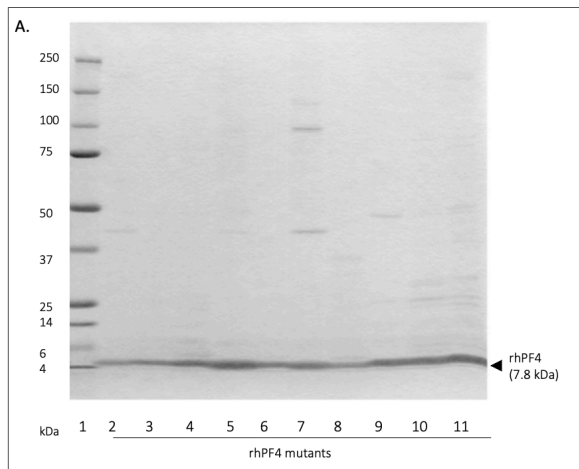
Figure 4. SDS-PAGE following purification of PF4 mutants from heparin columns using affinity chromatography. Lane 1 in A and MW in Figure B represents protein ladder standards. Lanes 2-11 in A and lanes 1-8 in B lanes 1-8 represent each of purified PF4 mutants E2A-C11A and L12A-Q19A, respectively. The arrowhead indicates protein band that corresponds to rhPF4 (approx. 7.8kDa). All protein samples were run on a denaturing 18% SDS-polyacrylamide gel.
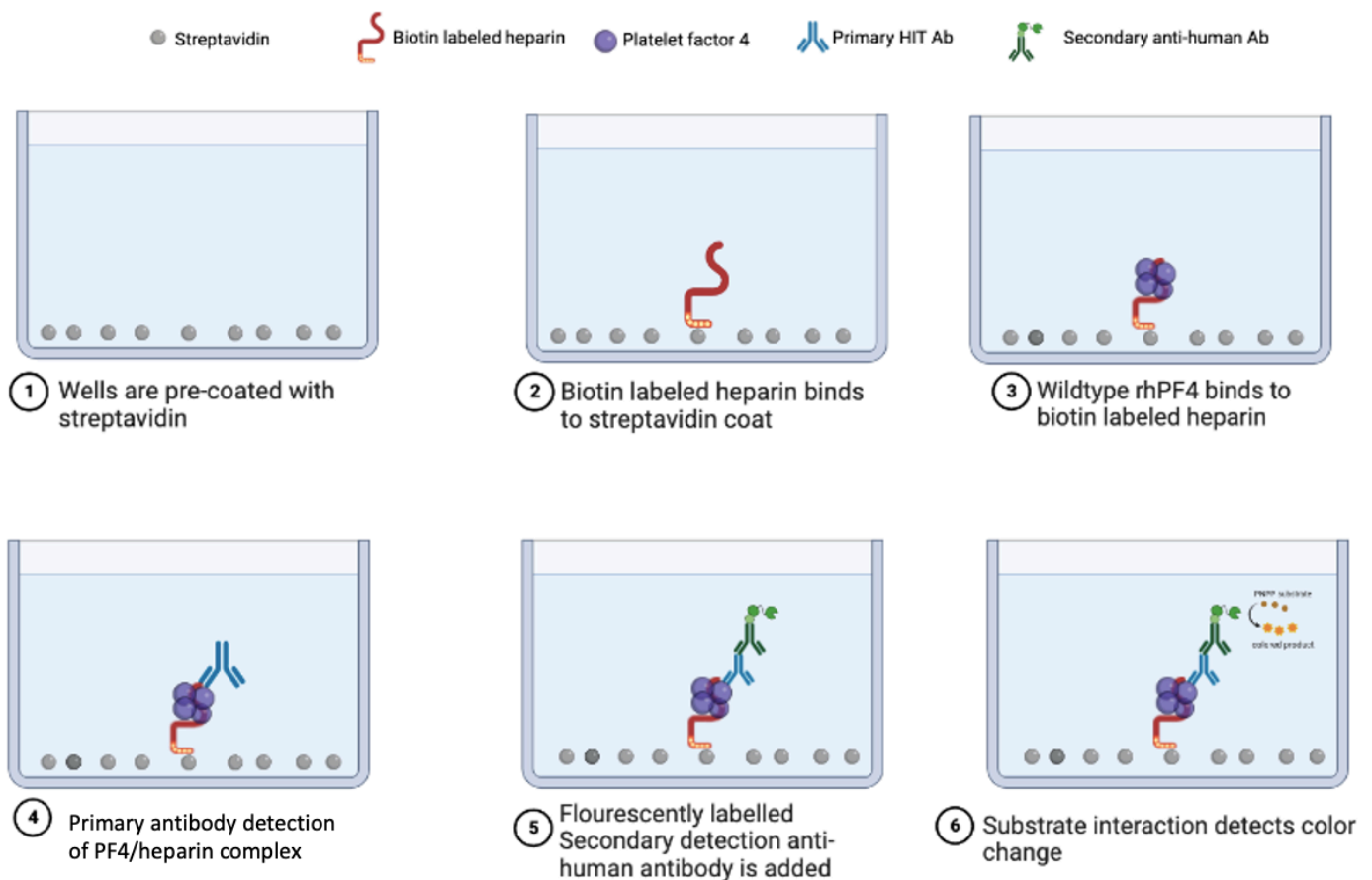
Figure 5. Schematic diagram of steps involved in detecting PF4/heparin specific antibodies from HIT patients in an EIA. Biotinylated heparin is immobilized on wells pre-coated with streptavidin. Either wildtype PF4 or a PF4 mutant is then added to wells to bind heparin and form mutant PF4/heparin complexes This is then followed by the addition of HIT antibodies (ie. KKO, antibodies from HIT samples, and HIT-like monoclonal antibodies) and detected by a labelled secondary antibody.
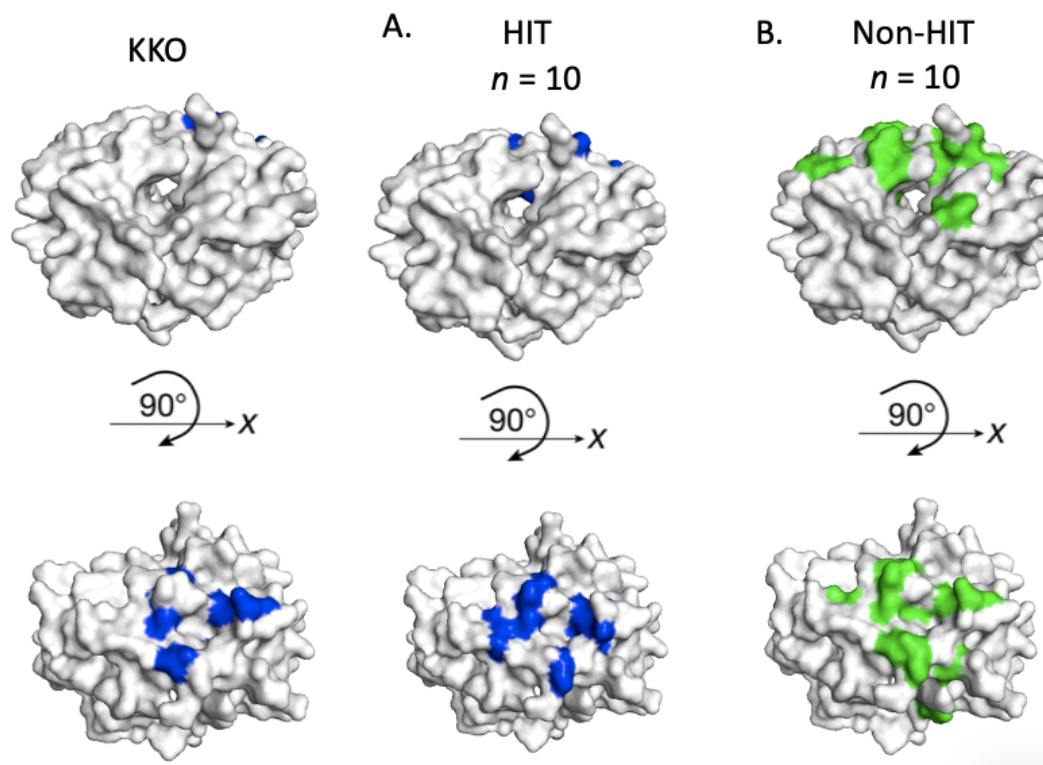
KKO: EAEEDGDLQCLCVKTTSQVRPRHITSLEVIKAGPHCPTAQLIATLKNGRKICLDLQAPLYKKIIKKLLES

