

Of HIT, Scissors and Rocks – Mechanistic basis of GPVI shedding in heparin-induced thrombocytopenia

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The work presented in this thesis is original and the result of full-time study from July 2016 to August 2019 at the John Curtin School of Medical Research, The Australian National University. This thesis contains no material previously published by another person except where due reference is made in the text of this thesis.

Christine Shu Mei Lee

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*To Mum, my biggest fan, for her endless support, unconditional love,
continuous encouragement and for believing that I can achieve anything I set my mind to.*

“Regardless if it is a celebration or a struggle, this too shall pass.”

“Doing it is easy, thinking about it is the hard part.”

- Ajahn Brahmavamso –

2004 John Curtin Medal recipient

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Lee, C.S.M., Montague, S.J., Hicks, S., Choi, P., Andrews, R.K. & Gardiner, E.E. *Regulation of platelet ADAM10 activity by tissue inhibitors of metalloproteinases (TIMPs)*.

Abstract

The most famous role for platelets is in thrombosis and haemostasis. Glycoprotein (GP) VI is a platelet-specific collagen and fibrin receptor which controls thrombus formation and growth. Human GPVI is stable on resting platelets but is rapidly metalloproteolysed by A-Disintegrin-And-Metalloproteinase (ADAM) 10 on activated platelets to release soluble GPVI (sGPVI). Regulation of platelet ADAM10 metalloproteolytic activity remains unclear. Active ADAM10 are detectable on resting human platelets but GPVI levels remain stable implying that there are mechanisms that control GPVI cleavage. Four tissue-inhibitor-of-metalloproteinases (TIMPs) regulate vascular metalloproteinase activity, but a role in controlling platelet ADAMs activity has not been described.

Heparin-induced thrombocytopenia (HIT) is a drug-induced, autoimmune disorder with potentially fatal outcomes. HIT occurs in a subset of patients exposed to heparin due to platelet-activating antibodies binding an antigenic complex of heparin and platelet factor 4 (PF4). This immune complex engages platelet Fc γ R1a and triggers signalling via immunoreceptor tyrosine-activation motifs (ITAMs) to induce platelet activation, aggregation and clearance. Engagement of Fc γ R1a also induces ectodomain shedding of platelet GPVI. However, the mechanistic basis of GPVI shedding induced by HIT immune complex remains to be explored. Identifying patients at risk of developing HIT remains clinically challenging because not all patients with detectable anti-PF4/heparin antibody develop HIT. Plasma sGPVI levels may indicate the presence of an activating anti-PF4/heparin antibody. Polymorphisms of Fc γ R1a may influence receptor function and platelet reactivity, and potentially modulate the risk of HIT.

This study aimed to (i) characterise changes in platelet surface GPVI and sGPVI levels upon engagement of FcγRIIa by HIT immune complexes; (ii) assess the utility of sGPVI to inform on pathological PF4/heparin autoantibodies in a prospective clinical cohort at risk of HIT; (iii) evaluate the haplotype-based association between FcγRIIa polymorphisms, Q27W and H131R, and HIT susceptibility using a long-read sequencing technology; and (iv) explore levels of TIMPs on platelets and the potential for TIMPs to regulate platelet ADAM10 activity.

Engagement of healthy donor platelet FcγRIIa by HIT patient immunoglobulins (Ig) induced cleavage of GPVI that was dependent on heparin, divalent cations, ITAM signalling and FcγRIIa, as measured by flow cytometry and ELISA. Inclusion of patient plasma sGPVI improved the overall accuracy, specificity and positive predictive value when evaluating risk of developing HIT. Higher plasma sGPVI was observed among individuals carrying at least one R allele at position 131. The haplotype combination of *FCGR2A* at positions 27 and 131 was not significantly associated with HIT susceptibility. TIMPs were detected on circulating platelets and in plasma, and surface levels increased upon platelet activation. TIMP1 and TIMP3 reduced resting platelet ADAM10 activity while only TIMP3 significantly reduced ADAM10 activity when platelets were stimulated.

Patient sGPVI levels may provide patient-specific information on platelet-activating HIT antibodies while the *FCGR2A* genotype may determine the state of activity of platelet in response to activation. This is the first study to assess the polymorphisms of *FCGR2A* in-phase. Dynamic platelet-associated TIMP levels may modulate ADAM10 activity and maintain platelet patency by stabilising GPVI levels. The findings in this thesis extended our knowledge of the molecular pathway involved in HIT Ig-mediated GPVI shedding and presented a new mechanism for regulating platelet ADAM10 activity.

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List of Abbreviations

ACD	Acid citrate dextrose
ADAM	A-Disintegrin-And-Metalloproteinase
ADP	Adenosine diphosphate
APS	Ammonium persulphate
AUC	Area under the curve
BAY61	Bay61-3606
BHQ2	Black hole quencher 2
bp	Base pair
BSA	Bovine serum albumin
<i>BstUI</i>	<i>Bacillus stearothermophilus</i> U458
chr	Chromosome
CLIA	Chemiluminescence immunoassay
CRP-xL	Crosslinked collagen-related peptide
Cy3	Cyanine 3
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
dsDNA	Double-stranded DNA
DTT	Dithiothreitol
dTTP	Deoxythymidine triphosphate
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
Fc	Fragment crystallisable
<i>FCGR2A</i>	Gene encoding FcγRIIa
FcRγ-chain	Fc receptor gamma-chain
FcγRIIa	Fc gamma receptor IIa
FPLC	Fast protein liquid chromatography
FRET	Fluorescent resonance energy transfer

gDNA	Genomic DNA
GI	GI254023X
GM	GM6001
GPVI	Glycoprotein VI
HEP	HIT expert probability
HIPA	Heparin-induced platelet aggregation
HIT	Heparin-induced thrombocytopenia
HITT	Heparin-induced thrombocytopenia with thrombosis
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
ICU	Intensive care unit
Ig	Immunoglobulin
ITAM	Immunoreceptor tyrosine-based activation motif
ITP	Immune thrombocytopenia
kDa	Kilo Dalton
KKO-I	KKO-inhibition
LAA	Long amplicon analysis
LFIA	Lateral flow immunoassay
LIA	Latex particle-enhanced immunoturbidimetry assay
MMP	Matrix metalloproteinase
NEM	N-ethylmaleimide
NPV	Negative predictive value
ns	Not significant
NT	Not treated
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PaGIA	Particle gel immunoassay
PAR	Protease-activated receptor
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline with Tween-20
PCR	Polymerase chain reaction
PEA	PF4-dependent P-selectin expression assay
PF4	Platelet factor 4

PIFA	Particle immunofiltration anti-PF4 assay
PLC γ 2	Phospholipase C γ 2
PMA	Phorbol myristate acetate
PPP	Platelet-poor plasma
PPV	Positive predictive value
PRP	Platelet-rich plasma
PVA	Platelet viability assay
PVDF	Polyvinylidene difluoride
RBC	Red blood cell
RFLP	Restriction fragment length polymorphism
RFU	Relative fluorescence units
rh	Recombinant human
RNA	Ribonucleic acid
ROC	Receiver operating characteristics
RT	Room temperature
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SFK	Src family kinase
sGPVI	Soluble GPVI
SH2	Src homology 2
SLE	Systemic lupus erythematosus
SLP-76	SH2 domain-containing leukocyte phosphoprotein of 76 kDa
SMRT	Single molecule real-time
SNP	Single nucleotide polymorphism
SRA	Serotonin-release assay
Syk	Spleen tyrosine kinase
TAE	Tris acetate EDTA
<i>Taq</i>	<i>Thermus aquaticus</i>
TBS	Tris buffered saline
TBS-T	Tris buffered saline with Tween-20
TCP	Non-HIT thrombocytopenic
TEMED	N,N,N',N'-Tetramethylethylenediamine
TIMP	Tissue inhibitor of metalloproteinase

TLR	Toll-like receptor
VWF	Von Willebrand factor
WBC	White blood cell
ZMW	Zero-mode waveguide

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Chapter 1 Introduction

1.1 Platelets and their multifaceted roles

1.1.1 Overview of platelet production and composition

Platelets are 2-3 μm wide, disc-shaped, anucleate cellular fragments of a nucleated precursor cell, the megakaryocyte. Accounting for $\sim 0.01\%$ of bone marrow cells, megakaryocytes are large (50-100 μm in diameter), multilobed residents of the bone marrow shown to circulate through the lungs (Lefrancais et al., 2017) and are capable of generating 2000-4000 platelets each (Machlus and Italiano, 2013, Li et al., 2017). Although megakaryocytes are one of the rarest cells in the bone marrow, platelets are the most abundant blood component. Among healthy individuals, the number of circulating platelets ranges from $150\text{-}400 \times 10^9$ per litre of whole blood with approximately 10^{11} platelets produced daily through a process called thrombopoiesis (Hartwig and Italiano, 2003).

Proplatelets, the cytoplasmic projections of terminally-differentiated megakaryocytes, extend between endothelial cells into the marrow sinusoids and undergo extensive microtubule and actin cytoskeleton rearrangements to release platelets from the ends of proplatelets (Wright, 1910, Radley and Scurfield, 1980, Ghalloussi et al., 2019). Platelets mature from the tips of proplatelets whereby platelet granules and organelles are transported via microtubules as individual vesicles from the megakaryocyte cell body along the length of the proplatelets (Italiano et al., 1999, Richardson et al., 2005). Human platelets typically have a lifespan of 5-10 days before being sequestered and cleared in the spleen and liver by macrophages and hepatocytes, respectively. The multidimensional mechanisms of platelet clearance involve

platelet receptors and surface glycan modifications, apoptotic machinery and opsonisation by anti-platelet antibodies (Grozovsky et al., 2015, Andrews and Gardiner, 2016).

Despite the absence of a nucleus, platelets carry the machinery for protein production and are metabolically active. Platelets contain endoplasmic reticulum, Golgi apparatus, mitochondria, messenger ribonucleic acid (RNA) and ribosomes which allow synthesis *de novo* of some molecules such as the integrin $\alpha_{IIb}\beta_3$, interleukin-1 β , B-cell lymphoma 3 and cyclooxygenase-1, either constitutively or in response to activating signals (Weyrich et al., 2009). Intracellular granules in platelets are filled with bioactive molecules such as platelet factor 4 (PF4), serotonin, adenosine diphosphate (ADP) and thromboxane A₂, which are either synthesised *de novo* in platelets, synthesised by megakaryocytes (e.g., PF4) or endocytosed from the blood plasma (e.g., fibrinogen) (Harrison et al., 1989). These granules contain over 300 mediators (Pagel et al., 2017) with opposing effects, for example, the co-occurrence of coagulant and anticoagulant factors (e.g., prothrombin and antithrombin), and angiogenic and antiangiogenic factors (e.g., vascular endothelial growth factor (VEGF) and PF4) within the alpha-granules (Italiano et al., 2008, Li et al., 2017). The compartmentalisation and selective secretion (Jonnalagadda et al., 2012) of these bioactive compounds are likely to contribute to the multifaceted functions of platelets in physiology and pathology.

1.1.2 Platelets in haemostasis and thrombosis

Owing to their small size, abundance and manoeuvrability due to the lack of a nucleus, platelets are the sentinels of the bloodstream capable of navigating through the narrowest of capillaries. Platelets survey the vasculature and are the first responders in case of a breach. These physical properties contribute to the primary function of platelets in minimising blood loss and maintaining blood volume. The chain reaction from platelet rolling, adhesion and eventually thrombus formation at damaged sites of the vascular endothelium involves an orchestra of platelet receptors and bioactive molecules contained in platelet granules as illustrated in Figure 1.1.

Platelet recruitment to an injured vasculature is initiated by a complex interplay between platelet surface molecules, exposed extracellular matrix and endothelial cells. P-selectin expressed on activated endothelium interacts with the platelet-specific glycoprotein (GP) Ib α of the GPIb-IX-V complex to engage the stream of flowing platelets in the blood vessel (Romo et al., 1999) (Figure 1.1A). P-selectin exposure on activated endothelium allows platelets to roll on the endothelial surface (Frenette et al., 1995) and facilitate platelet adherence to exposed extracellular matrix proteins namely von Willebrand factor (VWF) and collagen (Figure 1.1B). GPIIb/IIIa and GPIb-IX-V are the two crucial adhesion receptors which bind collagen and VWF, respectively, and initiate intracellular signalling pathways that convert the integrins $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$ into their high affinity forms for interaction with collagen and VWF to form stable adhesion (Li et al., 2010). Ligand-binding of these receptors also triggers platelet activation, degranulation and secretion of bioactive molecules. Platelets undergo cytoskeletal rearrangement resulting in a shape change and release of intracellular granule contents such as ADP and serotonin from the dense granules, and PF4 and P-selectin from the alpha-granules

(Offermanns, 2006, Amelot et al., 2007) (Figure 1.1C). These granule releasates act as a positive feedback to platelet activation leading to further platelet recruitment and aggregate formation mediated by active $\alpha_{IIb}\beta_3$ integrin which binds arginine-glycine-aspartic acid (RGD) sequences within VWF, fibrinogen as well as other RGD-containing plasma and matrix proteins (Figure 1.1D).

Activated platelets secrete coagulation factors and undergo flipping of the plasma membranes to expose negatively-charged phosphatidylserine residues which provide a procoagulant surface that supports and accelerates thrombin generation via activation of the coagulation cascade. Thrombin converts fibrinogen to fibrin which is crosslinked by active Factor XIII leading to the formation of a fibrin network, thrombus stabilisation and wound occlusion at a site of injury (Figure 1.1E). Several anticoagulant factors contained within platelet granules are secreted by activated platelets which may have a role in modulating thrombus formation (Swieringa et al., 2018).

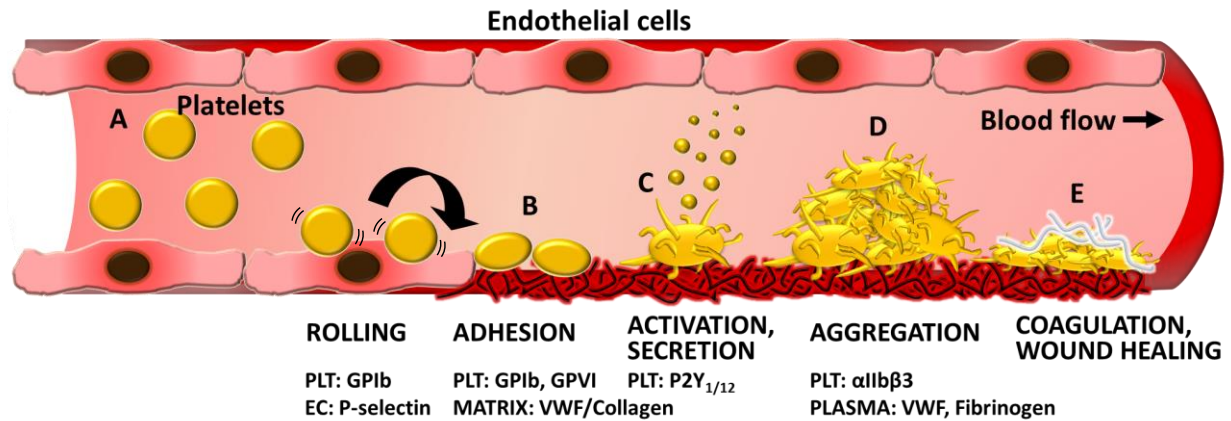


Figure 1.1 Platelet activation and thrombus formation.

(A) Circulating platelets roll on activated endothelial cell via GPIb/P-selectin interactions. (B) Initial adhesion of platelets to injured vessel wall is mediated by GPIb and GPVI through interaction with VWF and collagen in the exposed extracellular matrix. (C) These interactions activate platelets leading to secretion of secondary mediators (ADP, thromboxane A₂) and platelet spreading. (D) These secondary mediators recruit platelets to the site of injury while activation of integrin α IIb β 3 enables binding of fibrinogen and platelet aggregation leading to thrombus growth. (E) Activation of the coagulation cascade and thrombin generation converts fibrinogen to fibrin which stabilises the thrombus, culminating in wound occlusion thereby preventing blood loss.

1.1.3 Platelets in innate immunity and inflammation

The ubiquity in platelet number enables full coverage of the vascular system, a trait that facilitates immune surveillance against pathogens. The role of platelets in immunity has emerged following the discovery of immunologically-related receptors on platelets. Platelets express Toll-like receptors (TLR), complement receptors and immunoglobulin (Ig) receptors which play a role in the body's first line of defence against pathogens (Cox et al., 2011, Del Conde et al., 2005, Arbesu et al., 2016, Arman and Krauel, 2015). Release of cytokines from platelet granules and expression of adhesive receptors facilitate platelet/leukocyte interaction which can promote a pro-inflammatory response.

Platelets recognise opsonised pathogens and become activated to promote recruitment of immune cells by secreting chemokines and cytokines, and upregulation of the adhesive molecule, P-selectin, to capture leukocytes to the site of infection (Semple and Freedman, 2010). Platelets can prevent pathogen dissemination in the blood by secreting antimicrobial compounds or physically trap pathogens by encapsulating them. For example, PF4 has been reported to mediate killing of the plasmodium parasites during the blood stage of malaria infection in humans, supporting a role for platelets in controlling parasite load as part of the innate defence mechanism (Kho et al., 2018). Recently, Gaertner and colleagues (2017) demonstrated that adherent platelets scavenge and bundle up bacteria, and promote neutrophil activation, leading to the formation of "extracellular traps" of DNA decorated with citrullinated histones and assorted proteinases which exacerbates tissue damage in inflammatory conditions like sepsis. These observations add to a growing body of evidence for the role of platelets in innate immunity and inflammation.

1.2 Platelet receptors and signalling

1.2.1 Overview

Platelets express a plethora of receptors that are critical for platelet roles in haemostasis, thrombosis, innate immunity and inflammation. Platelet surface receptors can be broadly categorised into adhesion receptors involved in platelet activation (e.g., integrin $\alpha_{IIb}\beta_3$ and GPIb-IX-V), amplification of platelet activation and regulation of thrombus formation (e.g., GPVI and protease-activated receptors (PAR)1 and PAR4), receptors for soluble agonists (e.g., P2Y₁ and P2Y₁₂), pattern recognition receptors (e.g., TLRs 1-4), chemokine receptors (e.g., CCR1, CXCR4), Ig receptors (e.g., Fc γ RIIa and Fc ϵ RI) and hormone receptors (e.g., c-Mpl, 5-HT_{2A}). These receptors execute the multifaceted role of platelets. Among the signalling receptors on platelets, GPVI and Fc γ RIIa are of relevance to the work presented here.

1.2.2 GPVI

GPVI is a 62 kDa sialomucin and a member of the Ig-like receptor superfamily with two Ig-like extracellular domains, a heavily O-glycosylated mucin-like stem, a transmembrane region and a cytoplasmic tail harbouring a calmodulin binding site and SH3 domains for binding of Src family kinases (SFK), Fyn and Lyn (Horii et al., 2006, Andrews and Gardiner, 2016). GPVI expression is exclusive to megakaryocytes and platelets at ~9600 copies per platelet (Burkhart et al., 2012). GPVI was first discovered as the major collagen receptor (Moroi et al., 1989) on platelets but has been recently reported to also bind fibrin (Alshehri et al., 2015, Mammadova-Bach et al., 2015) and fibrinogen (Induruwa et al., 2018, Mangin et al., 2018), consistent with a role for GPVI in thrombus formation and stabilisation. Other physiological ligands of GPVI include laminin to support platelet adhesion and spreading (Inoue et al., 2006), fibronectin to

facilitate platelet adhesion to atherosclerotic plaque (Bultmann et al., 2010) and vitronectin for interaction with activated endothelial cells (Schonberger et al., 2012). Ligand-binding causes intracellular signalling leading to platelet activation and mediates platelet interactions with endothelial cells and monocytes. A variety of exogenous molecules can also activate GPVI, including the snake venom toxin convulxin, diesel exhaust particles, polysulfated sugars and antisense oligonucleotides (Rayes et al., 2019).

The D1 domain of GPVI recognises a glycine-proline-hydroxyproline (GPO) sequence which matches a sequence within collagen types I and III (Smethurst et al., 2004, Horii et al., 2006). Crosslinked collagen-related peptide (CRP-xL) is a synthetic peptide containing the GPO repeats to mimic collagen-induced platelet activation specifically via GPVI (Knight et al., 1999). As collagen engages multiple receptors on platelets (GPV, GPIb α , $\alpha_2\beta_1$, $\alpha_{IIb}\beta_3$) in addition to GPVI, CRP-xL is widely used as a GPVI-specific activator.

GPVI is expressed on the platelet surface in a non-covalent complex with Fc receptor γ -chain (FcR γ) dimer which is required for GPVI expression and signalling (Berlangu et al., 2002). The cytoplasmic region of FcR γ -chain contains an immunoreceptor tyrosine based-activation motif (ITAM) and ligand-binding to GPVI triggers a signalling cascade involving phosphorylation of tyrosine within the ITAM domain (Gibbins et al., 1997). Various studies provided evidence for an increase in dimeric forms of GPVI upon platelet activation (Jung et al., 2012, Loyau et al., 2012), which has been shown to strengthen collagen-induced signalling (Poulter et al., 2017). A crystallography study by Horii and colleagues (2006) revealed that dimeric GPVI forms a groove in which CRP-xL binds (Figure 1.2). The spacing and orientation of the groove match the dimensions of triple helices in collagen, suggesting a probable mechanism by which dimeric GPVI recognises collagen.

Loss of function in GPVI has been described in numerous patients since the first report of a thrombocytopenic Japanese female with impaired collagen-induced platelet aggregation (Sugiyama et al., 1987). GPVI functional deficiency may arise from causes including congenital defects (Arai et al., 1995), autoantibody exposure (Boylan et al., 2004, Rabbolini et al., 2017), GPVI signalling defect (Bellucci et al., 2005, Dunkley et al., 2007) or *GP6* gene mutations (Matus et al., 2013). These patients present with bleeding tendencies, thrombocytopenia, absence or diminished platelet aggregation in response to GPVI agonists (collagen, CRP-xL, convulxin) and/or reduced platelet adhesion or spreading on immobilised collagen. GPVI levels were reduced or undetectable in some of these patients when assessed by immunoblotting or flow cytometry (Sugiyama et al., 1987, Moroi et al., 1989, Boylan et al., 2004, Rabbolini et al., 2017).



Figure 1.2 Computational docking of CRP-xL onto dimeric GPVI.

Model of the GPVI extracellular IgG-like domains as dimers in complex with collagen-related peptide (CRP-xL), predicted by two computational algorithms (PatchDock and FTDock). Predicted binding sites for CRP-xL (red) within grooves of the D1 domains of two GPVI molecules (blue and green) which are likely to interact non-covalently at respective D2 domains. Figure adapted from Horii and colleagues (2006).

1.2.3 FcγRIIIa

A second ITAM-containing receptor on platelets is the low affinity Fcγ receptor FcγRIIIa which recognises the constant (Fc) region of IgG and was first reported on human platelets by Rosenfeld and colleagues (1985). FcγRIIIa is a 40 kDa Type I transmembrane protein made up of two extracellular Ig-like domains with a calmodulin-binding site on the intracellular tail and signals via an ITAM-dependent pathway. Human platelets express approximately 1000 molecules of FcγRIIIa per platelet (Burkhart et al., 2012). Although FcγRIIIa, also known as CD32a, is expressed at ~300-fold higher density per unit surface area in leukocytes including neutrophils, eosinophils and macrophages, human platelets form the largest pool of FcγRIIIa owing to the sheer abundance of platelets (Karas et al., 1982). FcγRIIIa is also the only activating platelet Fcγ receptor. FcγRIIIa is not expressed in mice, thus an FcγRIIIa-transgenic mouse model was generated by McKenzie and colleagues (1999) which has enabled many studies on FcγRIIIa-mediated pathologies. The absence of a mouse homologue contributed to an underappreciation of the involvement of FcγRIIIa in assessment of human pathologies using mouse models, for example in IgG-dependent anaphylaxis (Beutier et al., 2018).

FcγRIIIa binds with low affinity to monomeric IgG but more avidly to IgG associated with immune complexes (Karas et al., 1982). Human platelet FcγRIIIa ITAM signalling can be activated by a variety of ligands including aggregated IgGs (Mueller-Eckhardt and Luscher, 1968), the Fc portion of anti-platelet antibodies (Horsewood et al., 1991), bacteria and influenza viruses opsonised by IgG (Cox et al., 2011, Boilard et al., 2014) and immune complexes (Chong et al., 1989, Sun et al., 2013). Engagement of FcγRIIIa triggers phosphorylation of its ITAM domain composed of two YXXL (X corresponds to a variable residue) consensus amino acid sequences separated by 12 amino acids (Brooks et al., 1989),

leading to activation of the ITAM-dependent signalling cascade detailed in Section 1.2.5 and shown in Figure 1.4. Similar to GPVI, ligand engagement can trigger dimerization of FcγRIIIa as supported by data gained from the crystal structure of dimeric FcγRIIIa in complex with Fc domain of human IgG1 (Ramsland et al., 2011). The effector function elicited via FcγRIIIa engagement varies with the type of agonist as reviewed by Arman and Krauel (2015). Activation of human platelet FcγRIIIa by anti-platelet antibodies leads to calcium mobilisation (Qi et al., 1996), alpha- and dense granule secretion (Chong et al., 1989, Tomiyama et al., 1992), intraplatelet generation of reactive oxygen species (Arthur et al., 2012) and integrin-dependent platelet aggregation (Horsewood et al., 1991, De Reys et al., 1993). In addition, calpain-mediated intracellular cleavage of the ITAM domain upon engagement of FcγRIIIa has also been reported (Gardiner et al., 2008b).

1.2.4 FcγRIIIa polymorphisms and autoimmune disorders

Human FcγRIIIa is encoded by seven exons within the *FCGR2A* gene located on the long arm of chromosome 1 at position 23.3 (1q23.3) (Grundy et al., 1989) and has three polymorphic sites located at amino acids 27, 127 and 131. Figure 1.3 shows a crystal structure of a dimeric FcγRIIIa highlighting the polymorphic residues 27 and 131. The most widely studied non-synonymous single nucleotide polymorphism (SNP) at nucleotide 519 (519G>A, rs1801274) on exon 4 causes the replacement of arginine (R) by histidine (H) at amino acid 131. Both alleles are co-dominantly expressed in the human population at a genotype frequency of 29.2%, 49.7% and 21.1% for HH131, HR131 and RR131 genotypes, respectively (Nagelkerke et al., 2019). According to the crystal structure, amino acid 131 sits in the contact interface between the receptor and its ligand (Fc region of IgG) in the second Ig-like domain (Figure 1.3). The substitution of a weak base (histidine) for a strong base (arginine) does not impact on protein

expression levels (Anderson et al., 1987, Brandt et al., 1995) but alters the affinity of FcγRIIIa for its ligand. Both variants do not bind monomeric IgG (Bruhns et al., 2009) but showed considerable differences in binding IgG-bound immune complexes. While the H131 variant binds both human IgG1 and IgG2, the R131 variant binds IgG1 but has a poor affinity for IgG2 (Anderson et al., 1987, Warmerdam et al., 1991, Bruhns et al., 2009). This differential binding affinity may be critical as the R131 allele has been implicated with increased risk of thrombosis in patients with adverse drug reactions to heparin (Rollin et al., 2015). In addition, a greater prevalence of atherosclerosis and increased platelet reactivity were observed among patients with systemic lupus erythematosus (SLE) who carried the R131 allele (Clancy et al., 2019).

A dinucleotide substitution (CA>TG, rs201218628) on exon 3 of *FCGR2A* replaces a glutamine (Q) with tryptophan (W) at amino acid 27 which is located within the first Ig-like domain situated at the interface between two FcγRIIIa monomers (Figure 1.3, Table 1.1). As this polymorphism requires substitution of two nucleotides, a low occurrence of the alternative allele might be expected. Nagelkerke and colleagues (2019) recently reported ethnicity differences in the allele frequency of W27 in extensive cohorts of 919 Europeans, 428 Chinese and 508 Africans at 0.12, 0.00 and 0.11, respectively, with a virtually homogenous expression of the Q27 allele among the Han-Chinese (Table 1.1). Q27W was reported to have no effect on receptor function or IgG binding when studied in isolation however no examination of IgG isotype-specific binding has been performed (Warmerdam et al., 1990). In human peripheral blood mononuclear cells, there was no apparent difference in FcγRIIIa surface expression between individuals carrying the polar glutamine residue or the non-polar tryptophan residue at position 27 (Flinsenberg et al., 2014). However, the change from a polar glutamine residue to a non-polar tryptophan residue interrupted downstream signalling transduction when the variant form was overexpressed in the IIA1.6 cells (Flinsenberg et al., 2014). Consistent with

defective FcγRIIIa signalling, the authors reported an association between the W27 variant and increased prevalence of common variable immune deficiency in children. A higher frequency of the W27 variant allele was observed among 405 patients with Kawasaki disease which is a paediatric condition whereby medium-sized arteries become inflamed (Nagelkerke et al., 2019).

Norris and colleagues (1998) uncovered a novel *FCGR2A* polymorphic site in exon 4 encoding amino acid residue 127 in a healthy female who was homozygous for the R allele at position 131. This individual carried a SNP (C>A, rs139202723) which resulted in a non-synonymous glutamine (Q) to lysine (K) substitution at codon 127 in the ligand-binding domain of FcγRIIIa, proximal to H131R. Q127K is a rare variant with a minor allele frequency of 0.0002-0.0005, depending on the sequencing project (The Genomes Project, 2017). Interestingly, the presence of the positively-charged lysine residue three amino acids apart restored the binding affinity of R131 for IgG2 whereby the allele combination of K127 and R131 on FcγRIIIa enabled binding of both IgG1 and IgG2 (Norris et al., 1998). This observation highlighted the functional significance of a combinatorial effect across distinct polymorphic sites. To date, the impact of the combination of polymorphic sites within *FCGR2A* on receptor function has not been evaluated.

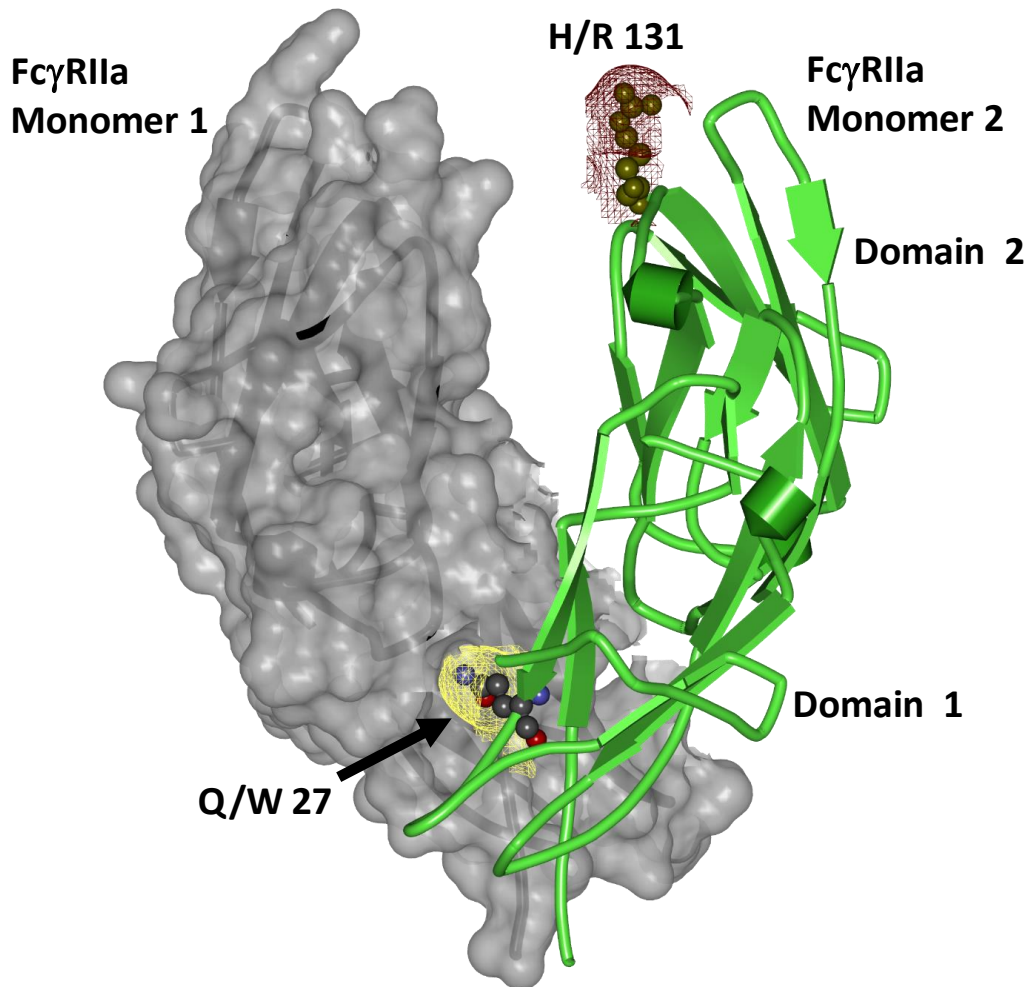


Figure 1.3 Crystal structure of FcγRIIIa dimer.

The crystal structure of FcγRIIIa homodimer viewed parallel to the membrane. Monomer 1 (surface rendered) and monomer 2 (α -carbon backbone) dimerises to form a ligand-binding pocket. The Q27W and H131R polymorphic sites are shown. Polymorphic residue 27 is located in the first Ig-like domain at the dimer interface between residues T23 and R30 which influence receptor dimerisation (Ramsland et al., 2011). H131R sits in the second Ig-like domain which is the contact interface between the receptor and its ligand, the constant region of IgG.

Table 1.1 The Q27W *FCGR2A* polymorphism.

Aspects	Literature
Amino acid variant	Glutamine (Q) to tryptophan (W) at amino acid 27
Alleles	CA>TG rs201218628
W27 allele frequency	European (n=919): 0.12 Chinese (n=428): 0.00 African (n=508): 0.11
IgG binding capacity	No effect on IgG binding but no study on IgG isotype-specific binding
Effect on FcγRIIa expression	No difference in surface expression between genotypes
Signalling transduction	Overexpression of the W27 variant resulted in defective FcγRIIa signalling
Disease implications	Increased prevalence of the W27 variant in common variable immune deficiency and Kawasaki disease in children

1.2.5 ITAM-dependent signalling via GPVI/FcR γ -chain and Fc γ RIIa

The ITAM is a sequence of amino acids in the cytoplasmic domain of certain immunoreceptors comprising two tyrosine (Y) residues within a conserved YXXL (X corresponds to a variable residue) sequence which play a critical role in receptor signalling leading to leukocyte activation (Boulaftali et al., 2014). The two platelet receptors that are of relevance to this thesis, GPVI and Fc γ RIIa, activate platelets via an ITAM-dependent signalling cascade. A schematic diagram of the GPVI/FcR γ -chain and Fc γ RIIa signal transduction is shown in Figure 1.4. Fc γ RIIa harbours the ITAM motif on its cytoplasmic tail while GPVI forms a non-covalent complex with the ITAM-bearing FcR γ -chain (Figure 1.4) to enable signal transduction. GPVI associates with SFK such as Fyn and Lyn, via the proline-rich domain of GPVI cytoplasmic tail (Ezumi et al., 1998, Suzuki-Inoue et al., 2002).

Upon ligand engagement and clustering of GPVI, ITAM in the associated FcR γ -chain is phosphorylated by SFK (Ezumi et al., 1998, Quek et al., 2000). Similarly, engagement of Fc γ RIIa triggers receptor dimerization and phosphorylation of the cytoplasmic YXXL motifs (Ramsland et al., 2011, Boulaftali et al., 2014). This enables recruitment and activation of the spleen tyrosine kinase (Syk) through its dual Src homology 2 (SH2) domains. Activated Syk initiates a cascade of events involving adaptor molecules and kinases including SH2 domain-containing leukocyte phosphoprotein of 76 kDa (SLP-76) (Gross et al., 1999) and linker for activated T-cells (LAT) (Pasquet et al., 1999) (Figure 1.4). Consequently, phospholipase C γ 2 (PLC γ 2) is translocated to the plasma membrane and becomes activated upon phosphorylation, leading to subsequent activation of protein kinase C and culminating in calcium mobilisation, integrin activation and granule secretion which are the hallmarks of platelet activation (Watson et al., 2005).

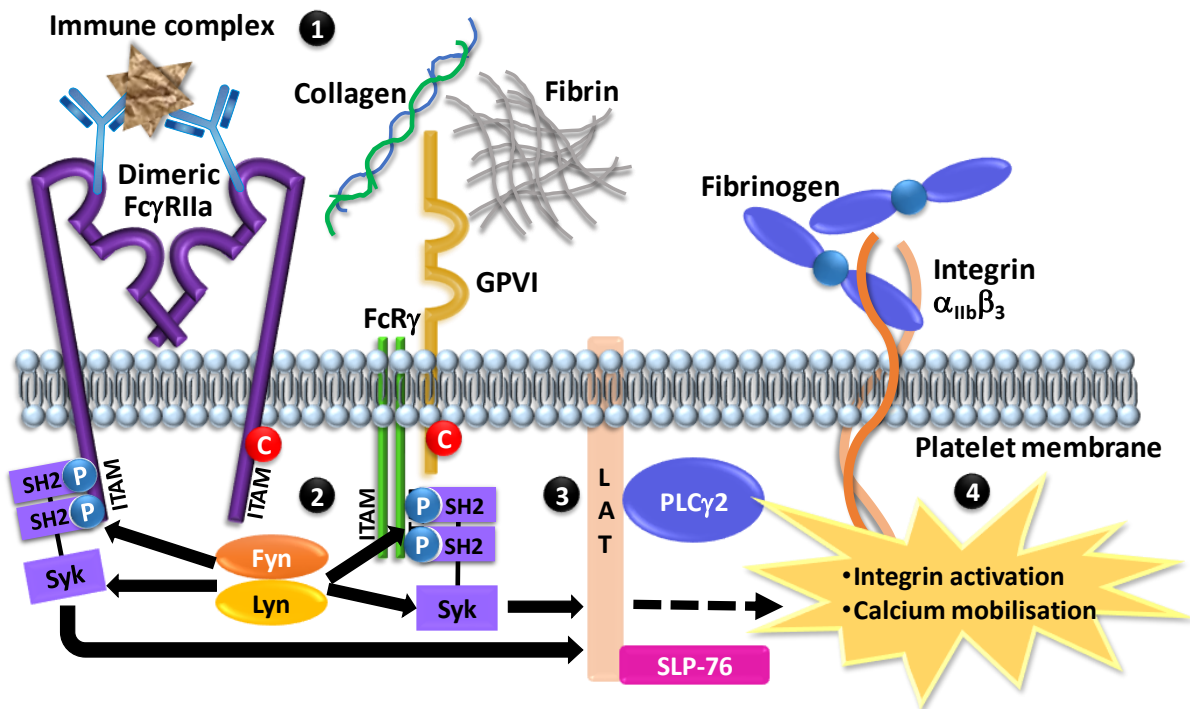


Figure 1.4 ITAM-dependent signalling via GPVI/FcR γ -chain and Fc γ RIIa in platelets.

(1) Ligand of Fc γ RIIa by immune complexes or GPVI by collagen or fibrin leads to tyrosine phosphorylation of ITAM within the cytoplasmic tails of Fc γ RIIa and FcR γ -chain (associated with GPVI) by SFKs including Fyn and Lyn. (2) This enables recruitment and activation of Syk through its dual Src homology 2 (SH2) domains. (3) Activated Syk initiates downstream activation of LAT and PLC γ 2 resulting in (4) activation of phosphoinositide 3-kinase, cytoskeletal reorganisation, $\alpha_{IIb}\beta_3$ integrin activation, calcium mobilisation and degranulation. Calmodulin-binding sites on Fc γ RIIa and GPVI are indicated by red circles (C). Syk, spleen tyrosine kinase; ITAM, immunoreceptor tyrosine-based activation motif; LAT, linker for activation of T-cells; SLP-76, SH2 domain-containing leukocyte phosphoprotein of 76 kDa; PLC γ 2, phospholipase γ 2.

1.3 Heparin-induced thrombocytopenia

1.3.1 A historical perspective of HIT

Fidlar and Jaques (1948) first described acute thrombocytopenia occurring rapidly following heparin administration in humans. Chong and Ismail (1989) later identified the platelet-aggregating effects of heparin which is historically referred to as type I heparin-induced thrombocytopenia (HIT) (Chong and Berndt, 1989). Heparin, a highly sulphated glycosaminoglycan discovered 101 years ago (Howell and Holt, 1918), is commonly administered to hospitalised patients and is the anticoagulant of choice among surgeons due to its immediate anticoagulant effect, reversibility, well-characterised pharmacokinetics and affordability (Kelton and Warkentin, 2008). The initial observations of HIT were made by a group of surgeons. A decade after the first report, Weismann and Tobin (1958) described the potentially fatal immune complication of heparin therapy, known as type II HIT, in 10 patients who developed thrombosis while receiving heparin, which proved fatal in six patients while two survivors suffered loss of limbs. Several years later, Roberts and colleagues (1964) observed similar episodes in 11 patients and hypothesised that this syndrome may result from an antigen-antibody mechanism. These observations exemplify HIT as a clinical paradox of the co-occurrence of thrombocytopenia and thrombosis consequent of heparin administration meant to prevent thrombosis. Rhodes and colleagues (1973) described the clinical features of HIT which include simultaneous manifestations of thrombocytopenia and thrombosis following heparin exposure, improvement in platelet counts following heparin cessation, presence of platelet-aggregating heparin-dependent antibodies during the acute phase and recurrent thrombocytopenia upon heparin re-exposure.

1.3.2 Pathogenesis

HIT is caused by antibodies, typically of the IgG class, recognising PF4 in complex with polyanionic heparin (Amiral et al., 1992). HIT pathogenicity occurs when platelet-activating antibodies in complex with PF4/heparin engage platelet Fc γ RIIa, as well as Fc receptors on monocytes (Rauova et al., 2010, Kasthuri et al., 2012) and neutrophils (Gollomp et al., 2018, Perdomo et al., 2019), and subsequently trigger platelet activation and aggregation via an ITAM-dependent signalling cascade (Section 1.2.5), resulting in the manifestation of thrombocytopenia and thrombosis in a subset of patients. Complement-mediated activation of endothelial cells and release of “extracellular traps” by activated neutrophils may further contribute to a prothrombotic vascular environment (Khandelwal et al., 2018, Rauova et al., 2018). HIT pathology requires anti-PF4/heparin antibodies and Fc γ RIIa expression as demonstrated in a transgenic mouse model (Reilly et al., 2001). As such, Fc γ RIIa is regarded as the pathological HIT receptor. Among the cells involved in the pathogenesis of HIT, platelets form the largest pool of Fc γ RIIa (Karas et al., 1982).

A typical autoantibody immune reaction is characterised by IgM formation during the initial phase of the immune response followed by subclass switching processes leading to the generation of immunoglobulins belonging to A, G, D or E subclasses (Janeway et al., 2001). Interestingly, in HIT, the pathological antibody responsible for the initial thrombocytopenia is almost always of IgG subclass, which is often seen in a secondary immune response. This antibody is generally detectable four to eight days after heparin exposure which is atypical of an autoimmune reaction (Warkentin et al., 2009). Nevertheless, Krauel and colleagues (2016) reported the formation of anti-PF4/heparin IgM antibodies in paediatric patients and mice without prior exposure to the antigen which may suggest the involvement of the innate

immune response. Moreover, there is discord between the frequency of HIT antibody formation and the development of HIT (see Section 1.3.4). For these reasons, significant efforts have been spent on characterising the PF4/heparin immunogenic complex and the consequences for platelets exposed to this immune complex.