HIT: EAEEDGDLQCLCVKTTSQVRPRHITSLEVIKAGPHCPTAQLIATLKNGRKICLDLQAPLYKKIIKKLLES

Non-HIT: EAEEDGDLQCLCVKTTSQVRPRHITSLEVIKAGPHCPTAQLIATLKNGRKICLDLQAPLYKKIIKKLLES

Figure 6. Identification of amino acids on PF4 that are critical for the binding of HIT antibodies using alanine-scanning mutagenesis. Figure A) shows the binding site for antibodies from 10 HIT patients (colored blue). 4 amino acids in the binding site recognized by the antibodies in 10 HIT patients overlap with amino acids that makeup the KKO binding site on PF4. Figure B) shows the binding site of antibodies in 10 non-HIT patients, the amino acids identified were arbitrary and were not restricted to a particular region on PF4.
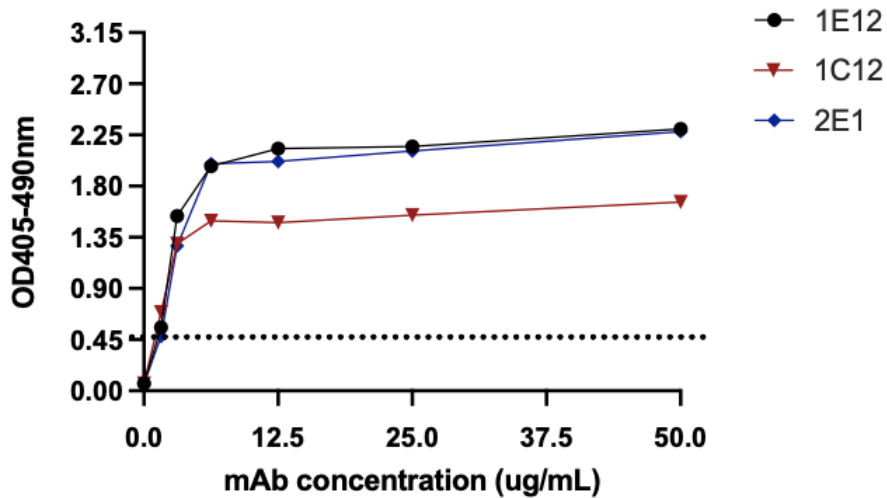
Figure 7. Binding of monoclonal Antibodies1E12, 1C12, 2E1 to PF4/heparin complexes in an Enzyme immunoassay. The OD values from incubating each monoclonal antibody with wildtype PF4/heparin complexes at varying concentrations of 1.6 ug/mL, 3.1ug/mL, 6.3 ug/mL, 12.5 ug/mL, 25 ug/mL and 50 ug/mL are presented by dilution curves. The pre-established cut-off for positivity is indicated by the black dotted line. OD values equal to or above 0.45 are considered positive in binding to and recognizing wildtype PF4/heparin complexes, while values below 0.45 are considered negative. We observed positive OD values at concentrations of 1E12 (black), 1C12 (red), and 2E (blue) 1 as low as 1.625 ug/mL suggesting their high affinity for PF4/heparin complexes.
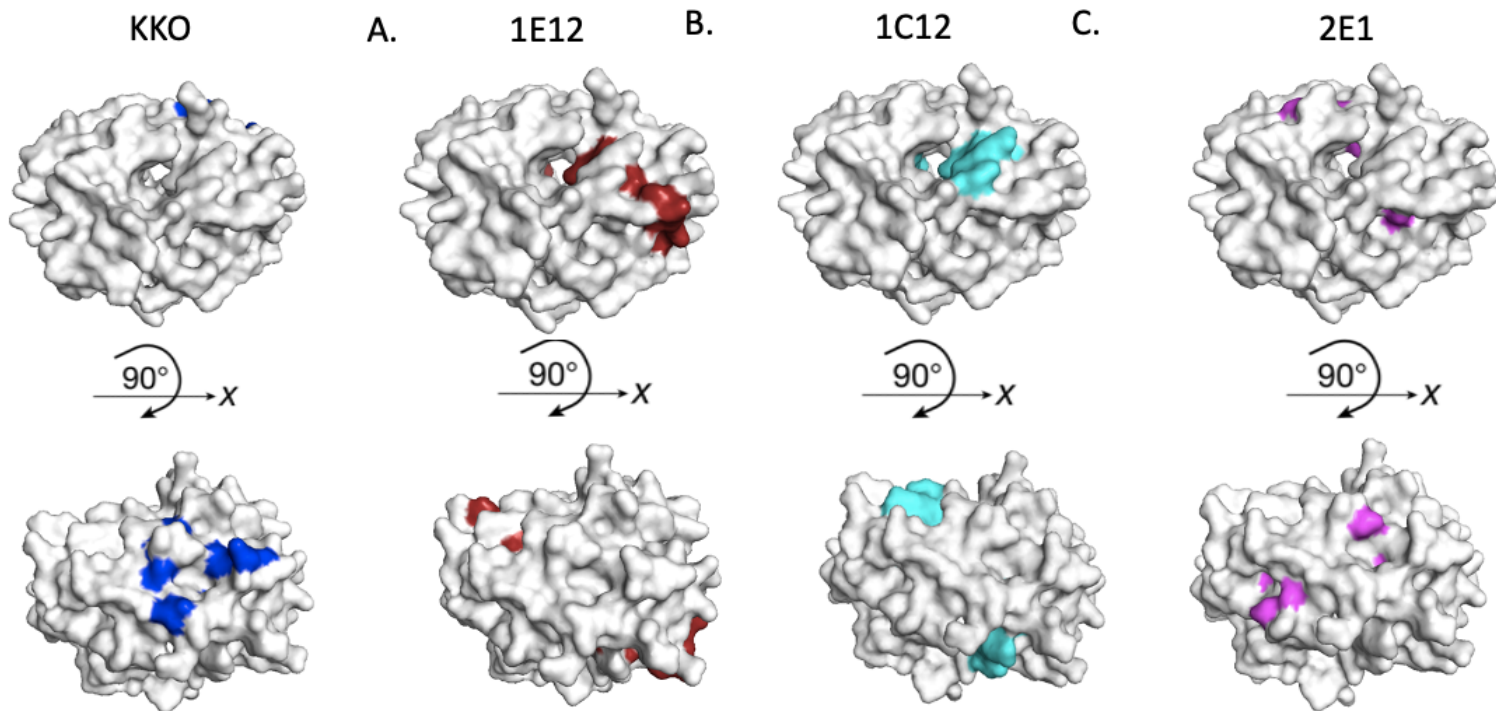
Figure 8. Identification of amino acids on PF4 that are critical for the binding of monoclonal HIT antibodies 1E12, 1C12, and 2E1 using alanine-scanning mutagenesis.
A,B,C) Three humanized Fc monoclonal antibodies derived from mice with the ability to activate platelets in the presence and absence of heparin like HIT antibodies. A,B) the amino acids that comprise the binding site for 1E12 and 1C12 are located in the same region as the VITT binding site. C) the amino acids that comprise the binding site for 2E1 are in a similar region as the KKO binding site on PF4.
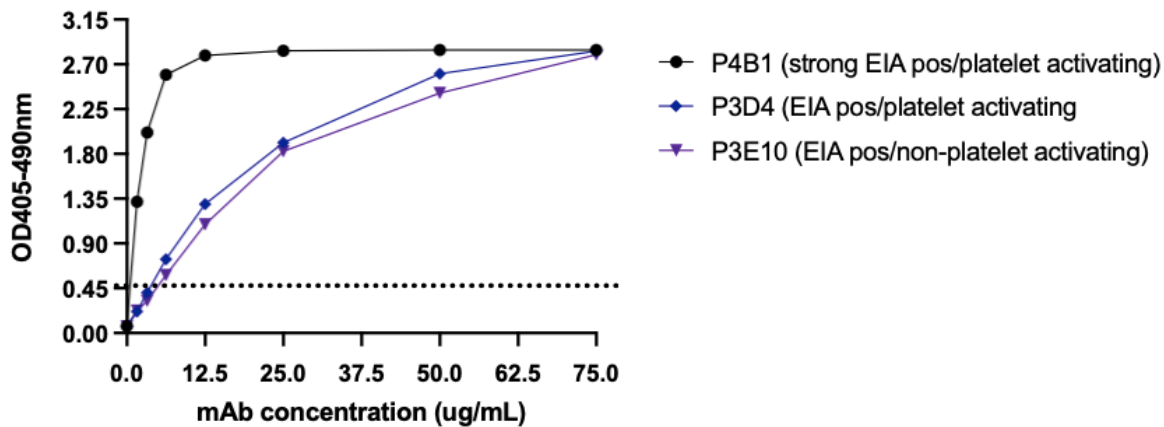
Figure 9. Binding of monoclonal AntibodiesP4B1, P3D4, and P3E10 to PF4/heparin complexes in an enzyme immunoassay. The OD values from incubating each monoclonal antibody with wildtype PF4/heparin complexes at varying concentrations of 1.6 ug/mL, 3.3 ug/mL, 6.3 ug/mL, 12.5 ug/mL, 25 ug/mL, 50 ug/mL and 75 ug/mL are presented by dilution curves. The pre-established cut-off for positivity is indicated by the black dotted line. OD values equal to or above 0.45 are considered positive in binding to and recognizing PF4/heparin complexes, while values below 0.45 are considered negative. We observed positive OD values at varying concentrations of 1.6ugmL for P4B1 (black), 6.3ug/mL for P3D4 (blue) and P3E10 (purple). Suggesting P4B1 has a higher affinity for PF4/heparin complexes while suggesting that P3D4 and P3E10 have lower affinities for PF4/heparin complexes.
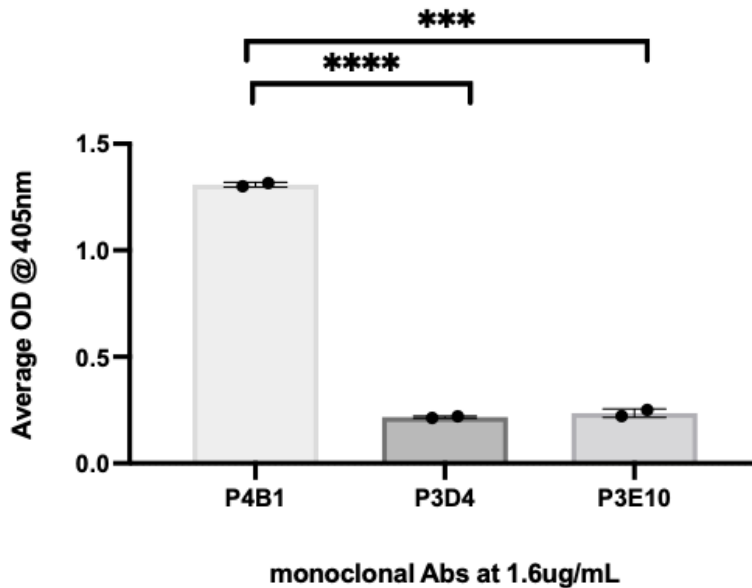
Figure 10. Statistical analysis of P4B1 from P3D4 and P3E10 at a concentration of 1.6 ug/mL in the EIA. We averaged the OD values from testing P4B1, P3D4, and P3E10 at concentrations of 1.6 ug/mL against PF4/heparin complexes and represented them as mean ± SD of 2 replicates. OD values equal to or above 0.45 are considered positive in binding to and recognizing PF4/heparin complexes, while values below 0.45 are considered negative. Mean OD values of 1.3, 0.2 and 0.2 were observed for P4B1, P3D4 and P3E10 at concentrations of 1.6 ug/mL, respectively. Suggesting P4B1 has a higher affinity for PF4/heparin complexes while suggesting that P3D4 and P3E10 have lower affinities for PF4/heparin complexes. Student's t-test was performed to determine the significant differences between P4B1 and P3D4, and P4B1 and P3E10. At a concentration of 1.6ug/mL, P4B1 could be distinguished from P3D4 and P3E10 with statistical significance ****$p<0.0001$,***$p<0.0002$.
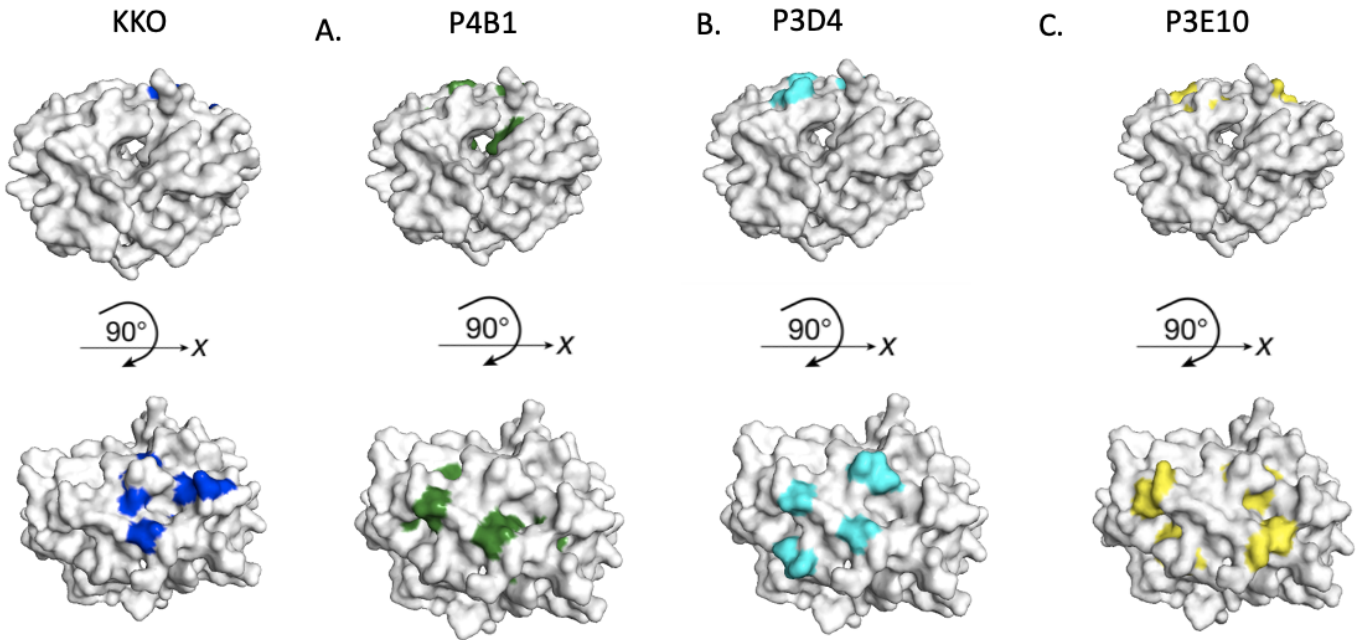
A. P4B1: EAEEDGDLQCLCVKTTSQVRPRHITSLEVIKAGPHCPTAQLIATLKNGRKICLDLQAPLYKKIIKKLLES
B. P3D4: EAEEDGDLQCLCVKTTSQVRPRHITSLEVIKAGPHCPTAQLIATLKNGRKICLDLQAPLYKKIIKKLLES
C. P3E10: EAEEDGDLQCLCVKTTSQVRPRHITSLEVIKAGPHCPTAQLIATLKNGRKICLDLQAPLYKKIIKKLLES