1.3.3 Defining the HIT antigen

Depending on the pH of the solution, PF4 exists as a heterogeneous mixture of monomers, dimers and tetramers (Mayo and Chen, 1989). PF4 tetramer forms a particularly stable and large complex with heparin through an electrostatic interaction over a narrow stoichiometric ratio of PF4:heparin that is associated with charge neutralisation (Rauova et al., 2005). A recent crystallography study revealed binding of a low-molecular-weight-heparin, fondaparinux, via its anionic sulphate groups to a cationic groove conferred by lysine, histidine and arginine residues on the 'closed' side of the asymmetric PF4 tetramer (Cai et al., 2015). These electrostatic interactions formed a stable PF4/fondaparinux complex which in turn stabilised the 'open' side of the PF4 tetramer and exposed the epitope for the HIT-like antibody KKO. Together, the stepwise interactions between heparin and PF4 tetramer, then between the PF4/heparin complex and KKO or HIT Ig culminate in the formation of a stable, platelet-activating ternary immune complex (Cai et al., 2016). In a single-amino-acid-mutagenesis study, five amino acid residues on PF4 were identified to be important for the binding of platelet-activating HIT antibodies from confirmed HIT patient sera (Huynh et al., 2019). Four of the five amino acids were cysteine residues, which when mutated to alanine, will disrupt disulphide bonds and potentially alter the structure of PF4, thus resulting in the loss of antibody binding.

1.3.4 Epidemiology in adult patients

HIT is the most common and potentially fatal, iatrogenic complication of heparin anticoagulation with an incidence rate of <0.1% to 7% among heparinised patients (Smythe et al., 2007). Annually, heparin is administered to an estimated 12 million hospitalised patients in the United States alone (Rice, 2004). HIT prevalence is influenced by heparin preparation (unfractionated more so than low-molecular-weight heparin) (Martel et al., 2005), patient type (more prevalent among orthopaedic patients than cardiac patients) (Warkentin et al., 2000) and length of heparin administration (more prevalent among patients with longer heparin exposure) (Lindhoff-Last et al., 2002). Thrombotic complications, which may lead to limb amputation or death in patients with HIT, occur in 30-60% of cases (Greinacher et al., 2005). Clearly, HIT poses a significant clinical complication that warrants accurate and timely diagnosis.

Anti-PF4/heparin antibodies are extremely rare in healthy individuals with a prevalence of 0.3-0.5% as assessed in blood bank donors (Hursting et al., 2010, Krauel et al., 2011). The use of unfractionated heparin and cardiac surgery have been significantly associated with the formation of anti-PF4/heparin antibodies. However, there is a discrepancy between HIT antibody formation and the risk for HIT development. Up to 50% of cardiac patients were positive for HIT antibody compared to 14.1% among orthopaedic patients (Warkentin et al., 2000). However, among those with detectable anti-PF4/heparin antibodies, orthopaedic patients (52.6%) were more likely to develop HIT than cardiac patients (5%), suggesting a potential contribution from impaired mobility. This discord also suggests heterogeneity in the ability of anti-PF4/heparin antibodies to activate platelets and lead to the manifestation of the syndrome associated with HIT.

1.3.5 The strengths and limitations of current diagnostic tools

The diagnosis of HIT typically relies on the combination of clinical assessment and laboratory testing to detect anti-PF4/heparin antibodies and subsequently evaluate the platelet-activating capacity of the antibodies. The strengths and limitations of current tools including clinical scoring systems, immunoassays and functional assays are summarised in Table 1.2.

1.3.5.1 Clinical scoring systems

The 4Ts score is a pre-test 'bedside' scoring system to quickly assess a patient's risk of HIT to guide initial clinical management and further testing. Developed by Lo and colleagues (2006), it is based on four criteria – extent of thrombocytopenia, timing of drop in platelet count relative to heparin exposure, thrombosis, and possibility of other causes of thrombocytopenia. This scoring system generates a whole number between 0 and 8 whereby scores of 0-3, 4-5 and 6-8 constitute low, intermediate and high probability of HIT, respectively (Warkentin and Heddle, 2003, Lo et al., 2006). A comprehensive meta-analysis performed by Cuker and colleagues (2012) revealed the excellent negative predictive value (NPV) of a low 4Ts score at 0.998 and this performance was independent of the operator, HIT prevalence or patient population. The strong NPV associated with a low 4Ts score allows clinicians to confidently exclude HIT as a likely diagnosis and not pursue HIT laboratory testing. In contrast, patients assigned with intermediate or high 4Ts scores require further evaluation due to the modest positive predictive value (PPV) of 0.14 and 0.64, respectively, in a meta-analysis involving a combination of 3068 patients (Cuker et al., 2012).

Although 4Ts score is relatively easy to apply for initial evaluation of HIT likelihood, there are two main limitations of this scoring system. Firstly, significant variability between operators with different levels of experience often occurs whereby different scores were assigned to the

same patient, therefore undermining the aim of the tool to guide further testing (Cuker et al., 2010, Nagler et al., 2012, Pishko et al., 2018). Secondly, as a pre-test HIT probability test, this clinical tool does not assess the pathology of the disease. Indeed, the 4Ts score is limited by its low PPV and has a poor performance when used in the setting of critically ill patients (Crowther et al., 2014). Fiorenza and colleagues (2014) summarised that thrombocytopenia observed in more than a quarter of intensive care unit (ICU) patients can result from multiple causes including treatment with antibiotic and other therapeutics, inflammation, malignancy and use of invasive medical devices. The presence of other reasons for thrombocytopenia introduces ambiguity in 4Ts scores assigned to critically ill patients due to the fourth criteria of the scoring system. As ICU patients are likely to experience thrombocytopenia for reasons not related to HIT, they are more likely to be assigned with lower scores, which may exclude them from further testing.

To improve on some of the limitations of 4Ts score, the HIT Expert Probability (HEP) score was designed. A comparative study in a prospective cohort of 292 patients revealed comparable performance characteristics between HEP score and 4Ts score but HEP score performed better when evaluating critically ill patients (Pishko et al., 2018). Like the 4Ts score, HEP score lacks specificity as it evaluates pre-test probability of HIT and does not include clinical laboratory measures of a patient's platelet reactivity nor the presence of HIT antibody.

1.3.5.2 Immunoassays

Patients assigned with intermediate or high 4Ts scores are commonly referred for PF4/heparin antibody testing. Significant efforts have been expended to develop assays for the detection of HIT antibodies in patient plasma or serum samples. Most immunoassays utilise a solid phase, pre-bound with PF4 in complex with heparin or other polyanionic molecules to detect

HIT antibodies through an enzyme-linked immunosorbent assay (ELISA). This technique provides information on antibody titre based on optical density (OD) values. The commercial IgG-specific or polyspecific HIT ELISA (detects any of the three main Ig classes, IgG, IgA and IgM) are commonly used in HIT laboratories.

HIT immunoassays are known for their excellent sensitivity in informing on the presence of anti-PF4/heparin antibody. However, these immunoassays do not discriminate between pathological and non-platelet-activating antibodies. As such, the detection of anti-PF4/heparin antibodies in a thrombocytopenic patient does not equate to a diagnosis of HIT, consequently resulting in the high false positive rates associated with HIT immunoassays (Lo et al., 2007, Nazi et al., 2015). In addition, patient plasma or serum samples are batch analysed for PF4/heparin antibodies at reference laboratories meaning significant delays in acquisition of results. In reality, clinicians often rely only on clinical scoring systems and intuition for the timely and appropriate management of anticoagulation in patients suspected of HIT, without the benefit of serological tests due to the delay in obtaining results. Rapid immunoassays such as the particle gel immunoassay (PaGIA) and lateral flow immunoassay (LFIA) were developed to enable a rapid bedside assessment of HIT antibody levels. Andrews and colleagues (2013) reported the use of the particle immunofiltration anti-PF4 assay (PIFA), a polyspecific rapid immunoassay, in ICU patients. Unfortunately, these rapid assays also demonstrated unfavourable positive predictive values due to poor specificities for platelet-activating antibodies similar to the conventional HIT immunoassays (Sachs et al., 2011, Leroux et al., 2014). The automated chemiluminescence immunoassay (CLIA) demonstrated superior sensitivity and specificity compared to conventional HIT ELISA but false negative results have been reported (Nagler et al., 2016, Warkentin et al., 2018).

The lack of specificity of HIT immunoassays has fuelled researchers to develop new diagnostic assays. Recently, Wines and colleagues (2018) demonstrated the use of a unique recombinant dimeric FcγRIIa extracellular region, the receptor mediating platelet activation in HIT (Sections 1.2.4 and 1.3.2). When immobilised, the recombinant FcγRIIa bound pathogenic antibodies with high affinity when compared with clinically irrelevant, non-pathogenic antibodies. This ELISA demonstrated improved specificity over HIT immunoassays but requires further evaluation to explain the false negative data observed in some HIT-positive samples and needs to be validated in a larger cohort. Again, this technique does not inform on patient-specific platelet reactivity.

Following the generation of the HIT-like monoclonal antibody, KKO, immunoassays based on KKO inhibition have been developed. Latex particle-enhanced immunoturbidimetry assay (LIA) (Warkentin et al., 2017) and KKO-inhibition (KKO-I) ELISA (Cuker et al., 2013) detect anti-PF4/heparin antibodies that recognise the same or similar epitopes as KKO. Both assays demonstrate high negative predictive value while KKO-I showed improved sensitivity and specificity compared to the conventional HIT ELISA. The rapid turnaround time of the LIA is afforded by its fully automated sample testing procedure. However, false negative results were observed in both assays, potentially due to activating antibodies binding to epitopes which are distinct from the antigen recognition sites of KKO.

1.3.5.3 Functional assays to assess pathology in HIT samples

Functional assays are used to detect platelet-activating capacity of anti-PF4/heparin antibodies. High responding donor washed platelets in the presence of heparin and some cases, PF4, are used in platelet-activation assays. The serotonin-release assay (SRA) measures the ability of patient plasma or serum to liberate radioisotope-labelled serotonin from donor

platelets as a readout of platelet activation (Sheridan et al., 1986). The SRA is a confirmatory test for HIT diagnosis and is regarded as the gold standard owing to its high sensitivity and specificity of 100% and 97%, respectively (Warkentin et al., 2005). Also, SRA often generates binary results, that is, a clear negative or strong positive result with few borderline outcomes (Warkentin et al., 2008). However, the utility of SRA is limited by its requirement for high responding healthy donor platelets, radioactivity and an extended turnaround time. The SRA is also restricted to specialised laboratories as it is technically demanding to perform. These limitations make SRA less accessible to the majority of clinicians and the delay in obtaining SRA results means clinicians often must change patient anticoagulation without this information.

To circumvent the use of radioactivity, the heparin-induced platelet aggregation (HIPA) assay was developed (Chong et al., 1993a). Like the SRA, reactive donor platelets must be washed and mixed with patient plasma or serum to test for platelet-activation in the presence of low and high dose of heparin. HIPA demonstrated high specificity but was less sensitive than the SRA in a direct comparative study (Look et al., 1997). However, the HIPA is also technically challenging, not standardised and also run by batch analysis. The use of a cell-permeable viability dye to assess platelet activation based on fluorescence and cell size has also been explored in the platelet viability assay (PVA) (Ivetic et al., 2019). The utility of PVA in a prospective cohort remains to be validated. Additionally, its requirement for donor platelets precluded the assessment of patient platelet-specific factors.

Attempts have also been made to assess upregulation of the activation marker, P-selectin, to inform on platelet-activating antibodies in donor platelets. Padmanabhan and colleagues (2016a) reported that the PF4-dependent P-selectin expression assay (PEA) improved

diagnostic accuracy compared to the SRA based on an alternative definition of HIT which only considers the 4Ts score and HIT ELISA. The PEA also detected platelet-activating antibodies earlier than SRA whereby samples that were PEA-positive only gave a positive result by SRA 30 hours later, and could potentially be used to monitor treatment responses (Padmanabhan et al., 2016b). However, the requirement for exogenous PF4 and donor washed platelets may impede the implementation of this assay as a routine test in regular laboratories.

The utility of FcγRIIIa proteolysis for identifying platelet-activating antibodies has also been evaluated. Nazi and colleagues (2013) demonstrated that densitometry analysis of nitrocellulose membranes after Western blotting for FcγRIIIa proteolysis in donor platelets yielded comparable specificity to the SRA. Of note, this assay was able to resolve samples that were inconclusive by SRA. As with many functional assays, the requirement for donor washed platelets poses a significant barrier to obtaining results in a routine, standardised and timely manner. To circumvent the use of donor platelets, Cuker and colleagues (2013) developed a luciferase assay using an FcγRIIIa-expressing DT40 cell line to detect FcγRIIIa-dependent cellular activation triggered by PF4/heparin antibodies which presented favourable diagnostic characteristics. This cell line requires routine cell culturing for maintenance and is not widely available. Data gained using this cell line also included some false negative results in 5 of 21 HIT-positive patients. Further evaluation of this cell-line based assay is warranted.

Table 1.2 Strengths and limitations of available tools for HIT evaluation and diagnosis.

Tools	Basis of evaluation	Advantages	Limitations
Clinical scoring systems			
4Ts score	Clinical features	Bedside evaluation, high negative predictive value for low 4Ts score	Inter-operator variability, low positive predictive value, not optimised for critically ill patients (e.g., ICU)
HIT Expert Probability (HEP) score	Clinical features	Bedside evaluation, better accuracy than 4Ts score among less experienced clinicians, performed better than 4Ts score when assessing patients in ICU	Inter-operator variability, longer time to perform due to extensive clinical features to be considered
Immunoassays			
Polyspecific or IgG-specific HIT ELISA	Detection of anti-PF4/heparin antibodies	High sensitivity, low per-test cost	High false positive rate, time-consuming, no information on patient's platelet status, batched analysed, access restricted to HIT testing laboratories
Lateral flow immunoassay (LFIA) (IgG-specific)	Detection of anti-PF4/heparin antibodies	High negative predictive value, rapid turnaround, does not require specialised equipment or expert knowledge	High false positive rate, higher per-test cost than ELISA, no information on patient's platelet status
Particle gel immunoassay (PaGIA) (polyspecific)	Detection of anti-PF4/heparin antibodies	High negative predictive value, rapid turnaround, does not require specialised equipment or expert knowledge	High false positive rate, higher per-test cost than ELISA, no information on patient's platelet status
Latex particle-enhanced immunoturbidimetry	Detection of anti-PF4/heparin antibodies with the same or similar	High negative predictive value, fully-automated, rapid turnaround, interlaboratory reproducibility	Low positive predictive value, false negative results in 1 in 40 SRA-positive patients

Tools	Basis of evaluation	Advantages	Limitations
assay (LIA) (polyspecific)	antigen recognition site as KKO		
Particle immunofiltration anti- PF4 assay (PIFA) (polyspecific)	Detection of anti- PF4/heparin antibodies	High negative predictive value evaluated in critically ill patients, rapid turnaround	Low positive predictive value, high false positive rate
Chemiluminescence immunoassay (CLIA) (poly- and IgG-specific)	Detection of anti- PF4/heparin antibodies	Higher sensitivity and specificity than HIT ELISA, rapid turnaround, automated	False negative results in 4 in 166 HIT-positive patients
Dimeric recombinant soluble FcγRIIIa ectodomain ELISA (IgG- specific)	Detection of FcγRIIIa-binding anti-PF4/heparin antibodies	Higher specificity than HIT ELISA	Lower sensitivity than HIT ELISA, false negative results in 2 of 27 SRA-positive patients
KKO-inhibition (KKO-I) ELISA	Detection of anti- PF4/heparin antibodies with the same or similar antigen recognition site as KKO	Higher sensitivity and specificity than HIT ELISA	False negative results in 1 in 10 HIT-positive patients, further evaluation warranted
Functional assays			
Serotonin release assay (SRA)	Detection of platelet-activating antibodies	Excellent sensitivity and specificity	Require high responding donor platelets and radioisotope, slow turnaround, technically challenging, does not inform on patient's

Tools	Basis of evaluation	Advantages	Limitations
Heparin-induced platelet aggregation assay (HIPA)	Detection of platelet-activating antibodies	Excellent sensitivity and specificity, does not require radioactivity	platelet activity, only available at specialised laboratories Requirement for high responding donor platelets, slow turnaround, technically challenging, does not inform on patient's platelet activity, only available at specialised laboratories
FcγRIIa proteolysis	Detection of platelet FcγRIIa proteolysis	Able to resolve inconclusive SRA samples, comparable specificity to SRA, does not require radioactivity	Require donor platelets, slow turnaround, further evaluation in larger cohort warranted
PF4-dependent P-selectin expression assay (PEA)	Detection of platelet-activating antibodies	Able to detect platelet-activating antibodies earlier than SRA, higher diagnostic accuracy than SRA, less technically-demanding than SRA, does not require radioactivity, potential to monitor treatment response	Require donor platelets, slow turnaround
Platelet viability assay (PVA)	Detection of platelet-activating antibodies	High sensitivity and specificity, does not require radioactivity, uses cost-effective viability dye	Require donor platelets, further study in prospective cohort warranted
DT40-luciferase assay	Detection of FcγRIIa-dependent cellular activation	Higher sensitivity and specificity than HIT ELISA, does not require radioactivity and donor platelets	False negative results in 5 of 21 HIT-positive patients, further evaluation warranted

1.3.6 Genetic studies on HIT susceptibility

Despite the extensive use of heparin and high morbidity of HIT, there is a lack of clinical tools to evaluate HIT risk prior to heparin administration. The inability to predict HIT remains a liability associated with heparin therapy. Genomic biomarkers have the potential to be used prior to heparin administration as a screening tool to avoid or reduce the length of heparin administration in patients at risk of HIT. Additionally, such tools may be valuable in risk stratification to identify patients who would benefit from additional monitoring and early treatment to manage thrombotic complications. Genetic studies of HIT are commonly under-powered due to the scarcity of true HIT cases which is compounded by the patient- and drug-dependent factors that contribute to HIT risk, resulting in a small effect size. Consequently, efforts to assess the genetic susceptibility of HIT have been hampered by disparate associations, small cohort size, lack of replication cohorts and the varied definition of true HIT between studies (Karnes, 2018). Despite these limitations, several genetic loci that were significantly associated with HIT development, anti-PF4/heparin antibody production and/or occurrence of HIT with thrombosis (HITT) have been described.

A genome-wide association study (GWAS) of HIT patients and heparinised controls by Karnes and colleagues (2015) identified two variants within the T cell death associated gene 8, *TDAG8* (*GPR65*) which encodes a G-protein-coupled receptor that has been implicated in T cell malignancies (Justus et al., 2017). These SNPs, rs1887289 (intronic region) and rs3742704 (I231L missense mutation), were associated with antibody production in 67 HIT cases compared to 884 matched, heparin-treated controls. However, functional assays were not performed to confirm the HIT cases. Nevertheless, this association of *TDAG8* SNPs may suggest a role for T cells in modulating HIT susceptibility. A recent GWAS study in 96 HIPA-

positive HIT cases uncovered a HIT susceptibility locus located in a non-coding intergenic region in chromosome 5 (Witten et al., 2018). The lack of functional annotation makes it challenging to hypothesize on the clinical implications of this variant.

Most studies on the genetic risk factors for HIT focused on immune-related genes generally thought to play a role in HIT pathogenesis, such as the Fc γ receptors as reviewed by Rollin and colleagues (2016). Several studies investigated the impact of a functional variant, H131R (rs1801274), within *FCGR2A*, which has been associated with multiple autoimmune conditions (see Section 1.2.4). The H131R polymorphism yielded inconsistent association with HIT. Four studies noted an overrepresentation of the H131 variant among patients with HIT (Burgess et al., 1995, Brandt et al., 1995, Denomme et al., 1997, Bachelot-Loza et al., 1998). In contrast, Carlsson and colleagues (1998) reported an association between the R131 variant and HIT in a large cohort of 389 patients who developed HIT. However, two studies found no association between the H131R polymorphism and HIT susceptibility (Arepally et al., 1997, Rollin et al., 2015). The variation in cohort sizes, definition of HIT-positivity and patient populations may account for the inconsistencies observed.

Three polymorphic sites within the *PTPRJ* gene which encodes the CD148 membrane protein tyrosine phosphatase, were implicated with HIT susceptibility. These non-synonymous substitutions (Q276P, R326Q, D872E) were associated with anti-PF4/heparin antibody levels and HIT development (Rollin et al., 2012). Of note, the P276 and Q326 variants were found to associate with lower risk of HIT and their expression resulted in reduced reactivity to anti-PF4/heparin antibodies with delayed phosphorylation of LAT and PLC γ 2 within the intracellular signalling cascade.

Although the R131 allele was not associated with HIT development, this variant was overrepresented among HIT patients who developed thrombosis within the same cohort, suggesting that R131 may be a risk allele for thrombosis (Rollin et al., 2015). In addition, the PIA2 polymorphism within *ITGB3* which encodes a platelet integrin, was also associated with HIT in a cohort of 66 HIT patients (Harris et al., 2008). Interestingly, the combination of the three SNPs, *FCGR2A* H131R, *ITGB3* PIA2 and *PECAM1* L125V, resulted in a significant association with HIT (Pamela et al., 2013). *PECAM1* encodes the platelet and endothelial cell adhesion molecule while *ITGB3* encodes the β_3 subunit of platelet $\alpha_{IIb}\beta_3$ integrin. The associations observed with *PECAM1* and *ITGB3* SNPs highlight the contributing role of platelet receptors in HIT.

1.4 Platelet receptor shedding (GPVI shedding)

1.4.1 Focusing on GPVI shedding

Platelet receptors play a central role in mediating platelet functions. Ectodomain shedding of platelet surface molecules is a common phenomenon. Platelets have been reported to release 69 distinct membrane proteins upon treatment with protein kinase C activator, phorbol myristate acetate (PMA) which results in activation of metalloproteinases (Fong et al., 2011). Metalloproteolytic shedding of GPVI has been extensively investigated over the past 15 years since the first report on GPVI release in human platelets by Gardiner and colleagues (2004).

The extracellular domain of GPVI is irreversibly cleaved under conditions where GPVI is exposed to its ligands (Gardiner et al., 2004, Montague et al., 2018), pathophysiological shear stress (Al-Tamimi et al., 2012b), anti-GPVI antibodies (Al-Tamimi et al., 2009b, Rabbolini et al., 2017), FcγRIIa-engaging anti-platelet antibodies from patients with HIT and ITP (Gardiner et al., 2008b, Gardiner et al., 2008a, Boylan et al., 2004) or active coagulation factors (Al-Tamimi et al., 2011b). Cleavage of GPVI within an extracellular juxtamembrane region generates plasma soluble GPVI (sGPVI) which is a ~50-55 kDa fragment of the GPVI ectodomain, leaving behind a ~10 kDa membrane-associated cytoplasmic domain. In addition to physiological triggers, GPVI release can also be induced with experimental reagents. Treatment with PMA (Gardiner et al., 2007, Al-Tamimi et al., 2011b), calmodulin inhibitor (W7) (Gardiner et al., 2004), a generic activator of metalloproteinases N-ethylmaleimide (NEM) (Al-Tamimi et al., 2009a), mitochondrial aging reagents such as carbonyl cyanide m-chlorophenyl hydrazine (CCCP) (Gardiner et al., 2007), Bcl-2 homology 3 (BH3) mimetics (ABT-737, ABT-263) (Schoenwaelder et al., 2011), calcium ionophore (A23187) (Montague et al., 2018, Howes et

al., 2018) and the snake venom protein convulxin (Gardiner et al., 2004, Stephens et al., 2005) have been shown to induce GPVI release.

Shedding of GPVI may represent a mechanism by which platelets alter surface reactivity by changing receptor density, thereby modifying thrombotic propensity. GPVI surface density can modulate GPVI dimerization (Arthur et al., 2007, Jung et al., 2012, Loyau et al., 2012) which influences GPVI signalling (Sarratt et al., 2005, Poulter et al., 2017) and hence, may dictate platelet activation and function. In addition, GPVI facilitates thrombin generation on the surface of platelets and contributes to thrombus stability through interactions with fibrin (Lecut et al., 2005, Alshehri et al., 2015, Mammadova-Bach et al., 2015).

Under pathologic conditions involving vascular injury or exposure to agonists, GPVI proteolysis may protect from thrombosis. For example, GPVI-deficient platelets demonstrated impaired thrombus formation on collagen and reduced adhesion on VWF under flow conditions (Goto et al., 2002). Additionally, released sGPVI may have anti-thrombotic properties as a soluble, dimeric form of the extracellular domain of GPVI inhibited platelet adhesion to collagen under flow and dramatically diminished platelet recruitment and thrombus formation in mice following vascular injury *in vivo* (Massberg et al., 2004). Taken together, loss of the ligand-binding ectodomain of GPVI leads to significant perturbations on the haemostatic and thrombotic functions of platelets.

1.4.2 Soluble GPVI as a marker of platelet activation

Although GPVI expression varies significantly among healthy individuals (Best et al., 2003, Samaha et al., 2004), the resting level on circulating platelets remains stable with negligible signs of shedding (Gardiner et al., 2007). Hence, proteolytic shedding of GPVI following platelet activation supports the utility of increased sGPVI levels as an indicator of pathology.

Since the development of an ELISA to measure sGPVI (Al-Tamimi et al., 2009a), plasma sGPVI has emerged as a platelet-specific activation marker in various clinical settings including stroke (Al-Tamimi et al., 2011a, Wurster et al., 2013), disseminated intravascular coagulation (Al-Tamimi et al., 2011b), acute myocardial infarction (Bigalke et al., 2008, Bigalke et al., 2011), sepsis (Montague et al., 2018) and in people exposed to mechanical circulatory support (Lukito et al., 2016).

Additionally, plasma sGPVI holds the potential to inform on the state of platelet activity over time. For example, the temporal profile of plasma sGPVI monitored for up to 12 months in a cohort of trauma patients demonstrated a rise in plasma sGPVI levels with sepsis onset following thermal injury which gradually tapered off to levels comparable to healthy donors within two months post-injury (Montague et al., 2018). Furthermore, cynomolgus monkeys injected with a recombinant fragment of human GPVI ectodomain closely tracked platelet activation following lipopolysaccharide injection and returned to baseline levels 24 hours later (Naitoh et al., 2015). As such, plasma sGPVI may be valuable in monitoring disease progressions or treatment responses. These observations highlight the potential value of sGPVI as a diagnostic and/or prognostic marker in the setting of thrombocoagulopathy.

1.4.3 Mechanisms regulating GPVI shedding – the biological scissors

Proteolysis in the vasculature is commonly mediated by metalloproteinases which form a diverse class of catalytic enzymes (Gardiner, 2018). The A-Disintegrin-And-Metalloproteinase (ADAM) family of metalloproteinases comprise more than 40 members. Metalloproteinases that are expressed on the platelet membrane include ADAM10 and ADAM17. Human GPVI is cleaved primarily by ADAM10 (Gardiner et al., 2007). The structure of ADAM10 consists of an N-terminal pro-domain followed by a catalytic, a disintegrin and a cysteine-rich domain

connected to a single pass transmembrane domain and a short cytoplasmic tail (Dreymueller et al., 2012). The pro-domain acts as an autoinhibitor and keeps the enzyme in its dormant form via a cysteine switch mechanism (Van Wart and Birkedal-Hansen, 1990). Proteolytic activity is inhibited through coordination of the zinc ion in the catalytic site by a cysteine residue within the pro-domain. Through post-translational processing in the Golgi apparatus, the pro-domain is removed from immature ADAM10 before being trafficked to the cell surface as catalytically active proteins (Giebeler and Zigrino, 2016). Facey and colleagues (2016) demonstrated the constitutive presence of mature ADAM10 with detectable proteolytic activity on the resting platelet surface. The crystal structure of the ADAM10 ectodomain revealed that the C-terminal cysteine-rich domain partially blocked the metalloproteinase active site in the mature protein from accessing its substrate (Seegar et al., 2017). This observation suggests the presence of a control mechanism for modulating mature ADAM10 activity on the membrane.

The ADAM responsible for GPVI shedding in mice is dependent on the stimulants used to trigger GPVI release. While ADAM10 was the predominant enzyme for shedding of GPVI induced by calmodulin inhibition, ADAM17 was the major GPVI sheddase when platelets were stimulated with the mitochondrial aging reagent, CCCP (Bender et al., 2010). Interestingly, anti-GPVI antibody-induced release of GPVI did not appear to involve either ADAM10 or ADAM17 when examined in mice lacking functional ADAM10 and ADAM17 (Bender et al., 2010), alluding to the possibility for a third protease capable of cleaving GPVI *in vivo*. A recent study by Baaten and colleagues (2018) revealed the existence of platelet heterogeneity in terms of receptor shedding following activation. Platelet thrombi formed by perfusing whole blood over collagen-coated chambers showed selective loss of GPVI (and GPIb α) that was dependent on phosphatidylserine-exposure. It is important to understand the molecular

regulation of GPVI surface density which may lead to novel therapeutic avenues to control platelet activity considering the recent evidence that loss of surface glycoprotein transitioned platelets from a less adhesive to a more procoagulant state (Baaten et al., 2018).

1.5 Regulation of platelet ADAM10 activity

The molecular mechanism governing receptor levels on platelets remains to be fully understood. As the process is commonly mediated by metalloproteinases, control of receptor proteolysis could be via direct inhibition of the catalytic process or by modulating substrate availability.

1.5.1 Tetraspanins

ADAMs metalloproteolytic activity is modulated by tetraspanins at the membrane surface. Tetraspanins comprise a family of 33 evolutionarily conserved proteins with four transmembrane domains that can assemble at the cell surface with one another or with non-tetraspanin partner proteins into microclusters of tetraspanin-rich regions in the membrane (Charrin et al., 2014). Tetraspanins have been reported to associate with ADAM10 (Dornier et al., 2012, Noy et al., 2016, Matthews et al., 2017), platelet integrin $\alpha_{IIb}\beta_3$ (Lau et al., 2004) and members of the immunoglobulin-like superfamily (Charrin et al., 2014).

There is a growing body of evidence to suggest that ADAM10 functional pleiotropy is achievable via interaction with tetraspanins mainly from the TspanC8 subgroup which interact with the disintegrin and cysteine-rich domains of the biological scissors (Noy et al., 2016). ADAM10 activity and substrate selectivity appear to be regulated by the tetraspanin to which the metalloproteinase is partnered (Matthews et al., 2017). Of note, overexpression of Tspan14, a member of the TspanC8 subfamily present in human platelets, suppressed

ADAM10 proteolytic activity (Noy et al., 2016). This effect was not observed with other TspanC8 members. The authors hypothesised that Tspan14 elicits a protective effect on GPVI under resting conditions but may be triggered to promote shedding upon platelet activation, in line with the observation that GPVI levels are stable on resting platelets but rapidly cleaved when stimulated (Section 1.4.1). This functional interaction between Tspan14 and ADAM10 may provide a therapeutic avenue to target platelet ADAM10 activity to modulate thrombotic risk in patients.

1.5.2 Substrate accessibility

There is a plethora of substrates that can be cleaved by both ADAM10 and ADAM17 as well as other ADAM family members (Matthews et al., 2017). Identification of a consensus sequence of amino acids recognised by these proteases remains challenging although proteolysis often occurs within the substrate at a specific distance above the surface of a membrane. The regulation and substrate specificity of ADAMs may also be determined by the conformation of the substrate to allow optimal positioning of the metalloproteolytic domain for effective cleavage as demonstrated by Janes and colleagues (2009). Spatial distribution determined by substrate density may influence the rate of ADAM10 activity. Substrate accessibility may also be modulated by ligand binding or membrane disruption and rearrangement of microdomains by exposure to fluid shear stress as observed for the GPVI receptor. Exposure to elevated shear stress in the absence of any ligands can trigger ADAM10-mediated shedding of GPVI on platelets without changing the activity of ADAM10 implying that shear stress has altered the accessibility of ADAM10 for GPVI (Facey et al., 2016).

1.5.3 Tissue inhibitors of metalloproteinases – rocks to the biological scissors

Tissue inhibitors of metalloproteinases (TIMPs) are the endogenous inhibitors of ADAMs and matrix metalloproteinases (MMPs). TIMPs play critical roles in regulating extracellular matrix turnover, tissue remodelling and cellular behaviour by controlling metalloproteolytic activity in the vasculature (Brew and Nagase, 2010). In humans, there are four known TIMP isoforms with about 40% identity in their 184-194 amino acid sequences and each have different N- and C-terminal domains (Murphy, 2011). These small (22-28 kDa), secreted molecules are present in the plasma and interstitial spaces, and are widely expressed by many cell types. TIMPs possess some redundancy in their abilities to inhibit specific ADAMs (Table 1.3).

Megakaryocytes and platelets carry messenger RNA transcripts and detectable protein levels of all four TIMPs (Villeneuve et al., 2009). In resting platelets, TIMP1, 2 and 4 are detected in sub-membranous structures separate from the α -granules with minimal colocalization while TIMP3 appeared in α -granules. Interestingly, the levels of all four TIMPs increased in the supernatant of thrombin-stimulated platelets. Activation of platelets by PMA resulted in release of all TIMPs except for TIMP4 which was not detectable by mass spectroscopy (Fong et al., 2011). TIMP4 may be expressed at low levels as a platelet proteomics study detected all other TIMPs but not TIMP4 (Burkhart et al., 2012).

Crystallographic studies revealed that the N-terminal of human TIMPs interact with the catalytic domain of metalloproteinases in a 1:1 stoichiometry and inhibit proteolytic activity by chelating the divalent cation Zn^{2+} in the enzyme active site (Murphy, 2011, Khokha et al., 2013). ADAM10 is inhibited by TIMP1 and TIMP3 although TIMP1 exhibited a much lower inhibition constant (Amour et al., 2000). A functional role for TIMPs on platelet ADAMs involved in modulating platelet receptor levels remain to be explored. As shown in the

literature, TIMPs exert an inhibitory effect on ADAMs by engaging the ADAM catalytic site and sterically hindering access by the substrate. Plasma-borne and platelet-associated TIMPs may inhibit the catalytically active ADAM10 on resting platelets and contribute in stabilising GPVI levels observed on resting platelets. TIMPs may act as rocks that inhibit the biological scissors, i.e. ADAMs, on platelets.

Table 1.3 Properties of human TIMPs with a focus on platelet-associated characteristics.

Properties	TIMP1	TIMP2	TIMP3	TIMP4	References
Amino acid residues	184	194	188	194	Murphy (2011)
Molecular weight (kDa)	28	22	24	22	Murphy (2011)
ADAM inhibition	ADAM10	ADAM12	ADAM10, ADAM12, ADAM17, ADAM19, ADAM33, ADAMTS1, ADAMTS4, ADAMTS5, ADAMTS2 (weak)	ADAM17, ADAM28	Murphy (2011)
Present in plasma/serum	Yes	Yes	Yes	Yes	Murate et al. (1997), Hendrickson et al. (2018), Sandrim et al. (2018)
Platelet localisation (by immunofluorescence)	Sub-membranous	Sub-membranous	α -granules	Sub-membranous	Villeneuve et al. (2009)
Platelet proteome (by mass spectrometry)	Yes	Yes	Yes	Not detected	Burkhart et al. (2012)
Thrombin-activated platelets (by Western blot)	All four TIMPs were upregulated in platelet supernatant				Villeneuve et al. (2009)
PMA-activated platelets (by mass spectrometry)	Released	Released	Released	Not detected	Fong et al. (2011)
Cellular expression	Fibroblasts, osteoblasts, endothelial cells, granulosa cells, dendritic cells, vascular smooth muscle cells, adipocytes, monocytes, platelets/megakaryocytes				Gresele et al. (2017)

1.6 Aims of thesis

Platelets express a myriad of receptors which facilitate a range of platelet functions. Human GPVI is rapidly metalloproteolysed by ADAM10 to release sGPVI upon platelet activation including in the presence of anti-platelet PF4/heparin antibodies in the setting of HIT. However, the mechanistic basis of GPVI shedding induced by platelet-activating HIT antibodies and the utility of sGPVI as a diagnostic marker of platelet activation in HIT remain to be characterised. In addition, the haplotype contribution of FcγRIIa which mediates HIT pathology has yet to be explored in allelic phase. While platelet receptor shedding has been studied extensively, regulation of platelet ADAM10 metalloproteolytic activity is not well understood. TIMPs regulate vascular metalloproteinase activity but a role in controlling platelet ADAMs has not been described.

This thesis aimed to (i) characterise changes in platelet surface GPVI and sGPVI levels upon engagement of FcγRIIa by HIT immune complexes; (ii) assess the utility of patient plasma sGPVI to inform on the presence of pathological anti-PF4/heparin antibodies using a prospective clinical cohort at risk of developing HIT; (iii) evaluate the haplotype-based association between FcγRIIa polymorphisms, Q27W and H131R, and HIT susceptibility using a long-read sequencing technology; and (iv) explore the level of TIMPs on platelets and the role of TIMPs in regulating platelet ADAM10 activity.

Chapter 2 Materials and Methods

2.1 Materials

2.1.1 Agonists and inhibitors

Platelet agonists and inhibitors used in the course of this thesis are detailed in Table 2.1 and Table 2.2, respectively.

Table 2.1 Platelet agonists

Reagents	Source
Adenosine 5'-diphosphate (ADP)	Sigma-Aldrich (St. Louis, Missouri, USA), catalogue # 01905-1G-F
Collagen (Kollagenreagens Horm suspension)	Takeda Austria (Linz, Austria), catalogue # 1130630
Collagen-related peptide (CRP), GCO-[GPO]10-GCOG-NH ₂	Auspep (Tullamarine, Victoria, Australia)
Convulxin (CVX)	Enzo Life Sciences (Farmingdale, New York, USA), catalogue # ALX-350-100-C050, lot # 06061870
Crosslinked collagen-related peptide (CRP-xL)	CRP crosslinked in-house using succinimidyl 3-(2-pyridyldithio)propionate (SPDP)
Protease-activated receptor 1 (PAR-1) agonist (SFLLRN) (TRAP-6)	Tocris Bioscience (Bristol, United Kingdom), catalogue # 3497
Thrombin from bovine plasma	Sigma-Aldrich (St. Louis, Missouri, USA), catalogue # T4648

Table 2.2 Inhibitors

Reagents	Source
Bay 61-3606 hydrochloride (Syk kinase inhibitor)	Abcam (Cambridge, United Kingdom), catalogue # ab142106
Complete protease inhibitor cocktail (EDTA-free)	Roche, Sigma-Aldrich (St. Louis, Missouri, USA), catalogue # 11873580001
GI 254023X (Selective ADAM10 inhibitor)	Tocris Bioscience (Bristol, United Kingdom), catalogue # 3995
GM 6001, Ilomastat (broad spectrum metalloproteinase inhibitor)	Tocris Bioscience (Bristol, United Kingdom), catalogue # 2983
GRGDSP peptide ($\alpha_{IIb}\beta_3$ blocker)	Calbiochem, Merck (Kenilworth, New Jersey, USA), catalogue # 03-34-0035
PP1 (Src family kinase inhibitor)	Tocris Bioscience (Bristol, United Kingdom), catalogue # 1397
Soybean trypsin inhibitor	Sigma-Aldrich (St. Louis, Missouri, USA), catalogue # T9128

2.1.2 Antibodies

Details of antibodies used in this thesis are as listed in Table 2.3.

Table 2.3 Antibodies used and their applications

Antibodies	Applications	Source
1A12 anti-human GPVI mouse monoclonal	ELISA	Dr Robert Andrews (Melbourne, Australia)
1G5 anti-human GPVI mouse monoclonal	Flow cytometry	Purified in-house from supernatant of hybridoma
1G5 anti-human GPVI mouse monoclonal (Alexa Fluor 488)	Flow cytometry	Conjugated in-house
AK4 anti-CD62P mouse monoclonal (Phycoerythrin, PE)	Flow cytometry	Abcam (Cambridge, United Kingdom), catalogue # ab119104
Anti-human GPVI extracellular domain rabbit polyclonal	ELISA	Dr Robert Andrews (Melbourne, Australia)
Anti-human TIMP1 rabbit polyclonal	Flow cytometry, Western blot	Abcam (Cambridge, United Kingdom), catalogue # ab61224
Anti-human TIMP2 rabbit polyclonal	Flow cytometry, Western blot	Abcam (Cambridge, United Kingdom), catalogue # ab180630
Anti-human TIMP3 rabbit polyclonal	Flow cytometry, Western blot	Abcam (Cambridge, United Kingdom), catalogue # ab39184
Anti-human TIMP4 rabbit polyclonal	Flow cytometry, Western blot	Abcam (Cambridge, United Kingdom), catalogue # ab58425
Goat anti-mouse IgG (Alexa Fluor 488)	Flow cytometry	Abcam (Cambridge, United Kingdom), catalogue # ab150113
Goat anti-rabbit IgG (Alexa Fluor 488)	Flow cytometry	Abcam (Cambridge, United Kingdom), catalogue # ab150077
HRP-conjugated rabbit anti-mouse polyclonal (affinity-adsorbed against human and bovine Ig)	ELISA	Dako (Agilent Technologies, Santa Clara, California, USA), catalogue # P026002-2
IV.3 anti-human FcγRIIa (CD32) mouse monoclonal	FcγRIIa function blocker	Dr Robert Andrews (Melbourne, Australia)
KKO anti-PF4 mouse monoclonal	GPVI-shedding	Invitrogen, Thermo Fisher Scientific (Waltham, Massachusetts, USA), catalogue # MA5-17641

2.1.3 Recombinant proteins

Recombinant proteins used in the course of this thesis are listed in Table 2.4.

Table 2.4 Recombinant proteins

Recombinant proteins	Source
Recombinant human ADAM10	R&D Systems (Minneapolis, Minnesota, USA), catalogue # 936AD020
Recombinant human ADAM17	R&D Systems (Minneapolis, Minnesota, USA), catalogue # 930ADB010
Recombinant human TIMP1	R&D Systems (Minneapolis, Minnesota, USA), catalogue # 970TM010
Recombinant human TIMP2	R&D Systems (Minneapolis, Minnesota, USA), catalogue # 971TM010
Recombinant human TIMP3	R&D Systems (Minneapolis, Minnesota, USA), catalogue # 973TM010
Recombinant human TIMP4	R&D Systems (Minneapolis, Minnesota, USA), catalogue # 974TSF010

2.1.4 DNA primers

Primer sequences used in the course of this thesis are listed in Table 2.5. All primers were sourced from Sigma-Aldrich (St. Louis, Missouri, USA).