Figure 11. Identification of amino acids on PF4 that are critical for the binding of monoclonal HIT antibodies P4B1, P3D4, and P3E10 using alanine-scanning mutagenesis. A,B,C) Three monoclonal antibodies derived from HIT patients that bind to and recognize PF4/heparin as an antigen in the EIA similar to HIT antibodies. A,B), P4B1 and P3D4 are monoclonal antibodies that have the ability to activate platelets in the presence of heparin similar to HIT antibodies. A,B the amino acids that comprise the binding site for P4B1 and P3D4 are located in the same region as the KKO binding site on PF4. C, the amino acids that comprise the binding site for P3E10 are outside the binding site for KKO on PF4.
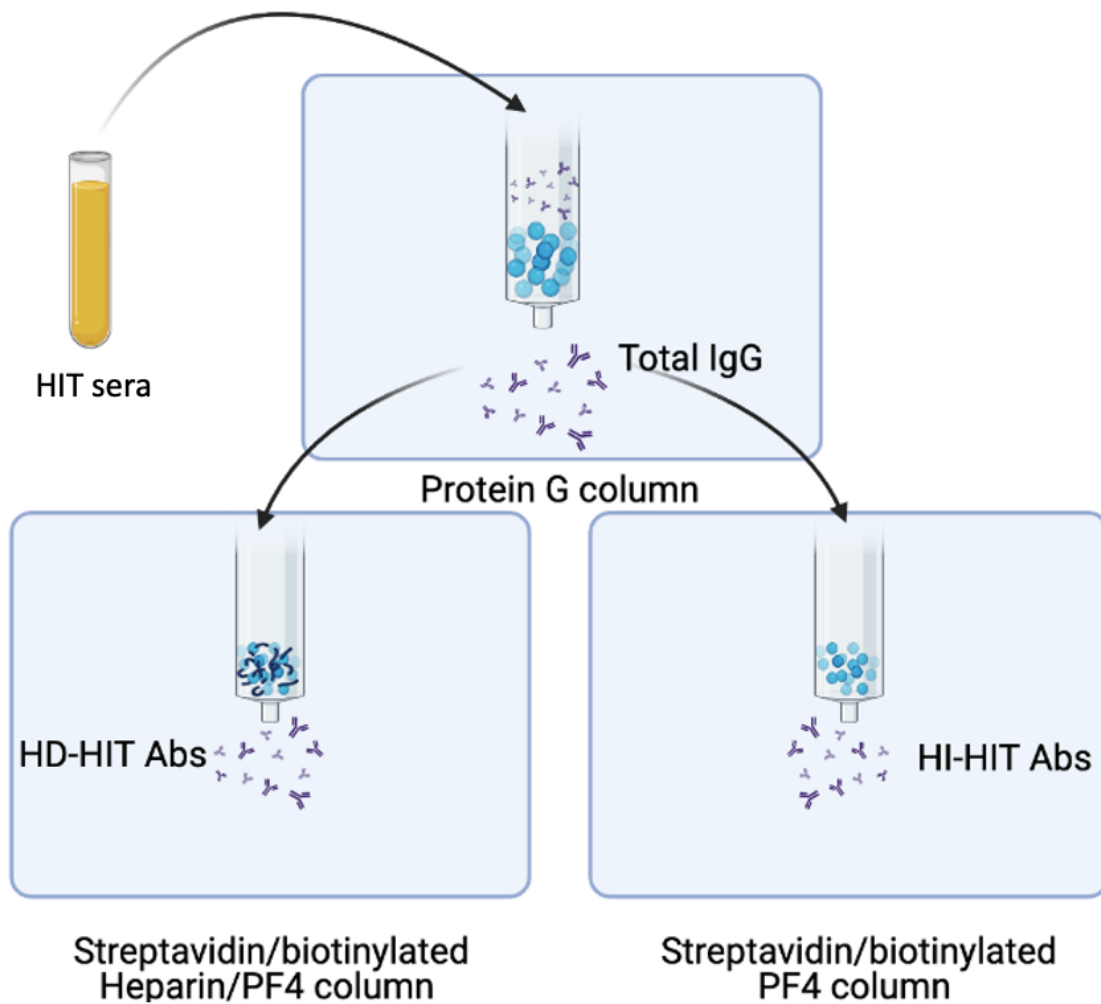
Figure 12. Schematic diagram of steps involved in isolating heparin-independent (HI) and heparin-dependent (HD) HIT antibodies from patient samples by method of affinity purification. A subset of HIT patients tested positive for both HI antibodies that bind PF4 alone and HD antibodies that bind PF4/heparin. A PF4 affinity column and PF4/heparin affinity column were created to purify HI and HD antibodies directly from HIT patients, respectively.
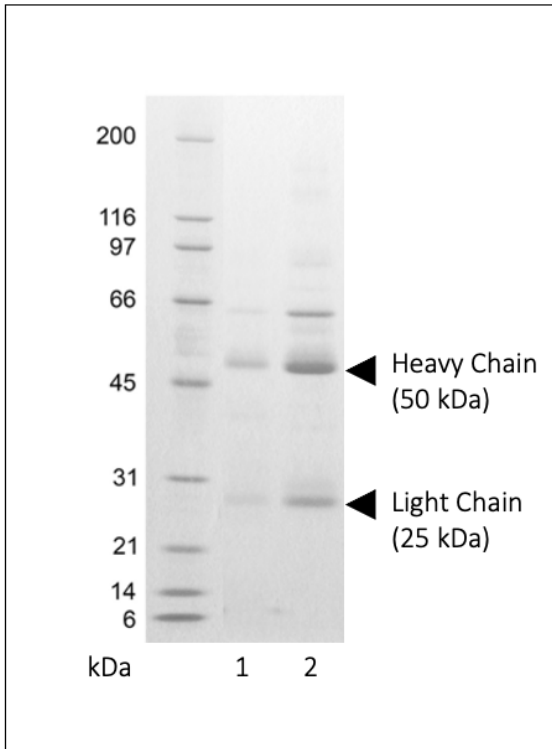
Figure 13. Representative SDS-PAGE showing affinity purified immunoglobulin G (IgG) antibodies from HIT patients. Lanes 1 and 2 consist of the total amount of IgG purified from pre-screened HIT patients through a protein G column by method of affinity purification. The bands at 50 kDa indicate the heavy chains and the bands at 25 kDa indicate the light chains of purified IgG class antibodies.
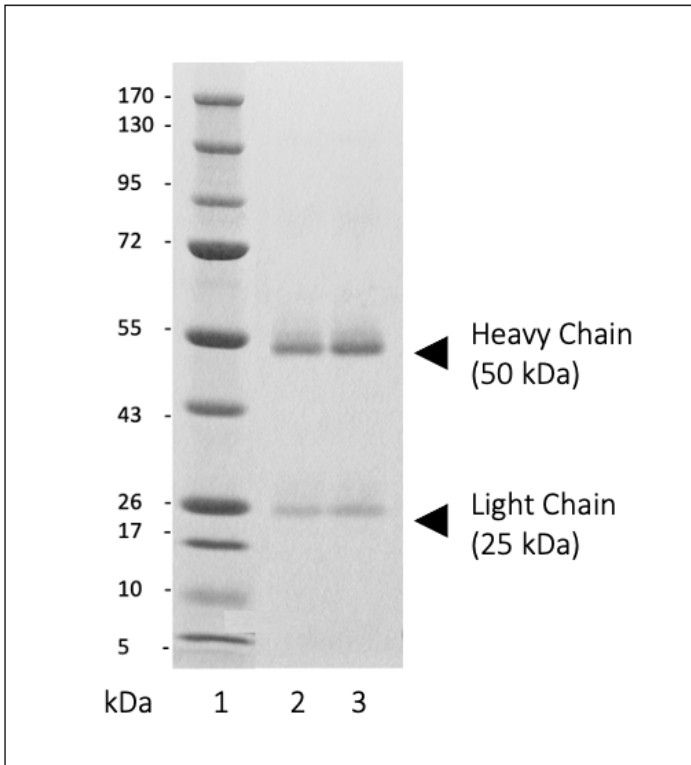
Figure 14. Representative SDS-PAGE analysis of affinity purified heparin dependent (HD) and heparin independent (HI) specific antibodies from HIT patients. HI and HD antibodies were purified from the total amount of IgG previously by method of PF4 column and PF4/heparin column affinity purification, respectively. Lane 1 represents the protein ladder standards. Lane 2 consists of the HI antibodies obtained from the PF4 column and Lane 3 consists of the HD antibodies obtained from the PF4/heparin column. The bands at 50 kDa indicate the heavy chains and the bands at 25 kDa indicate the light chains of purified IgG class antibodies.
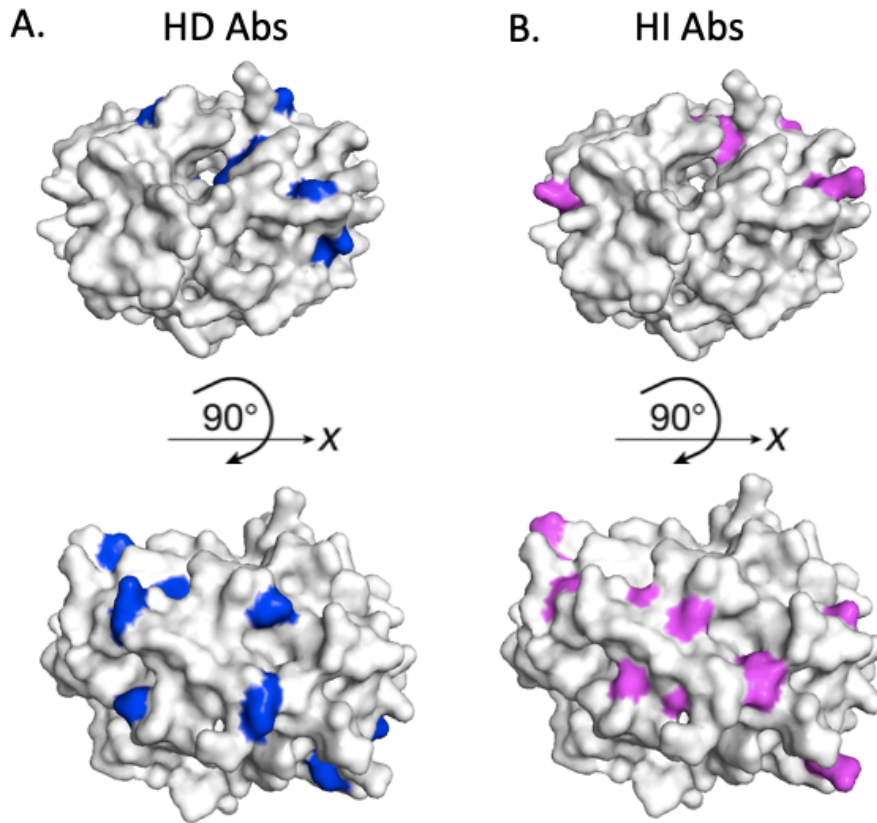
A. HD HIT Abs : EAEEDGDLQCLCVK**T**TSQVRPR**H**ITSLEV**I**KAGPHCP**T**AQLIATLKNGR**K**ICLDLQAPLYKKIIKK**K**LLES