Table 2.5 DNA primer sequences

Label	Primer sequences	Application	Purification method
<i>FCGR2A_H131R_F</i>	5'-GGAAAATCCCAGAAATTCTCGC	Forward primer for <i>FCGR2A_H131R</i> amplification for RFLP analysis (Jiang et al., 1996)	Desalt
<i>FCGR2A_H131R_R</i>	5'-CAACAGCCTGACTACCTATTACGCGGG	Reverse primer for <i>FCGR2A_H131R</i> amplification for RFLP analysis (Jiang et al., 1996); sequencing primer for Sanger sequencing validation	Desalt
<i>F_FCGR2A_27_131</i>	5'-[AmC6]- gcagtcgaacatgtagctgactcaggtcacTCCTTTGGCATCCACAGTCC	Forward primer for long range amplification of <i>FCGR2A</i> spanning exons 3 and 4, containing universal tagging sequence (in lowercase) for subsequent barcoding and 5' amino block. Template-specific sequence in uppercase.	HPLC
<i>R_FCGR2A_27_131</i>	5'-[AmC6]- tggatcactgtgcaagcatcacatcgtagCAACAGCCTGACTACCTATTAC	Reverse primer for long range amplification of <i>FCGR2A</i> spanning exons 3 and 4, containing universal tagging sequence (in lowercase) for subsequent barcoding and 5' amino block. Template-specific sequence in uppercase.	HPLC
<i>Q27W_F</i>	5'-TCCTTTGGCATCCACAGTCC	Sequencing primer for Sanger sequencing validation	Desalt

2.1.5 Barcode sequences used in phased haplotyping assay

The 16-base barcode sequence contained within each primer pair in the Barcoded Universal F/R Primers Plate-96 (Pacific Biosciences, Menlo Park, California, USA) are listed below:

Table 2.6 List of 16-base barcode sequences used to discriminate samples from each individual in phased haplotyping assay

Well ID	Barcode ID	Barcode Sequence
A1	lbc001	TCAGACGATGCGTCAT
B1	lbc002	CTATACATGACTCTGC
C1	lbc003	TACTAGAGTAGCACTC
D1	lbc004	TGTGTATCAGTACATG
E1	lbc005	ACACGCATGACACACT
F1	lbc006	GATCTCTACTATATGC
G1	lbc007	ACAGTCTATACTGCTG
H1	lbc008	ATGATGTGCTACATCT
A2	lbc009	CTGCGTGCTCTACGAC
B2	lbc010	GCGCGATACGATGACT
C2	lbc011	CGCGCTCAGCTGATCG
D2	lbc012	GCGCACGCACTACAGA
E2	lbc013	AACTGACGTCGCGAC
F2	lbc014	CGTCTATATACGTATA
G2	lbc015	ATAGAGACTCAGAGCT
H2	lbc016	TAGATGCGAGAGTAGA
A3	lbc017	CATAGCGACTATCGTG
B3	lbc018	CATCACTACGCTAGAT
C3	lbc019	CGCATCTGTGCATGCA
D3	lbc020	TATGTGATCGTCTCTC
E3	lbc021	GTACACGCTGTGACTA
F3	lbc022	CGTGTCGCGCATATCT
G3	lbc023	ATATCAGTCATGCATA
H3	lbc024	GAGATCGACAGTCTCG
A4	lbc025	CACGCACACACGCGCG

Well ID	Barcode ID	Barcode Sequence
B4	lbc026	CGAGCACGCGCGTGTG
C4	lbc027	GTAGTCTCGCACAGAT
D4	lbc028	GAGACTCTGTGCGCGT
E4	lbc029	GCTCGACTGTGAGAGA
F4	lbc030	AGAGATGTGTGATGAC
G4	lbc031	TACGACTACATATCAG
H4	lbc032	TATCTCTGTAGAGTCT
A5	lbc033	AGAGAGAGACATGCGC
B5	lbc034	ACTCTCGCTCTGTAGA
C5	lbc035	TCTATGTCTCAGTAGT
D5	lbc036	GCGTATATCTCATGCG
E5	lbc037	GTGCGTATGTCGCTAC
F5	lbc038	TGCTCGCAGTATCACA
G5	lbc039	CTGTGTGTGATAGAGT
H5	lbc040	CAGTGAGAGCGCGATA
A6	lbc041	GTACATATGCGTCTGT
B6	lbc042	GAGACTAGAGATAGTG
C6	lbc043	TACGCGTGTACGCAGA
D6	lbc044	TGTCACTCATCTGAGT
E6	lbc045	GCACATACACGCTCAC
F6	lbc046	GCTCGTCGCGCGCACA
G6	lbc047	ACAGTGCGCTGTCTAT
H6	lbc048	TCACACTCTAGAGCGA
A7	lbc049	TCACATATGTATACAT
B7	lbc050	CGCTGCGAGAGACAGT
C7	lbc051	ACACACAGACTGTGAG
D7	lbc052	GCAGACTCTCACACGC
E7	lbc053	TGCTCTCGTGTACTGT
F7	lbc054	GTGTGAGATATATATC
G7	lbc055	CTCAGTGTGACACATG
H7	lbc056	TGCGAGCGACTCTATC
A8	lbc057	GTCAGCTAGTGTGAGC

Well ID	Barcode ID	Barcode Sequence
B8	lbc058	AGATATCATCAGCGAG
C8	lbc059	GTGCAGTGATCGATGA
D8	lbc060	TGACTCGCTCATAGTC
E8	lbc061	ATGCTGATGACGCGCT
F8	lbc062	GACAGCATCTGCGCTC
G8	lbc063	AGCGTCTGACGTGAGT
H8	lbc064	TCGATATACGACGTGC
A9	lbc065	TCGTCATACGCTCTAG
B9	lbc066	CGACTACGTACAGTAG
C9	lbc067	GCGTAGACAGACTACA
D9	lbc068	ACAGTATGATGTACTC
E9	lbc069	GTCTGATAGATACAGA
F9	lbc070	CTGCGCAGTACGTGCA
G9	lbc071	TAGATCTCTGACTCAC
H9	lbc072	CTGATGCGCGCTGTAC
A10	lbc073	CACTCGTGACGATGC
B10	lbc074	TGACAGTATCACAGTG
C10	lbc075	GAGATACGCTGCAGTC
D10	lbc076	ACGTGAGCTCACTCGC
E10	lbc077	ATAGAGAGTGTCTCAG
F10	lbc078	CATAGAGAGATAGTAT
G10	lbc079	ATCTCGAGATGTAGCG
H10	lbc080	ACGATCACTCGTGTCA
A11	lbc081	GATCGACTCGAGCATC
B11	lbc082	ATGCTCACTACTACAT
C11	lbc083	CGTGACATCTATAGC
D11	lbc084	GACTGCACATGCACGA
E11	lbc085	TATGACTAGTGTACTA
F11	lbc086	GACGTGTCGTAGATAT
G11	lbc087	ATAGCGACGCGATATA
H11	lbc088	ATCGCTGTGTCTATAG
A12	lbc089	TCTCACTGATAGCGTG

Well ID	Barcode ID	Barcode Sequence
B12	lbc090	TGTCGTCTATCATGTA
C12	lbc091	CACACGAGATCTCATC
D12	lbc092	AGATACACATGATACT
E12	lbc093	CGTGAGTAGTCAGACG
F12	lbc094	TCTCGACTGCACATAT
G12	lbc095	TGAGTGACGTGTAGCG
H12	lbc096	GTGTGCACTCACACTC

2.1.6 Commercial kits

Commercial kits used in the course of this thesis are listed in Table 2.7.

Table 2.7 Commercial kits

Kits	Source
Alexa Fluor 488 Antibody Labeling Kit	Invitrogen, Thermo Fisher Scientific (Waltham, Massachusetts, USA), catalogue # A20181
GeneJET PCR Purification Kit	Thermo Fisher Scientific (Waltham, Massachusetts, USA), catalogue # K0702
Human TIMP Magnetic Luminex Performance Assay 4-plex Kit	R&D Systems (Minneapolis, Minnesota, USA), catalogue # LKTM003
Qubit 1X dsDNA HS (High-Sensitivity) Assay Kit	Invitrogen, Thermo Fisher Scientific (Waltham, Massachusetts, USA), catalogue # Q33231
Sequel Sequencing Kit v2.1	Pacific Biosciences (Menlo Park, California, USA), catalogue # 101-310-500
SMRTbell Template Preparation Kit 1.0 SPv3	Pacific Biosciences (Menlo Park, California, USA), catalogue # 100-259-100

2.1.7 Other reagents

Other reagents used in the course of this thesis are listed in Table 2.8.

Table 2.8 Other reagents

Reagents	Source
1 Kb Plus DNA ladder	Invitrogen, Thermo Fisher Scientific (Waltham, Massachusetts, USA), catalogue # 10787018
2-mercaptoethanol	Sigma-Aldrich (St. Louis, Missouri, USA), catalogue # M3148
Acrylamide	Sigma-Aldrich (St. Louis, Missouri, USA), catalogue # A8887
Amersham ECL rainbow marker – full range	GE Healthcare (Chicago, Illinois, USA), catalogue # RPN800E
Ammonium persulphate (APS)	VWR (Radnor, Pennsylvania, USA), catalogue # 0486-100G
Bovine serum albumin (BSA)	Sigma-Aldrich (St. Louis, Missouri, USA), catalogue # A1933
Brij-35 (Brij L23 solution)	Sigma-Aldrich (St. Louis, Missouri, USA), catalogue # B4184
Brilliant Blue R	Sigma-Aldrich (St. Louis, Missouri, USA), catalogue # B7920
Bromophenol blue	Schwarz/Mann Biotech (Cleveland, Ohio, USA), catalogue # 805732
<i>Bst</i> UI restriction enzyme	New England Biolabs (Ipswich, Massachusetts, USA), catalogue # R0518L
Calcium chloride dihydrate, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	AnalaR, BDH (Kilsyth, Victoria, Australia), catalogue # 10070
Carbonate-bicarbonate buffer capsule	Sigma-Aldrich (St. Louis, Missouri, USA), catalogue # C3041
Cell Lysis Solution	Qiagen (Hilden, Germany), catalogue # 158906
Chloroform:isoamyl alcohol 24:1	BioUltra, Fluka (Seelze, Germany), catalogue # 25666
Citric acid	AnalaR, BDH Chemicals (Port Fairy, Victoria, Australia), catalogue # 10081
Color Prestained Protein Standard, Broad Range (11–245 kDa)	New England Biolabs (Ipswich, Massachusetts, USA), catalogue # P7712S

Reagents	Source
CutSmart buffer	New England Biolabs (Ipswich, Massachusetts, USA), catalogue # R0518L
Deoxynucleotide (dNTP) solution mix	New England Biolabs (Ipswich, Massachusetts, USA), catalogue # N0447S
D-glucose, C ₆ H ₁₂ O ₆	Univar, Ajax Finechem (Taren Point, New South Wales, Australia), catalogue # 783
Dimethyl sulphoxide (DMSO)	Sigma-Aldrich (St. Louis, Missouri, USA), catalogue # D2650
di-Sodium hydrogen orthophosphate, Na ₂ HPO ₄	Univar, Ajax Finechem (Taren Point, New South Wales, Australia), catalogue # 621
DL-Dithiothreitol (DTT)	Sigma-Aldrich (St. Louis, Missouri, USA), catalogue # D0632
DNA Hydration Solution	Qiagen (Hilden, Germany), catalogue # 158914
Ethanol, absolute	Univar, Ajax Finechem (Taren Point, New South Wales, Australia), catalogue # AJA214
Ethyl alcohol denatured	Fisher Scientific (Hampton, New Hampshire, USA), catalogue # A407-4
Ethyl alcohol, pure	Sigma-Aldrich (St. Louis, Missouri, USA), catalogue # 459844
Ethylenediaminetetraacetic acid, EDTA	AnalaR, BDH (Kilsyth, Victoria, Australia), catalogue # 10093
Glacial acetic acid, H ₃ CCOOH	AnalaR NORMAPUR, VWR Chemicals (Radnor, Pennsylvania, USA), catalogue # 20104.334
GPVI-Cy3 FRET substrate (236-248 GPVI FRET 540/590)	Auspep (Tullamarine, Victoria, Australia)
Heparin Sepharose 6 Fast Flow	GE Healthcare (Chicago, Illinois, USA), catalogue # 17099801
Heparin sodium salt from porcine intestinal mucosa	Sigma-Aldrich (St. Louis, Missouri, USA), catalogue # H3393
Human platelet factor 4	ChromaTec GmbH (BioRegion Greifswald-Rostock, Germany) (gift from Dr Brendan McMorran, Canberra, Australia)
Igepal CA-630	Sigma-Aldrich (St. Louis, Missouri, USA), catalogue # I8896
Isopropanol (2-Propanol)	VWR (Radnor, Pennsylvania, USA), catalogue # 0918
Magnesium chloride hexahydrate, MgCl ₂ .6H ₂ O	AnalaR, BDH (Kilsyth, Victoria, Australia), catalogue # 10149

Reagents	Source
Methanol	Labchem, Ajax Finechem (Taren Point, New South Wales, Australia), catalogue # AJA5005
N,N,N',N'-Tetramethylethylenediamine (TEMED)	VWR (Radnor, Pennsylvania, USA), catalogue # 443083G
N,N'-Methylenebisacrylamide	Sigma-Aldrich (St. Louis, Missouri, USA), catalogue # M7279
N-ethylmaleimide, C ₆ H ₇ NO ₂	Sigma-Aldrich (St. Louis, Missouri, USA), catalogue # E1271
PageRuler unstained broad range protein ladder	Thermo Fisher Scientific (Waltham, Massachusetts, USA), catalogue # 26630
Phenol:chloroform:isoamyl alcohol 25:24:1	Sigma-Aldrich (St. Louis, Missouri, USA), catalogue # P2069
Potassium chloride, KCl	Univar, Ajax Finechem (Taren Point, New South Wales, Australia), catalogue # 383
PrimeSTAR GXL DNA polymerase	Takara Bio Inc. (Kusatsu, Shiga Prefecture, Japan), catalogue # R050A
Protein A Sepharose CL-4B	GE Healthcare (Chicago, Illinois, USA), catalogue # 17078001
Protein Precipitation Solution	Qiagen (Hilden, Germany), catalogue # 158910
RBC Lysis Solution	Qiagen (Hilden, Germany), catalogue # 158902
Sodium chloride, NaCl	Chem-Supply (Gillman, South Australia, Australia), catalogue # SA046
Sodium dihydrogen orthophosphate, NaH ₂ PO ₄	Chem-Supply (Gillman, South Australia, Australia), catalogue # SL061
Sodium dodecyl sulphate (SDS)	Sigma-Aldrich (St. Louis, Missouri, USA), catalogue # L3771
Sodium hydrogen carbonate, NaHCO ₃	Univar, Ajax Finechem (Taren Point, New South Wales, Australia), catalogue # 475
SPDP (succinimidyl 3-(2-pyridyldithio)propionate)	Thermo Fisher Scientific (Waltham, Massachusetts, USA), catalogue # 21857
Superdex 200 HR 10/300 column	Amersham Biosciences (GE Healthcare, Chicago, Illinois, USA)
SuperSignal ELISA Pico Chemiluminescent substrate	Thermo Fisher Scientific (Waltham, Massachusetts, USA), catalogue # 37069
SYBR Safe DNA gel stain	Invitrogen, Thermo Fisher Scientific (Waltham, Massachusetts, USA), catalogue # S33102

Reagents	Source
Taq DNA polymerase	New England Biolabs (Ipswich, Massachusetts, USA), catalogue # M0267S
ThermoPol reaction buffer	New England Biolabs (Ipswich, Massachusetts, USA), catalogue # M0267S
Tris base (Trizma)	Sigma-Aldrich (St. Louis, Missouri, USA), catalogue # T1503
Trisodium citrate dihydrate, $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$	Sigma-Aldrich (St. Louis, Missouri, USA), catalogue # C8532
Tween-20	BioXtra, Sigma-Aldrich (St. Louis, Missouri, USA), catalogue # P7949
UltraPure agarose	Invitrogen, Thermo Fisher Scientific (Waltham, Massachusetts, USA), catalogue # 16500500
UltraPure DNase/RNase-Free distilled water	Invitrogen, Thermo Fisher Scientific (Waltham, Massachusetts, USA), catalogue # 10977023
Xylene cyanol FF	Schwarz/Mann Biotech (Cleveland, Ohio, USA), catalogue # 806801.12
Zinc sulphate, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	Univar, Ajax Chemicals (Auburn, New South Wales, Australia), catalogue # 583

2.2 Methods

2.2.1 Blood collection

Blood collection was conducted after obtaining informed consent from healthy individuals between 18-60 years old who were not previously diagnosed with a bleeding disorder and not using anticoagulant or antiplatelet medications, using methods approved by the Australian National University Human Research Ethics Committee (Protocol 2016/317). Venesection was performed by trained phlebotomists using a 19-gauge winged infusion needle. The first 5 mL of venous blood was collected in syringes for serum isolation, followed by syringes containing either 3.2% (w/v) trisodium citrate (TSC) (9:1 ratio of blood to TSC) or acid-citrate-dextrose (ACD – 97 mM TSC, 111 mM glucose, 78 mM citric acid) (6:1 ratio of blood to ACD) anticoagulants for platelet rich plasma or washed platelet preparation, respectively. Anticoagulants were prepared fresh on the day of blood collection. Blood collected into syringes containing anticoagulants was mixed by gentle inversion.

2.2.2 Platelet isolation

2.2.2.1 Platelet rich plasma preparation

Anticoagulated whole blood (Section 2.2.1) was transferred into 10 mL polypropylene flat-bottomed tubes (Techno Plas, Adelaide, South Australia, Australia) and fractionated by centrifugation at 110 *g* for 20 min at room temperature (RT) with no brake (Beckman Coulter Allegra X-12R, Brea, California, USA). The upper platelet rich plasma (PRP) layer was carefully collected by aspiration, avoiding the buffy coat fraction. Whole blood cell count and PRP count was obtained using a Cell-Dyn Emerald 22 haematology analyser (Abbott Diagnostics, Chicago, Illinois, USA).

2.2.2.2 Washed platelet preparation

PRP from ACD-anticoagulated blood (Section 2.2.1) was isolated (Section 2.2.2.1) and combined then centrifuged in a V-bottomed centrifuge tube (Corning Falcon, Corning, New York, USA) at 1270 *g* for 15 min at RT with brake function enabled. Platelet-poor plasma (PPP) was removed and the platelet pellet was washed by gentle resuspension in 123 mM NaCl containing 13 mM TSC and 33.3 mM glucose, pH 7.0 (CGS buffer) and centrifugation at 1270 *g* for 15 min at RT with brakes on. This process was repeated twice then the platelet pellet was resuspended at 5×10^8 platelets/mL in 5.5 mM glucose, 137 mM NaCl, 12 mM NaHCO₃, 1.8 mM CaCl₂, 0.49 mM MgCl₂·6H₂O, 2.7 mM KCl, 0.36 mM NaH₂PO₄·H₂O, pH 7.4 (Tyrode's buffer) to obtain washed platelets.

2.2.3 Plasma isolation

2.2.3.1 Healthy donors

Double spun plasma was obtained by two centrifugation steps of PPP at 21,000 *g* for 5 min at RT in a microcentrifuge (IEC Micromax, Thermo Electron Corporation, Waltham, Massachusetts, USA) whereby the supernatant from each centrifugation step was transferred to a fresh 1.5 mL microcentrifuge tube. Double spun PPP was stored in barcoded cryovials (Fluidx, BioTools, Loganholme, Queensland, Australia) at -80°C for batch analysis of plasma components.

2.2.3.2 Patients suspected of HIT

4.5 mL of blood from each patient was collected in citrate vacutainer tubes at the Hospital of the University of Pennsylvania and centrifuged at 2,000 *g* for 10 min. Supernatant containing

PPP was transferred to a 5 mL conical tube and centrifuged again at 2000 *g* for 10 min. The double spun PPP supernatant was aliquoted into tubes and stored frozen at -80°C.

2.2.4 SDS-PAGE (Polyacrylamide Gel Electrophoresis)

2.2.4.1 Sample preparation

Washed platelet suspensions were mixed with equivolumes of ice-cold lysis buffer (20 mM Tris, pH 7.4, 5 mM EDTA, 1% Triton, all final concentrations) containing Complete Protease Inhibitor cocktail. Purified proteins (10-20 µg), recombinant proteins (0.2-0.4 µg), total proteins (100 µg) or washed platelet lysate (20-100 µg) were mixed with 50 mM Tris-HCl, pH 6.8, 2% (w/v) sodium dodecyl sulphate (SDS), 10% (v/v) glycerol (SDS sample buffer). For samples in reducing conditions, 10 mM DTT was added. All samples were heated at 100°C for 5 min and then centrifuged at 16,000 *g* for 1 min before loading into polyacrylamide gels.

2.2.4.2 SDS polyacrylamide gel casting and electrophoresis

The Protean II xi system (Bio-Rad Laboratories, Hercules, California, USA) for casting 16 x 20 cm gels was assembled according to manufacturer's instructions. A single percentage polyacrylamide resolving gel mixture containing final concentrations of 0.375 M Tris-HCl buffer pH 8.8, 0.1% (v/v) SDS, 1.4% (v/v) glycerol, 0.04% (v/v) N, N, N', N'-tetramethylethylenediamine (TEMED), 0.075% (v/v) ammonium persulphate (APS, freshly made) and variable volumes of 30% (w/v) acrylamide stock solution depending on the desired percentage, was made up to 35 mL (for a single maxi gel) with Milli-Q H₂O. The resolving gel mixture was poured into the assembled Protean II xi system with an overlay of Milli-Q H₂O and allowed to polymerise at RT overnight.

A 10% stacking gel mixture containing final concentrations of 0.125 M Tris-HCl buffer (pH 6.8), 0.1% (v/v) SDS, 0.08% (v/v) TEMED, 0.075% (v/v) APS and 30% (w/v) acrylamide stock solution was prepared. The overlay was removed from the resolving gel and the stacking gel was poured onto the polymerised resolving gel, followed by insertion of a 1.5 mm thick 15-lane Teflon comb (Bio-Rad Laboratories, Hercules, California, USA).

Once the stacking gel had set, the gel sandwich was assembled onto the central cooling core and the assembly was transferred into a Protean II xi Cell (Bio-Rad Laboratories, Hercules, California, USA) containing the gel running buffer (25 mM Tris, 190 mM glycine, 0.1% (w/v) SDS, pH 8.3, all final concentrations) in the lower chamber. The upper chamber was then filled with the gel running buffer and the comb was removed. Prepared samples (Section 2.2.4.1) and protein ladder were loaded into the wells. Electrophoresis was performed at a constant current of 0.03 A for day run (~5 h) or maintained at 50-60 V (depending on gel percentage) for an overnight run (~16 h) at RT until the dye front reached the bottom of the plate.

2.2.4.3 Coomassie staining

Once electrophoresis was complete, the gel was removed from the assembly and fixed in fixing solution (50% (v/v) methanol, 10% (v/v) glacial acetic acid) for at least 1 h at RT on a rocker. In order to visualise protein bands, the gel was immersed in Coomassie staining solution (50% (v/v) methanol, 10% (v/v) glacial acetic acid, 0.1% (w/v) Brilliant Blue R, filtered through Whatman Grade 2) for 1 h at RT on a rocker, followed by de-staining in de-staining solution (10% (v/v) methanol, 10% (v/v) glacial acetic acid) until the background was clear. The Coomassie-stained gel was imaged using a Bio-Rad ChemiDoc MP Imaging System (Bio-Rad Laboratories, Hercules, California, USA) and the associated Image Lab version 5.0 (Bio-Rad Laboratories, Hercules, California, USA) software.

2.2.4.4 Western blotting

Electrophoresed protein was transferred onto methanol-activated 0.2 μ m polyvinylidene difluoride (PVDF) membrane (Amersham Hybond P Western blotting membrane, GE Healthcare, Chicago, Illinois, USA), via wet transfer in a Bio-Rad Trans-Blot Cell (Bio-Rad Laboratories, Hercules, California, USA) containing pre-chilled 25 mM Tris, 190 mM glycine, 20% (v/v) methanol, pH 8.3 (transfer buffer). Electroelution of proteins to the membrane was conducted at 100 V for 1 h. Membranes were blocked in 5% (w/v) skim milk in Tris-buffered saline containing Tween-20 (TBS-T) (20 mM Tris, 137 mM NaCl, 0.1% (v/v) Tween-20, pH 7.6) for 1 h at RT or overnight at 4°C on a rocker, followed by incubation with 0.5-5 μ g/mL primary antibody diluted in BSA, for 1 h at RT or overnight at 4°C on a rocker. Membranes were washed four times with TBS-T for 10 min each wash on a rocker to remove excess antibodies, and then incubated with appropriate HRP-conjugated secondary antibody diluted 500-10,000-fold in TBS-T for 1 h at RT on a rocker. Wash steps were repeated to remove excess antibodies. Bound antibody was visualised by applying enhanced chemiluminescence (ECL) substrate (Amersham ECL Western Blotting Detection Reagent, GE Healthcare, Chicago, Illinois, USA) to the membrane and chemiluminescence was captured using a Bio-Rad ChemiDoc MP Imaging System at exposure times appropriate for the signal intensity detected, using Image Lab version 5.0 software.

2.2.5 Purification of human PF4 from platelet transfusion bags by chromatography

Native human platelet factor 4 (PF4) was purified from expired blood group O irradiated platelet transfusion bags collected from The Canberra Hospital Blood Bank using an established method first described by Medici and colleagues (1989). This method has been

used in multiple studies to assess platelet activation by HIT antibodies (Visentin et al., 1994, Padmanabhan et al., 2015, Padmanabhan et al., 2016a, Choi, 2016).

Platelet bags were stored at -80°C until needed. Each platelet bag was approximately 340 mL (pooled from 4 donations), of which 70% of total component volume was SSP+ platelet additive solution. Constituents of SSP+ platelet additive solution are listed in Table 2.9 (Australian Red Cross, 2015).

Table 2.9 Constituents of SSP+ additive solution in platelet transfusion bag

Components	Concentration (mg/mL)
Sodium chloride	4.05
Sodium acetate trihydrate	4.42
Sodium citrate dihydrate	3.18
Sodium dihydrogenophosphate	1.05
Disodium hydrogenophosphate	3.05
Potassium chloride	0.37
Magnesium chloride	0.30

Frozen platelet bags were thawed within a plastic containment bag in a 37°C water bath. The contents was transferred into Beckman Coulter 500 mL polypropylene centrifuge bottles (Brea, California, USA) and centrifuged at 6000 *g* for 30 min at 4°C in a F500 rotor (S/N: 611096) in a Beckman Coulter Avanti J-E (Brea, California, USA) high speed centrifuge at full acceleration but centrifuge brakes set to off. The volume of supernatant collected was measured and appropriate mass of NaCl was added to adjust the NaCl concentration in the supernatant to 0.5 M (calculations based on the NaCl concentration in SSP+ solution and supernatant yield). Mixture was stirred on a magnetic stirrer at 4°C overnight to dissolve the NaCl. No salt precipitate was observed after overnight stirring.

5 mL of Heparin Sepharose 6 Fast Flow (GE Healthcare, Chicago, Illinois, USA) was washed and equilibrated in sterile-filtered equilibration buffer (500 mM NaCl, 10 mM Na₂HPO₄, 2.7 mM KCl, 1.8 mM KH₂PO₄, pH 7.4), according to manufacturer's instructions. Sepharose beads were washed and equilibrated in 25 mL of equilibration buffer in a 50 mL Falcon tube, and left on a roller for 3-5 min before centrifuging at 700 *g* for 5 min without centrifuge brakes to sediment the beads and remove the supernatant. This wash and equilibration step was performed three times.

The following steps were performed at 4°C in a cold room. Equilibrated Heparin Sepharose beads were added to supernatant from platelet lysate (adjusted to contain 0.5 M NaCl) and left to stir on a magnetic stirrer overnight at 4°C to allow binding of PF4 to the beads. To collect the unbound fraction, the mixture was aliquoted into 50 mL Falcon tubes and centrifuged at 700 *g* for 5 min at 4°C without centrifuge brakes. Supernatant containing unbound samples were removed and this step was repeated until all the beads were recovered.

Beads containing bound material were rinsed with 10 column volumes of equilibration buffer and then resuspended in the same buffer before loading into a glass Econo-Column Chromatography Column, 1 x 10 cm (Bio-Rad Laboratories, Hercules, California, USA). A bed volume of approximately 5 mL was obtained and washed with at least 10 bed volumes (~50 mL) of equilibration buffer.

Bound material was eluted from the column with elution buffer (2.0 M NaCl, 10 mM Na₂HPO₄, 2.7 mM KCl, 1.8 mM KH₂PO₄, pH 7.4) and 1 mL fractions were collected. Protein levels in fractions were estimated using a NanoDrop 2000c spectrophotometer (Thermo Scientific, Waltham, Massachusetts, USA) at 280 nm. Samples were visualised by SDS-PAGE (Section 2.2.4).

To exchange eluted material into physiological buffer, pooled fractions containing 0.549 mg of protein (3 mL) were dialysed against phosphate buffered saline (PBS – 137 mM NaCl, 10 mM Na₂HPO₄, 2.7 mM KCl, 1.8 mM KH₂PO₄, pH 7.4) (3 changes x 2 L) in a Spectra/Por Cellulose Ester Float-A-Lyzer (molecular weight cut-off 1000 Da, Spectrum Labs, Repligen, Waltham, Massachusetts, USA) at 4°C overnight. The material was then concentrated three-fold using an Amicon Ultra-15 Centrifugal Filter Unit with Ultracel-3 regenerated cellulose membrane (molecular weight cut-off 3000 Da, Millipore, Merck, Kenilworth, New Jersey, USA) according to manufacturer's instructions.

Previous work has shown that using this procedure, material purified is predominantly PF4, however a major contaminant is antithrombin which is present in platelet transfusion bags and can avidly bind Heparin Sepharose (Niewiarowski et al., 1979). As the molecular weights of PF4 and antithrombin are 7.8 kDa and 58 kDa, respectively, size exclusion chromatography was used to separate PF4 from high molecular weight materials obtained from affinity chromatography on Heparin Sepharose. Size exclusion chromatography was performed on a column of Superdex 200 HR 10/300 column (30 cm x 1 cm diameter) with a bed volume of 24 mL (Amersham Biosciences, GE Healthcare, Chicago, Illinois, USA) on the ÄKTA Explorer fast protein liquid chromatography (FPLC) system (GE Healthcare, Chicago, Illinois, USA). The column was equilibrated with PBS, pH 7.4. 1 mL of the protein concentrate (0.28 mg/mL) was loaded onto the equilibrated column at a flow rate of 0.3 mL/min. 1 mL fractions were collected. Fractions containing protein which eluted at a molecular weight consistent with native human PF4 (peak at 50 min) or with antithrombin (peak at 39 min) were pooled, concentrated and quantified as described above.

2.2.6 Affinity purification of Ig from human plasma

Immobilised Protein A is a standard reagent used to isolate immunoglobulins (predominantly IgG classes) from complex mixtures. 0.1 g of lyophilised Protein A Sepharose CL-4B (GE Healthcare, Chicago, Illinois, USA) was swollen by gentle swirling in 5 mL distilled H₂O to generate a slurry of approximately 0.7 mL bed volume. Beads were settled by centrifugation at 350 *g* for 1 min and the supernatant was removed. The beads were washed in 5 mL of distilled H₂O with mild agitation for 5 min then settled by centrifugation as described above. This procedure was repeated three more times. Washed slurry was resuspended in 8 mL of a solution of 10 mM Tris, 0.15 M NaCl, pH 7.4 (binding buffer) with mild agitation for 5 min then pelleted by centrifugation at 350 *g* for 1 min. This process was repeated three more times.

0.5 mL of either citrate- or ACD-anticoagulated plasma was thawed at 37°C in a heat block for 10 min and mixed with the beads (1:1 volume bead:plasma ratio). The bead/plasma suspension was incubated at RT for 2.5 h on a roller to allow binding of Ig (primarily IgG) and the beads were pelleted by centrifugation at 350 *g* for 1 min. Supernatant containing unbound material was removed by aspiration and the beads containing bound IgG were washed by resuspension in 10 mL of binding buffer and agitated on a roller for 10 min at RT. The suspension was centrifuged at 350 *g* for 1 min at RT, and the supernatant was discarded by aspiration. This process was repeated once more.

Bound IgG was eluted by addition of 300 µL of 0.1 M glycine, pH 2.4 to the Protein A Sepharose beads and agitated on a roller for 20 min at RT. The suspension was centrifuged and the supernatant containing the eluted IgG was immediately collected and neutralised with 75 µL of 1 M Tris, pH 8.5. This elution step was repeated two more times to collect a total of three

fractions which were quantified for protein by spectrophotometry as described in Section 2.2.5 and stored at 4°C.

2.2.7 Exposure of healthy donor platelets to HIT antibodies

PRP and washed platelets from healthy donors were prepared as described in Sections 2.2.2.1 and 2.2.2.2, respectively. 50 µL of PRP or 60 µL of washed platelets at 5×10^8 platelets/mL were pre-treated at RT for 30 min with inhibitors (10 µM Bay 61-3606, 10 µM PP1, 0.2% DMSO vehicle control, 50 mM EDTA or 10 µg/mL IV.3 monoclonal antibody, all final concentrations in 100 µL reaction volume) followed by addition of 10 µg/mL native human PF4 (either purified in-house (Section 2.2.5) or commercially prepared (ChromaTec GmbH, BioRegion Greifswald-Rostock, Germany) and 0.5 U/mL heparin (therapeutic dose) or 100 U/mL heparin (saturating dose). 0.1-0.5 U/mL heparin is the therapeutic heparin concentration used in HIT assays (Warkentin, 2019, Bakchoul et al., 2014, Warkentin et al., 2015). The mixture was incubated for 10 min at RT to allow for antigenic heparin/PF4 complex formation. Then, 50 µg/mL KKO anti-PF4 monoclonal antibody or 30 µL plasma or 100 µg/mL affinity purified Ig (Section 2.2.6) from healthy controls, non-HIT thrombocytopenic controls or confirmed HIT patients was added. Each sample was made up to 100 µL reaction volume with 10 mM Tris, 150 mM NaCl, pH 7.4 (Tris saline buffer).

Samples were incubated at RT for 2 h (for HIT and control plasma experiments) or 4 h (for affinity-purified Ig experiments). For experiments using KKO antibody, samples were incubated at 37°C for 50 min. Reactions were stopped with addition of 50 mM EDTA final concentration. 10 µL aliquots were taken for flow cytometry analysis of surface receptors (Section 2.2.8). The remaining material was centrifuged at 21,000 *g* for 3 min to pellet platelets and the supernatant was stored at -80°C for sGPVI measurements (Section 2.2.9).

2.2.8 Analysis of platelet surface molecule levels by flow cytometry

Levels of platelet surface molecules on resting or activated washed platelets or PRP were assessed by flow cytometry. Platelet samples were made up to a final volume of 100 μ L with Tris saline buffer containing 5 mM EDTA (TS-EDTA) for antibody binding. Samples were incubated with primary antibodies (detailed in Section 2.1.2, Table 2.3); 1 μ g/mL anti-human GPVI monoclonal antibody, 1G5, or 1 μ g/mL Alexa Fluor 488-conjugated anti-human GPVI monoclonal antibody, 1G5, or 0.6 μ g/mL PE-conjugated mouse anti-CD62P monoclonal antibody, AK4, 3 μ g/mL anti-human TIMPs 1, 2, 3, or 4, or 0.6 μ g/mL PE-conjugated mouse IgG1 isotype control, and incubated at RT for 30 min. For unconjugated primary antibodies, a further 30 min incubation in the presence of Alexa Fluor 488-conjugated secondary antibody (1 μ g/mL goat anti-mouse Ig for 1G5, 2 μ g/mL goat anti-rabbit Ig for anti-human TIMPs 1-4) was performed at RT protected from light. Finally, the platelet suspension was diluted by addition of 400 μ L TS-EDTA buffer and samples were analysed within 3 h using an LSRFortessa X-20 cytometer (BD Biosciences, Franklin Lakes, New Jersey, USA). Fluorescence associated with 10,000 platelet events were recorded. Post-acquisition flow cytometry analysis was performed using FlowJo version 10.4 (FlowJo, LLC, Ashland, Oregon, USA).

2.2.9 Measurements of plasma and serum components

2.2.9.1 sGPVI ELISA

sGPVI levels in double spun PPP prepared as described in Section 2.2.3 or supernatant collected from washed platelet experiments were measured by an enzyme-linked immunosorbent assay (ELISA) as described (Al-Tamimi et al., 2009a). This established method has been extensively used to measure levels of sGPVI clinically and experimentally (Gardiner

et al., 2008a, Al-Tamimi et al., 2011a, Al-Tamimi et al., 2011b). Wells of a white, flat-bottomed 96-well microtitre plate (Nunc MaxiSorp, Thermo Fisher Scientific, Waltham, Massachusetts, USA) were coated with 1 µg/mL rabbit anti-human GPVI extracellular domain polyclonal capture antibody in 100 µL 0.05 M sodium carbonate-bicarbonate buffer, pH 9.6 (coating buffer) per well and incubated overnight at 4°C. Wells were washed six times with PBS, pH 7.4, containing 0.2% (v/v) Tween-20 (PBS-T), then blocked with 1% (w/v) BSA in PBS (200 µL per well) for 1 h at RT and washed six times with PBS-T.

Standards consisting of 10% (v/v) supernatant from NEM-treated PRP (with known sGPVI amount) in 10% (v/v) GPVI-depleted plasma in PBS were included in every plate to generate a standard curve from which sGPVI levels were interpolated. All assays included samples of plasma and serum with a known concentration of sGPVI to serve as an internal control and to monitor plate-to-plate variation (coefficient of variation for control plasma and serum were 10.9% and 3.8%, respectively). Test samples diluted 1 in 10 in PBS were added in duplicates in the majority of experiments, and singlets in some experiments. After a 1 h incubation at RT, wells were washed six times with PBS-T followed by addition of 100 µL of 1 µg/mL murine anti-human GPVI monoclonal antibody, 1A12, in PBS.

After a further 1 h incubation at RT and six washes with PBS-T, 100 µL of 2.6 µg/mL HRP-conjugated polyclonal rabbit anti-mouse secondary antibody (affinity-adsorbed against human and bovine Ig, stock at 1.3 mg/mL, Dako) in PBS was added followed by another 1 h incubation at RT and six washes in PBS-T. Equal parts of SuperSignal ELISA Pico Luminol/Enhancer and SuperSignal Pico Stable Peroxide solution (Pierce) were mixed and added at 100 µL per well, then incubated for 1 min at RT before measuring light emission using a Tecan Infinite 200 PRO (Männedorf, Zürich, Switzerland) plate reader.

2.2.9.2 Magnetic bead-based ELISA measurements of TIMPs

Human plasma and serum TIMPs 1-4 levels were quantified using the Human TIMP Magnetic Luminex Performance Assay 4-plex Kit according to manufacturer's protocol. EDTA-anticoagulated plasma, citrated plasma and serum, stored at -80°C, were thawed and centrifuged at 16,000 *g* for 4 min immediately before use. All reagents were brought to RT before use. All samples were diluted 50-fold in Calibrator Diluent RD6-48 and thoroughly mixed. The Standard Cocktail provided was reconstituted in Calibrator Diluent RD6-48 and a 3-fold dilution series was performed to produce seven standards of known TIMPs concentrations. Microparticle Cocktail was prepared in Microparticle Diluent within 30 min of use and kept in the dark.

All standards and samples were assayed in duplicate in the 96-well microplate provided. 50 µL of the prepared standards or samples were added per well followed by addition of 50 µL of diluted Microparticle Cocktail to each well and incubated for 2 h at RT on a horizontal orbital microplate shaker set at 800 rpm. The plate was placed on a microplate magnetic device for 1 min to hold the microparticle at the bottom of the wells before proceeding with wash procedure. Wash procedure was performed three times with 100 µL Wash Buffer per well. 50 µL of diluted Biotin-Antibody Cocktail was then added to all wells and incubated for 1 h at RT on the microplate shaker. Wash steps were repeated followed by addition of 50 µL of diluted Streptavidin-PE and further incubation at RT for 30 min on the shaker. After further washing, the microparticles were resuspended in 100 µL of Wash Buffer per well and incubated for 2 min on the shaker at RT. Plate was read within 90 min using the Luminex MAGPIX analyser with xPONENT 4.2 system (Luminex Corporation, Austin, Texas, USA).

2.2.10 *FCGR2A* genotype and haplotype studies

2.2.10.1 Genomic DNA isolation

For healthy donors and non-HIT thrombocytopenic patients, genomic DNA (gDNA) was isolated from leucocytes as described by John and colleagues (1991) with modifications. 5 mL of blood (mixture of buffy coat layer and haematocrit after PRP isolation, Section 2.2.2.1) was made up to 10 mL with 10 mM Tris pH 7.6, 10 mM KCl, 10 mM MgCl₂ (solution I) in a 15 mL V-bottomed tube and 120 µL of Igepal CA-630 was added to lyse the cells. After inverting several times to mix, the nuclear pellet was pelleted by centrifugation at 1000 *g* for 10 min. The supernatant was carefully decanted without disrupting the nuclear pellet. Isolated nuclear pellets were stored at -20°C for batch processing.

Frozen nuclear pellets were thawed at RT followed by gentle resuspension in 800 µL of 10 mM Tris pH 7.6, 10 mM KCl, 10 mM MgCl₂, 0.5 M NaCl, 0.5% (v/v) SDS, 2 mM EDTA (solution II) to lyse the nuclei. 400 µL of phenol:chloroform:isoamyl alcohol (saturated with 10 mM Tris, pH 8.0, 1 mM EDTA) was added to the lysate in a 1.5 mL microcentrifuge tube and mixed well until emulsion was formed. After centrifuging at 10,000 *g* for 1 min, the upper phase was transferred to a fresh microcentrifuge tube and a further 400 µL phenol:chloroform:isoamyl alcohol (saturated with 10 mM Tris, pH 8.0, 1 mM EDTA) was added. After mixing and centrifuging as above, the upper phase was collected in a microcentrifuge tube. 700 µL of chloroform:isoamyl alcohol was added and extraction was repeated as above. After centrifuging, the upper aqueous phase was carefully transferred to a fresh microcentrifuge tube, while avoiding the interface. Two volumes of pure ethanol was added and mixed by inversion then stored at -20°C for 15 min to precipitate the DNA. DNA was pelleted by centrifugation at 10,000 *g* for 5 min and washed with 1 mL of 70% ethanol followed by a

further centrifugation at 10,000 *g* for 5 min. Ethanol was discarded and DNA pellet was air-dried in a 37°C heat block for 5-10 min with the lids opened, while being careful to not over-dry the DNA. DNA pellet was resuspended in 50-100 µL of ultrapure distilled H₂O (pre-warmed to 37°C) and quantified on a NanoDrop 2000c spectrophotometer (Thermo Scientific, Waltham, Massachusetts, USA) at 230 nm, 260 nm and 280 nm. The quality of gDNA was defined by the A_{260}/A_{280} ratio of 1.7-2.0 and the A_{260}/A_{230} ratio of >1.5. Isolated gDNA was also assessed for integrity by visualising on agarose gel (Section 2.2.10.2).

For patients with clinical suspicion of HIT, gDNA was extracted from blood samples as follows. After removal of PRP (Section 2.2.3), a volume of PBS equivalent to the volume of PRP removed was mixed with the lower red cell fraction and 3 mL was transferred to a 15 mL conical tube containing 9 mL red blood cell (RBC) Lysis Solution. The cell suspension was inverted then incubated at RT for 10 min. The tube was inverted again at least once during incubation. WBC were pelleted by centrifugation at 2000 *g* for 10 min and the supernatant containing cellular debris was decanted, ensuring less than 200 µL residual liquid remained. The WBC pellet was lysed by resuspending in 3 mL of Cell Lysis Solution and vortexed at the highest setting for 10 s. If cell clumps were visible, the mixture was incubated at 37°C in a water bath until a homogeneous solution was obtained.

To extract protein from the cell lysate, 1 mL of Protein Precipitation Solution was added and mixed by vortexing at high speed for 20 s. The mixture was centrifuged at 2000 *g* for 10 min to obtain a dark brown precipitated protein pellet. If a loose pellet was formed, the mixture was vortexed at high speed for 20 s and incubated on ice for 5 min, then centrifuged again. The supernatant, containing DNA, was decanted into a 15 mL conical tube containing 3 mL of 100% isopropanol and mixed by gentle inversion until white threads of DNA formed a visible

clump. The mixture was centrifuged at 2000 *g* for 3 min to obtain a small white DNA pellet. The supernatant was removed, and the tube was briefly drained on a paper towel before 3 mL of 70% ethanol was added. The tube was inverted several times to wash the DNA pellet followed by centrifugation at 2000 *g* for 1 min. The ethanol was carefully decanted, and the tube was drained on a paper towel then air-dried for 10-15 min. Care was taken to avoid over-drying the DNA pellet.

Depending on the size of the pellet, a volume of (~250 μ L) DNA Hydration Solution was added and incubated at 65°C for 1 h. The tube was periodically agitated to help dissolve the DNA then further rehydrated overnight at RT and transferred to a storage tube and kept at -80°C.

2.2.10.2 DNA agarose gel electrophoresis

Samples for DNA electrophoresis were prepared in DNA loading dye containing 5% (v/v) glycerol, 0.025% (w/v) bromophenol blue and 0.025% (w/v) xylene cyanol FF in Tris-acetate-EDTA (TAE) buffer (40 mM Tris pH 7.6, 20 mM acetic acid, 1 mM EDTA in distilled H₂O). 500 ng of 1 Kb Plus DNA ladder diluted in DNA loading dye was included in at least one well for every DNA agarose gel.

UltraPure agarose powder was heated in a microwave oven to dissolve in TAE buffer containing SYBR Safe DNA gel stain. For analysis of gDNA, PCR amplicon or restriction fragment length polymorphism (RFLP), 0.8%, 1.2% or 4% agarose gel were used, respectively. Molten agarose was poured into a UV-transparent removable tray fixed on a Gel Caster (Bio-Rad Laboratories, Hercules, California, USA) with the appropriate well-forming comb in place and left to solidify at RT. The comb was carefully removed, and the tray was placed onto the base of the Sub-Cell horizontal electrophoresis system (Bio-Rad Laboratories, Hercules, California, USA) containing the TAE buffer, ensuring the solidified gel was submerged 3-6 mm beneath

the buffer. Prepared samples and a sample containing a DNA size ladder were loaded into the gel wells and samples were subjected to electrophoresis at a constant voltage of 80 V until the desired separation was achieved.

2.2.10.3 Genotyping of *FCGR2A* H131R by RFLP

RFLP analysis of *FCGR2A* H131R polymorphism was modified from the published assay by Jiang and colleagues (1996). Genomic DNA (Section 2.2.10.1) was amplified by polymerase chain reaction (PCR) using the *FCGR2A*_H131R_F forward and *FCGR2A*_H131R_R reverse primers for RFLP listed in Table 2.5. PCR amplification was conducted in a 50 μ L reaction volume containing 100 ng gDNA, 0.5 μ M of each primer, 200 μ M of NEB deoxynucleotide (dNTP) solution mix (containing equimolar concentrations of dATP, dCTP, dGTP and dTTP), and 1.25 U Taq DNA polymerase in 20 mM Tris-HCl, 10 mM $(\text{NH}_4)_2\text{SO}_4$, 10 mM KCl, 2 mM MgSO_4 , 0.1% Triton X-100, pH 8.8 (ThermoPol buffer). The thermal cycling program was 94°C for 3 min for polymerase activation and DNA denaturation followed by 35 cycles of 94°C for 15 s, 60°C for 30 s and 72°C for 1 min, followed by a final extension step at 72°C for 5 min then a hold at 10°C step. This program was executed by a T100 Thermal Cycler (Bio-Rad Laboratories, Hercules, California, USA). The lid temperature was set to 105°C. A control tube containing no gDNA template was included in every PCR run to confirm reagents were free of contamination.

The nature of the amino acid at 131 of Fc γ R11a was evaluated by restriction enzyme digestion of PCR-amplified sequences performed in a 40 μ L reaction volume containing 20 μ L of PCR product, 2 U of *Bst*UI restriction enzyme (2 μ L of 1000 U/mL stock) and 50 mM $\text{C}_2\text{H}_3\text{KO}_2$, 20 mM Tris-acetate pH 7.9, 10 mM $\text{C}_4\text{H}_{14}\text{MgO}_8$, 100 μ g/mL BSA (CutSmart buffer) in UltraPure distilled H_2O . The reaction mixture was incubated in a 60°C heat block, overnight (~16 h) for restriction digest. Undigested and *Bst*UI-digested PCR products were visualised on 4% TAE

agarose electrophoresis gels (Section 2.2.10.2) to ascertain the genotype at amino acid 131 of *FCGR2A*. The restriction digestion pattern and its corresponding genotype are shown in Figure 2.1.

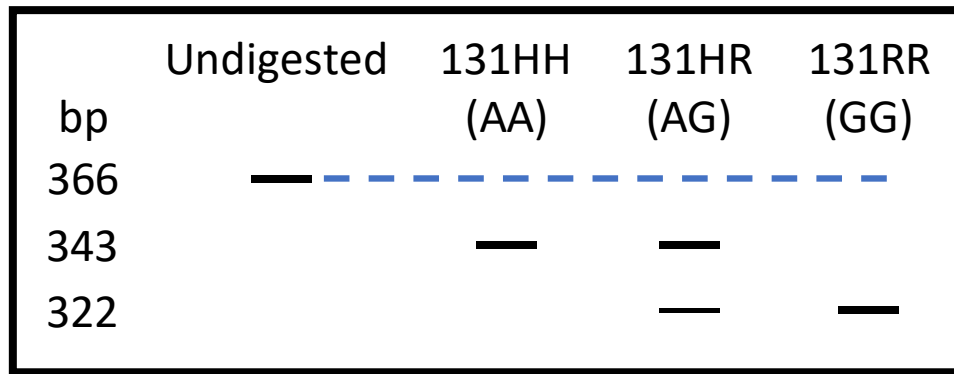


Figure 2.1 Restriction digest patterns for *FCGR2A* H31R genotyping by PCR-RFLP.

A stylised representation of the expected bands on agarose gel electrophoresis for an undigested PCR product of 366 base pairs (bp), and *Bst*UI-digested samples for each genotype. Individuals homozygous for 131HH and 131RR will produce single *Bst*UI cleavage products at 343 bp and 322 bp, respectively, while heterozygous 131HR genotype will produce two bands (343 bp and 322 bp).

2.2.10.4 Long range PCR amplification of *FCGR2A* spanning exons 3 and 4

To obtain allele-specific haplotype information of *FCGR2A* across the two polymorphic sites of interest, H131R (519A>G, rs1801274) and Q27W (CA>TG, rs201218628), F_*FCGR2A*_27_131 forward and R_*FCGR2A*_27_131 reverse primers (Table 2.5) were designed to amplify a region of *FCGR2A* spanning exons 3 and 4. The primers contained a 30-nucleotide universal tagging sequence on the 5' ends to enable subsequent barcoding. Figure 2.2 shows the graphical locations of the polymorphic sites and primer annealing regions relative to the *FCGR2A* gene, and the expected size of PCR products generated from long range PCR amplification.

PCR amplification was performed in a 50 µL reaction volume containing 50 ng gDNA, 0.2 µM of each primer, 200 µM of Takara dNTP solution mix (equimolar concentrations of dATP, dCTP, dGTP and dTTP), PrimeSTAR GXL buffer (components are proprietary information) and 1.25 U of high-fidelity PrimeSTAR GXL DNA polymerase in UltraPure distilled H₂O. The thermal cycling profile was 98°C for 1 min for polymerase activation and initial DNA denaturation, followed by 30 cycles of 98°C for 10 s, 60°C for 15 s and 68°C for 4 min, followed by a final extension step at 68°C for 10 min then a hold at 4°C step. This program was performed by a T100 Thermal Cycler (Bio-Rad Laboratories, Hercules, California, USA). The lid temperature was set to 105°C. A control tube without gDNA template was included in every PCR run to confirm reagents were free of contamination. Every PCR product was assessed for yield, quality and specificity by DNA gel electrophoresis on a 1.2% agarose gel, as described (Section 2.2.10.2). The specificity of the PCR product was also validated by Sanger sequencing as described (Section 2.2.10.8).

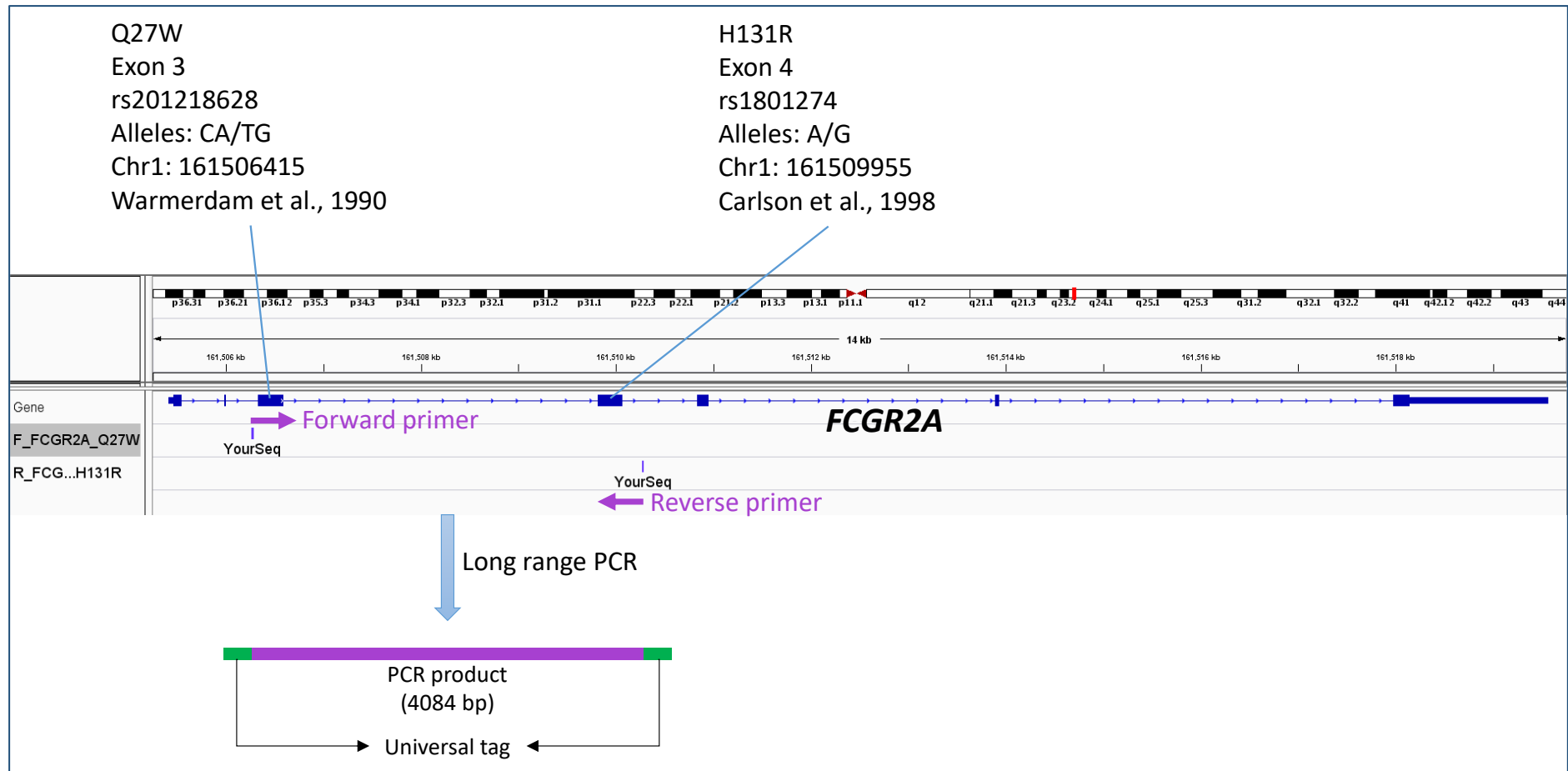


Figure 2.2 Graphical locations of *FCGR2A* polymorphic sites of interest.

The Q27W dinucleotide substitution is located on exon 3 of *FCGR2A* while the H131R single nucleotide polymorphism is located on exon 4. The two polymorphic sites are 3540 nucleotides apart. The relative annealing sites of primers designed for long range PCR amplification to generate a 4084 bp PCR product spanning exons 3 and 4 of *FCGR2A* are shown by the purple arrows. Universal tagging sequences for downstream barcoding of PCR products are shown in green.

2.2.10.5 Amplicon library preparation

A second PCR amplification step was required to attach unique barcodes to the PCR products containing the universal tagging sequence generated in Section 2.2.10.4. Inclusion of unique barcodes for PCR products amplified from each individual enabled the pooling of samples prior to amplicon sequencing on a PacBio Sequel platform (Pacific Biosciences, Menlo Park, California, USA). Amplicons from the first round of PCR were purified and subsequently quantified using the Qubit quantitation system (Thermo Fisher Scientific, Waltham, Massachusetts, USA) to ensure equimolar amounts of PCR amplicon templates for the second round of PCR.

2.2.10.5.1 Purification of PCR amplicons

PCR amplicons were purified using a GeneJET PCR Purification Kit and the manufacturer's protocol, in order to remove excess primers, unincorporated dNTPs, primer dimers, enzymes and salts present in the reaction mixture which may interfere with downstream quantitation and subsequent PCR. An equal volume of binding buffer was added to the PCR amplicon mixture, and mixed thoroughly and loaded onto the GeneJET purification column and centrifuged at 15,000 *g* for 45 s. After discarding the flow-through, 700 μ L of wash buffer (diluted in ethanol as per manufacturer's instructions) was added to the column and centrifuged at 15,000 *g* for 45 s. The flow-through was discarded and the empty column was centrifuged for an additional 1 min at 15,000 *g* to remove residual wash buffer. The column was then placed into a fresh 1.5 mL microcentrifuge tube. 30 μ L of UltraPure distilled H₂O (pre-warmed to 37°C) was added to the column, and incubated for 1 min at RT before centrifugation at 15,000 *g* for 1 min to elute the purified PCR amplicon. Purified products were stored at -20°C for batch quantitation (Section 2.2.10.5.2).

2.2.10.5.2 Quantitation of double-stranded DNA (dsDNA)

The Qubit 1X dsDNA High-Sensitivity (HS) Assay Kit was used with the Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) to quantify dsDNA content in the purified amplicons (Section 2.2.10.5.1), according to manufacturer's instructions. This assay uses a fluorescent dye that binds selectively to dsDNA (Thermo Fisher Scientific, 2016). To ensure the concentrations measured fall within the dynamic range of this assay (1-500 ng/mL), samples were diluted 1 in 200 in Qubit 1X dsDNA HS Working Solution to a final volume of 200 μ L in thin-walled Qubit Assay tubes (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Samples were thoroughly mixed by vortexing for 2-3 s and incubated for at least 2 min at RT to ensure optimal fluorescence before readings were taken on the Qubit 3.0 Fluorometer. All readings were taken within 20 min of sample preparation.

2.2.10.5.3 Barcoded PCR products – second round of PCR

Barcoded Universal Forward and Reverse Primers Plate-96 (Pacific Biosciences, Menlo Park, California, USA) were used to attach unique barcodes to dsDNA amplicons from each individual (Sections 2.2.10.5.1 and 2.2.10.5.2). For each sample, a forward and reverse primer pair contain the same barcode with a 3' universal tagging sequence. The sequence of the 96 unique 16-base barcodes are listed in Table 2.6.

To generate barcoded PCR products, 50 ng of purified amplicon was added to a solution containing 0.2 μ M Barcoded Universal Primers, 200 μ M of Takara dNTP solution mix (equimolar concentrations of dATP, dCTP, dGTP and dTTP), PrimeSTAR GXL buffer (components are proprietary information) and 0.625 U of high-fidelity PrimeSTAR GXL DNA polymerase in UltraPure distilled H₂O in a 25 μ L total reaction volume. The thermal cycling profile was 98°C for 1 min for polymerase activation and initial DNA denaturation, 25 cycles

of 98°C for 10 s, 64°C for 15 s and 68°C for 4 min, followed by a final extension step at 68°C for 10 min then a hold step at 4°C, was performed in a T100 Thermal Cycler (Bio-Rad Laboratories, Hercules, California, USA). The lid temperature was set to 105°C. A control without DNA template was included in every PCR run to confirm reagents were free of contamination. All PCR products were visualised on 0.7% agarose gel to evaluate successful amplification prior to purification and quantitation using a GeneJET PCR Purification Kit followed by quantitation on a Qubit system (Sections 2.2.10.5.1 and 2.2.10.5.2).

2.2.10.5.4 Pooling of amplicons

In preparation for in-phase sequencing of *FCGR2A*, uniquely barcoded amplicons were pooled in equimolar amounts to yield 8 µg of total amplicons per pool to ensure adequate and equal representation of each sample within each pool. In the first plate, 92 samples were pooled at 87 ng per sample while the second plate which consisted of 93 samples were pooled at 86 ng per sample. SMRTbell library construction for each pool was performed at the Ramaciotti Centre for Genomics (University of New South Wales, Sydney, Australia).

2.2.10.6 SMRTbell library construction and sequencing

The PacBio Sequel system uses Single Molecule Real-Time (SMRT) Sequencing technology which enables long-read sequencing with high consensus accuracy. Acquisition of a greater depth of genetic information is made possible by the creation of the zero-mode waveguides (ZMWs) which are 70 nm wide, with a volume of 20×10^{-21} L each, held on a SMRT Cell chip (each containing one million ZMWs). Immobilisation of individual DNA polymerases on the bottom of each ZMW chamber permits single molecule sequencing whereby the fluorescence signal from each ZMW corresponds to the sequence of one DNA molecule. Each nucleotide has a fluorophore attached to its phosphate group. When incorporated, the fluorophore is

released and emits a signal which identifies the nucleotide that was incorporated based on the template DNA molecule in each ZMW.

The PacBio Sequel system generates read lengths of up to 30 kb on average with a >99.999% consensus accuracy (PacBio, 2019). The long-read sequencing with high consensus accuracy stems from the SMRTbell template library design. The target DNA molecule is ligated into SMRTbell adaptors on each end to form a circular fragment which a DNA polymerase then reads continuously. The number of loops each polymerase makes depends on the size of the DNA template, that is, the shorter the template sequence, the greater the number of passes. A consensus sequence is generated based on multiple passes made by the polymerase through each DNA template. This design of circular sequencing significantly reduces the overall error rate (Lou et al., 2013) to overcome the high error rate of ~15% per nucleotide (English et al., 2014).

SMRTbell library preparation, polymerase binding and amplicon sequencing on the PacBio Sequel system were performed at the Ramaciotti Centre for Genomics (University of New South Wales, Sydney, NSW, Australia). The PacBio SMRTbell Template Preparation Kit 1.0 SPv3 was used for template preparation. SMRTbell library preparation included DNA damage repair to the pooled samples (Section 2.2.10.5) caused by prolonged exposure to high temperatures during PCR amplification. Pooled barcoded amplicons were treated to form blunt ends to which SMRTbell hairpin adapters were ligated to generate circular fragments which form the template for circular sequencing. Exonuclease treatment was performed after ligation to remove molecules with free ends which did not have the SMRTbell adapter ligated. The sequencing primer was then annealed to the SMRTbell adapters. AMPure bead purification steps were used throughout library construction to remove off-target contaminating products

which were <1.5 kb, and for buffer exchange between procedures. A summary of quality control checks for the two pools of samples is included in Appendix 1.

Amplicon sequencing was performed using a Sequel Sequencing Kit v2.1 with two SMRT cells containing a pool of 92 individuals on one SMRT cell and 93 individuals on the other. The starting input for the respective pools were 2.6 µg and 2.4 µg.

2.2.10.7 Haplotype phasing

Analysis of raw sequencing data and phasing of haplotypes were performed with assistance from Dr Ignatius Pang (School of Biotechnology and Biomolecular Sciences, The University of New South Wales, Sydney, NSW, Australia) and Dr Tonia Russell (Ramaciotti Centre for Genomics, University of New South Wales, Sydney, NSW, Australia). Raw sequence reads for each pool were first demultiplexed to identify individual samples based on the unique barcode sequences. A Long Amplicon Analysis (LAA) pipeline was used to generate subread consensus sequences for each individual using the SMRT Link software version 6.0.0.47841. A minimum subread length filter of 3000 bp was set to select for reads that were sufficiently long to contribute to phasing, as the genomic distance between the two polymorphic sites of interest is 3540 bp (Q27W chr1:161506415, H131R chr1:161509955, Figure 2.2). Coarse and waterfall clustering were performed to cluster similar subreads within each individual to generate a consensus sequence. The LAA reports on the two pools of samples are attached in Appendices 2 and 3.

Subreads consensus sequence generated using the SMRT Link LAA software was mapped onto the Genome Reference Consortium Human Build 38 (hg38) human genome. The top two consensus reads (based on read counts) were used to interrogate the phased haplotypes for each individual. Both paternal and maternal alleles for every individual were amplified and

sequenced using PacBio's long-read technology which captured the entire length of the target region as a single strand (target region ~4000 bp, that is the length of barcoded PCR amplicons in each pool). This enables phasing of the paternal and maternal haplotypes to obtain allele-specific information and to determine the genotype at each polymorphic site of interest.

2.2.10.8 Validation by Sanger sequencing

In order to validate the genotypes identified by PCR-RFLP and PacBio long-read sequencing, Sanger sequencing was performed on an Applied Biosystems 3730xl DNA Analyzer (Foster City, California, USA) at the Genome Discovery Unit in the ACRF Biomolecular Resource Facility, The John Curtin School of Medical Research, The Australian National University. For each sample to be validated, 32 μ L of unbarcoded, purified PCR amplicon (Section 2.2.10.4) at 100 ng/ μ L and 15 μ L of each sequencing primer at 1.6 pmol/ μ L were submitted for sequencing. Primers Q27W_F and *FCGR2A*_H131R_R (Table 2.5) were used as sequencing primers to validate genotypes at amino acids position 27 and 131 of *FCGR2A*, respectively, according to the manufacturer's protocol (Thermo Fisher Scientific, 2014). Chromatogram traces were viewed on Sequence Scanner Software 2 (Applied Biosystems, Foster City, California, USA) to determine sequencing quality and genotype of samples. Quality control report for a subset of samples is included in Appendix 4.

2.2.11 ADAM10 activity measurements

Recombinant human (rh) ADAM10, rhADAM17 or native human platelet ADAM10 activity was measured using an established fluorescence resonance energy transfer (FRET) assay (Facey et al., 2016). This assay utilised a GPVI-Cy3 substrate consisting of a peptide with sequence matching the amino acid residues 236-248 of GPVI that is cleaved by ADAM10, C-terminally conjugated to a Black Hole Quencher (BHQ) 2 and N-terminally conjugated to a cyanine (Cy) 3

fluorophore via lysine (K) residues, as illustrated in Figure 2.3. Cleavage of the peptide sequence releases the BHQ2 quencher from the Cy3 fluorophore, thus enabling the detection of fluorescence signal at excitation at 540 nm and emission at 590 nm. All the buffers used in ADAM10 activity assays were prepared in UltraPure distilled water.

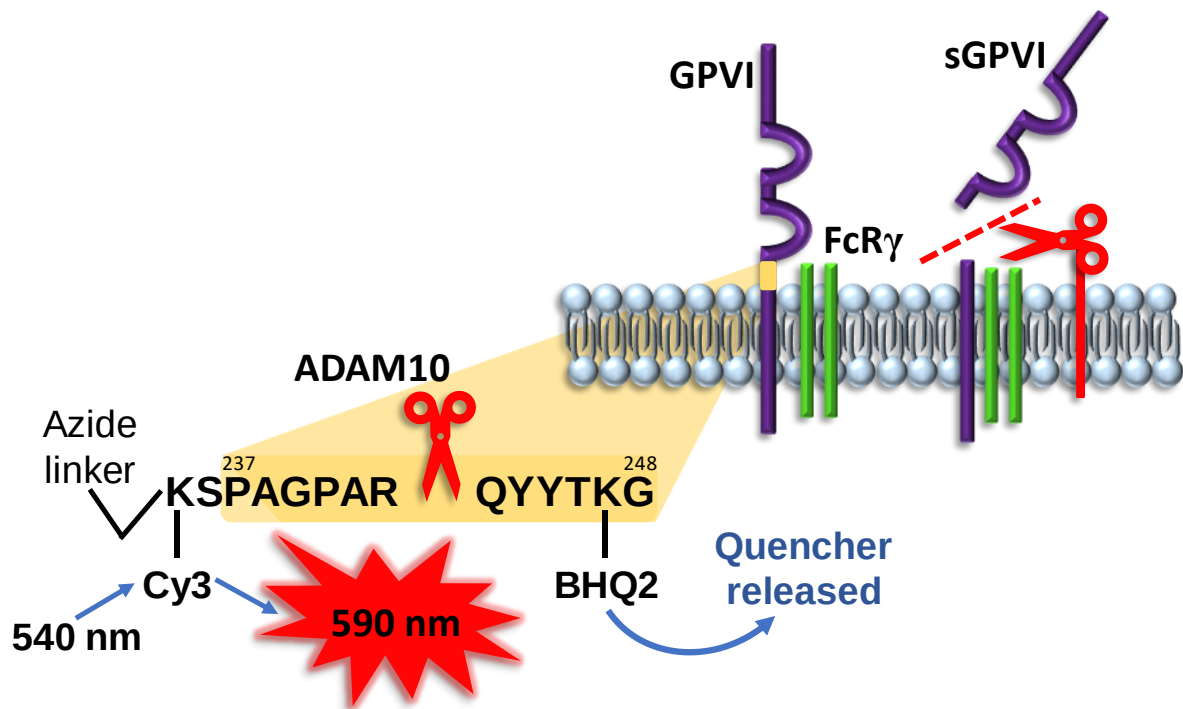


Figure 2.3 Schematic of GPVI-Cy3 ADAM10 substrate for ADAM10 activity measurements.

The GPVI-Cy3 substrate carries a GPVI peptide sequence that is predominantly cleaved by ADAM10. Cleavage of this substrate releases the BHQ2 quencher from the Cy3 fluorophore, resulting in fluorescence emission that is detectable at 590 nm.

2.2.11.1 Recombinant human ADAMs activity

rhADAM10 and rhADAM17 were used to evaluate the specificity of the GPVI-Cy3 substrate. In an OptiPlate-96 White Opaque 96-well microplate (PerkinElmer, Waltham, Massachusetts, USA), 10-90 nM of rhADAM10 or rhADAM17 were mixed with 1 μ M GPVI-Cy3 substrate and made up to 100 μ L with 100 mM Tris pH 7.4, 150 mM NaCl, 5 μ M ZnSO₄ and 0.005% (v/v) Brij-35. Brij-35 is a non-ionic detergent included in the reaction buffer to maintain the solubility and stability of the recombinant proteins. Fluorescence energy was quantified by a Tecan Infinite 200 PRO plate reader set to 37°C and continuous kinetic reads at an excitation wavelength of 540 nm and an emission wavelength of 590 nm, with a gain set at 80. A 1 s orbital shaking step with an amplitude of 1 mm was programmed for every 29 s and fluorescence (top reading mode) from each well was monitored every 29 s for 2 h. Increase in fluorescence over time expressed as relative fluorescence unit (RFU) was plotted after subtracting background fluorescence contributed by GPVI-Cy3 substrate in buffer without recombinant enzymes. In some experiments, rhADAM10 or rhADAM17 were pre-treated with metalloproteinase inhibitors (50 mM EDTA, 100 μ M GM6001, 2 μ M GI254023X or 1 μ g/mL rhTIMPs 1-4) for 10-15 min at RT before the addition of 5 mM NEM, 10 μ g/mL human PF4, 0.5 U/mL heparin or 100 U/mL heparin, and finally the GPVI-Cy3 substrate.

2.2.11.2 Measuring platelet ADAM10 activity by FRET

To quantify ADAM10 activity on resting and treated platelets, washed platelets were prepared as described (Section 2.2.2.2) and resuspended at 5×10^8 platelets/mL in Tyrode's buffer. Test samples in 250 μ L reaction volumes were prepared in wells of an OptiPlate-96 White Opaque 96-well microplate (PerkinElmer, Waltham, Massachusetts, USA). Unless otherwise specified, 20 μ L of washed platelets (10^7 platelets per well) were mixed with 100 μ L of 100 mM Tris

buffer pH 7.4 containing 5 μ M ZnSO₄, and pre-treated with inhibitors for 30 min at RT before addition of agonists. The inhibitors used in ADAM10 activity assays were 50 mM EDTA, 100 μ M GM6001, 2 μ M G1254023X, 10 μ M Bay 61-3606, 10 μ M PP1, 10 μ g/mL IV.3 monoclonal antibody or 1 μ g/mL rhTIMPs 1-4, all final concentrations. 5 mM NEM, 10 μ M A23187 calcium ionophore, 1-10 μ g/mL collagen or 10-30 μ g/mL CRP-xL were used as agonists in washed platelet assays. In experiments to test the effect of HIT immune complex on platelet ADAM10 activity, 0.5 U/mL or 100 U/mL heparin, 10 μ g/mL human PF4, or 100 μ g/mL of plasma Ig isolated from confirmed HIT or non-HIT thrombocytopenic patients (Section 2.2.6), were added to the platelets. All samples were made up to 250 μ L with 150 mM NaCl. Each assay plate was equilibrated at 37°C for 3 min before addition of 1 μ M of the GPVI-Cy3 substrate and change in fluorescence was measured as described in Section 2.2.11.1. The relative change in relative fluorescence unit (RFU) was calculated by subtraction of background fluorescence in wells containing all reagents except platelets. The rate of activity was compared at the 20 min timepoint whereby the greatest rate of change was observed.

2.2.12 Statistical analyses

The statistical package in GraphPad Prism version 7.03 (San Diego, California, USA) was used to compute the following statistical analyses. Shapiro-Wilk's normality test was applied to evaluate the data distribution. Student's *t*-test was used to compare differences between two groups of normally distributed datasets while the Mann-Whitney test was performed on non-parametric datasets. Comparisons between more than two groups were performed using one-way ANOVA with Tukey's multiple comparisons post-hoc test for parametric datasets while Kruskal-Wallis with Dunn's multiple comparisons post-hoc test was computed for non-parametric datasets. Two-way ANOVA with Tukey's post-hoc test for parametric datasets or

Sidak's test for non-parametric datasets was used to test the effect of two independent variables and presence of interaction, on the dependent variable. Whiskers and outliers in box-and-whisker plots were plotted using Tukey's method whereby outliers were identified as greater than or less than 1.5 times the interquartile range (75th percentile – 25th percentile). To assess the correlation between normally distributed parameters, Pearson's correlation was computed, otherwise Spearman's correlation was performed. When comparing the genotype proportions between groups, Pearson's Chi-square test for independence was performed. Fisher's exact test was calculated to compare allelic frequencies between groups. IBM SPSS Statistics version 24 (Armonk, New York, USA) was used to perform Pearson's Chi-square analysis of the association between patient characteristics and SRA outcome, and plasma sGPVI levels. Two-tailed *p*-value of <0.05 was considered statistically significant. Where relevant, *p*-values were adjusted for multiple hypotheses testing using the Benjamini-Hochberg method (Benjamini and Hochberg, 1995).

Chapter 3 Consequences of engagement of platelets by anti-platelet autoantibodies

3.1 Background

Platelet activation leading to thrombocytopenia is a common event in a number of autoimmune disorders such as SLE, rheumatoid arthritis, Sjogren's syndrome and anti-phospholipid syndrome (Habets et al., 2013, Łukasik et al., 2018, Linge et al., 2018, Olumuyiwa-Akeredolu et al., 2019), and is a signature event in platelet-based autoimmune diseases such as HIT and ITP. It is imperative to understand how platelet activation is triggered at a molecular level in a disease-specific manner in order to design targeted therapeutic interventions, to better understand and predict bleeding or thrombotic propensity, and to improve the clinical management of patients.

Platelet receptors such as GPIb α (Section 1.1.2) and GPVI (Section 1.2.2) have been demonstrated to have roles in autoimmune-based platelet pathologies. Interestingly, most negatively charged anticoagulants have some ability to potentiate the activation of platelets by agonists such as ADP, generally when platelets are permitted to adhere to surfaces coated with the negatively charged material. This interaction requires active $\alpha_{IIb}\beta_3$ (Gao et al., 2011). The low affinity receptor for IgG, Fc γ RIIa is an additional receptor on human platelets that plays a key role in the pathogenesis of several platelet-based autoimmune disorders (Section 1.2.4), however the mechanism(s) leading to platelet activation via Fc γ RIIa have not been fully elucidated. Work described in this chapter will define some of the key steps in the molecular pathway that are triggered upon ligand-based engagement of Fc γ RIIa that lead to platelet activation and receptor shedding.

3.1.1 Platelet FcγRIIa

Although FcγRIIa is expressed in cells including platelets, neutrophils, eosinophils and macrophages, it is the only activating Fcγ receptor on platelets. FcγRIIa binds with low affinity to monomeric IgG but more avidly to IgG within immune complexes (Karas et al., 1982). FcγRIIa is made up of two extracellular Ig-like domains, a single transmembrane domain and a cytoplasmic tail containing a juxtamembranous calmodulin-binding site. FcγRIIa signals via the ITAM motif harboured within the cytoplasmic region as illustrated in Figure 3.1.

The mouse monoclonal antibody, IV.3, generated by Looney and colleagues (1986), is widely used in platelet studies due to its ability to functionally block human FcγRIIa. The intact monomeric form of IV.3 recognises amino acid residues 132-137 located in the second Ig-like domain of FcγRIIa (Ramsland et al., 2011). Thus IV.3 blocks IgG engagement of the FcγRIIa ligand-binding domain which includes amino acid 131 (Tate et al., 1992). In this manner, IV.3 is extensively used as a non-activating function blocker of FcγRIIa. However, crosslinking of IV.3 with anti-mouse F(ab')₂ fragments triggers clustering of FcγRIIa leading to platelet activation (Rosenfeld et al., 1985).

3.1.2 A role for FcγRIIa in platelet-related autoimmune disorders

Human platelet FcγRIIa can be activated by Ig in a variety of forms including aggregated IgG, anti-platelet antibodies, pathogens opsonised by IgG and immune complexes (Arman and Krauel, 2015). Platelet FcγRIIa has been implicated in numerous autoimmune conditions as discussed in Section 1.2.4, including HIT (Kelton et al., 1988, Reilly et al., 2001, Gardiner et al., 2008b). HIT is a drug-induced, autoimmune disorder caused by platelet-activating antibodies, typically of the IgG class, binding an antigenic complex of polyanionic heparin and cationic PF4

as detailed in Section 1.3. As the only Fc receptor present on human platelets, Fc γ R1la is regarded as the major mediator of platelet pathology in HIT.

3.1.3 Focusing on GPVI

GPVI is expressed on the platelet surface in a non-covalent complex with FcR γ -chain dimer which is required for GPVI signalling (Berlango et al., 2002). The cytoplasmic region of FcR γ -chain contains an ITAM and ligand-binding to GPVI triggers a signalling cascade involving phosphorylation of tyrosine residues within the ITAM domain (Gibbins et al., 1997), as depicted in Figure 3.1. The sequential phosphorylation of Src family kinases (SFK, Fyn and Lyn) to the conserved ITAM motif (YXXL) within FcR γ -chain results in recruitment and activation of spleen tyrosine kinase (Syk) which in turn activates numerous downstream proteins, culminating in integrin activation, calcium mobilisation and platelet activation (Nieswandt and Watson, 2003). Details of the ITAM signalling cascade is discussed in Section 1.2.5.

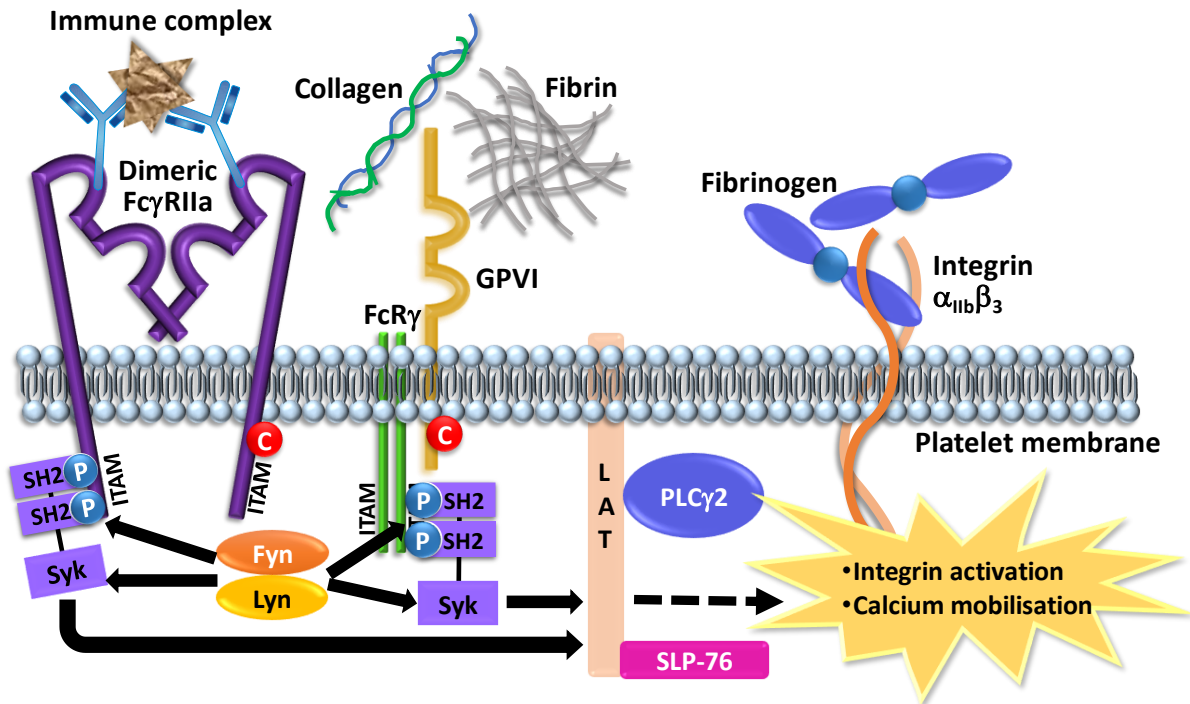


Figure 3.1 ITAM signalling mediated by GPVI/FcR γ -chain and Fc γ RIIa.

Ligation of Fc γ RIIa by immune complexes or GPVI by collagen or fibrin leads to tyrosine phosphorylation of ITAM motifs within the cytoplasmic tails of Fc γ RIIa and FcR γ -chain (associated with GPVI) and phosphorylation of SFKs including Fyn, Lyn and Syk. This then initiates downstream activation of LAT and PLC γ 2 resulting in activation of PI3 kinase, cytoskeletal reorganisation, $\alpha_{IIb}\beta_3$ integrin activation, calcium mobilisation and platelet aggregation. Calmodulin-binding sites on Fc γ RIIa and GPVI are indicated by red circles (C). Syk, spleen tyrosine kinase; ITAM, immunoreceptor tyrosine-based activation motif; LAT, linker for activation of T-cells; SLP-76, SH2-domain-containing leukocyte protein of 76kDa; PLC γ 2, phospholipase γ 2.

3.1.4 Ligand engagement of GPVI and FcγRIIa

FcγRIIa and GPVI share many common features which extend beyond their structural properties (Qiao et al., 2015). Gardiner and colleagues (2008b) demonstrated that treatment of platelets with GPVI ligands resulted in a calpain-mediated proteolytic cleavage of the FcγRIIa cytoplasmic domain. Platelets treated with sera from HIT patients caused a similar proteolysis of FcγRIIa that was concordant with a positive SRA which is a confirmatory diagnostic assay for HIT (Nazi et al., 2013). Engagement of FcγRIIa by the Fc portion of a number of monoclonal antibodies, for example VM58 against CD36, 1G5 against GPVI or 14A2 against tetraspanin CD151, activated an ADAM10-mediated metalloproteolytic shedding of the GPVI ectodomain and calpain-mediated cleavage of the FcγRIIa cytoplasmic tail (Gardiner et al., 2008b, Al-Tamimi et al., 2009b). Detachment of calmodulin from cytoplasmic binding sites by treatment of platelets with calmodulin inhibitors also triggered proteolysis of GPVI and FcγRIIa. Proteolysis of both receptors was EDTA-inhibitable, indicating a requirement for divalent cations in the proteolysis. Irreversible removal of the ITAM motif from FcγRIIa and the ligand-binding region of GPVI is proposed to be a mechanism for down-regulation of intracellular signalling and platelet reactivity (Nieswandt et al., 2001, Gardiner et al., 2004, Bergmeier et al., 2004, Gardiner et al., 2008b, Bender et al., 2013).

Work to date has shown that antibodies isolated from the sera of patients with confirmed HIT resulted in the same dual proteolysis of FcγRIIa and GPVI from donor washed platelets in a Western blot analysis of platelet lysates (Gardiner et al., 2008b). These studies provided evidence for a role for FcγRIIa engagement in mediating a loss of GPVI and imply that release of sGPVI could signal engagement, particularly by autoantibodies, of the FcγRIIa receptor. To

assess the possibility that sGPVI could be a biomarker for FcγRIIa activation, an analysis of the molecular pathway leading to GPVI proteolysis is warranted.

3.1.5 Studies of PF4 conformation in the formation of the HIT immune complex

Depending on the pH of the solution, PF4 exists as a heterogeneous mixture of monomers, dimers and tetramers (Mayo and Chen, 1989). PF4 tetramer forms a particularly stable and large complex with heparin through electrostatic interaction over a narrow stoichiometric ratio of PF4:heparin (Rauova et al., 2005). A recent crystallography study revealed binding of a low-molecular-weight-heparin, fondaparinux, via its anionic sulphate groups to a cationic groove on PF4 tetramer conferred by lysine, histidine and arginine residues (Cai et al., 2015). These electrostatic interactions formed a stable PF4/fondaparinux complex and exposed the epitope for the HIT-like antibody KKO. Together, the stepwise interactions between heparin and PF4 tetramer, then between the PF4/heparin complex and KKO or HIT Ig culminate in the formation of a stable, platelet-activating ternary immune complex (Cai et al., 2016). In a single-amino-acid-mutagenesis study, five amino acid residues on PF4 were identified to be important for the binding of platelet-activating HIT antibodies from confirmed HIT patient sera (Huynh et al., 2019). Four of the five amino acids were cysteine residues, which when mutated to alanine, will disrupt disulphide bonds and potentially alter the structure of PF4, thus resulting in the loss of antibody binding.