B. HI HIT Abs : EAEEDGD**L**Q**C**LCVK**T**TSQV**R**PRHITSLEVIKAGPHCPTAQLIATLKNGRKI**C**LDLQAPLY**K**KIIKK**L**LES

Figure 15. Identification of amino acids on PF4 that are critical for the binding of heparin-dependent (HI) and heparin dependent (HD) and HIT antibodies using alanine-scanning mutagenesis. Figure A) shows the binding site comprised of 7 amino acids on PF4 for HI antibodies purified from 4 HIT patients (colored blue). The HI binding site shares a total of 3 amino acids with the monoclonal HIT antibodies 1C12 and 2E1 that display heparin-independent reactivity. Figure B) shows the binding site comprised of 6 amino acids on PF4 for HD antibodies purified from 4 HIT patients (colored pink). The HI binding site shares a total of 2 amino acids with the monoclonal HIT antibodies P3D4 and P4B1 that display heparin-independent reactivity.
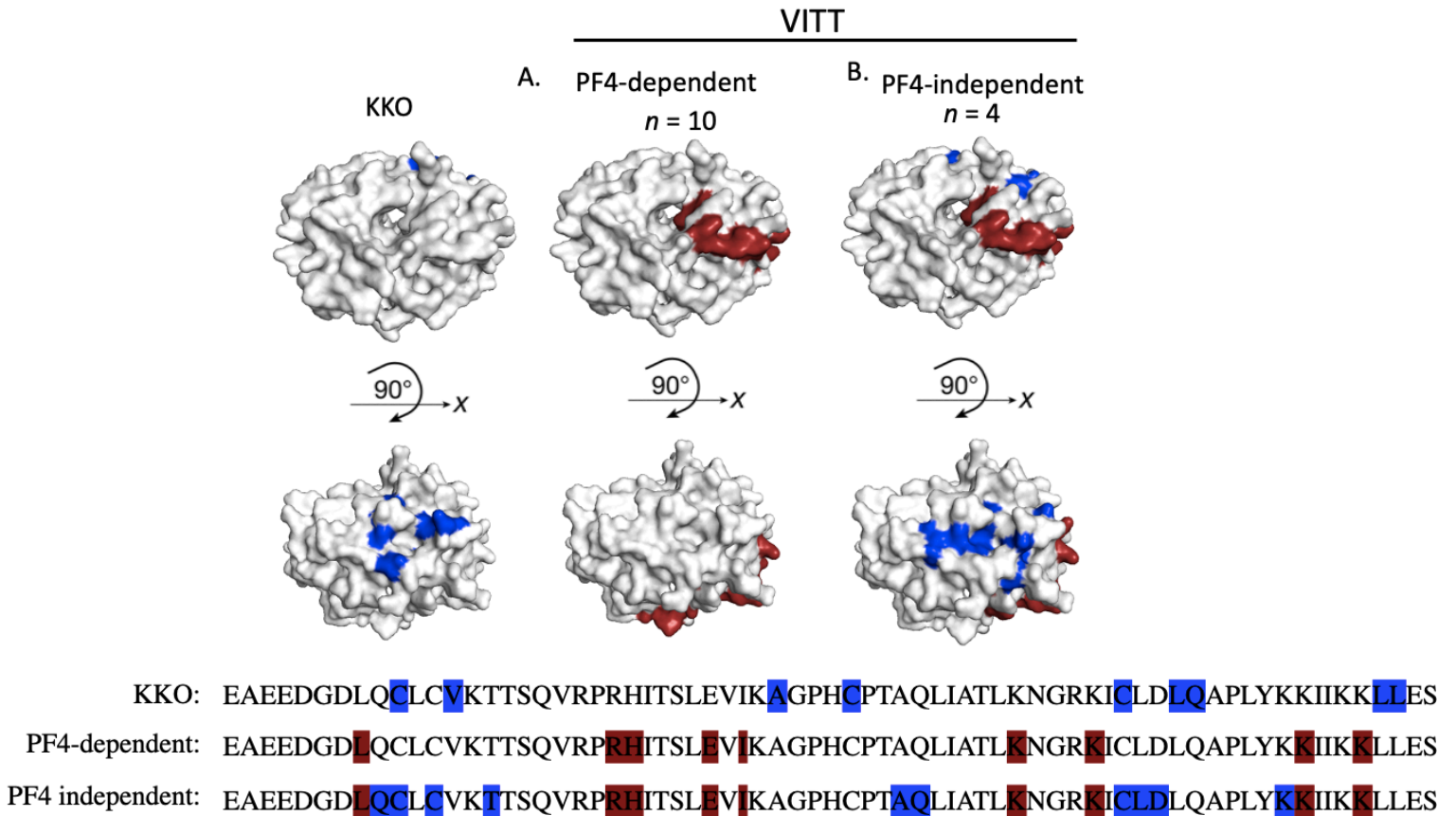
Figure 16. Identification of amino acids on PF4 that are critical for the binding of VITT antibodies using alanine-scanning mutagenesis. A, B) For VITT samples (n=14): A) the binding site (colored red) of the VITT patients with PF4-dependent activity aligns with the previously published VITT site by Huynh *et al*. and within the heparin binding region on PF4. B) a subset of VITT patient's samples (n=4) aligns with the amino acids of the published VITT sites with an additional 10 amino acids that align with the KKO site on PF4.
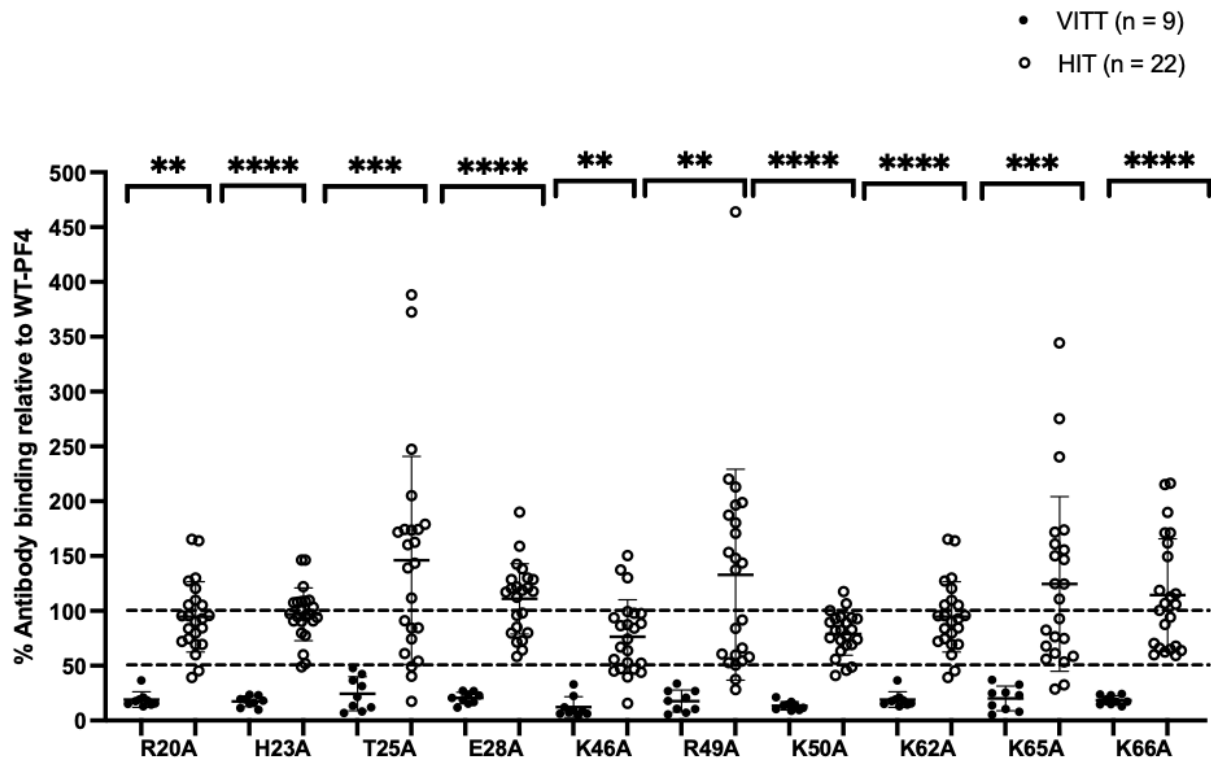
Figure 17. Selected PF4 mutants that differentiate VITT from HIT antibodies. The 10 PF4 mutants selected, are those of which the corresponding amino acids are important either for VITT antibody binding in the first alanine scanning mutagenesis screen (Figure 16) or the heparin binding on