3.1.6 Key questions to be addressed

To date, platelet ITAM-mediated signalling in HIT pathogenesis has not been extensively evaluated. It is hypothesised that treatment of platelets with a HIT antibody in complex with PF4/heparin causes platelet activation and this is dependent on FcγRIIa. Further, by engagement of the ITAM signalling pathways, platelet-activating HIT antibodies can induce

rapid GPVI shedding in a heparin- and metalloproteinase-dependent manner. A role for ADAM10 in this pathway has not been fully addressed and it is hypothesised that exposure of platelets to PF4/heparin immune complexes will alter ADAM10 activity and release of sGPVI.

Figure 3.2 highlights aspects of HIT pathogenic pathways that will be addressed in this chapter. Shedding of GPVI was evaluated by flow cytometry to assess levels of platelet GPVI and by an established ELISA that quantifies the released sGPVI fragment. Experimental conditions to activate washed platelets with the KKO monoclonal antibody and induce P-selectin expression were created and applied in experiments to evaluate consequences of purified HIT Ig or control Ig on levels of donor platelet GPVI in the presence of low or high concentrations of heparin, and PF4 with inhibitors of Syk or Src. Ig purified from plasma samples from different HIT patients provided an opportunity to investigate differences in capacity of the Ig to cause GPVI shedding in donor platelets. An evaluation of the impact of a HIT antibody in complex with PF4/heparin on platelet ADAM10 activity was attempted using a FRET activity assay.

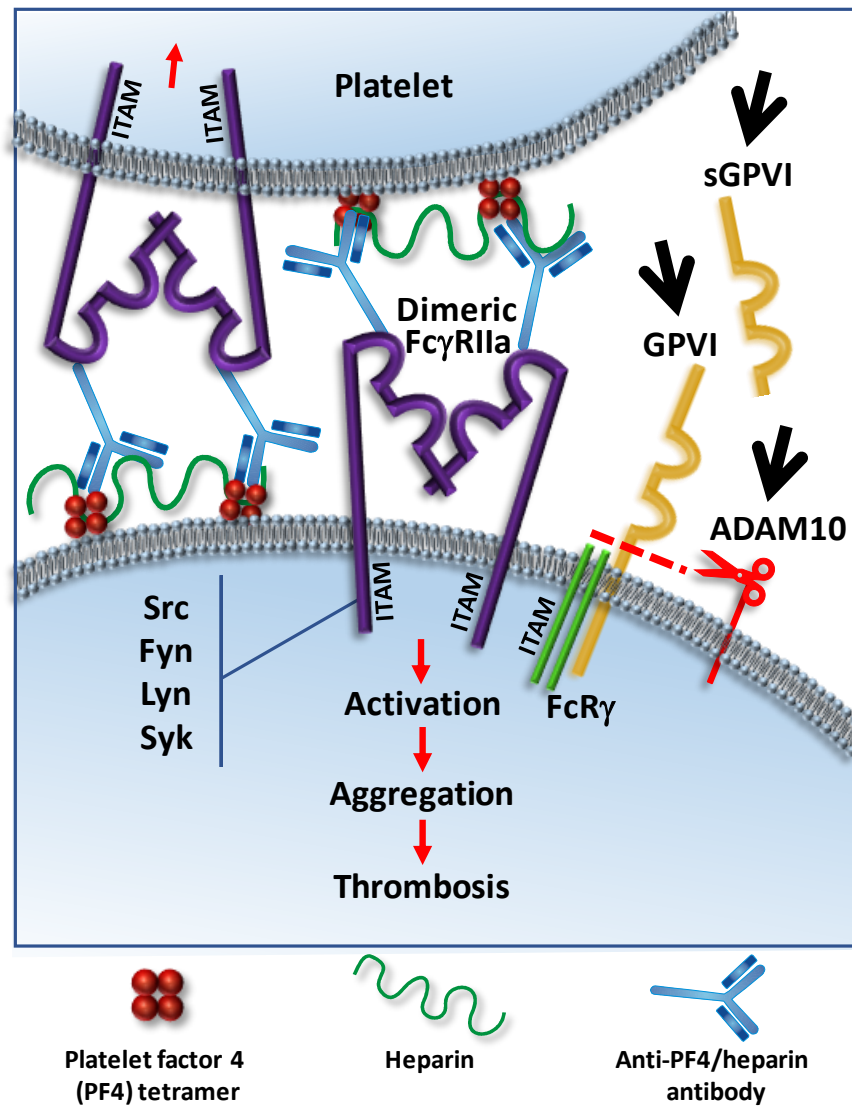


Figure 3.2 Consequences of engagement of platelets by anti-platelet autoantibodies.

This chapter will focus on the consequence of HIT Ig on donor platelet surface GPVI levels and release of sGPVI, as indicated by black arrows. A role for platelet ADAM10 will also be explored.

3.2 Aims

To explore the HIT pathogenic pathways in platelets from healthy donors using purified native human PF4 in combination with either KKO antibody, or plasma samples or Ig purified from patients with confirmed HIT, and to assess the requirement for ITAM signalling and platelet ADAM10 activity in HIT Ig-induced GPVI shedding.

3.3 Results

3.3.1 Purification of native human PF4

Sufficient purified, native human PF4 was required for experiments to form a complex with heparin and create the antigenic complex recognised by HIT Ig. Thus, PF4 was purified from expired platelet transfusion bags using affinity chromatography followed by size exclusion chromatography as described (Section 2.2.5) using a method essentially the same as described by Medici and colleagues (1989).

In order to evaluate purity, aliquots of starting material (supernatant of platelet lysate) and various column fractions collected across the heparin-affinity chromatography step were electrophoresed on a 15% SDS-PAGE under reducing conditions and the gel was stained with Coomassie blue solution for visualisation of proteins (Figure 3.3). Supernatant fractions contained the total protein present after lysis of platelets and fractions labelled unbound contained proteins that did not bind to the beads. As expected, these fractions contained many proteins with a variety of molecular weights. Proteins that bound non-specifically were eluted during the wash steps (wash start and end) and samples labelled Eluate 1 and 2 contained proteins eluted in buffer containing 2 M NaCl. Eluate fractions were dialysed against PBS, then concentrated three-fold using an ultracentrifugal filter device, and aliquots were assayed by SDS-PAGE. A protein band of approximately 58 kDa and a lower molecular weight (<10 kDa) band were visible in the eluate fractions. These bands represent antithrombin (58 kDa) and monomeric PF4 (7.8 kDa) both of which are known to bind to Heparin Sepharose beads (Niewiarowski et al., 1979, Medici et al., 1989).

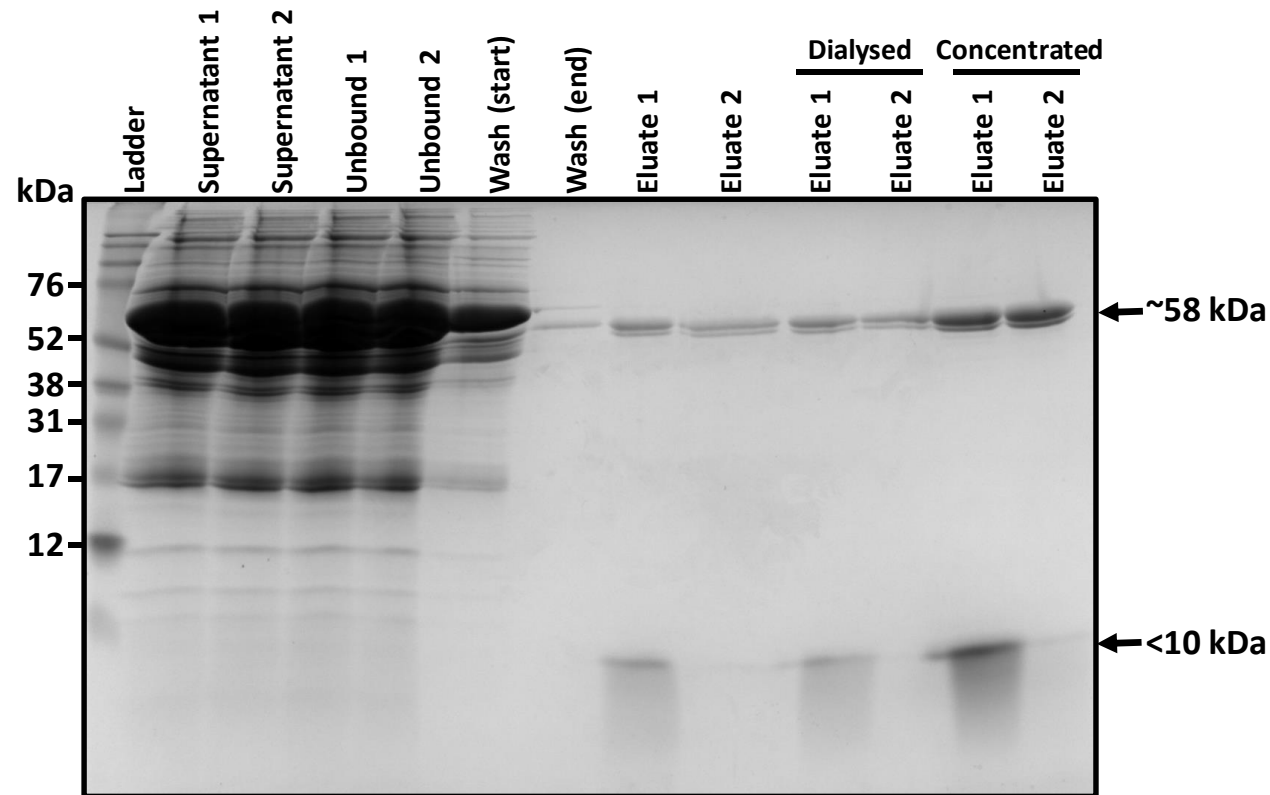


Figure 3.3 Isolation of native human PF4 by affinity chromatography on Heparin Sepharose.

Platelets were isolated from an expired platelet transfusion bag and treated as described in Section 2.2.5 to form platelet lysates, then subjected to affinity chromatography on Heparin Sepharose (Section 2.2.5). Proteins that bound to the resin were eluted using phosphate buffer containing 2 M NaCl. All fractions were dialysed into PBS and aliquots were subjected to electrophoresis under reducing conditions on a 15% SDS-acrylamide gel which was stained with Coomassie blue (Sections 2.2.4.3). A protein band of approximately 58 kDa and a lower molecular weight (<10 kDa) band were visible. These bands represent antithrombin (58 kDa) and PF4 (7.8 kDa for monomeric PF4). The amounts of protein loaded, based on absorbance at 280 nm were as follows: 100 µg supernatant, 100 µg unbound, 39 µg wash (start), 0.2 µg wash (end), 10 µg Eluate 1, 0.9 µg Eluate 2, 4.1 µg dialysed Eluate 1, 1.8 µg dialysed Eluate 2, 10 µg concentrated Eluate 1 and 7.5 µg concentrated Eluate 2.

To separate the lower molecular weight band away from other proteins in the eluate fractions, Superdex 200 size exclusion chromatography was employed as the resolution range was appropriate to separate proteins of less than 10 kDa (retained by the column) away from proteins of 50 kDa or greater. Eluate 1 and 2 isolated in Section 2.2.5 were pooled and concentrated three-fold to 0.28 mg/mL and applied to a Superdex 200 column using an ÄKTA Explorer FPLC system as described (Section 2.2.5). Figure 3.4 displays the UV absorbance values at 215 nm and 280 nm of fractions eluted from the column. Two peaks were observed, which represent the 58 kDa protein (peak 1) and the lower molecular weight protein (peak 2). Figure 3.5 shows a Coomassie-stained 10% acrylamide SDS gel of fractions collected during size exclusion chromatography. The starting material contained a mixture of high and low molecular weight proteins. Pooled fractions A12 and A13 corresponded to components of peak 1, while pooled fractions A15 and B1 were the composition of peak 2 in the elution profile in Figure 3.4. A band greater than 52 kDa, representing antithrombin (58 kDa), was seen in fractions A12 and A13. A <10 kDa band, representing monomeric PF4 under reducing conditions (7.8 kDa), was observed in fractions A15 and B1.

106 µg of a low-molecular-weight protein of <10 kDa, representing PF4, was isolated from 320 mL of platelet lysate from 240×10^9 platelets. The amount of protein was estimated using the Beer-Lambert Law equation based on the molecular weight of monomeric PF4 (7769 g/mol) at a molar extinction coefficient of $1740 \text{ M}^{-1}\text{cm}^{-1}$ (non-reduced) and absorbance at 280 nm with a path length of 1 cm. The estimated yield of PF4 was 3.7% of the expected yield as the physiological level of PF4 was estimated to be $12 \pm 5 \text{ ng per } 10^6 \text{ platelets}$ (mean $\pm$ SD) in healthy donors (Peterson et al., 2010).

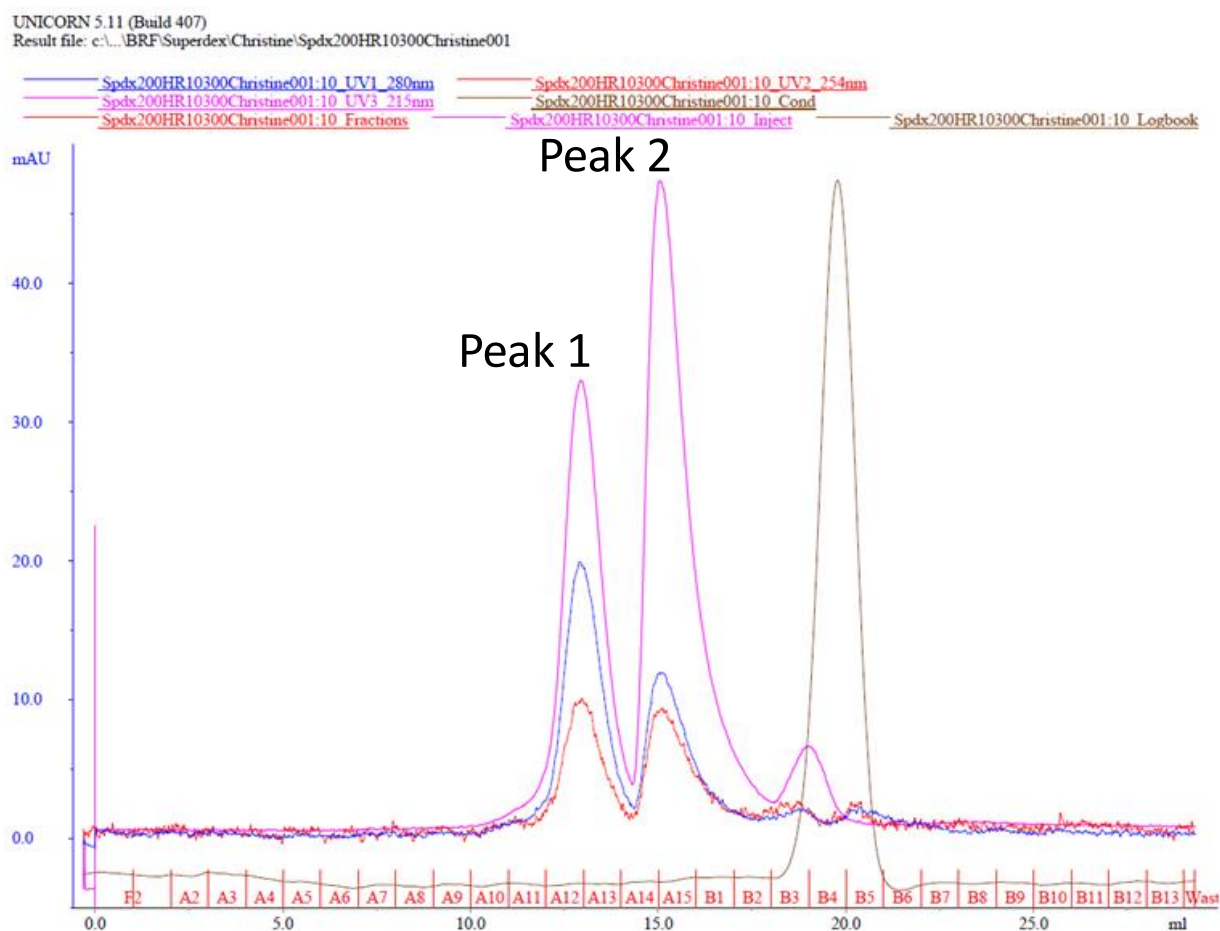


Figure 3.4 Elution profile of proteins present in the eluted material from Heparin Sepharose affinity chromatography of platelet lysate.

Protein in the eluate fractions obtained from affinity chromatography of platelet lysate on Heparin Sepharose (Section 2.2.5) was subjected to size exclusion chromatography on a Superdex 200 media to separate the lower molecular weight band away from other proteins seen in Eluate 1 and 2 in Figure 3.3. Eluate 1 and 2 were combined and concentrated three-fold to 0.28 mg/mL and applied onto a Superdex 200 column using a ÄKTA Explorer FPLC system at a flow rate of 0.3 mL/min (Section 2.2.5). 1 mL fractions were collected. Peak 1 which eluted at 39 min (peak at 13.0 mL) represent the higher molecular weight band of approximately 58 kDa while peak 2 which eluted at 50 min (peak at 15.5 mL) represent the <10 kDa band observed in Figure 3.3. Ultraviolet absorbance at 280 nm (blue trace) is shown in mAU on the y-axis. The x-axis shows the fractions collected (in red) and elution volume (in black). The pink, red and brown traces represent absorbance at 215 nm, absorbance at 254 nm and conductivity, respectively.

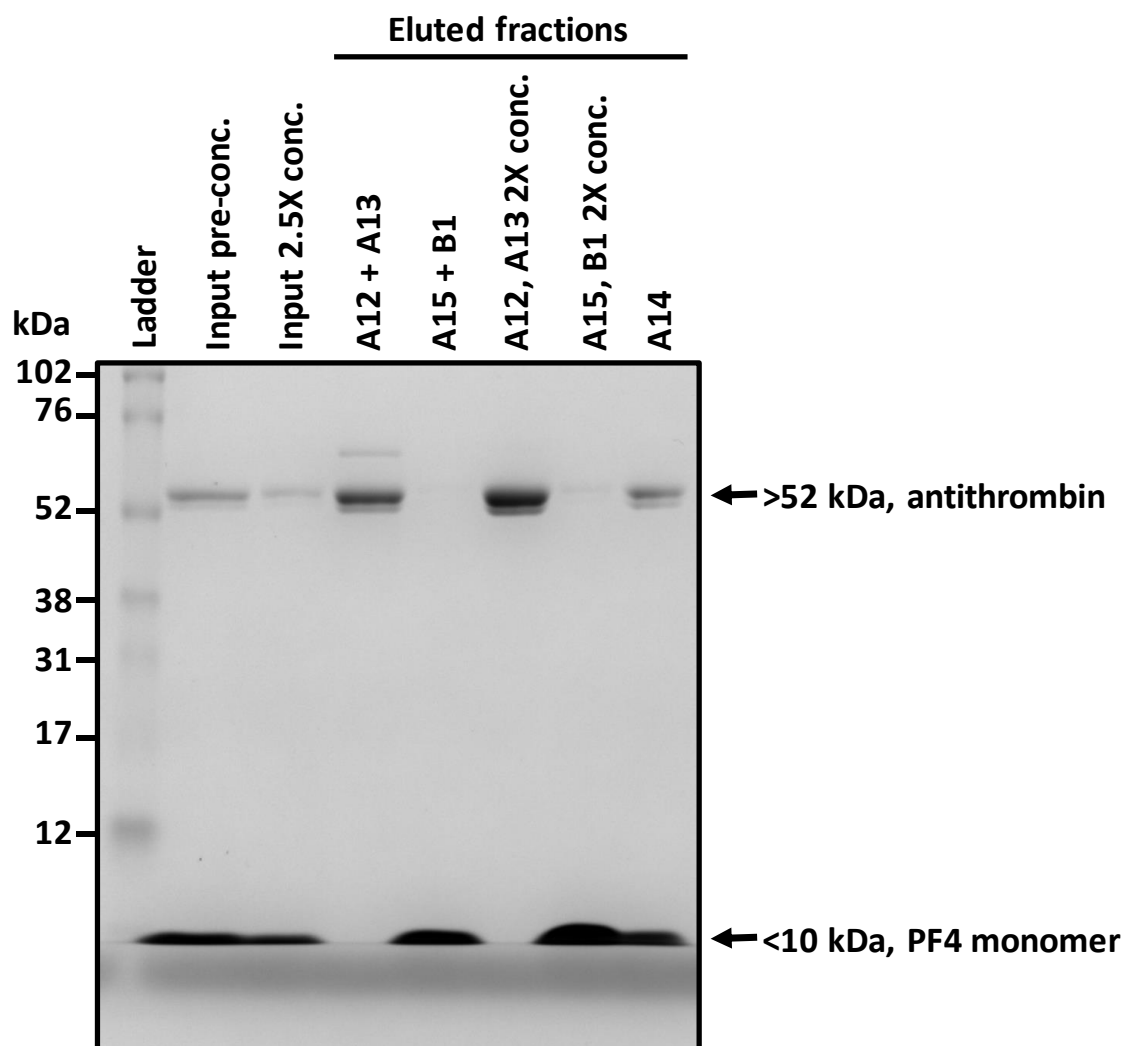


Figure 3.5 Isolation of human PF4 by size exclusion chromatography on Superdex 200.

Protein in the eluate fractions obtained from affinity chromatography of platelet lysate on Heparin Sepharose (Section 2.2.5) was subjected to size exclusion chromatography on a Superdex 200 media to separate the lower molecular weight band away from other proteins seen in Eluate 1 and 2 in Figure 3.3. Eluate 1 and 2 were combined and concentrated three-fold to 0.28 mg/mL and applied onto a Superdex 200 column (Section 2.2.5). The input material and fractions corresponding to the ultraviolet absorbance peaks seen in Figure 3.4 were subjected to electrophoresis on a 10% reducing SDS-acrylamide gel (Section 2.2.4.2) which was stained with Coomassie blue (Section 2.2.4.3). Fractions A12 and A13 corresponded to peak 1 while fractions A15 and B1 corresponded to peak 2 in Figure 3.4. A >52 kDa protein band representing antithrombin (58 kDa), was visible in fractions A12 and A13 which corresponded to peak 1 in Figure 3.4. A lower molecular weight (<10 kDa) band representing monomeric PF4 (7.8 kDa), was observed in fractions A15 and B1 which corresponded to peak 2 in Figure 3.4. Both the >52kDa and <10 kDa bands were visible in fraction A14 which contains sample collected at the interface between peak 1 and peak 2. The amounts of protein loaded based on absorbance at 280 nm were as follows: 2.9 μ g input pre-conc., 2.8 μ g input 2.5-fold conc., 2.0 μ g A12+A13 fractions, 1.3 μ g A15+B1 fractions, 3.9 μ g 2-fold conc. A12+A13 fractions, 2.4 μ g 2-fold conc. A15+B1 fractions and 1.7 μ g A14 fraction. conc. denotes concentrated samples.

3.3.2 KKO antibody caused variable extents of platelet activation in donor platelets

The mouse anti-human PF4 monoclonal antibody, KKO was used to design a set of experimental conditions where the platelet-activating pathways triggered by HIT antibodies could be evaluated in donor platelets. Washed platelets from healthy donors were mixed with 10 µg/mL native human PF4 (Figure 3.5) and 50 µg/mL KKO antibody in the presence of low (0.2 U/mL) or high (100 U/mL) heparin as described in Section 2.2.7. Some samples were pre-treated with 10 µg/mL FcγRIIa function blocking antibody, IV.3 and all platelet suspensions were held at 37°C for 50 min. The extent of degranulation as measured by levels of P-selectin was evaluated by flow cytometry using a PE-conjugated anti-P-selectin monoclonal antibody (Section 2.2.8).

Figure 3.7 illustrates changes in P-selectin levels on donor washed platelets (n=3 unique healthy individuals) expressed as a fold-change in CD62P-binding relative to untreated samples for each donor. Treatment of platelets with high or low amounts of heparin, or purified human PF4 or KKO antibody alone did not activate degranulation. Mixing heparin with PF4 also did not cause platelet activation but the addition of KKO antibody to PF4 or low dose heparin induced platelet activation in all three donors, to various extents as seen in the histograms on Figure 3.6. The combination of KKO together with low dose heparin and PF4 triggered the greatest increase in P-selectin expression. As expected, P-selectin upregulation was abolished when 100 U/mL heparin was included as high doses of heparin engage PF4 and disrupt PF4-containing antigenic complexes, potentially disengaging the complex from FcγRIIa. P-selectin upregulation was also prevented when FcγRIIa was blocked by IV.3 monoclonal antibody. Although the differences in P-selectin levels between treatment groups did not achieve statistical significance (paired t test relative to untreated group, $p>0.05$, n=3), the data

clearly showed that anti-PF4 antibody KKO in the presence of PF4 can activate platelet degranulation and this was probably via engagement of Fc γ R1la. Responses from each donor whilst variable, consistently showed KKO-mediated platelet degranulation that required PF4 and functional Fc γ R1la. This data confirmed that purified PF4 was intact (recognised by KKO) and functional.

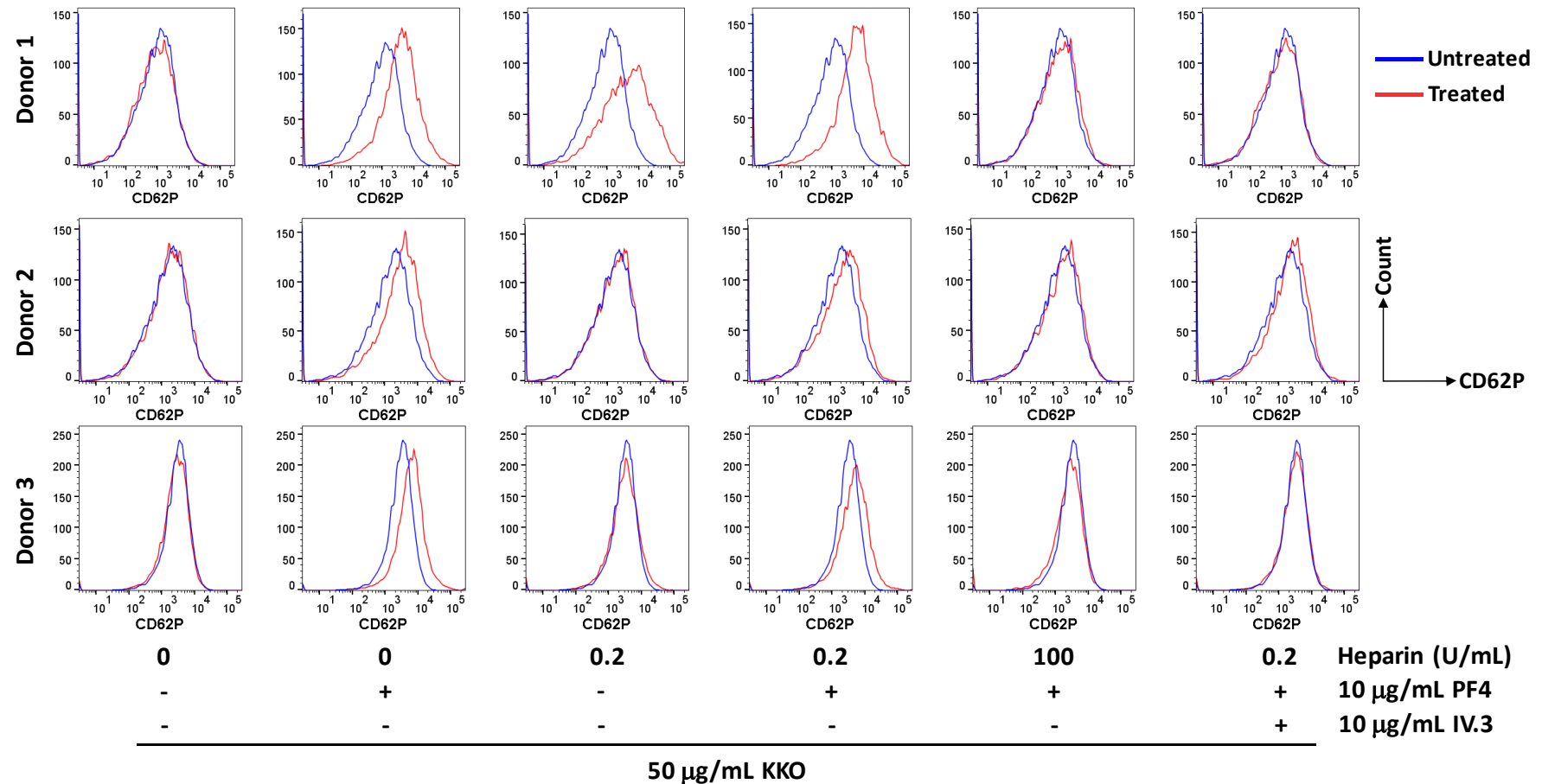


Figure 3.6 Variability in donor platelet responses to KKO antibody measured by P-selectin (CD62P) expression.

Healthy donor washed platelets were mixed with low (0.2 U/mL) or high (100 U/mL) doses of heparin, 10 µg/mL human PF4 or pre-treated with 10 µg/mL FcγRIIa function blocking antibody, IV.3, in the presence or absence of 50 µg/mL KKO mouse monoclonal antibody as described (Section 2.2.7). After 50 min at 37°C, platelets were labelled with a PE-conjugated anti-P-selectin monoclonal antibody to evaluate the extent of degranulation by flow cytometry (Section 2.2.8) as shown in Figure 3.7. Levels of P-selectin on treated platelets (red histogram) were compared to that of resting (untreated) platelets (blue histogram) for the corresponding donor.

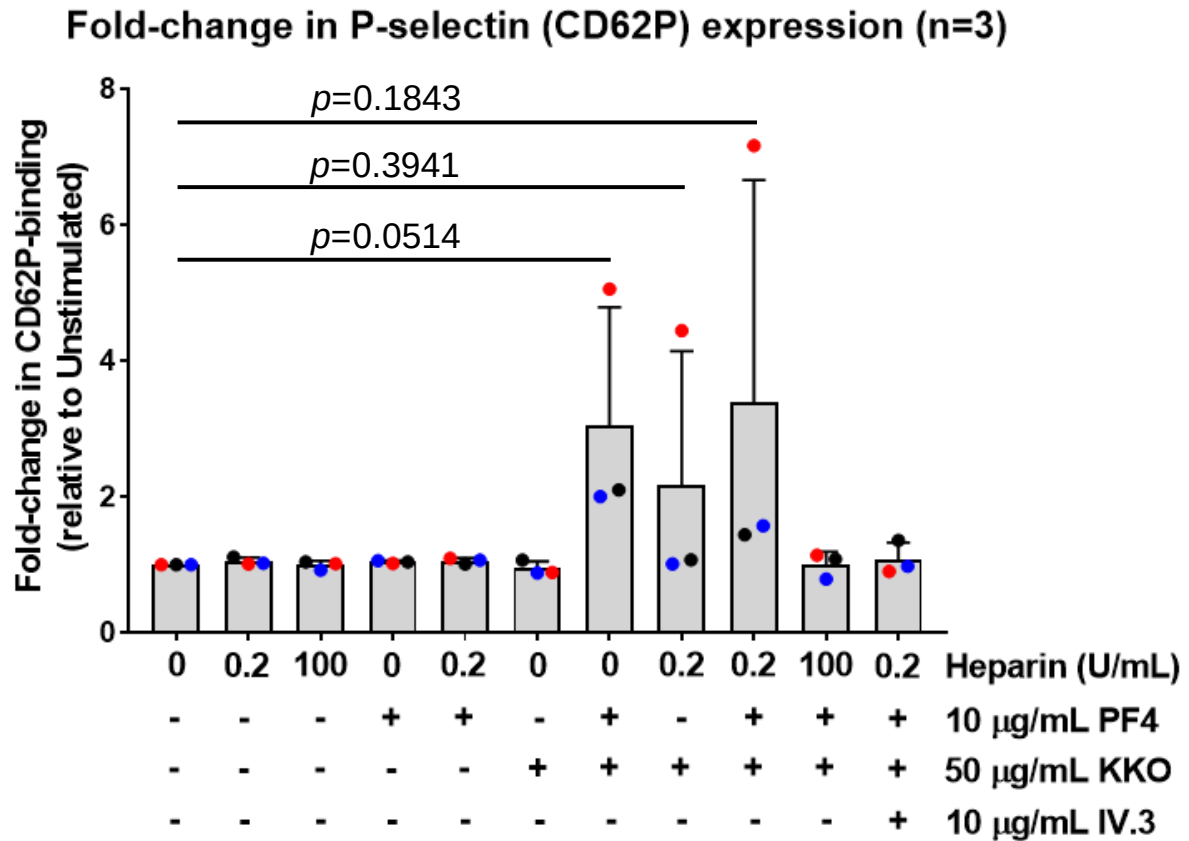


Figure 3.7 KKO antibody induced variable P-selectin (CD62P) expression in donor platelets.

Healthy donor washed platelets were mixed with low (0.2 U/mL) or high (100 U/mL) doses of heparin, 10 µg/mL human PF4 or pre-treated with 10 µg/mL FcγRIIa function blocking antibody, IV.3, in the presence or absence of 50 µg/mL KKO mouse monoclonal antibody as described (Section 2.2.7). After 50 min at 37°C, levels of P-selectin were assessed by flow cytometry (Section 2.2.8). Each coloured circle represents a unique donor (n=3). Changes in P-selectin surface expression on treated platelets were represented by a fold-change in P-selectin antibody binding (mean ± SD) relative to untreated platelets. No statistically significant differences in P-selectin surface levels between treatment groups were observed (paired t test relative to untreated group, $p>0.05$).

3.3.3 Effect of HIT plasma on donor platelet GPVI expression

To validate that the platelet activating effect of KKO on healthy donor platelets shown in Figure 3.7 can be achieved with plasma samples from confirmed HIT patients, the following experiment was performed using GPVI shedding as a measure of platelet activation. Citrated plasma from a confirmed HIT patient (HIT #1) or healthy control was isolated as described (Section 2.2.3) and incubated with healthy donor PRP in the presence of low (0.5 U/mL) or high (100 U/mL) heparin concentrations, or pre-treated with 50 mM EDTA as described in Section 2.2.7. After a 2 h incubation at RT, platelets were labelled with a 1G5 anti-human GPVI monoclonal antibody followed by staining with an Alexa Fluor 488-conjugated anti-mouse secondary antibody to enable evaluation of GPVI surface levels by flow cytometry (Section 2.2.8). The gating strategy to identify GPVI-positive population was based on fluorescence above antibody control to account for background fluorescence contributed by secondary antibody as illustrated in Figure 3.8.

Percent loss of GPVI was reported relative to unstimulated platelets in one healthy donor (Figure 3.9). In donor PRP incubated with control plasma, GPVI surface levels remained relatively stable at all heparin concentrations, with 3.0% loss of GPVI at low heparin dose (0.5 U/mL) and 3.1% at high heparin dose (100 U/mL) (Figure 3.9). When incubated with HIT plasma, a 5.9% loss of GPVI was observed at low heparin concentration which was reduced to a level comparable to no heparin at high heparin dose (2.9% at 100 U/mL heparin compared to 3.5% without heparin). In both control and HIT plasma, loss of GPVI was the lowest in the presence of EDTA which chelates divalent cation and is likely to imply metalloproteolytic shedding of GPVI.

The marginal loss of GPVI (6%) observed with HIT plasma may be due to the presence of plasma components which can interfere with the engagement of HIT Ig to donor platelets. For example, recent studies reported the influence of normal plasma Ig (Jones et al., 2018) and plasma fibronectin (Krauel et al., 2018) on platelet activation induced by HIT antibody. The functional assays for HIT diagnosis, SRA and HIPA, utilise donor washed platelets to detect platelet-activating antibodies. In addition, washed platelet systems have been reported to have greater sensitivity in platelet-activation assays compared to PRP assays for HIT testing (Warkentin et al., 2015). For these reasons, the experiment was subsequently performed using affinity-purified plasma Ig and donor washed platelets to minimise the presence of plasma components which may interfere with the assay.

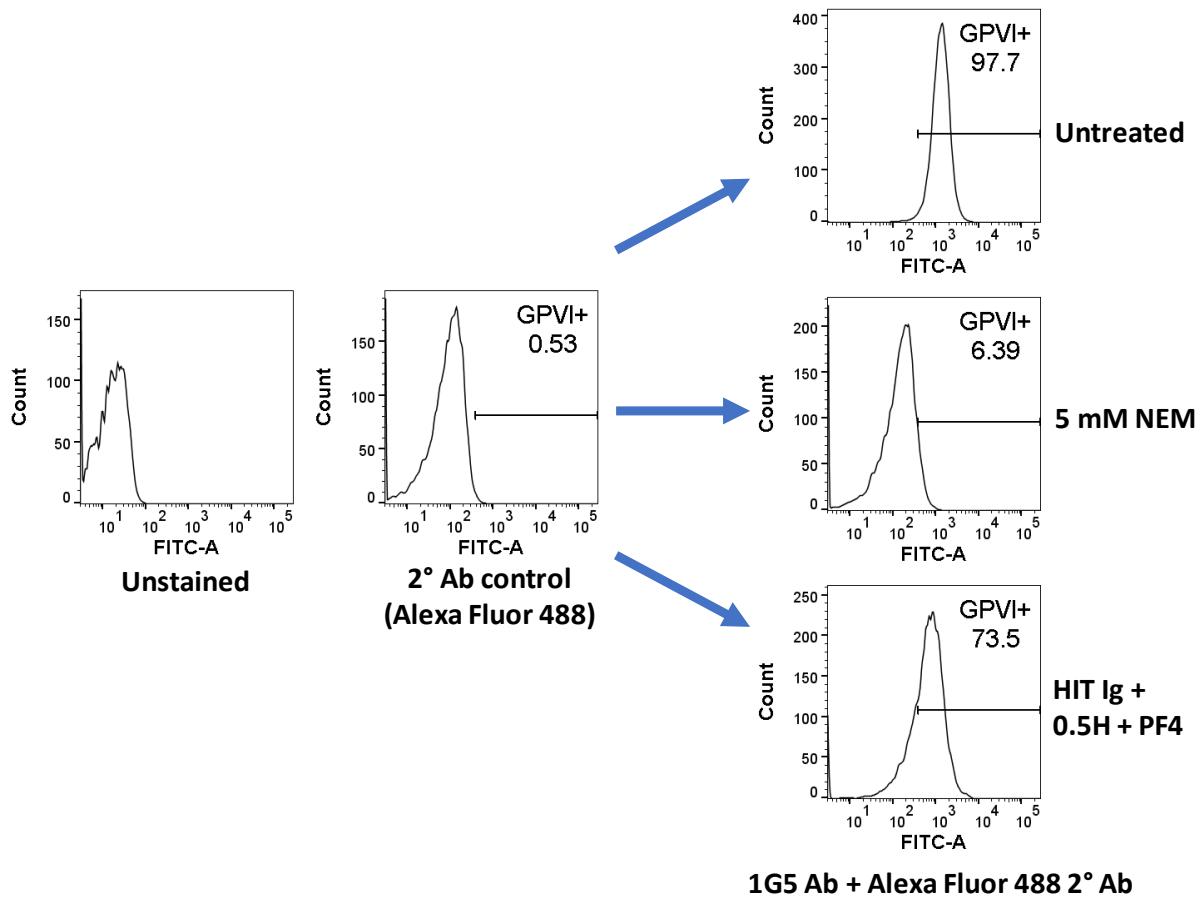


Figure 3.8 Gating strategy to define GPVI-positive platelet population.

GPVI-positive gate was set based on fluorescence detected on platelets stained with secondary (2°) antibody (Ab) control (Alexa Fluor 488-conjugated goat anti-mouse Ig). This gate was applied to all samples labelled with an unconjugated 1G5 anti-human GPVI monoclonal antibody, and an Alexa Fluor 488-conjugated anti-mouse secondary antibody. Example levels of GPVI on untreated platelets or platelets treated with 5 mM NEM or Ig from a HIT patient in the presence of PF4/heparin (0.5H + PF4) complex are shown on the right. NEM, N-ethylmaleimide, is a potent activator of GPVI shedding.

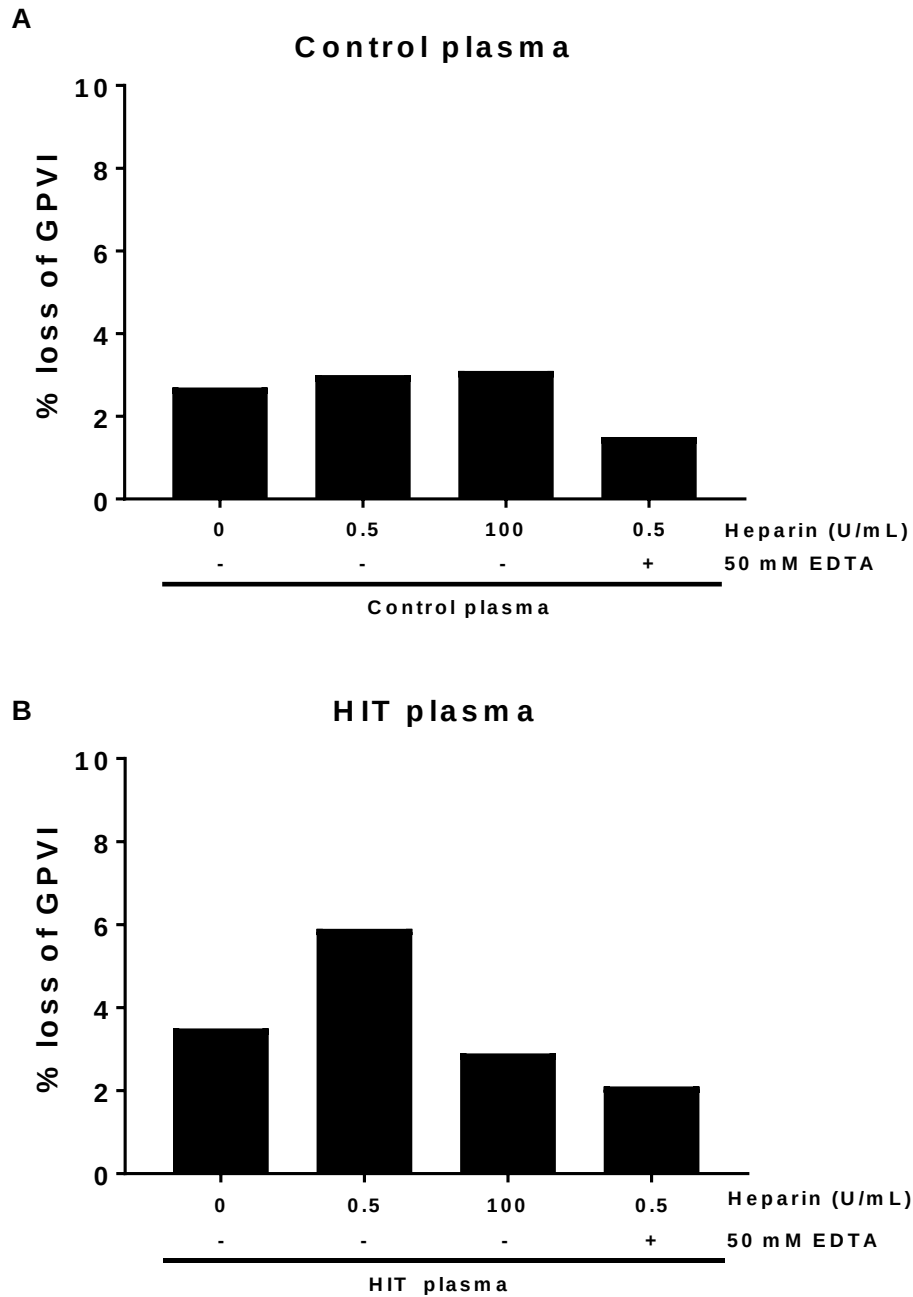


Figure 3.9 Effect of HIT plasma on donor platelet surface GPVI levels.

50 μ L of healthy donor PRP (Section 2.2.2.1) was mixed with 30 μ L of citrated plasma from confirmed HIT patient (HIT #1) or healthy control in the presence of low (0.5 U/mL) or high (100 U/mL) heparin concentrations, or pre-treated with 50 mM EDTA as described (Section 2.2.7). After a 2 h incubation at RT, platelets were labelled with a 1G5 anti-human GPVI monoclonal antibody followed by staining with an Alexa Fluor 488-conjugated anti-mouse secondary antibody to enable evaluation of GPVI surface levels by flow cytometry (Section 2.2.8). GPVI-positive platelet population was determined as described in Figure 3.8. Percent loss of GPVI was expressed relative to unstimulated platelets. This experiment was performed in n=1 healthy donor.

3.3.4 Isolation of Ig from human plasma

In order to validate that the platelet-activating effect of KKO shown in Figure 3.7 can be achieved with Ig from HIT patients and to minimise the presence of plasma components which may interfere with the assay as observed in Section 3.3.3, plasma Ig from three healthy controls, seven non-HIT thrombocytopenic controls and four confirmed HIT patients were affinity-purified. These purified Ig samples were subsequently tested on healthy donor washed platelets in the presence of low or high doses of heparin, and native human PF4 (Figure 3.5) to evaluate platelet activation measured by GPVI shedding.

All plasma samples were collected between years 2017 and 2018 except for one confirmed HIT patient plasma (HIT #1) which was collected in year 2007. For each sample, the total plasma Ig, predominantly IgG, was isolated by affinity chromatography on Protein A Sepharose as described in Sections 2.2.3 and 2.2.6. Aliquots of the fractions were analysed by SDS-PAGE under reducing and non-reducing conditions (Section 2.2.4.2), and the isolated proteins were visualised by Coomassie staining (Section 2.2.4.3).

Figures 3.10-3.13 show representative Coomassie blue-stained gels that were used to verify the molecular weights of purified Ig proteins. Ig was isolated from a total of seven batches of plasma: pooled ACD-anticoagulated plasma from three healthy donors (Figure 3.10), citrated plasma from four confirmed HIT patients (HIT #1 in Figure 3.10, HIT #8 in Figure 3.11, HIT #5 in Figure 3.12, HIT #2 in Figure 3.13), pooled citrated plasma from seven non-HIT thrombocytopenic patients (Figure 3.11), and citrated plasma from a thrombocytopenic donor (TCP in Figure 3.12). For each purification, two bands at ~50 kDa and ~25 kDa were visible in the eluate fractions under reducing conditions, and 150 kDa under non-reducing conditions (Figure 3.10, Figure 3.11, Figure 3.12 and Figure 3.13) strongly implying that these

predominant bands were the Ig heavy (50 kDa) and light (25 kDa) chains, that migrated as a single band of high molecular weight (>150 kDa) when disulphide bonds were intact. Approximately 6 mg of IgG was recovered from 1 mL of plasma for each batch as estimated by absorbance at 280 nm.

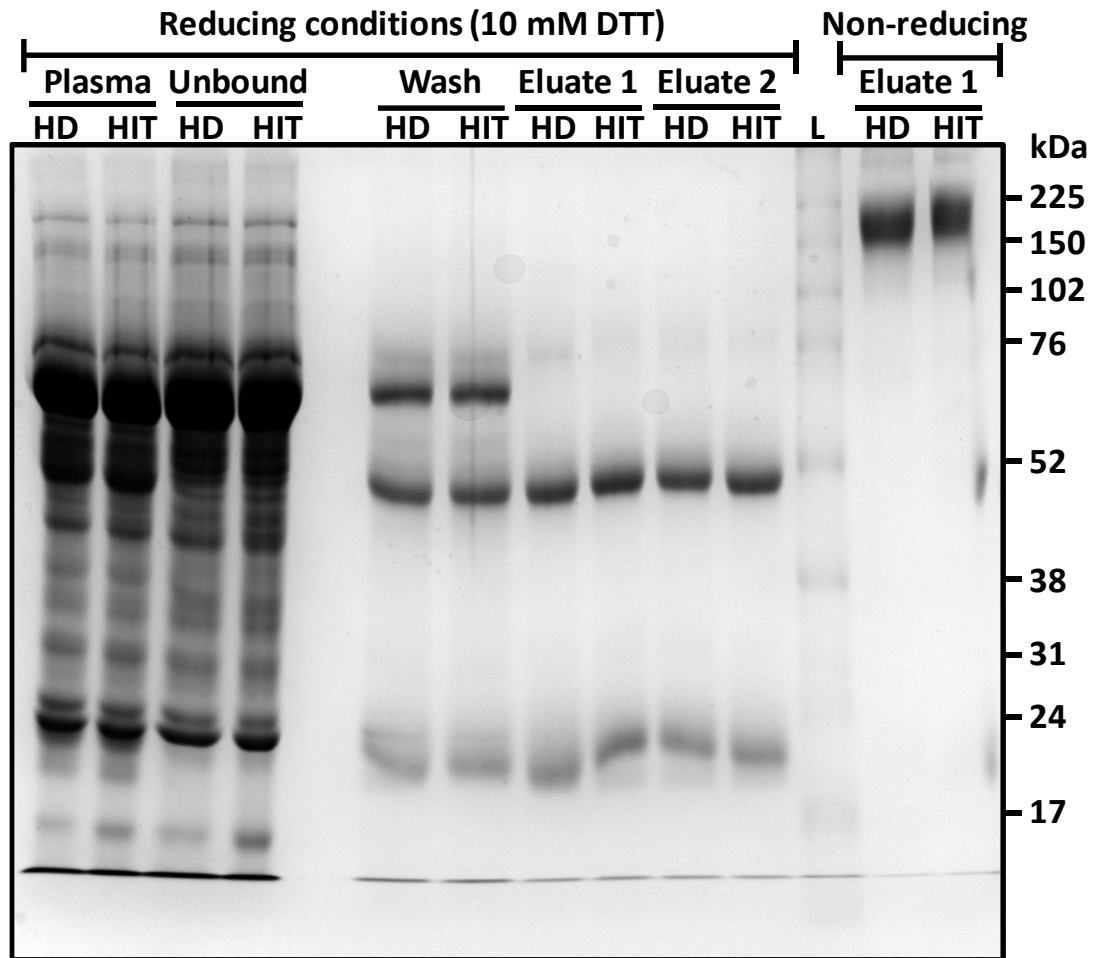


Figure 3.10 SDS-acrylamide gel stained with Coomassie blue of plasma and purified Ig from healthy donors (HD) and a confirmed HIT patient (HIT #1).

Ig were isolated from plasma by affinity chromatography on Protein A Sepharose as described in Section 2.2.6 from three healthy donors or a confirmed HIT patient. Aliquots of fractions were analysed on a 10% SDS-acrylamide gel, under reducing and non-reducing conditions (Section 2.2.4.2), and proteins were visualised by staining the gel with Coomassie blue (Section 2.2.4.3). Under reducing conditions, two bands at approximately 50 kDa and between 17-24 kDa, likely to represent the Ig heavy (50 kDa) and light (25 kDa) chains, were visible in the eluate fractions. These bands migrated as a single band between 150-225 kDa under non-reducing conditions which is characteristic of an intact Ig molecule (~150 kDa). The amounts of protein loaded were 100 µg total protein in plasma or unbound samples, or 20 µg total protein in wash or eluate fractions, based on absorbance at 280 nm. L = protein ladder.

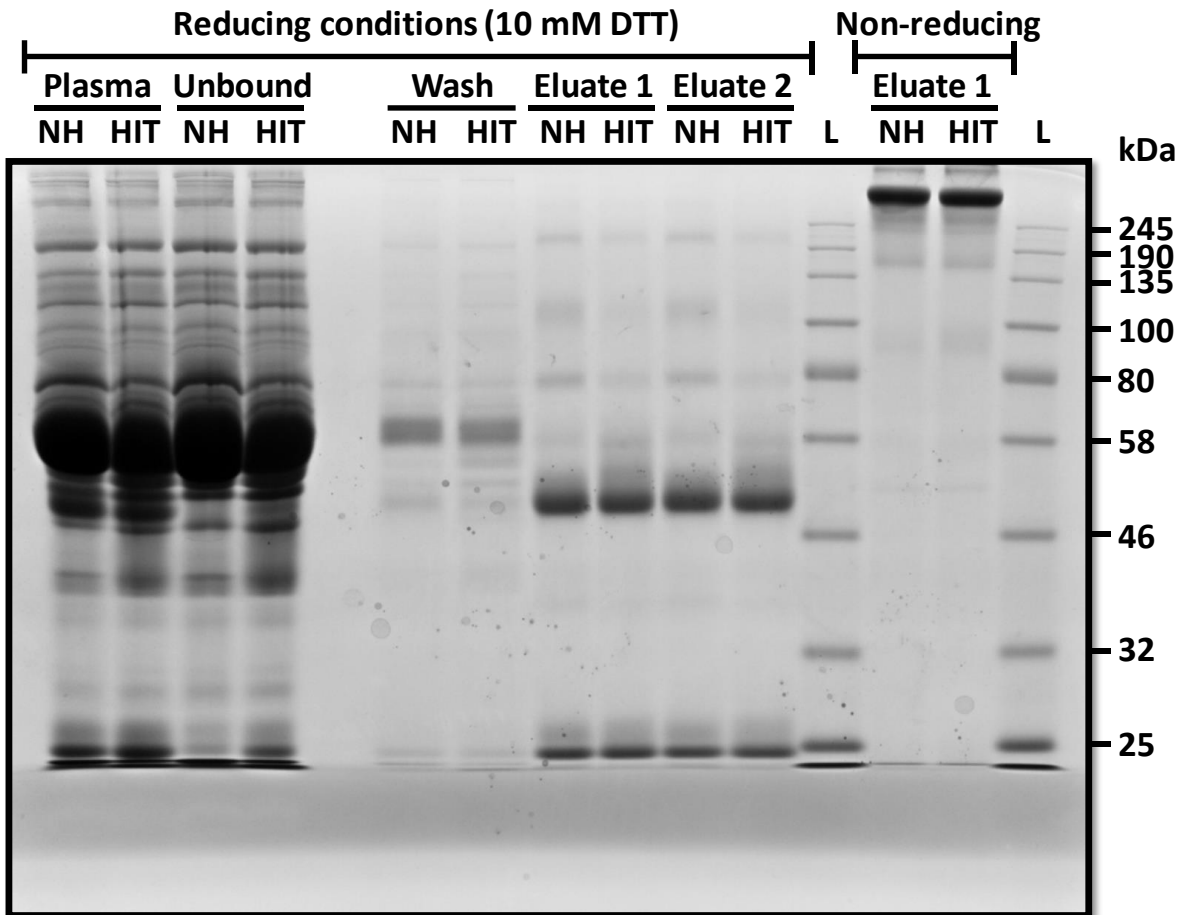


Figure 3.11 SDS-acrylamide gel stained with Coomassie blue of plasma and purified Ig from non-HIT thrombocytopenic patients (NH) and a confirmed HIT patient (HIT #8).

Ig were isolated from plasma by affinity chromatography on Protein A Sepharose as described in Section 2.2.6 from seven non-HIT thrombocytopenic patients or a confirmed HIT patient. Aliquots of fractions were analysed on a 10% SDS-acrylamide gel, under reducing and non-reducing conditions (Section 2.2.4.2), and proteins were visualised by staining the gel with Coomassie blue (Section 2.2.4.3). Under reducing conditions, two bands at approximately 50 kDa and 25 kDa, likely to represent the Ig heavy and light chains, were visible in the eluate fractions. These bands migrated as a high molecular weight band (>245 kDa) under non-reducing conditions which is characteristic of an intact Ig molecule (~150 kDa). The amounts of protein loaded were 100 µg total protein in plasma or unbound samples, or 20 µg total protein in wash or eluate fractions, based on absorbance at 280 nm. L = protein ladder.

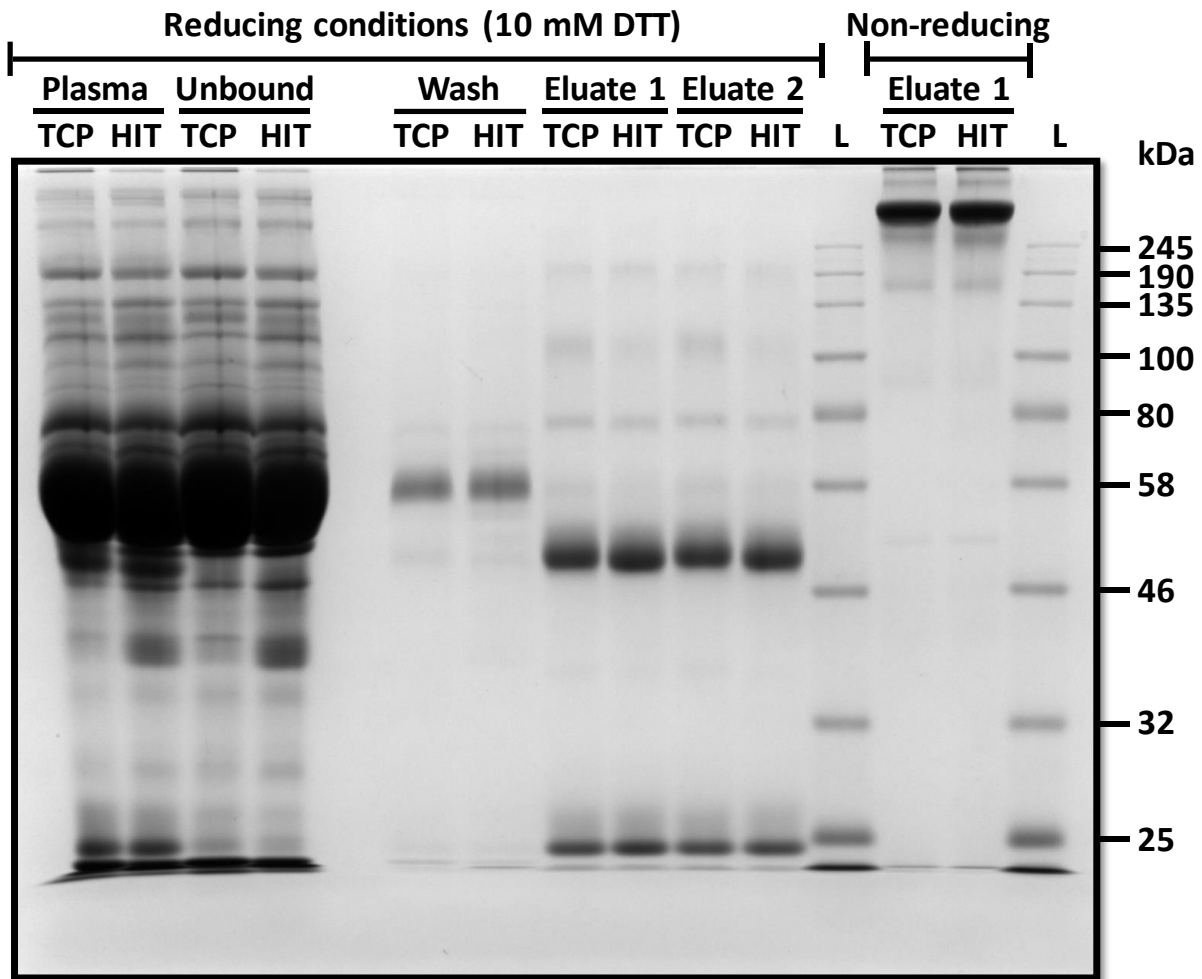


Figure 3.12 SDS-acrylamide gel stained with Coomassie blue of plasma and purified Ig from a non-HIT thrombocytopenic patient (TCP) and confirmed HIT patient (HIT #5).

Ig were isolated from plasma by affinity chromatography on Protein A Sepharose as described in Section 2.2.6 from a non-HIT thrombocytopenic patient or a confirmed HIT patient. Aliquots of fractions were analysed on a 10% SDS-acrylamide gel, under reducing and non-reducing conditions (Section 2.2.4.2), and proteins were visualised by staining the gel with Coomassie blue (Section 2.2.4.3). Under reducing conditions, two bands at approximately 50 kDa and 25 kDa, likely to represent the Ig heavy and light chains, were visible in the eluate fractions. These bands migrated as a high molecular weight band (>245 kDa) under non-reducing conditions which is characteristic of an intact Ig molecule (~150 kDa). The amounts of protein loaded were 100 µg total protein in plasma or unbound samples, or 20 µg total protein in wash or eluate fractions, based on absorbance at 280 nm. L = protein ladder.

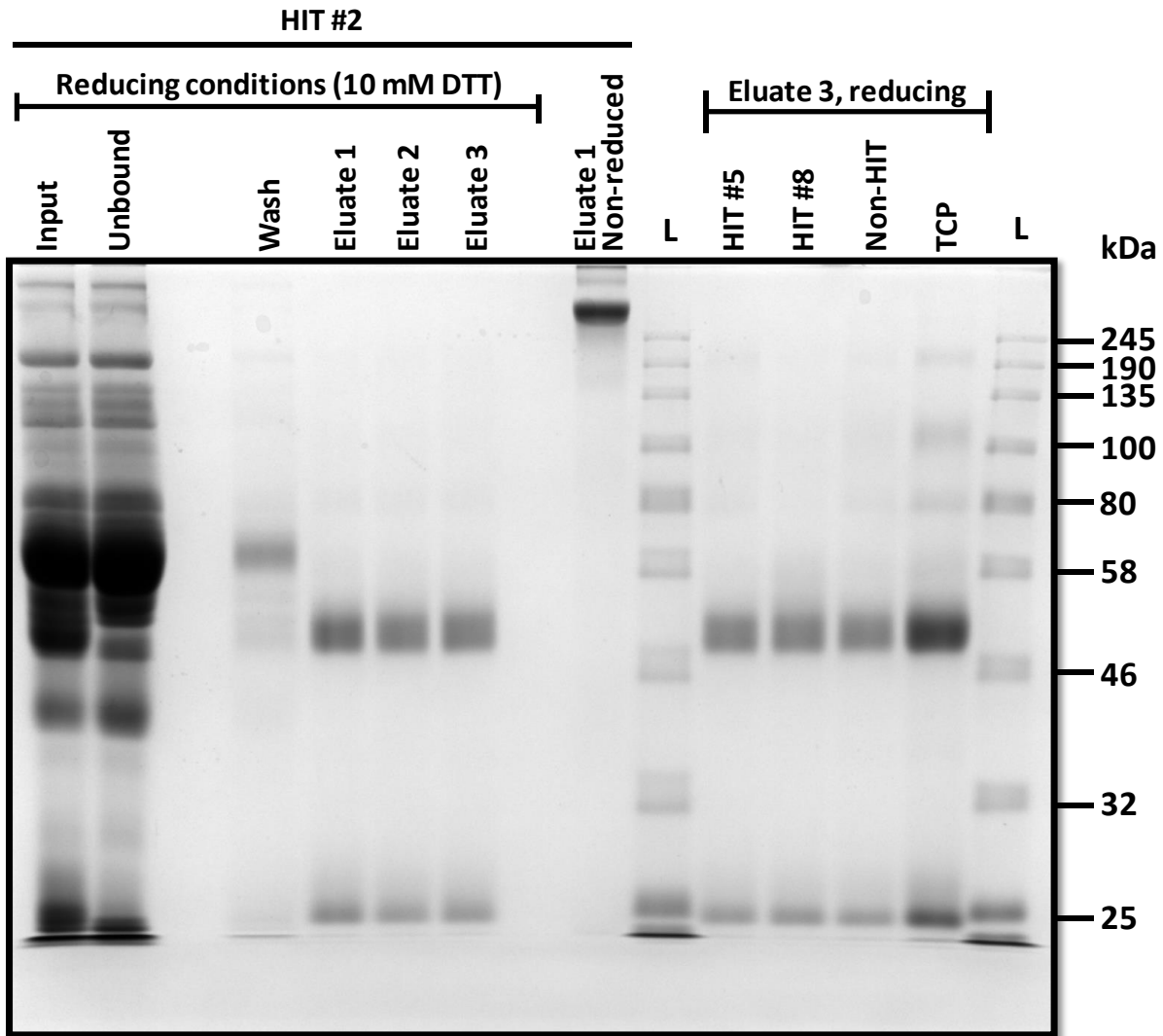


Figure 3.13 SDS-acrylamide gel stained with Coomassie blue of plasma and purified Ig from three confirmed HIT patients (HIT #2, HIT #5, HIT #8) and two non-HIT thrombocytopenic patients (Non-HIT, TCP).

Ig were isolated from plasma by affinity chromatography on Protein A Sepharose as described in Section 2.2.6 from three confirmed HIT patients or two non-HIT thrombocytopenic patients. Aliquots of fractions were analysed on a 10% SDS-acrylamide gel, under reducing and non-reducing conditions (Section 2.2.4.2), and proteins were visualised by staining the gel with Coomassie blue (Section 2.2.4.3). Under reducing conditions, two bands at approximately 50 kDa and 25 kDa, likely to represent the Ig heavy and light chains, were visible in the eluate fractions. These bands migrated as a high molecular weight band (>245 kDa) under non-reducing conditions which is characteristic of an intact Ig molecule (~150 kDa). The amounts of protein loaded were 100 µg total protein in plasma or unbound samples, or 20 µg total protein in wash or eluate fractions, based on absorbance at 280 nm. L = protein ladder.

3.3.5 Establishing an optimal reaction time to detect an effect of HIT Ig on donor washed platelet GPVI levels

Ig isolated from citrated plasma of a confirmed HIT patient (HIT #1) shown in Figure 3.10 was used to determine the optimal incubation period to detect an effect of HIT Ig on donor platelet GPVI. The following experiment was performed as described in Section 2.2.7 using human PF4 isolated in Figure 3.5. Healthy donor washed platelets were incubated with 100 µg/mL healthy donor control or HIT Ig in the presence of low (0.5 U/mL) or high (100 U/mL) doses of heparin, and 10 µg/mL human PF4. Some platelets were pre-treated with 50 mM EDTA. GPVI levels on donor platelets were assessed by flow cytometry using 1G5 anti-human GPVI monoclonal antibody as described in Section 2.2.8.

Figure 3.14 compares GPVI surface levels on donor platelets after 30 min, 1 h, 2 h and 4 h incubation periods (panels A, B, C, and D, respectively). Panels on the left show the proportion of GPVI lost from donor platelets based on the difference between percent GPVI positive platelets in untreated (NT) and treated sample. Percent GPVI-positive population was gated based on fluorescence above antibody control (background fluorescence contributed by secondary antibody) as described in Figure 3.8. Fluorescence geomeans representing GPVI surface levels (1G5-binding) are shown on the right panels.

In general, the greatest loss of GPVI in donor platelets treated with healthy donor control or HIT Ig was observed at the 4 h time point. At earlier time points, the mean loss of GPVI was less than 5% across the treatment groups. In contrast, PF4 alone or therapeutic heparin dose (0.5 U/mL) plus PF4 induced >5% loss in the presence of HIT Ig and this effect was reduced with EDTA pre-treatment. As the 4 h time point was only performed in one individual, the variability in responses cannot be determined. The 4 h incubation period was used in

*Establishing an optimal reaction time to detect an effect of HIT Ig on donor washed platelet
GPVI levels*

subsequent experiments to increase the probability of detecting an effect of HIT Ig on donor
platelet GPVI levels.

Establishing an optimal reaction time to detect an effect of HIT Ig on donor washed platelet GPVI levels

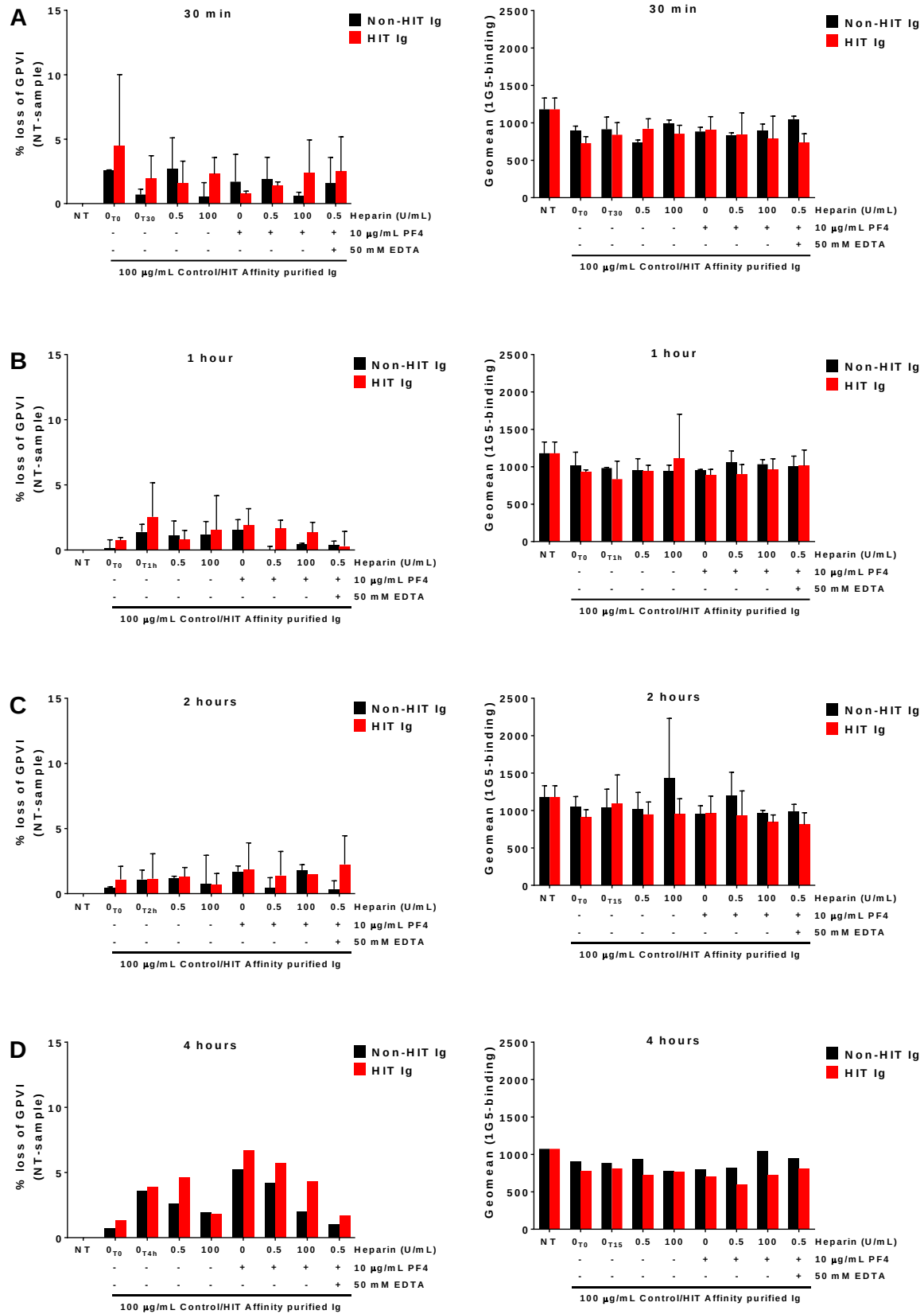


Figure 3.14 Time-course experiment to determine the optimal reaction time to detect an effect of HIT Ig on donor platelet GPVI levels.

Healthy donor washed platelets were mixed with 100 µg/mL affinity-purified healthy donor Ig (control) or HIT Ig in the presence of 0.5 U/mL heparin, 100 U/mL heparin, 10 µg/mL human PF4 or pre-treated with 50 mM EDTA and incubated at RT for 30 min, 1 h, 2 h or 4 h (panels A, B, C, and D, respectively) as described in Section 2.2.7. Platelets were labelled with 1G5 anti-human GPVI monoclonal antibody followed by staining with 1 µg/mL of Alexa Fluor 488-conjugated anti-mouse secondary antibody to allow evaluation of GPVI surface levels by flow cytometry (Section 2.2.8). Panels on the left show the proportion of GPVI lost from donor platelets based on the difference between percent GPVI positive platelets in untreated (NT) and treated sample. Percent GPVI-positive population was gated based on fluorescence above antibody control as illustrated in Figure 3.8. Fluorescence geomean representing GPVI surface levels (1G5-binding) are reported on the right panels. Experiment was performed with 1-2 healthy donors and the mean ± SD are shown for experiments with two donors.

3.3.6 Effect of affinity-purified Ig from a HIT patient (HIT #1) versus healthy control Ig on GPVI levels in donor platelets

Healthy donor washed platelets were treated with affinity-purified Ig from a HIT patient (HIT #1) or healthy controls (Figure 3.10) in the presence of 0.5 U/mL heparin, 100 U/mL heparin, or 10 µg/mL human PF4, for 4 h at RT (determined in Figure 3.14), as described in Section 2.2.7. To assess the requirement for active metalloproteinase and ITAM signalling proteins (Syk and Src) in HIT Ig-mediated GPVI shedding, some platelets were pre-treated for 30 min at RT with 50 mM EDTA, 10 µM Bay61-3606 (BAY61, Syk inhibitor) or 10 µM PP1 (Src inhibitor). GPVI levels on donor platelets labelled with 1G5 anti-human GPVI monoclonal antibody were assessed by flow cytometry (Section 2.2.8) while sGPVI levels in the supernatant of each sample were quantified by ELISA (Section 2.2.9.1).

3.3.6.1 Loss of platelet surface GPVI induced by HIT Ig in donor platelets

In Figure 3.15, the proportion of GPVI lost from donor platelets was expressed as the difference between percent GPVI positive platelets in untreated (NT, washed platelets alone) and treated sample. Percent GPVI-positive population was gated based on fluorescence above antibody control to account for background fluorescence contributed by the secondary antibody. Variable loss of surface GPVI in six unique healthy donors in response to healthy control or HIT Ig were observed (Figure 3.15). In the presence of the low dose heparin and PF4, HIT Ig induced a significant loss of surface GPVI compared to time-zero sample ($p=0.012$, Figure 3.15B), which was not observed with healthy control Ig ($p=0.320$, Figure 3.15A). Addition of a high heparin dose reduced GPVI loss in four out of six donors (red, dark pink, light pink and yellow circles in Figure 3.15B) but did not achieve statistical significance

*Effect of affinity-purified Ig from a HIT patient (HIT #1) versus healthy control Ig on GPVI levels
in donor platelets*

($p=0.589$ with HIT Ig versus $p=0.940$ with control Ig). The loss of GPVI induced by HIT Ig was blocked by EDTA ($p=0.026$), indicating the requirement for divalent cations in GPVI shedding.

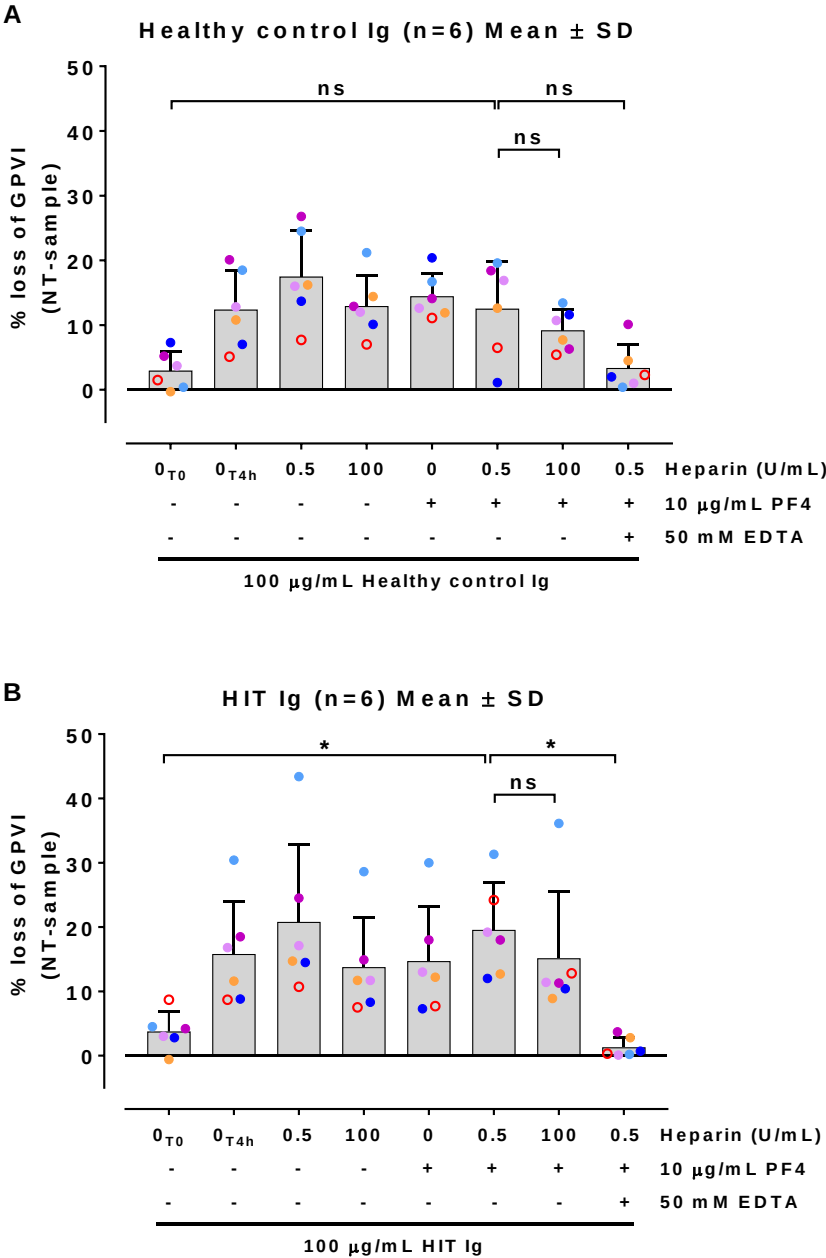


Figure 3.15 Effect of HIT Ig (HIT #1) on GPVI surface levels in donor platelets.

Healthy donor washed platelets were mixed with 100 μ g/mL affinity-purified healthy control Ig (A) or HIT Ig (B) in the presence of 0.5 U/mL heparin, 100 U/mL heparin, 10 μ g/mL human PF4 or pre-treated with 50 mM EDTA and incubated at RT for 4 h as detailed in Section 2.2.7. Platelets were labelled with a 1G5 anti-human GPVI monoclonal antibody followed by staining with an Alexa Fluor 488-conjugated anti-mouse secondary antibody to allow evaluation of GPVI surface levels by flow cytometry (Section 2.2.8). The percent GPVI-positive population was determined based on fluorescence above antibody control (Figure 3.8). The proportion of GPVI lost from donor platelets represents the difference between percent GPVI-positive platelets in untreated (NT) and treated sample. The corresponding release of sGPVI is presented in Figure 3.16. * p <0.05, one-way ANOVA with Tukey's multiple comparisons in six unique healthy donor washed platelets. Subscript T0 represents sample at time-zero while T4h indicates sample after 4 h incubation. ns, not significant.

3.3.6.2 Release of sGPVI mediated by HIT Ig in donor platelets

Level of sGPVI released was employed as an additional approach to quantify loss of GPVI. sGPVI levels in the supernatant of samples presented in Figure 3.15 were measured by ELISA (Section 2.2.9.1). Change in sGPVI levels were obtained by normalising to untreated washed platelets alone (NT) as baseline for each donor (Figure 3.16). NEM-treated washed platelets were included as a positive control for sGPVI release. All treatments resulted in an increase in sGPVI compared to untreated ($p < 0.05$, $n = 6$ per group), apart from EDTA pre-treated samples. In the presence of low dose heparin and PF4, HIT Ig triggered a greater average increase of sGPVI at 48.65 ± 18.36 ng/mL (mean $\pm$ SD, $p = 0.0009$ versus NT; Figure 3.16B) compared to that induced by control Ig (38.25 ± 16.35 ng/mL, $p = 0.015$; Figure 3.16A). This increase in sGPVI was inhibited by EDTA, ($p = 0.0064$, $n = 6$), which is likely to indicate a role for metalloproteinase-mediated shedding of GPVI. Addition of a high heparin concentration significantly attenuated the release of sGPVI induced by HIT Ig at low dose heparin ($p = 0.015$ versus $p = 0.173$ in control Ig), indicating a heparin-dependent effect that is unique to HIT Ig.

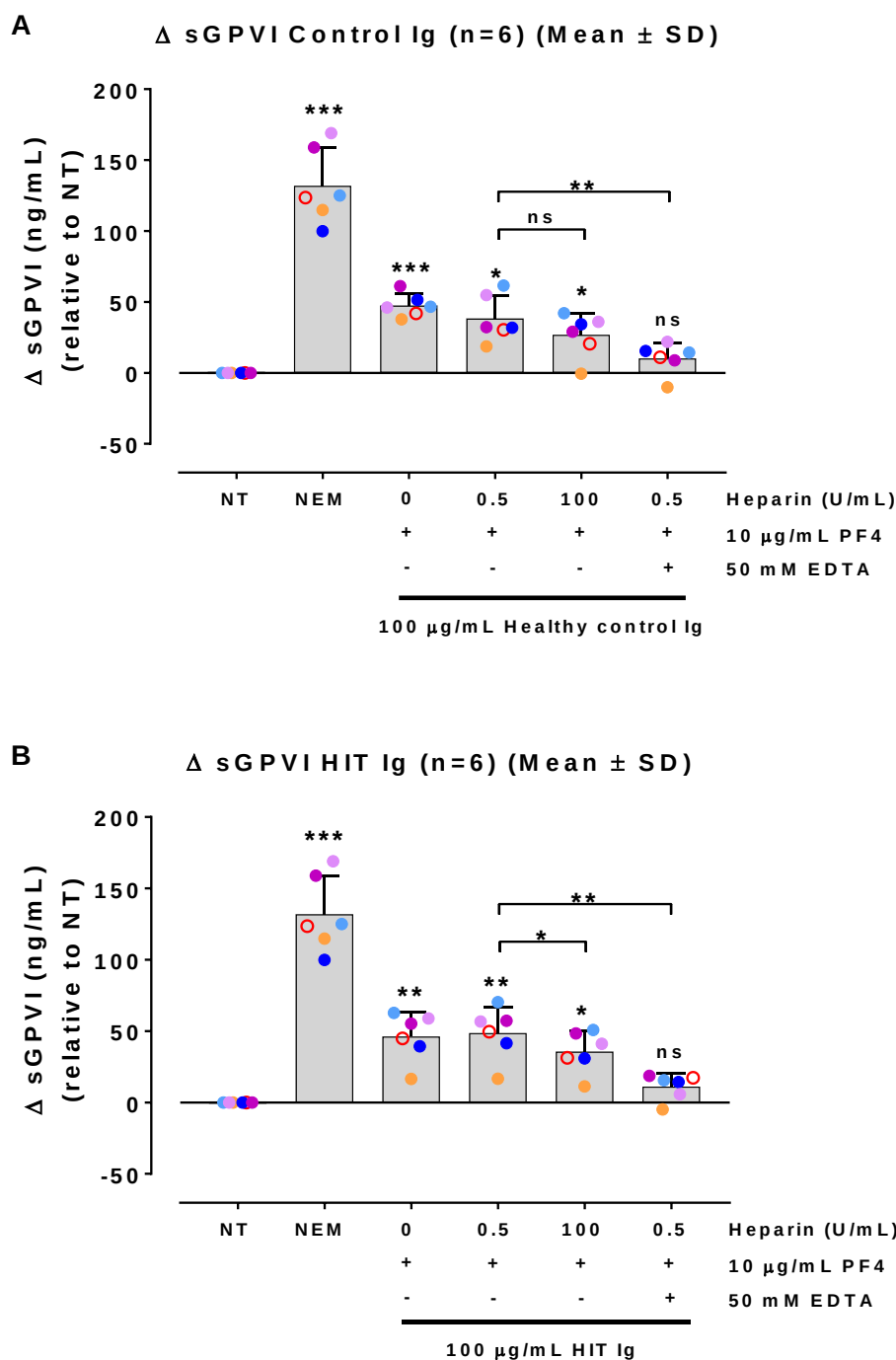


Figure 3.16 Release of sGPVI induced by healthy control or HIT Ig (HIT #1).

Donor washed platelets were mixed with 100 µg/mL affinity purified healthy control Ig (A) or HIT Ig (B) in the presence of 0.5 U/mL heparin, 100 U/mL heparin, 10 µg/mL human PF4 or pre-treated with 50 mM EDTA and incubated at RT for 4 h, as detailed in Section 2.2.7. sGPVI levels in sample supernatant were measured by ELISA (Section 2.2.9.1) and expressed as a change relative to baseline untreated washed platelets (NT) for each donor. The corresponding surface GPVI levels are presented in Figure 3.15. Asterisks refer to significance relative to NT unless indicated by black horizontal bars. * p <0.05, ** p <0.01, *** p <0.001, one-way ANOVA with Tukey's multiple comparisons in six unique healthy donors. NEM = N-ethylmaleimide, as positive control for sGPVI release. ns, not significant.

3.3.6.3 Effect of signalling inhibitors on HIT Ig-mediated GPVI shedding

As described in Section 3.3.2 and Figure 3.7, FcγRIIa was required for KKO-mediated platelet activation. As FcγRIIa activates platelets via the ITAM motif on the cytoplasmic domain, the role of ITAM signalling proteins in HIT Ig-mediated GPVI shedding was explored. The experiment in Sections 3.3.6.1 and 3.3.6.2 were repeated by pre-treating donor washed platelets with Syk inhibitor (10 μM BAY61) or Src inhibitor (10 μM PP1) before incubating with native human PF4, low heparin dose and healthy control or HIT Ig for 4 h at RT (Section 2.2.7). These concentrations of signalling inhibitors were effective at inhibiting collagen-mediated GPVI shedding on washed platelets as measured by flow cytometry in Figure 3.17 ($p=0.0010$ with 10 μM BAY61, $p=0.0002$ with 10 μM PP1, compared to no inhibitor, $n=5$). Importantly, the vehicle control, 0.2% DMSO, showed no significant difference in GPVI levels compared to no inhibitor ($p=0.1775$), indicating that the inhibitory effect was not due to the solvent.