PF4. Each point represents the binding ability of VITT (n = 9) and HIT (n = 23) serum samples to 10 PF4 mutants (H23A, E28A, R20A, T26A, K46A, R49A, K50A, K62A, K65A, K66A) relative to wild-type PF4. These 10 mutants were found to have a significant difference in binding between VITT and HIT antibodies. Data reported as mean PF4 mutant binding relative to wild-type PF4 and error bars correspond to the standard deviation. Black closed circles represent the binding of VITT patient sera and the black open circles represent HIT patient sera. Student's t-test was performed to determine the significant differences between the two antibody groups. **p<0.01, ***p<0.001, ****p<0.0001.
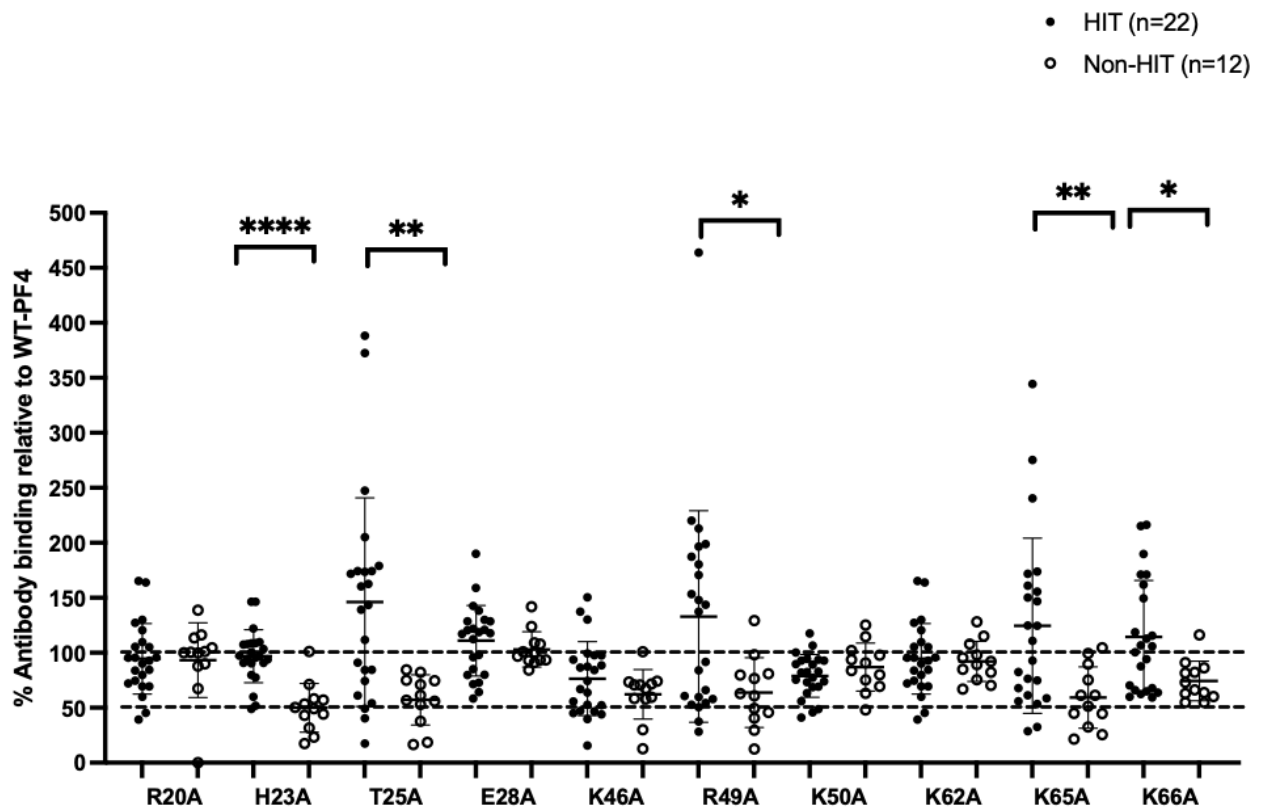
Figure 18. Selected PF4 mutants that differentiate HIT from non-HIT antibodies. The 10 PF4 mutants selected, are those of which the corresponding amino acids are important either for VITT antibody binding in the first alanine scanning mutagenesis screen (Figure 16) or the heparin binding on PF4. Each point represents the binding ability of HIT (n = 22) and HIT (n = 12) serum samples to 10 PF4 mutants (H23A, E28A, R20A, T26A, K46A, R49A, K50A, K62A, K65A, K66A) relative to wild-type PF4. 5 out of 10 of these mutants were found to have a significant difference in binding between HIT and non-HIT antibodies (H23A, T25A, R49A, K65A, K66A). Data reported as mean PF4 mutant binding relative to wild-type PF4 and error bars correspond to the standard deviation. Black closed circles represent the binding of HIT patient sera and the black open circles represent non-HIT patient sera. Student's t-test was performed to determine the significant differences between the two antibody groups. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