Figure 3.18 illustrates the effect of ITAM signalling inhibition on GPVI shedding induced by HIT Ig in the presence of PF4 and low dose heparin. Syk and Src inhibition did not significantly change the extent of GPVI lost (Figure 3.18 A(i)) or release of sGPVI (Figure 3.18 A(ii)) with healthy control Ig. Although none of the signalling inhibitors significantly rescue the loss of GPVI or release of sGPVI, Syk and Src inhibition, on average, reduced loss of surface GPVI induced by HIT Ig in all three donors (5.17% versus 7.83%, $p=0.3508$ versus $p=0.4190$, respectively, Figure 3.18 B(i)), with a greater inhibitory effect exerted by the Syk inhibitor, BAY61. Similarly, all donor platelets treated with HIT Ig displayed a reduction in sGPVI release with Syk inhibition (17.5 ± 10.3 ng/mL, $p=0.2091$) and a smaller effect was observed with Src inhibition (24.3 ± 12.2 ng/mL, $p=0.3818$) compared to 35.9 ± 17.2 ng/mL without any signalling inhibitors or DMSO vehicle control (44.2 ± 9.7 ng/mL, $p=0.4359$), results reported as mean $\pm$

SD. Despite the variability in responses between donors, the data presented in Figures 3.15-3.18 shows that HIT Ig, and not healthy control Ig, can trigger shedding of GPVI in the presence of PF4 and low dose heparin, as evidenced by a reduction in surface GPVI levels and a corresponding increase in sGPVI release.

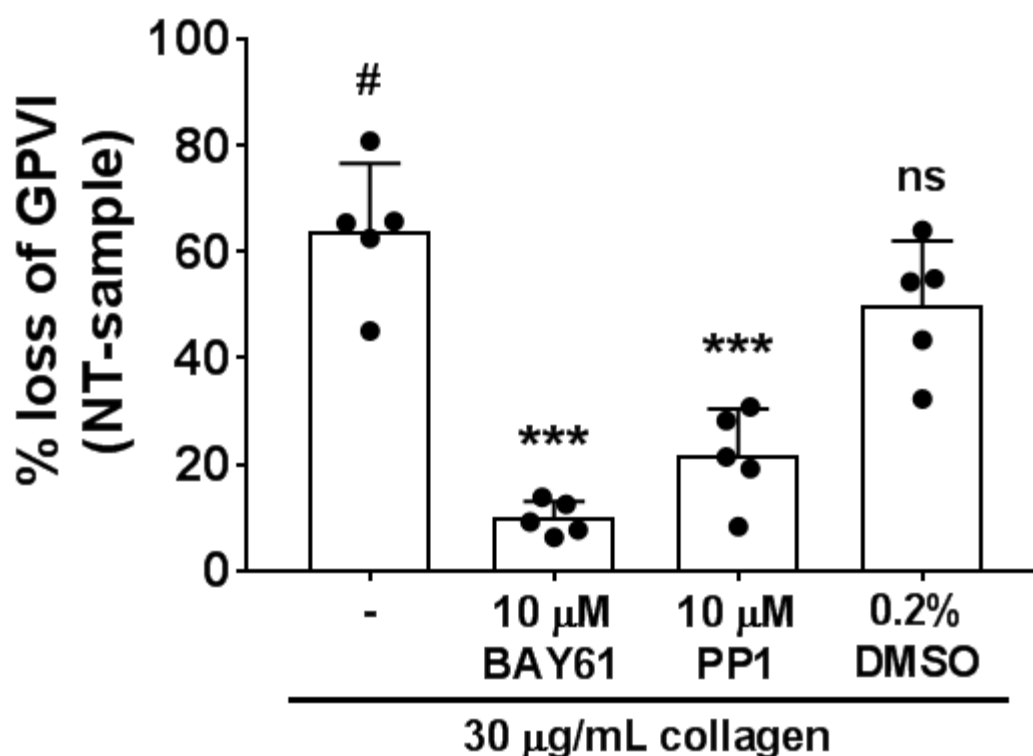


Figure 3.17 Effect of signalling inhibitors on collagen-mediated GPVI shedding.

Healthy donor washed platelets were pre-treated for 30 min at RT with Syk or Src family kinase inhibitors, 10 µM BAY61 or 10 µM PP1, respectively, or 0.2% DMSO vehicle control, before stimulating with 30 µg/mL collagen for 4 h at RT. After addition of 50 mM EDTA to stop the reaction, platelets were labelled with 1G5 anti-human GPVI mouse monoclonal antibody followed by staining with Alexa Fluor 488-conjugated anti-mouse secondary antibody to allow evaluation of GPVI surface levels by flow cytometry (Section 2.2.8). The percent GPVI-positive population was determined based on fluorescence above antibody control (Figure 3.8). GPVI levels were expressed as percent loss of GPVI relative to untreated (NT) platelets. One-way ANOVA with Tukey's multiple comparisons test was computed, and significance compared to no inhibitor (#) are shown. *** $p < 0.001$, data presented as mean $\pm$ SD, $n = 5$. ns, not significant.

Effect of affinity-purified Ig from a HIT patient (HIT #1) versus healthy control Ig on GPVI levels in donor platelets

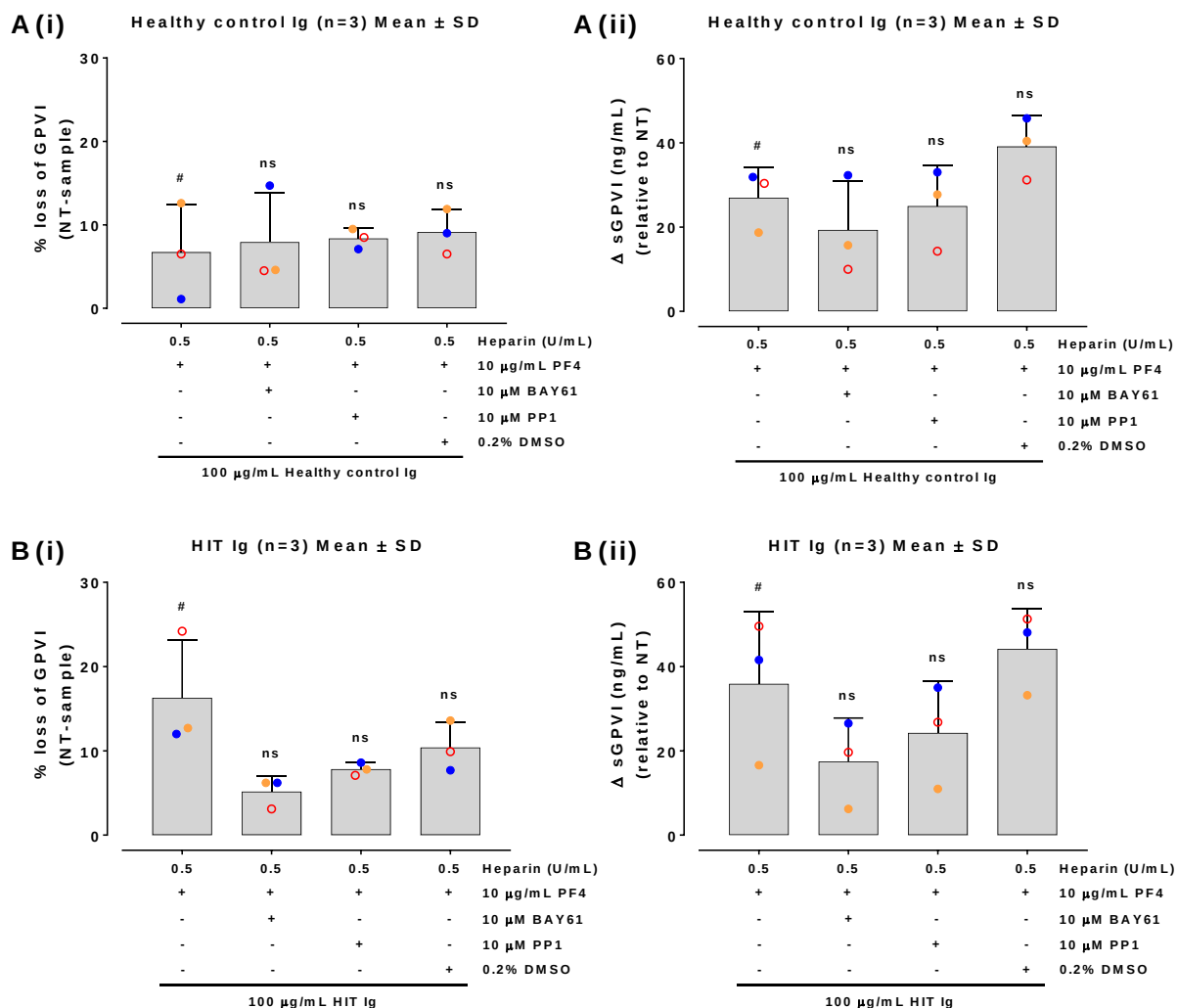


Figure 3.18 Effect of ITAM signalling inhibitors on GPVI shedding mediated by HIT Ig (HIT #1).

Healthy donor washed platelets were pre-treated with 10 μ M BAY61 (Syk inhibitor) or 10 μ M PP1 (Src inhibitor) before incubating for 4 h at RT with 0.5 U/mL heparin, 10 μ g/mL human PF4, and healthy control (A) or HIT (B) Ig (purified in Figure 3.10), as detailed in Section 2.2.7. After addition of 50 mM EDTA to stop the reaction, platelets were labelled with a 1G5 anti-human GPVI mouse monoclonal antibody followed by staining with an Alexa Fluor 488-conjugated anti-mouse secondary antibody to allow evaluation of GPVI surface levels by flow cytometry (Section 2.2.8). The percent GPVI-positive population was determined based on fluorescence above antibody control (Figure 3.8). The proportion of GPVI lost from donor platelets represents the difference between percent GPVI-positive platelets in untreated (NT) and treated sample shown on the left panels (A(i) and B(i)). sGPVI levels in the corresponding platelet supernatant were measured by ELISA (Section 2.2.9.1) and expressed as a change relative to baseline untreated washed platelets (NT) for each donor (A(ii) and B(ii)). One-way ANOVA with Tukey's multiple comparisons was computed in n=3 healthy donors. ns indicates not significant relative to no inhibitor (#).

3.3.7 Comparison of platelet-activating activity between Ig isolated from three HIT patients and non-HIT thrombocytopenic controls

The experimental conditions used in Section 3.3.6 were able to detect loss of GPVI induced by HIT Ig that was dependent on heparin and PF4. To validate the findings in Figures 3.15-3.18, the experiment was repeated using affinity-purified plasma Ig from three other confirmed HIT patients to investigate differences in capacity of the Ig to cause degranulation and GPVI shedding in donor platelets.

Ig from three confirmed HIT patients, a pool of seven non-HIT thrombocytopenic controls (Non-HIT) and a non-HIT thrombocytopenic patient (TCP) were affinity-purified from citrate-anticoagulated plasma as shown in Figures 3.11-3.13 (Section 3.3.4). Platelet count and plasma sGPVI levels (measured by ELISA as described in Section 2.2.9), in these patients are summarised in Table 3.1. Ig isolated from these patients were mixed with donor platelets to compare the effects on GPVI levels and platelet degranulation (measured by P-selectin expression).

Table 3.1 Platelet count and sGPVI levels in plasma samples from which human Ig were isolated.

Sample	Platelet count (10 ⁹ /L)	sGPVI (ng/mL)	Source
Non-HIT thrombocytopenic (n=7)	<100	21.74	Dr Jim Tiao (WACTH, Perth)
TCP non-HIT thrombocytopenic	78	73.56	JCSMR, Canberra
HIT #5	89	44.51	Dr Jim Tiao (WACTH, Perth)
HIT #8	61	28.59	Dr Jim Tiao (WACTH, Perth)
HIT #2	47	38.22	Dr Philip Choi (The Canberra Hospital)

Comparisons of the activity of Ig isolated from three HIT patients and non-HIT thrombocytopenic patients are presented in Figure 3.19, Figure 3.20 and Figure 3.21. Non-HIT Ig was isolated from a pool of seven non-HIT thrombocytopenic patients. All Ig samples were tested with washed platelets from one healthy donor to assess GPVI shedding and platelet activation by flow cytometry using 1G5 anti-human GPVI monoclonal antibody and AK4 anti-CD62P (P-selectin) monoclonal antibody, respectively. Donor washed platelets were mixed with 10 µg/mL human PF4, 0.5 U/mL or 100 U/mL heparin and treated with 100 µg/mL affinity-purified Ig from patients with either confirmed HIT or non-HIT-related thrombocytopenia for 4 h at RT as detailed in Section 2.2.7. Some platelets were pre-treated with 50 mM EDTA for 30 min at RT before exposure to PF4, heparin and human Ig. Some platelets were labelled with a 1G5 anti-human GPVI mouse monoclonal antibody followed by staining with an Alexa Fluor 488-conjugated anti-mouse secondary antibody to allow evaluation of GPVI surface levels by flow cytometry (Section 2.2.8). Separate aliquots of treated platelets were labelled with a PE-conjugated AK4 mouse anti-CD62P monoclonal antibody to assess P-selectin expression by flow cytometry (Section 2.2.8). P-selectin levels were assessed in non-EDTA-treated samples as EDTA interferes with P-selectin antibody binding.

Flow cytometry histograms in Figure 3.19 represent levels of surface GPVI on donor platelets upon treatment (red traces) with Ig isolated from non-HIT thrombocytopenic or HIT patients compared to baseline GPVI level on donor platelets (blue traces). GPVI levels remained stable at the start of experiment (time-zero, T0) and after 4 h in platelets that were pre-treated with EDTA to block metalloproteinase activity. Varying levels of GPVI shedding were evident in the presence of PF4 and low dose heparin (0.5 U/mL) plus PF4. The greatest extent of GPVI shedding occurred with HIT #5 and HIT #2 Ig, whereby two populations of platelets with low and high GPVI expression were seen. Shedding of GPVI was rescued at high heparin

concentration (100 U/mL) and presence of the metalloproteinase inhibitor, EDTA. P-selectin levels on donor platelets treated with the various Ig are represented by histogram overlays in Figure 3.20. No increase in P-selectin expression was observed.

Figure 3.21 A(i)-E(i) (left panels) report the percent loss of GPVI surface levels relative to untreated platelets (NT) and the fluorescence geomean of CD62P antibody binding were reported in Figure 3.21 A(ii)-E(ii) (right panels). Orange bars indicate treatment with low dose heparin (0.5 U/mL) and human PF4 (10 µg/mL) in the presence of HIT or non-HIT Ig (100 µg/mL). With this treatment, the two batches of non-HIT thrombocytopenic Ig (Figure 3.21 A(i) and B(i)) exerted a <5% loss of GPVI (2.9% with Non-HIT Ig, 3% with TCP Ig). In contrast, Ig from HIT patients induced 5-60% loss of GPVI with HIT #5 (59.4%) exhibiting the greatest activity followed by HIT #2 (6.3%) and HIT #8 being the least potent at 5%. This loss of GPVI mediated by all three HIT Ig was attenuated at high heparin concentration and EDTA, with HIT #5 showing a remarkable reduction from 59.4% at 0.5 U/mL heparin to 1.9% at 100 U/mL heparin and 1.2% with EDTA present, suggesting dependency on heparin concentration and metalloproteinase activity. Of note, GPVI shedding in HIT Ig was more prominent with the addition of human PF4 compared to heparin alone (0.5 U/mL), except for HIT #8. However, PF4 alone with no heparin reduced the extent of GPVI shedding in all three HIT Ig, indicating the requirement for the formation of the heparin/PF4 complex to trigger loss of GPVI.

A 1.6- to 2.2-fold increase in P-selectin expression was observed with the three HIT Ig (geomeans between 805 and 1117) compared to the two non-HIT thrombocytopenic Ig (geomeans of 693, Non-HIT Ig and 852, TCP Ig) at low dose heparin and PF4. The level of platelet activation as measured by P-selectin levels were reflective of the potency of the HIT Ig to cause GPVI shedding, that is, HIT #5 Ig induced the highest P-selectin expression

consistent with the greatest loss of GPVI while HIT #8 Ig triggered the lowest P-selectin expression and the least amount of GPVI shedding. Of interest, P-selectin levels remained relatively high at high heparin dose despite a diminished loss of GPVI, possibly suggesting a disparate pathway between P-selectin upregulation and GPVI shedding. However, in this experimental system, the possibility that some P-selectin could be exported from platelets via microvesicle release cannot be excluded.

In summary, the data shows that plasma Ig from different HIT patients differ in their capacity to cause GPVI shedding and platelet degranulation when assessed on donor platelets from a single individual. In the presence of PF4 and low dose of heparin, Ig from HIT patients caused a greater extent of GPVI shedding than Ig from non-HIT related thrombocytopenic patients which supports the hypothesis that HIT Ig can induce a metalloproteolytic loss of GPVI in a heparin- and PF4-dependent manner.

Comparison of platelet-activating activity between Ig isolated from three HIT patients and non-HIT thrombocytopenic controls

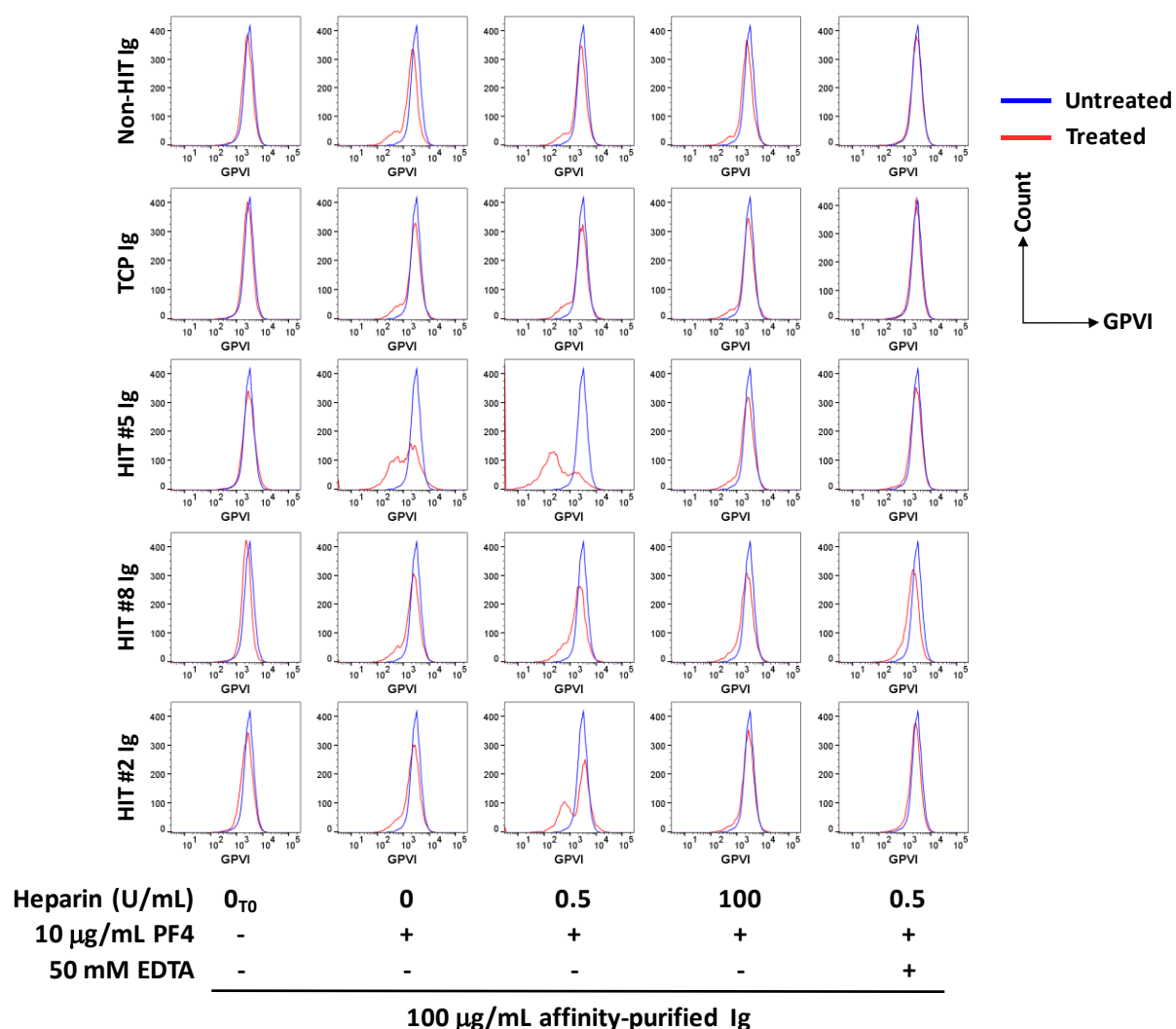


Figure 3.19 Comparison of GPVI surface expression on Non-HIT or HIT Ig-treated donor platelets.

Healthy donor washed platelets (n=1) were treated for 4 h at RT with 100 µg/mL affinity-purified non-HIT thrombocytopenic Ig (Non-HIT or TCP) or HIT Ig (HIT #5, HIT #8 or HIT #2), in the presence of PF4 (10 µg/mL), low heparin dose (0.5 U/mL), high heparin dose (100 U/mL) or pre-treated with 50 mM EDTA, as detailed in Section 2.2.7. Platelets were labelled with an anti-human GPVI monoclonal antibody followed by staining with an Alexa Fluor 488-conjugated anti-mouse secondary antibody to allow evaluation of GPVI surface levels by flow cytometry (Section 2.2.8). Baseline GPVI level on untreated donor platelets is shown in blue while red traces indicate GPVI expression after treatment. Subscript T0 indicates time-zero of experiment. Bar graphs comparing levels of GPVI across the treatment groups are shown in Figure 3.21.

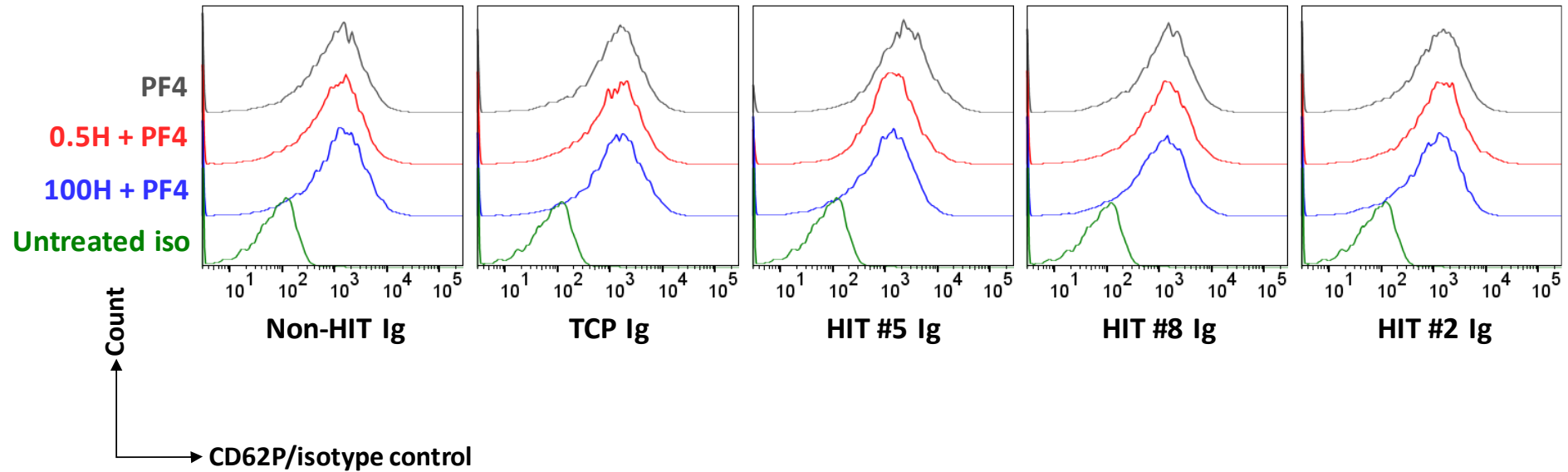
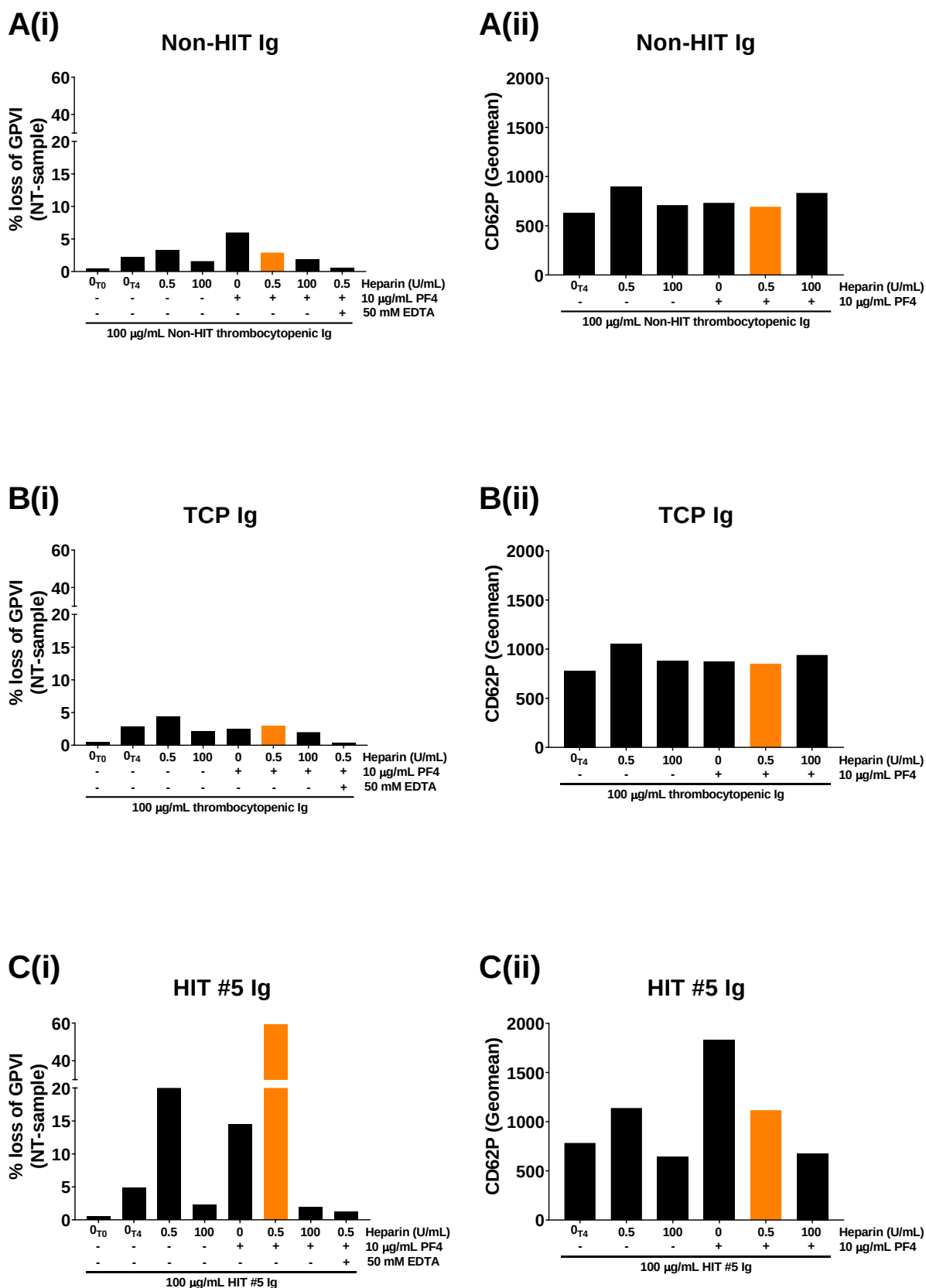


Figure 3.20 Comparison of CD62P (P-selectin) expression on Non-HIT or HIT Ig treated donor platelets.

Healthy donor washed platelets ($n=1$) were treated for 4 h at RT with 100 $\mu\text{g}/\text{mL}$ affinity-purified non-HIT thrombocytopenic Ig (Non-HIT or TCP) or HIT Ig (HIT #5, HIT #8 or HIT #2), in the presence of 10 $\mu\text{g}/\text{mL}$ PF4 (grey), PF4 plus 0.5 U/mL heparin (red) or PF4 plus 100 U/mL heparin (blue), as detailed in Section 2.2.7. Platelets were labelled with a PE-conjugated AK4 mouse anti-CD62P monoclonal antibody to assess P-selectin expression by flow cytometry (Section 2.2.8). Untreated donor platelets stained with a PE-conjugated isotype control for anti-CD62P antibody is shown in green. Histograms from each treatment group were overlaid to compare P-selectin levels. The level of fluorescence detected on the PE channel is represented on the x-axis while the height of a histogram represents platelet count (y-axis). Bar graphs of fluorescence geomeans contributed by P-selectin antibody binding are shown in Figure 3.21.

Comparison of platelet-activating activity between Ig isolated from three HIT patients and non-HIT thrombocytopenic controls



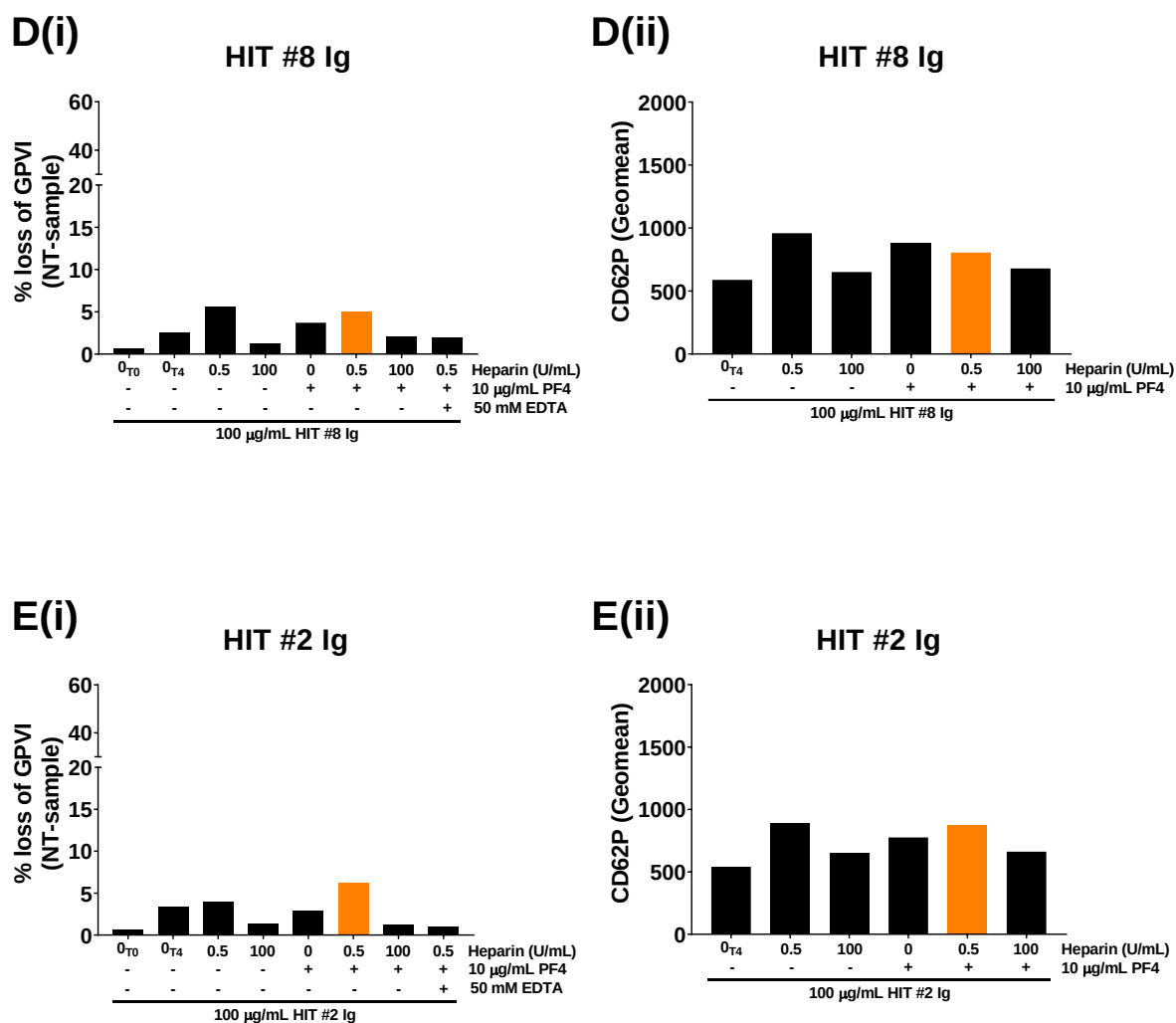


Figure 3.21 Comparison of the activity of Ig isolated from three HIT patients and non-HIT thrombocytopenic patients.

All Ig samples were tested with washed platelets from one healthy donor to assess GPVI shedding and platelet activation by flow cytometry using an anti-human GPVI and an anti-CD62P (P-selectin) antibody, respectively. Donor washed platelets were treated for 4 h at RT with 100 µg/mL affinity-purified Ig from non-HIT thrombocytopenic patients (Non-HIT or TCP) or HIT patients (HIT #5, HIT #8 or HIT #2), in the presence of PF4 (10 µg/mL), 0.5 U/mL heparin, 100 U/mL heparin or pre-treated with 50 mM EDTA as detailed in Section 2.2.7. Platelets were labelled with a 1G5 anti-human GPVI monoclonal antibody followed by staining with an Alexa Fluor 488-conjugated anti-mouse secondary antibody to evaluate GPVI surface levels by flow cytometry (Section 2.2.8). Separate aliquots of platelets were labelled with a PE-conjugated AK4 mouse anti-CD62P monoclonal antibody to assess P-selectin expression by flow cytometry (Section 2.2.8). P-selectin levels were only assessed in non-EDTA-treated samples as EDTA interferes with P-selectin antibody binding. Left panels A(i) to E(i) report percent loss of GPVI surface levels relative to untreated platelets (NT) while the fluorescence geomean of CD62P antibody binding were reported in panels A(ii) to E(ii) on the right. Orange bars indicate treatment with low dose heparin (0.5 U/mL) and human PF4 (10 µg/mL) in the presence of HIT or non-HIT Ig. The histogram traces representing fluorescence contributed by 1G5 or P-selectin antibody binding are in Figure 3.19 and Figure 3.20, respectively.

3.3.8 Evaluating differences in the capacity of Ig from different HIT patients to induce GPVI shedding

Variability in the ability of Ig isolated from different HIT patients to induce GPVI shedding in donor platelets as observed in Figure 3.21 was formally evaluated here using the experimental setup described in Section 2.2.7. Washed platelets were mixed with 10 µg/mL human PF4, plus 0.5 U/mL or 100 U/mL heparin and treated with 100 µg/mL affinity-purified Ig from patients with either confirmed HIT (HIT #5 or HIT #2 or HIT #1) or non-HIT-related thrombocytopenia, or healthy control for 4 h at RT. After addition of 50 mM EDTA to stop the reactions, platelets were labelled with a 1G5 anti-human GPVI mouse monoclonal antibody followed by staining with an Alexa Fluor 488-conjugated anti-mouse secondary antibody to evaluate GPVI surface levels by flow cytometry (Section 2.2.8). The percent GPVI-positive population was determined based on fluorescence above antibody control as explained in Figure 3.8. The proportion of GPVI lost from donor platelets represents the difference between percent GPVI-positive platelets in untreated (NT) and treated sample. sGPVI levels in the corresponding platelet supernatant were measured by ELISA (Section 2.2.9.1) and expressed as a change relative to baseline untreated washed platelets (NT) for each donor.

Both non-HIT and healthy control Ig did not result in a loss of GPVI or sGPVI release between low and high heparin doses in donor platelets ($p>0.05$, Figure 3.22). The extent of GPVI loss due to HIT Ig was higher at low heparin concentration, achieving statistical significance with HIT #5 Ig ($p=0.0046$, Figure 3.22A). In contrast, sGPVI release was significantly reduced at high dose heparin for all three HIT Ig ($p<0.05$, Figure 3.22C and D).

To evaluate the contribution of Ig and heparin to the variation observed in GPVI loss and sGPVI release, two-way ANOVA with Tukey's (Non-HIT vs HIT #5 vs HIT #2 Ig) or Sidak's (healthy

control vs HIT #1 Ig) multiple comparisons was computed as summarised in Table 3.2. Source of Ig and heparin concentration contributed significantly to the overall differences observed in GPVI loss and sGPVI release induced by HIT #5 and HIT #2 Ig compared to Non-HIT Ig ($p < 0.05$, Table 3.2A and B), consistent with the graphs in Figure 3.22A and C, with heparin concentration having a larger contribution to the total variation observed (35.10%, $p = 0.0205$ and 43.31%, $p = 0.0024$, of variation in GPVI loss and sGPVI release, respectively). A significant interaction component between source of Ig and heparin dose was also detected, accounting for 22.09% ($p = 0.0030$) and 17.00% ($p < 0.0001$) of total variation in GPVI loss and sGPVI release, respectively. This is consistent with the notion that the antigenic complex of heparin/PF4 forms at a particular stoichiometry to enable recognition by the anti-PF4/heparin HIT antibody and high concentrations of heparin disrupts the complex (Rauova et al., 2005). In contrast, neither Ig nor heparin effect was apparent in determining the loss of surface GPVI in healthy control vs HIT #1 Ig but were significant in explaining the variation in sGPVI release. This may be due to the differences in the sensitivity between the sGPVI assay and measuring surface GPVI by flow cytometry whereby a smaller effect size may be detectable by the sGPVI ELISA but not the latter.

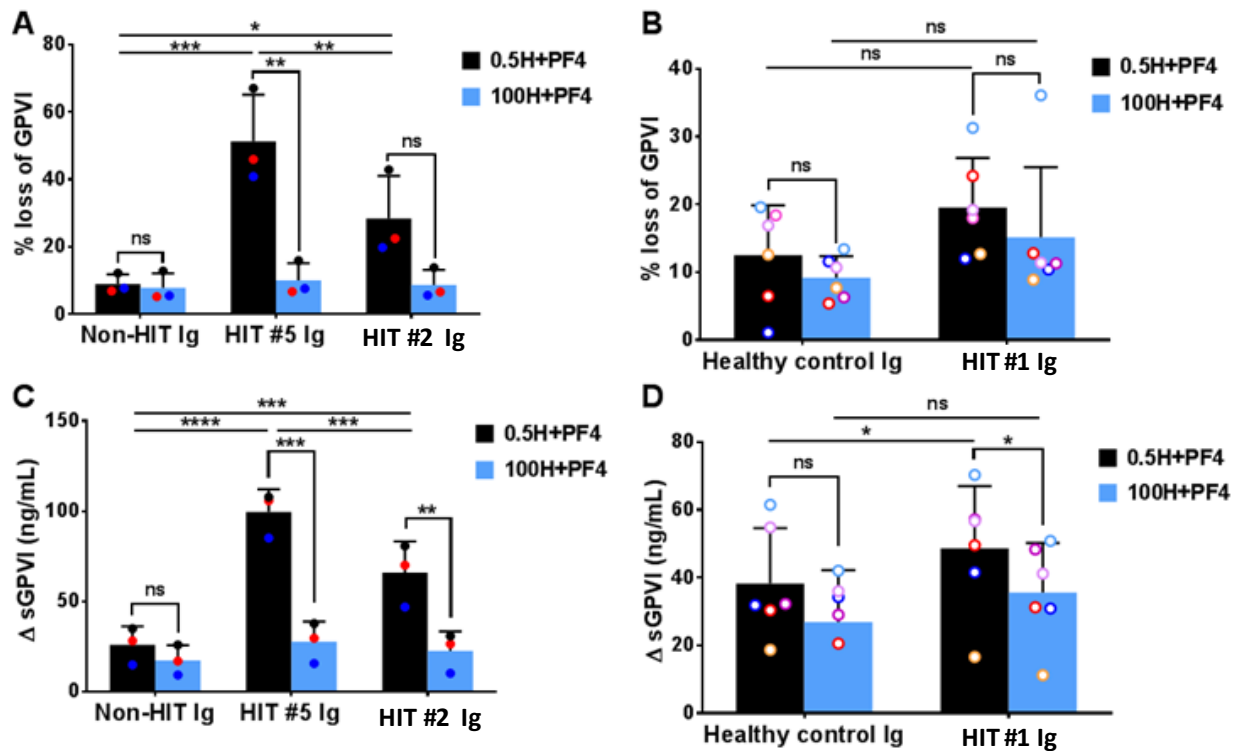


Figure 3.22 Differences between HIT Ig in mediating GPVI shedding.

Healthy donor washed platelets were treated for 4 h at RT with 100 $\mu\text{g}/\text{mL}$ affinity-purified Ig from non-HIT thrombocytopenic patients or HIT patients (HIT #5 or HIT #2) or healthy control, in the presence of PF4 (10 $\mu\text{g}/\text{mL}$) plus 0.5 U/mL heparin or 100 U/mL heparin, as detailed in Section 2.2.7. After addition of 50 mM EDTA to stop the reactions, platelets were labelled with a 1G5 anti-human GPVI monoclonal antibody followed by staining with an Alexa Fluor 488-conjugated anti-mouse secondary antibody to assess GPVI surface levels by flow cytometry (Section 2.2.8). The percent GPVI-positive population was determined based on fluorescence above antibody control (Figure 3.8). The proportion of GPVI lost from donor platelets represents the difference between percent GPVI-positive platelets in untreated (NT) and treated sample for each donor. sGPVI levels in the platelet supernatant were measured by ELISA (Section 2.2.9.1) and expressed as a change relative to baseline untreated washed platelets (NT) for each donor. Panels A and B show loss of GPVI in the presence of non-HIT Ig versus HIT Ig (HIT #5 and HIT #2) or healthy control Ig versus HIT #1 Ig, respectively. Panels C and D present changes in sGPVI levels (ΔsGPVI relative to unstimulated platelets) in samples from panels A and B, respectively. Error bars indicate mean $\pm$ SD with three healthy donors in A and C and six healthy donors in B and D. Repeated measure one-way ANOVA with Tukey's multiple comparisons test was computed for within group differences. Two-way ANOVA with Tukey's (A and C) or Sidak's (B and D) multiple comparisons test was performed for between group differences. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns indicates not significant, 0.5H denotes 0.5 U/mL heparin, 100H denotes 100 U/mL heparin.

Table 3.2 Analysis of Ig and heparin contribution to GPVI shedding in donor platelets.

Two-way ANOVA with Tukey's (Non-HIT vs HIT #5 vs HIT #2 Ig) or Sidak's (healthy control vs HIT #1 Ig) multiple comparisons to assess the effect and extent of contribution of Ig source and heparin on the variability in the loss of GPVI (A) and sGPVI release (B) observed in Figure 3.22. Significant *p*-values are in bold.