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Table 1. Key amino acids in PF4 for the binding of HIT antibodies (n=10).

Amino acids affected	Loss of binding compared to WT-PF4 (%)	Range (%)	# of HIT samples affected
L8	81.7	18.2-31.3	7/10
Q9	58.6	11.5-97.0	7/10
C10	80.6	11.2-25.4	9/10
K14	59.1	20.4-83.4	8/10
A32	50.0	18.5-111.6	6/10
C36	76.2	21.3-36.3	9/10
P37	52.9	17.1-98.0	7/10
L41	48.7	13.4-89.0	7/10
A43	60.5	17.3-81.5	8/10
L53	68.5	35.9-89.6	8/10
D54	61.5	17.9-24.5	8/10
L67	51.8	19.8-94.0	7/10

Table 2. Yield of total IgG antibody and EIA testing results following affinity purification of HD and HI antibodies from HIT patient serum samples.

Patients	Serum (OD405-490nm)	Total IgG affinity purification yield (mg/mL)	Total IgG (OD405-490nm)	HI Abs (OD405-490nm)	HD Abs (OD405-490nm)
1	3.6	5.7	0.9	0.3	0.1
2	3.3	8.8	2.0	0.45	0.7
3	2.3	9.0	0.5	0.2	0.09
4	3.1	12.0	0.7	1.1	1.3

Table 3. Key amino acids in PF4 for the binding of VITT antibodies from 10 VITT patients.

Amino acids affected	Loss of binding compared to WT-PF4 (%)	Range (%)	# of VITT samples affected	Domains on protein
L8	61.2	29.3-48.2	9/10	Beta strand
R22	73.1	11.8-42.0	9/10	Alpha helix
H23	60.7	34.7-43.9	9/10	Alpha helix
E28	69.0	27.2-34.7	9/10	Beta strand
K46	55.2	44.0-45.6	9/10	Beta strand
N47	69.85	28.1-32.2	8/10	Beta strand
K50	74.6	20.0-31.0	10/10	Beta strand
K62	63.5	32.9-40.2	10/10	Alpha helix
K66	76.1	9.8-38.0	10/10	Alpha helix

Table 4. Clinical characteristics of PF4-dependent vs. PF4 independent VITT patient samples.

Patient Demographics	PF4-dependent (n=10)	PF4-independent (n=4)	Significance (p-value
Female (%)	7/10 (70%)	3/10 (30%)	*
Mean Age (Range)	51 (30-65)	46 (43-48)	*
Vaccine Type			
ChAdOx1 nCoV-19 (AstraZeneca)	10/10 (100%)	3/4 (75%)	*
Ad26.COV2.S (Johnson & Johnson)	--	1/4 (25%)	*
Clinical Presentation			
Platelet counts	49.9	170.7	<0.001
Total Thrombosis	10/10 (100%)	3/3 (100%) ¶	*
Total Thrombocytopenia (<150x10^9/L)	10/10 (100%)	1/3 (33%)	*
Thrombocytopenia and Thrombosis	10/10 (100%)	3/3 (100%)	*
Thrombocytopenia alone	10/10 (100%)	1/3 (33%)	*
Thrombosis alone	10/10 (100%)	3/4 (75%)	*
Thrombotic Manifestations*			
Cerebral Venous Sinus Thrombosis (CVST)	4/10 (40%)	2/4 (50%)	*
Deep Vein Thrombosis (DVT)	2/10 (20%)	2/4 (50%)	*
Pulmonary Embolism (PE)	2/10 (20%)	2/4 (50%)	*

P<0.05 was considered statistically significant

¶ Change in denominator due to missing patient data

*Due to small sample size, hypothesis testing comparing significance could not be approximated for all groups

Table 5. Key amino acids in PF4 for the binding of PF4-independent VITT antibodies (n=4)

Amino acids affected	Loss of binding compared to WT-PF4 (%)	Range (%)	# of SRA pos VITT samples affected
C10	80.7	16.0-22.5	3/4
R22	75.8	17.0-31.4	4/4
H23	68.8	21.4-43.8	4/4
E28	82.6	11.3-29.3	4/4
I30	83.7	10.3-25.8	4/4
A32	77.6	16.2-33.9	4/4
K46	67.3	18.5-46.9	3/4
K50	83.2	24.1-42.8	3/4
C52	66.6	20.6-37.7	4/4
L53	71.0	15.4-31.8	4/4
K62	79.0	28.3-38.3	4/4
K66	79.5	10.7-14.7	4/4
L67	66.8	13.1-49.1	4/4

Table 6. PF4 mutants that affected binding of VITT, HIT and non-HIT antibodies in an EIA.

Disorder	PF4 Mutants Total Number of patients affected (OD < 0.45)									
	H23A	**R20A**	**T25A**	**E28A**	**K46A**	**R49A**	**K50A**	**K62A**	**K65A**	**K66A**
VITT (n=9)	2/9 (22.2%)	5/9 (55.6%)	4/9 (44.4%)	5/9 (55.6%)	6/9 (66.7%)	5/9 (55.6%)	7/9 (77.8%)	5/9 (55.6%)	5/9 (55.6%)	5/9 (55.6%)
HIT (n=22)	2/22 (9.1%)	6/22 (41.0%)	2/22 (9.1%)	1/22 (4.5%)	4/22 (18.2%)	3/22 (13.6%)	4/22 (18.2%)	3/22 (13.6%)	4/22 (18.2%)	3/22 (13.6%)
Non-HIT (n=12)	3/12 (25.0%)	7/12 (58.3%)	5/12 (41.7%)	2/12 (16.7%)	4/12 (33.3%)	5/12 (41.7%)	2/12 (16.7%)	3/12 (25.0%)	6/12 (50.0%)	4/12 (33.3%)

Table 7. Statistical analysis of PF4 mutants that distinguish VITT, HIT and non-HIT patients from each other.

	PF4 Mutants									
	H23A	**R20A**	**T25A**	**E28A**	**K46A**	**R49A**	**K50A**	**K62A**	**K65A**	**K66A**
PF4 Mutant separates VITT from HIT (Y/N)	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
VITT vs. HIT Significance (p-value)	<0.0001	0.0037	0.0007	<0.0001	0.0007	0.0013	<0.0001	<0.0001	0.0009	<0.0001
PF4 Mutant separates HIT from non-HIT (Y/N)	Y	N	Y	N	N	Y	N	N	Y	Y
HIT vs. non-HIT Significance (p-value)	<0.0001	0.9120	0.0032	0.4285	0.2009	0.0220	0.2734	0.8106	0.0100	0.0137

Table 8. Standard EIA and SRA results of VITT patient samples against wild-type PF4 and PF4 mutants.

VITT Patients	PF4-SRA result (^{14}C-serotonin ≥ 20%)	WT-PF4/heparin EIA OD$_{405-490nm}$ (OD ≥ 0.45)	Mutant PF4/heparin EIA Mean OD$_{405-490nm}$ ± SD									
			H23A	**R20A**	**T25A**	**E28A**	**K46A**	**R49A**	**K50A**	**K62A**	**K65A**	**K66A**
Patient 1	Positive	**1.721**	1.7635	0.4005	0.5385	0.354	0.567	0.464	0.233	0.3175	0.4295	0.2975
Patient 2	Positive	**1.812**	0.3915	0.318	0.217	0.2795	0.1555	0.1935	0.249	0.321	0.188	0.31
Patient 3	Positive	**2.182**	2.085	0.3135	0.2945	0.4145	0.2055	0.2285	0.3165	0.4185	0.3025	0.3285
Patient 4	Positive	**2.682**	1.854	0.9215	0.1825	0.614	0.168	0.19	0.569	0.567	0.214	0.5
Patient 5	Positive	**2.077**	1.9295	0.394	0.1695	0.247	0.1035	0.1145	0.2395	0.2705	0.1105	0.2695
Patient 6	Positive	**2.982**	0.8265	0.832	0.644	0.708	0.36	0.618	0.509	0.453	0.6865	0.4365
Patient 7	Positive	**2.527**	0.822	0.305	1.206	0.679	0.4175	0.6805	0.227	0.397	0.937	0.599
Patient 8	Positive	**2.143**	0.85	0.544	0.786	0.36	1.142	0.3835	0.222	0.7855	0.5455	0.492
Patient 9	Positive	**3.347**	1.0945	0.627	1.423	0.908	1.3075	1.129	0.3215	0.4865	1.098	0.8115

Table 9. Standard SRA and EIA results of HIT patient samples against wild-type PF4 and PF4 mutants.

HIT Patients	Standard SRA result (^{14}C-serotonin ≥ 20%)	WT-PF4/heparin EIA $OD_{405-490nm}$ (OD ≥ 0.45)	Mutant PF4/heparin EIA Mean $OD_{405-490nm}$ ± SD									
			H23A	R20A	T25A	E28A	K46A	R49A	K50A	K62A	K65A	K66A
Patient 1	Positive	**0.6425**	0.7085	0.178	0.1725	0.8995	0.2655	0.1385	0.1865	0.6095	0.1865	0.43
Patient 2	Positive	**1.616**	1.7865	0.8395	0.9275	1.648	0.979	0.813	1.296	1.9145	1.296	1.5135
Patient 3	Positive	**1.7315**	1.59	1.073	0.8445	1.333	0.871	0.7865	0.9955	1.811	0.9955	1.534
Patient 4	Positive	**2.1**	1.915	0.8595	0.849	1.769	0.779	0.986	0.8735	1.649	0.8735	1.8185
Patient 5	Positive	**0.796**	1.028	0.386	0.4585	0.576	0.888	0.579	0.966	0.726	0.966	0.671
Patient 6	Positive	**1.836**	1.292	0.4215	0.578	1.1315	0.549	0.844	0.998	1.087	0.998	1.365
Patient 7	Positive	**1.3085**	0.401	0.621	0.5285	1.0455	0.953	0.689	0.4255	0.7845	0.4255	0.7845
Patient 8	Positive	**0.9465**	0.6465	0.7125	0.5905	0.595	1.142	0.505	0.588	0.7855	0.588	0.553
Patient 9	Positive	**1.3505**	1.3075	0.8675	0.775	1.1535	1.1795	1.473	0.8675	1.283	0.8675	0.6245
Patient 10	Positive	**2.4095**	2.218	0.9875	1.6795	1.994	1.803	1.6285	1.6035	1.8555	1.6035	1.1195
Patient 11	Positive	**1.582**	1.2955	0.4295	0.9725	1.109	1.372	1.032	1.078	0.9645	1.078	0.977
Patient 12	Positive	**2.348**	1.604	1.576	1.312	1.236	1.1325	1.1945	0.853	1.42	0.853	1.3725
Patient 13	Positive	**0.8105**	0.874	0.4725	0.495	0.988	0.567	0.485	0.454	0.3175	0.454	0.5265
Patient 14	Positive	**0.7095**	0.7625	0.7805	0.988	0.836	0.1555	1.211	0.7855	0.321	0.7855	0.713
Patient 15	Positive	**1.1915**	1.1385	0.573	0.5855	0.8685	0.586	0.6915	0.6985	1.142	0.6985	1.261
Patient 16	Positive	**1.643**	1.652	0.542	0.8895	1.8455	1.266	1.0835	1.114	1.5235	1.114	1.547
Patient 17	Positive	**0.66**	0.643	0.685	1.1345	0.8495	1.1505	0.9485	0.822	0.4925	0.822	0.4125
Patient 18	Positive	**1.2645**	1.008	0.213	0.22	1.0765	0.3105	0.3575	0.3625	0.912	0.3625	1.107
Patient 19	Positive	**1.788**	1.679	0.966	1.511	2.156	1.5755	0.971	1.0945	1.613	1.0945	1.2555
Patient 20	Positive	**2.2815**	2.2045	1.4305	2.0775	2.188	1.783	1.383	1.741	1.9145	1.741	1.5065
Patient 21	Positive	**0.4815**	0.2885	0.196	1.87	0.309	0.418	0.2435	0.257	0.384	0.257	0.2855
Patient 22	Positive	**1.9945**	1.036	0.7125	1.4815	1.1625	1.499	0.7495	1.4935	1.3845	1.4935	1.2735

Table 10. Standard SRA and EIA results of non-HIT patient samples against wild-type PF4 and PF4 mutants.

Non-HIT Patients	Standard SRA result (^{14}C-serotonin ≥ 20%)	WT-PF4/heparin EIA OD$_{405-490nm}$ (OD ≥ 0.45)	Mutant PF4/heparin EIA Mean OD$_{405-490nm}$ ± SD									
			H23A	R20A	T25A	E28A	K46A	R49A	K50A	K62A	K65A	K66A
Patient 1	Negative	1.271	1.7635	0.4005	0.7095	1.5745	0.756	0.517	1.2975	1.628	0.3245	1.1555
Patient 2	Negative	0.446	0.3915	0.318	0.3665	0.4165	0.3305	0.3415	0.3565	0.3665	0.4	0.3845
Patient 3	Negative	1.794	2.085	0.3135	0.297	1.637	0.2265	0.223	1.2465	1.3535	0.384	1.189
Patient 4	Negative	1.833	1.854	0.9215	0.6905	1.712	1.0695	1.117	0.879	1.2905	0.9425	1.4955
Patient 5	Negative	1.699	1.9295	0.394	0.316	1.706	0.511	0.5035	1.28	1.442	0.553	1.3975
Patient 6	Negative	0.822	0.8265	0.832	0.694	0.692	0.829	1.0635	0.804	0.786	0.8595	0.9555
Patient 7	Negative	0.691	0.315	0.305	0.49	0.754	0.4895	0.316	0.628	0.463	0.308	0.416
Patient 8	Negative	1.2595	0.85	0.544	0.716	1.1725	0.7305	0.622	0.7955	1.161	0.5645	0.793
Patient 9	Negative	1.0975	1.0945	0.627	0.8175	1.063	0.7395	0.887	0.921	0.979	0.826	0.7005
Patient 10	Negative	0.414	0.4325	0.204	0.2195	0.4465	0.297	0.2605	0.388	0.4055	0.254	0.2265
Patient 11	Negative	0.856	0.8555	0.497	0.6415	0.8635	0.6285	0.84	0.981	0.9855	0.8505	0.469
Patient 12	Negative	0.4025	0.3615	0.215	0.2465	0.571	0.2845	0.3215	0.504	0.434	0.2465	0.294

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