A) Source of variation for GPVI loss	Non-HIT vs HIT #5 vs HIT #2		Healthy control vs HIT #1	
	% of total variation	<i>p</i> -value	% of total variation	<i>p</i> -value
Ig effect	27.30	0.0027	17.31	0.0658
Heparin effect	35.10	0.0205	6.14	0.1167
Interaction: Ig x Heparin effect	22.09	0.0030	0.12	0.7785

B) Source of variation for sGPVI release	Non-HIT vs HIT #5 vs HIT #2		Healthy control vs HIT #1	
	% of total variation	<i>p</i> -value	% of total variation	<i>p</i> -value
Ig effect	29.84	0.0001	8.20	0.0270
Heparin effect	43.31	0.0024	13.35	0.0044
Interaction: Ig x Heparin effect	17.00	<0.0001	0.06	0.6872

3.3.9 The role of FcγRIIa in HIT Ig-mediated GPVI shedding

As described in Section 3.3.2, KKO-mediated P-selectin upregulation in donor platelets was abolished when FcγRIIa was blocked with the IV.3 monoclonal antibody. A similar experiment was performed on donor platelets using affinity-purified Ig from HIT patients and non-HIT thrombocytopenic patients to investigate the role of FcγRIIa in GPVI shedding induced by HIT Ig. Washed platelets from three unique donors were mixed with low (0.5 U/mL) or high (100 U/mL) doses of heparin or 10 µg/mL human PF4 in the presence or absence of 10 µg/mL FcγRIIa function blocking antibody, IV.3, before exposure to 100 µg/mL of non-HIT, HIT #5 or HIT #2 Ig for 4 h at RT as detailed in Section 2.2.7. Unlike the experiments described before which used unconjugated GPVI antibody, platelets were labelled with an Alexa Fluor 488-conjugated 1G5 anti-human GPVI monoclonal antibody to evaluate GPVI surface levels by flow cytometry (Section 2.2.8). The use of directly conjugated 1G5 antibody eliminate the need for a secondary antibody to avoid cross-reactivity with the IV.3 mouse monoclonal antibody. sGPVI levels in the corresponding platelet supernatant were measured by ELISA (Section 2.2.9.1) and expressed as a change relative to baseline untreated washed platelets (NT) for each donor.

Figure 3.23 illustrates the shift in histogram shapes due to GPVI shedding events while Figure 3.24 summarises the effect of HIT Ig compared to non-HIT Ig on GPVI surface levels and release of sGPVI in donor platelets (n=2-3). GPVI-negative peaks corresponding to loss of surface GPVI were observed in samples incubated with HIT Ig at low heparin dose, but not in non-HIT control Ig, and was abolished at high dose of heparin or with FcγRIIa blockade (Figure 3.23).

Consistent with the shift in histogram, affinity-purified Ig from HIT patient plasma samples induced significant loss of GPVI at low heparin dose and PF4 ($p=0.0004$ with HIT #5, $p=0.0021$

with HIT #2, Figure 3.24 B(i) and C(i)) compared to time-zero of experiment, which was not observed with non-HIT thrombocytopenic control Ig ($p>0.9999$, Figure 3.24 A(i)). This loss of surface GPVI was accompanied with an increase in sGPVI release ($p<0.0001$ with HIT #5, $p<0.0001$ with HIT #2, Figure 3.24 B(ii) and C(ii)). In contrast, no significant release in sGPVI was detected with non-HIT Ig at low heparin dose and PF4 ($p=0.0696$, Figure 3.24 A(ii)). Of note, both HIT Ig was able to induce GPVI shedding at low dose heparin in the absence of PF4 (69.67 ± 19.74 ng/mL, $p<0.001$ with HIT #5; 38.29 ± 9.54 ng/mL, $p<0.05$ with HIT #2; Figure 3.24 B(ii) and C(ii)) but to a lesser extent than with the inclusion of PF4 (99.57 ± 12.55 ng/mL, $p<0.0001$ with HIT #5; 65.95 ± 17.26 ng/mL, $p<0.0001$ with HIT #2). Loss of GPVI and the corresponding sGPVI release elicited by HIT Ig were attenuated with high heparin dose ($p>0.05$) and blockade of FcγRIIIa ($p>0.05$), suggesting the effect was heparin-dependent and required functional FcγRIIIa. The extent of GPVI shedding varied between the two HIT Ig, possibly due to the differences in the levels of active anti-PF4/heparin antibodies in the total Ig isolated.

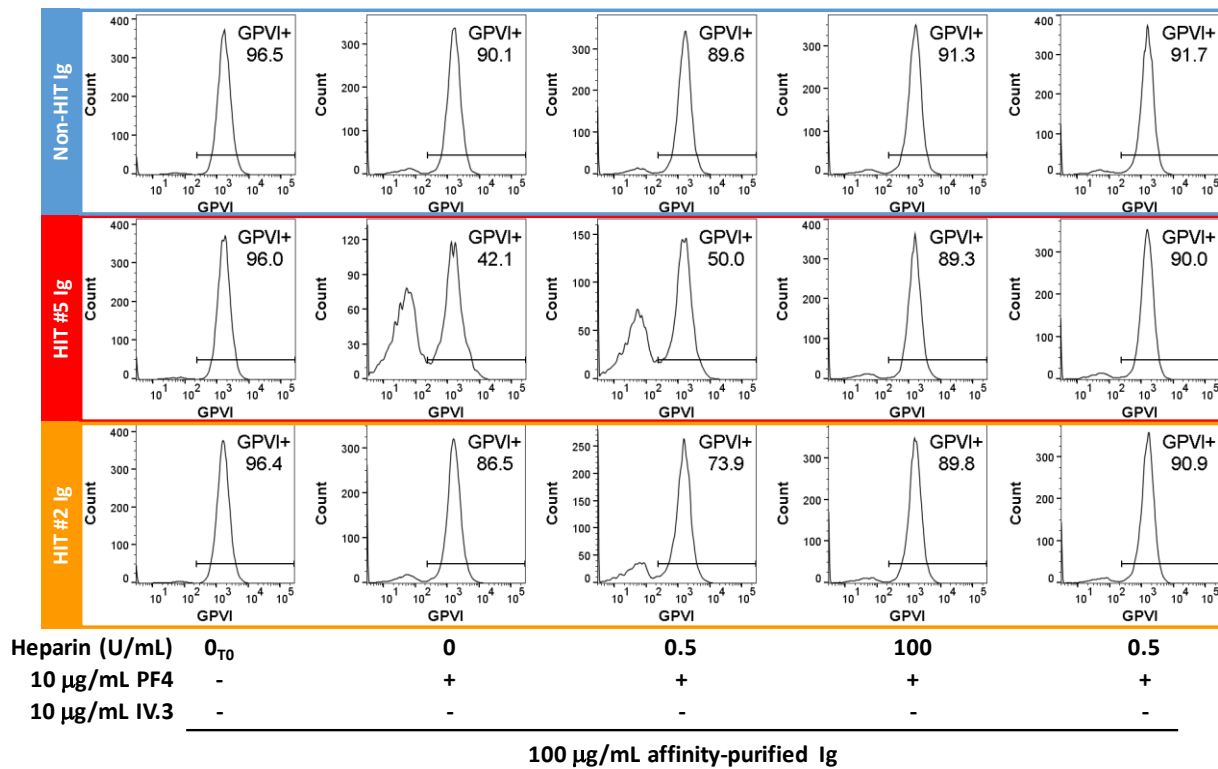


Figure 3.23 GPVI surface levels on donor platelets in response to non-HIT or HIT Ig.

Example histograms of GPVI levels on washed platelets from one donor to demonstrate GPVI shedding in response to 100 µg/mL of non-HIT or HIT Ig (HIT #5 or HIT #2) in the presence of 0.5 U/mL or 100 U/mL heparin or 10 µg/mL human PF4 or pre-treatment with 10 µg/mL FcγRIIIa function blocking antibody, IV.3, as described (Section 2.2.7). After 4 h at RT, 50 mM EDTA was added to stop the reactions and platelets were labelled with an Alexa Fluor 488-conjugated 1G5 anti-human GPVI monoclonal antibody to evaluate GPVI surface levels by flow cytometry (Section 2.2.8). Fluorescence due to binding of 1G5 antibody is shown on the x-axis while platelet event counts are on the y-axis. Horizontal bar overlaid on each histogram shows the GPVI-positive gate based on control antibody and percent GPVI-positive events are shown. A leftward shift and the formation of a second peak of lower fluorescence indicate GPVI shedding.

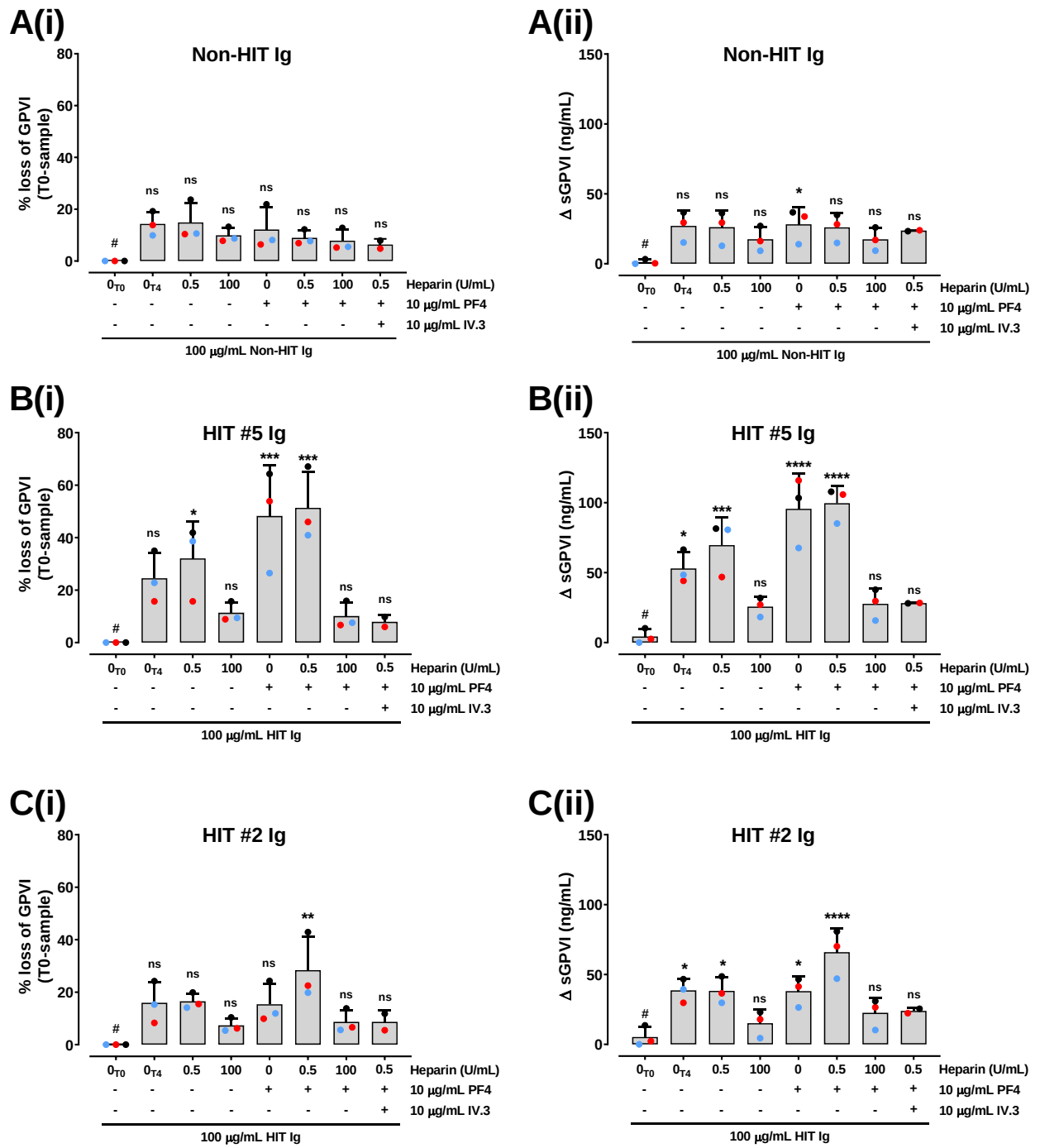


Figure 3.24 HIT Ig-induced shedding of GPVI in donor platelets was dependent on heparin concentration and mediated by FcγRIIIa.

Washed platelets from three unique donors were mixed with low (0.5 U/mL) or high (100 U/mL) doses of heparin or 10 µg/mL human PF4 in the presence or absence of 10 µg/mL FcγRIIIa function blocking antibody, IV.3, before exposure to 100 µg/mL of non-HIT, HIT #5 or HIT #2 Ig for 4 h at RT as detailed in Section 2.2.7. After addition of 50 mM EDTA to stop the reactions, platelets were labelled with an Alexa Fluor 488-conjugated 1G5 anti-human GPVI monoclonal antibody to evaluate GPVI surface levels by flow cytometry (Section 2.2.8). sGPVI levels in the corresponding platelet supernatant were measured by ELISA (Section 2.2.9.1). Changes in GPVI and sGPVI levels in donor platelets treated with non-HIT thrombocytopenic Ig (A) or HIT Ig (patients HIT #5 (B) and HIT #2 (C)) are shown. Left panels (A(i), B(i) and C(i)) present changes in surface GPVI levels expressed as percent loss of GPVI relative to time-zero (T0) of experiment while panels on the right (A(ii), B(ii) and C(ii)) show the corresponding change in sGPVI levels (Δ sGPVI calculated relative to unstimulated platelets) measured in the platelet supernatant. One-way ANOVA with Tukey's multiple comparisons test was computed and the statistical significance relative to # (time-zero of experiment) were reported. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, $n = 2-3$, mean $\pm$ SD shown. ns, not significant.

3.3.10 HIT Ig-induced GPVI shedding requires ITAM signalling and divalent cations

As shown in Figure 3.24, HIT Ig-mediated shedding of GPVI required functional FcγRIIa. As both GPVI and FcγRIIa signal via ITAM, the following experiments were performed to evaluate the importance of ITAM signalling in GPVI shedding mediated by HIT Ig from two HIT patients. The role of metalloproteinases in shedding of GPVI induced by HIT Ig was also investigated as ADAM10 is the predominant metalloproteinase in cleaving human GPVI (Gardiner et al., 2007).

Healthy donor platelets were pre-treated with Syk inhibitor, BAY61 (10 μM), Src family kinase inhibitor, PP1 (10 μM), vehicle control (0.2% DMSO) or divalent cation chelator, EDTA (50 mM) for 30 min at RT before incubating with 100 μg/mL of non-HIT thrombocytopenic control or HIT Ig (HIT #5 or HIT #2) in the presence of 0.5 U/mL heparin and 10 μg/mL PF4 for 4 h at RT as described in Section 2.2.7. Platelets were labelled with an Alexa Fluor 488-conjugated 1G5 anti-GPVI antibody to evaluate surface GPVI levels by flow cytometry (Section 2.2.8) while sGPVI levels in platelet supernatant were measured by ELISA (Section 2.2.9).

Figure 3.25 illustrates changes in the histogram profile of surface GPVI on platelets from a single donor upon treatment with signalling inhibitors or EDTA. Appearance of the GPVI-negative peaks in HIT Ig-treated platelets were abolished with Syk inhibition or depletion of divalent cations and reduced to a lesser extent by Src inhibition. The effect of signalling inhibition and divalent cation depletion in 2-3 unique donor platelets is summarised in Figure 3.26. In the presence of non-HIT thrombocytopenic control Ig, no appreciable changes in GPVI surface levels or sGPVI release was observed with Syk or Src inhibitor, or with EDTA (Figure 3.26 A(i) and A(ii)). On exposure to either HIT Ig, DMSO vehicle control was not significantly different from the no inhibitor control, indicating that any effect observed was truly the action

of the inhibitors (Figure 3.26 B and C). Pre-treatment with Syk inhibitor markedly reduced the loss of GPVI triggered by either HIT Ig (mean $\pm$ SD GPVI loss of $7.6 \pm 5.5\%$ versus $51.33 \pm 13.9\%$ without inhibitor in HIT #5, $7.7 \pm 3.5\%$ versus $28.4 \pm 12.6\%$ without inhibitor in HIT #2), although the difference was statistically significant only with HIT #5 Ig but not HIT #2 Ig ($p=0.0083$ for HIT #5, $p=0.1073$ for HIT #2; Figure 3.26 B(i) and C(i)). Nevertheless, sGPVI release was significantly reduced with both HIT Ig upon Syk blockade ($p=0.0007$ for HIT #5, $p=0.0039$ for HIT #2; Figure 3.26 B(ii) and C(ii)). In contrast, Src inhibition showed a mild reduction in loss of surface GPVI (mean $\pm$ SD GPVI loss of $22.6 \pm 0.2\%$ versus $51.33 \pm 13.9\%$ without inhibitor in HIT #5, $15.1 \pm 1.9\%$ versus $28.4 \pm 12.6\%$ without inhibitor in HIT #2) and sGPVI release (mean $\pm$ SD sGPVI release of 69.3 ± 20.2 ng/mL versus 99.6 ± 12.6 ng/mL without inhibitor in HIT #5, 43.7 ± 3.5 ng/mL versus 66.0 ± 17.3 ng/mL without inhibitor in HIT #2) which did not achieve statistical significance with either HIT Ig ($p>0.05$). The concentration of PP1 tested ($10 \mu\text{M}$) was effective at blocking GPVI shedding induced by $30 \mu\text{g/mL}$ of collagen ($p<0.001$, Figure 3.17). This observation suggests that Syk inhibition may exert a greater effect than Src inhibition in reducing platelet activation resulting in GPVI shedding consequent of Fc γ RIIa engagement by HIT, possibly due to compensatory mechanisms by other members of the Src family kinase. Also, cleavage of GPVI induced by HIT Ig required divalent cation as evidenced by the action of EDTA which diminished the loss of GPVI ($p=0.0031$ and $p=0.0353$ with HIT #5 and HIT #2, respectively) and release of sGPVI ($p=0.0001$ and $p=0.0006$ with HIT #5 and HIT #2, respectively).

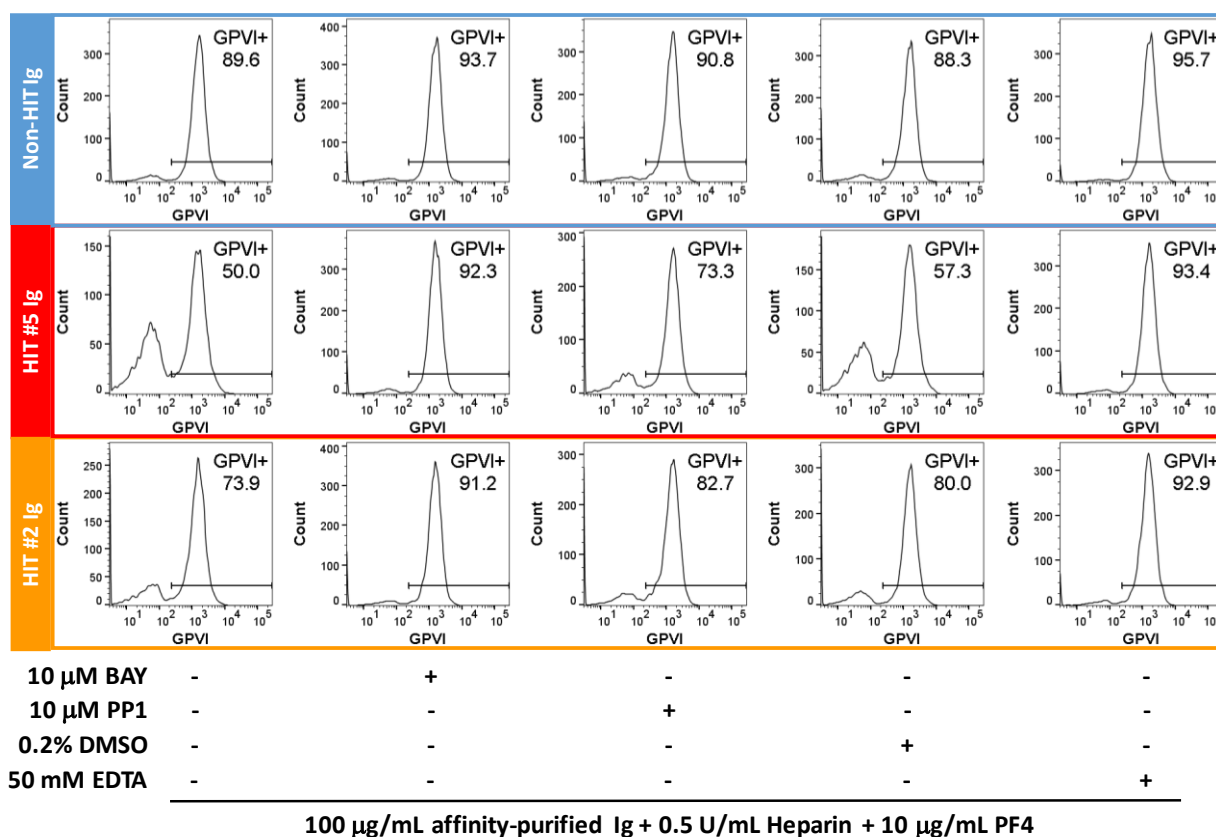
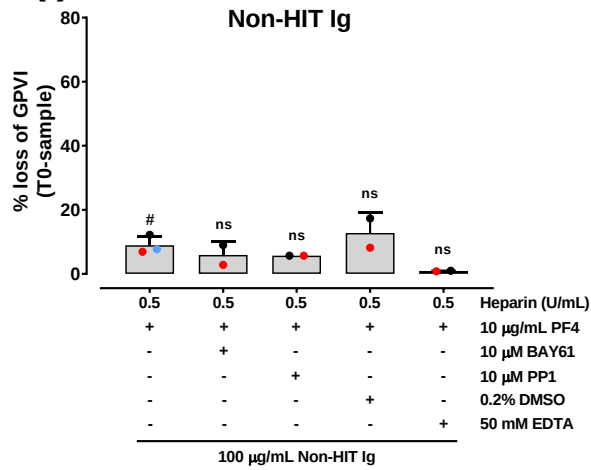


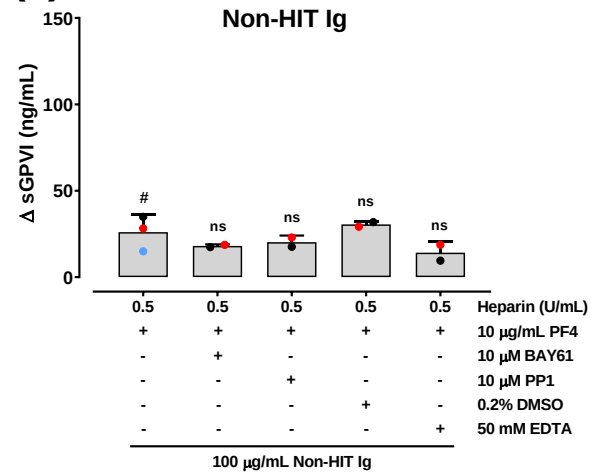
Figure 3.25 HIT Ig-mediated GPVI shedding can be modulated by Syk inhibition and divalent cation depletion.

Example histogram profile of GPVI levels on washed platelets from one donor to demonstrate GPVI levels in response to 100 μ g/mL of non-HIT or HIT Ig (HIT #5 or HIT #2) and 0.5 U/mL heparin and 10 μ g/mL PF4 in the presence or absence of 10 μ M Syk inhibitor (BAY), 10 μ M Src family inhibitor (PP1), 0.2% DMSO vehicle control or 50 mM divalent cation chelator (EDTA), as described (Section 2.2.7). After 4 h at RT, 50 mM EDTA was added to stop the reactions and platelets were labelled with an Alexa Fluor 488-conjugated 1G5 anti-human GPVI monoclonal antibody to evaluate GPVI surface levels by flow cytometry (Section 2.2.8). Fluorescence due to binding of 1G5 antibody is shown on the x-axis while platelet event counts are on the y-axis. The horizontal bar overlaid on each histogram shows the GPVI-positive gate based on control antibody and percent GPVI-positive events are reported. A leftward shift followed by the formation of a second peak of lower fluorescence indicates GPVI shedding.

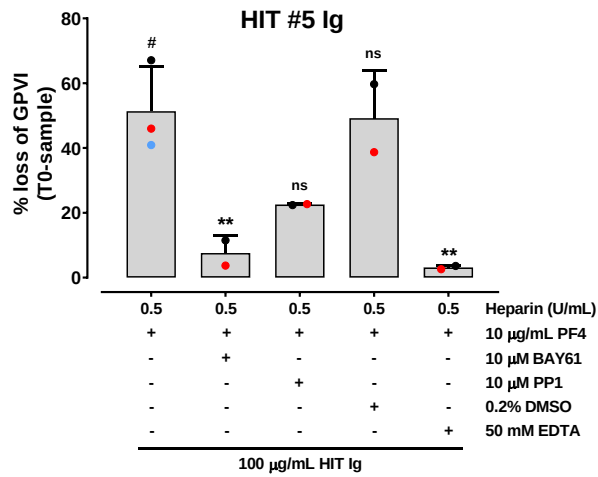
A(i)



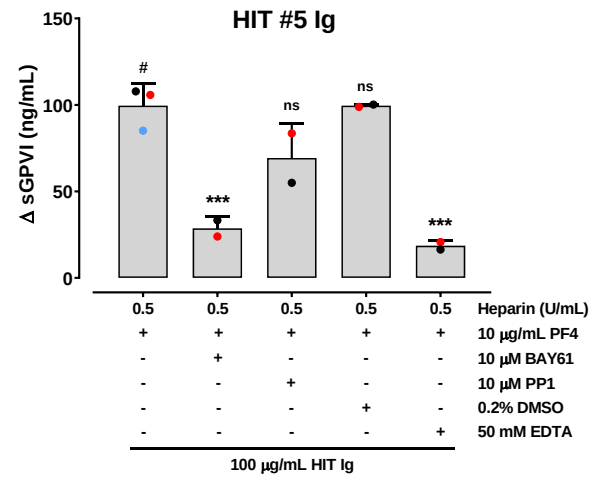
A(ii)



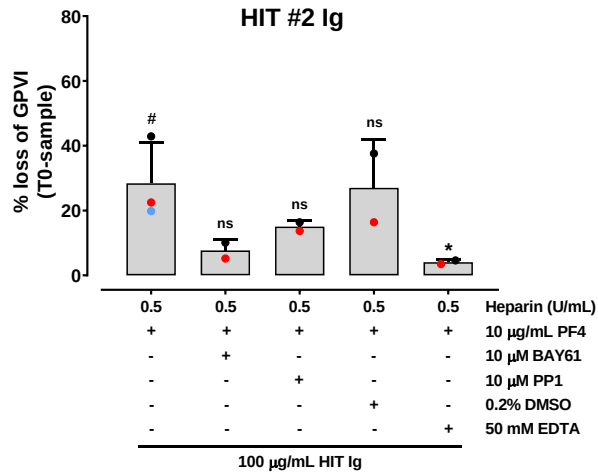
B(i)



B(ii)



C(i)



C(ii)

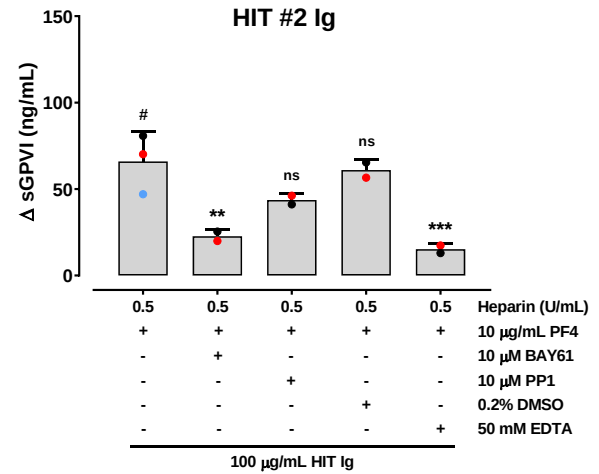


Figure 3.26 Syk kinase and divalent cations were required for HIT Ig-induced shedding of GPVI.

Healthy donor platelets (n=2-3) were pre-treated with Syk inhibitor, BAY61 (10 μ M), Src family kinase inhibitor, PP1 (10 μ M), vehicle control (0.2% DMSO) or divalent cation chelator, EDTA (50 mM) for 30 min at RT before incubating with 100 μ g/mL of non-HIT thrombocytopenic control or HIT Ig (HIT #5 or HIT #2) in the presence of 0.5 U/mL heparin and 10 μ g/mL PF4 for 4 h at RT as described in Section 2.2.7. Platelets were labelled with an Alexa Fluor 488-conjugated 1G5 anti-GPVI antibody to evaluate surface GPVI levels by flow cytometry (Section 2.2.8) while sGPVI levels in platelet supernatant were measured by ELISA (Section 2.2.9). Changes in GPVI and sGPVI levels in donor platelets treated with non-HIT thrombocytopenic Ig (A) or HIT Ig (patients HIT #5 (B) and HIT #2 (C)) are shown. Left panels (A(i), B(i) and C(i)) present changes in surface GPVI levels expressed as percent loss of GPVI relative to time-zero samples while panels on the right (A(ii), B(ii) and C(ii)) show the corresponding change in sGPVI levels (Δ sGPVI calculated relative to unstimulated platelets). One-way ANOVA with Tukey's multiple comparisons test was computed, statistical significance relative to no inhibitor (#), * p <0.05, ** p <0.01, *** p <0.001, mean $\pm$ SD shown. ns, not significant.

3.3.11 Measuring platelet ADAM10 activity on exposure to HIT Ig and PF4/heparin complex

Exposing donor platelets to plasma Ig isolated from confirmed HIT patients but not non-HIT thrombocytopenic patients, in the presence of PF4/heparin complex, triggered loss of surface GPVI and a consequent increase in the release of sGPVI, as shown in Figure 3.22 and Figure 3.24. As ADAM10 is the metalloproteinase responsible for cleavage of human GPVI (Gardiner et al., 2007), the impact of HIT Ig on platelet ADAM10 activity was investigated using a published FRET assay (Facey et al., 2016) described in Section 2.2.11.2. This approach utilised a GPVI-Cy3 substrate consisting of a peptide with sequence matching the amino acid residues 236-248 of GPVI that is cleaved by ADAM10, as illustrated in Figure 2.3. Cleavage of this peptide releases the BHQ2 quencher from the Cy3 fluorophore and the resulting fluorescence signal can be detected at excitation at 540 nm and emission at 590 nm.

To demonstrate that ADAM10 activity was detectable on resting platelets, equal concentration of GPVI-Cy3 substrate (1 μ M) was exposed to increasing number of platelets and the fluorescence signal was continuously captured for 1 h at 37°C, as described (Section 2.2.11.2). Increase in fluorescence, measured in relative fluorescence units (RFU), over time was corrected for background fluorescence detected in wells containing the substrate and buffer alone. Figure 3.27A represents the kinetic curves resulting from cleavage of the substrate in the presence of an increasing number of washed platelets. Firstly, the increase in fluorescence over time demonstrated that platelet surface ADAM10 were active and have a detectable amount of kinetic activity, consistent with the findings of Facey and colleagues (2016). Secondly, this increase in fluorescence activity increased proportionally with the number of platelets which is the source of the ADAM10 enzyme. Upon pre-treatment of platelets with a broad metalloproteinase inhibitor (100 μ M GM6001), an ADAM10 inhibitor (2

μM GI54023X) or a divalent cation chelator (50 mM EDTA) (described in Section 2.2.11.2), fluorescence activity was markedly reduced by greater than 1.5-fold (Figure 3.27B). The greatest reduction in activity was seen with EDTA which chelates divalent cations including Zn^{2+} , which is critical for metalloproteinase function. GM6001 displayed a greater inhibition of activity than GI254023X, at the concentrations tested. In contrast, platelets treated with a thiol-modifying reagent, N-ethylmaleimide (NEM, 5 mM) which activates metalloproteinases, an appreciable increase in fluorescence activity compared to resting platelets was observed (Figure 3.27B). These observations suggest that the fluorescence signal detected in this FRET-based assay was contributed by metalloproteolytic activity.

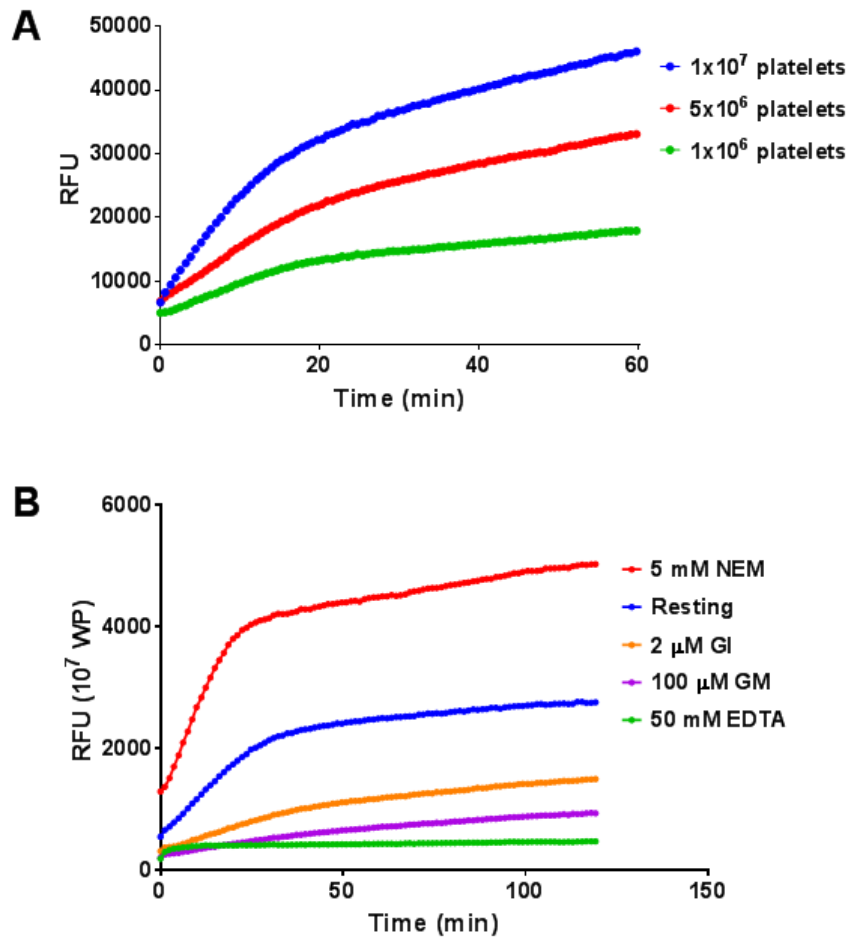


Figure 3.27 Fluorescence activity detected on washed platelets by a fluorescence resonance energy transfer (FRET) assay for ADAM10 activity measurements.

ADAM10 activity was measured using a FRET-based approach which utilised a GPVI-Cy3 substrate consisting of a peptide with sequence matching the amino acid residues 236-248 of GPVI that is cleaved by ADAM10, as illustrated in Figure 2.3. Cleavage of this peptide releases the BHQ2 quencher from the Cy3 fluorophore and the resulting fluorescence signal can be detected. Increase in fluorescence (measured in relative fluorescence units, RFU) over time was monitored and corrected for background fluorescence detected in wells containing the substrate and buffer alone. (A) Increase in fluorescence monitored over 1 h at increasing number of washed platelets exposed to equimolar concentrations of GPVI-Cy3 substrate (1 μ M). (B) 10^7 washed platelets were mixed with 1 μ M of the GPVI-Cy3 substrate in the presence or absence of a thiol-modifying reagent, 5 mM N-ethylmaleimide (NEM) which activates metalloproteinases, a broad metalloproteinase inhibitor, 100 μ M GM6001, an ADAM10 inhibitor, 2 μ M GI254023X or a divalent cation chelator, 50 mM EDTA as described (Section 2.2.11.2). Data is representative of experiments performed on washed platelets isolated from at least six healthy donors.

Having established a FRET-based assay for measuring ADAM10 activity on washed platelets, the effect of HIT Ig on platelet ADAM10 activity was investigated. As described in Section 2.2.11.2, 10^7 washed platelets were mixed with 100 $\mu\text{g/mL}$ of plasma Ig isolated from confirmed HIT (HIT #5 in Figure 3.12 or HIT #2 in Figure 3.13) or non-HIT thrombocytopenic patients (Figure 3.11), 0.5 U/mL or 100 U/mL heparin, and 10 $\mu\text{g/mL}$ of PF4 in the presence or absence of metalloproteinase inhibitors (100 μM GM6001 or 2 μM GI54023X), signalling inhibitors (10 μM Bay 61-3606 or 10 μM PP1), divalent cation chelator (50 mM EDTA) or Fc γ RIIa function inhibitor (10 $\mu\text{g/mL}$ IV.3 monoclonal antibody). The GPVI-Cy3 substrate was finally added to the platelet suspension and the fluorescence signal resulting from cleavage of the substrate was continuously monitored for 2 h at 37°C. Fc γ RIIa, Syk and Src inhibitors, and EDTA were included in the assay as the HIT Ig-induced cleavage of GPVI was mediated through engagement of Fc γ RIIa, required divalent cations and was dependent on ITAM signalling as presented in Figure 3.24 and Figure 3.26.

Figure 3.28A illustrates the effect of PF4 and heparin on washed platelet fluorescence activity due to cleavage of the ADAM10 sensor, GPVI-Cy3. Unexpectedly, the presence of PF4 increased the basal fluorescence activity observed on resting platelets by ~ 1.5 -fold at 2 h while the addition of heparin markedly reduced the activity in a concentration dependent manner whereby a greater inhibition of resting platelet activity was detected at 100 U/mL heparin (~ 5 -fold reduction) compared to 0.5 U/mL heparin (~ 2 -fold reduction). The kinetic curves of fluorescence activity monitored on platelets exposed to HIT Ig are shown on Figure 3.28B. The fluorescence activity of platelets upon addition of Ig isolated from a confirmed HIT patient was comparable to the resting platelet activity represented by the blue curves in Figure 3.28B versus Figure 3.28A, respectively. The increase in resting platelet fluorescence activity due to PF4 was also observed in the presence of HIT Ig. However, all heparin-treated platelets

showed diminished fluorescence activity (Figure 3.28B) that were similar to the heparin effect seen in Figure 3.28A in the absence of human Ig. These observations were representative of experiments with non-HIT thrombocytopenic Ig.

Figure 3.29 compares the rate of increase in fluorescence at 20 min on washed platelets treated with non-HIT thrombocytopenic Ig or HIT Ig (HIT #5 or HIT #2) in the presence of PF4 and low or high doses of heparin. Some platelets were pre-treated with metalloproteinase inhibitors (100 μ M GM6001 or 2 μ M GI54023X), signalling inhibitors (10 μ M Bay 61-3606 or 10 μ M PP1), divalent cation chelator (50 mM EDTA) or Fc γ R1a function inhibitor (10 μ g/mL IV.3 monoclonal antibody). The rate of activity (RFU/min) at 20 min was calculated by subtracting the RFU at time zero from the RFU at 20 min and dividing the difference by 20 min. In the presence of Ig alone, higher rates of activity at 50.7 RFU/min (Figure 3.29B) and 66.0 RFU/min (Figure 3.29C) were observed with HIT #5 and HIT #2 HIT Ig, respectively, compared to non-HIT Ig at 43.8 RFU/min (Figure 3.29A). Unfortunately, the presence of heparin consistently inhibited all fluorescence activity, in line with the heparin effect observed in Figure 3.28A. Hence, any increase in ADAM10 activity potentially contributed by HIT Ig in the presence of PF4 and heparin cannot be measured using this approach.

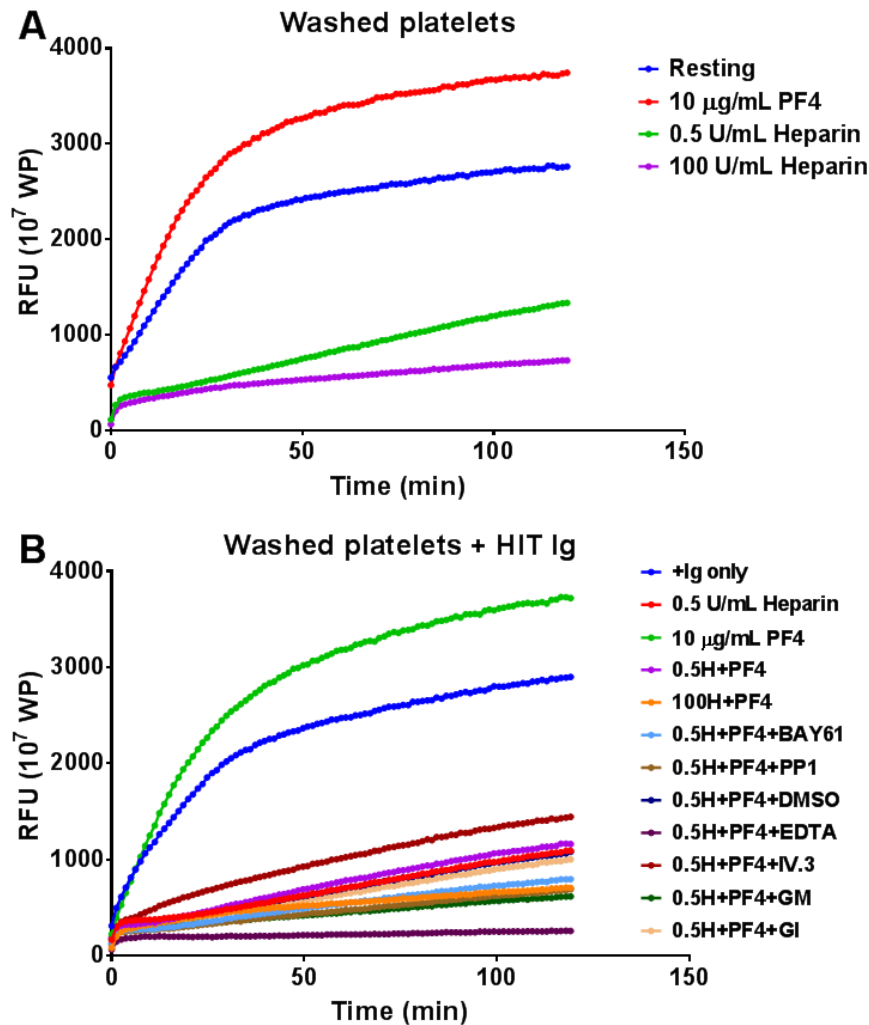


Figure 3.28 Fluorescence activity detected on washed platelets using the ADAM10 sensor, GPVI-Cy3 substrate, is interfered by PF4 and heparin.

10^7 washed platelets were mixed with 100 µg/mL of plasma Ig isolated from a confirmed HIT patient (HIT #2), 0.5 U/mL or 100 U/mL heparin, and 10 µg/mL of PF4 in the presence or absence of metalloproteinase inhibitors (100 µM GM6001 or 2 µM GI54023X), signalling inhibitors (10 µM Bay 61-3606 or 10 µM PP1), divalent cation chelator (50 mM EDTA) or FcγRIIa function inhibitor (10 µg/mL IV.3 monoclonal antibody) as detailed in Section 2.2.11.2. 1 µM of the GPVI-Cy3 substrate was finally added to the platelet suspension and the fluorescence signal resulting from cleavage of the substrate was continuously monitored for 2 h at 37°C. Increase in fluorescence (relative fluorescence units, RFU) over time was corrected for background fluorescence detected in wells containing the substrate and buffer alone. (A) Change in fluorescence activity monitored over 2 h on washed platelets treated with PF4 or heparin compared to untreated resting platelets. (B) Fluorescence activity on washed platelets treated with Ig isolated from a confirmed HIT patient (HIT #2) in the presence of PF4 or heparin or the PF4/heparin complex plus metalloproteinases inhibitors, signalling inhibitors, EDTA or FcγRIIa inhibitor. Kinetic curves are representative of experiments performed with plasma Ig isolated from non-HIT thrombocytopenic patients or another confirmed HIT patient (HIT #5), described in Figure 3.29. 0.5H denotes 0.5 U/mL heparin, 100H denotes 100 U/mL heparin.

Rate of activity at 20 min

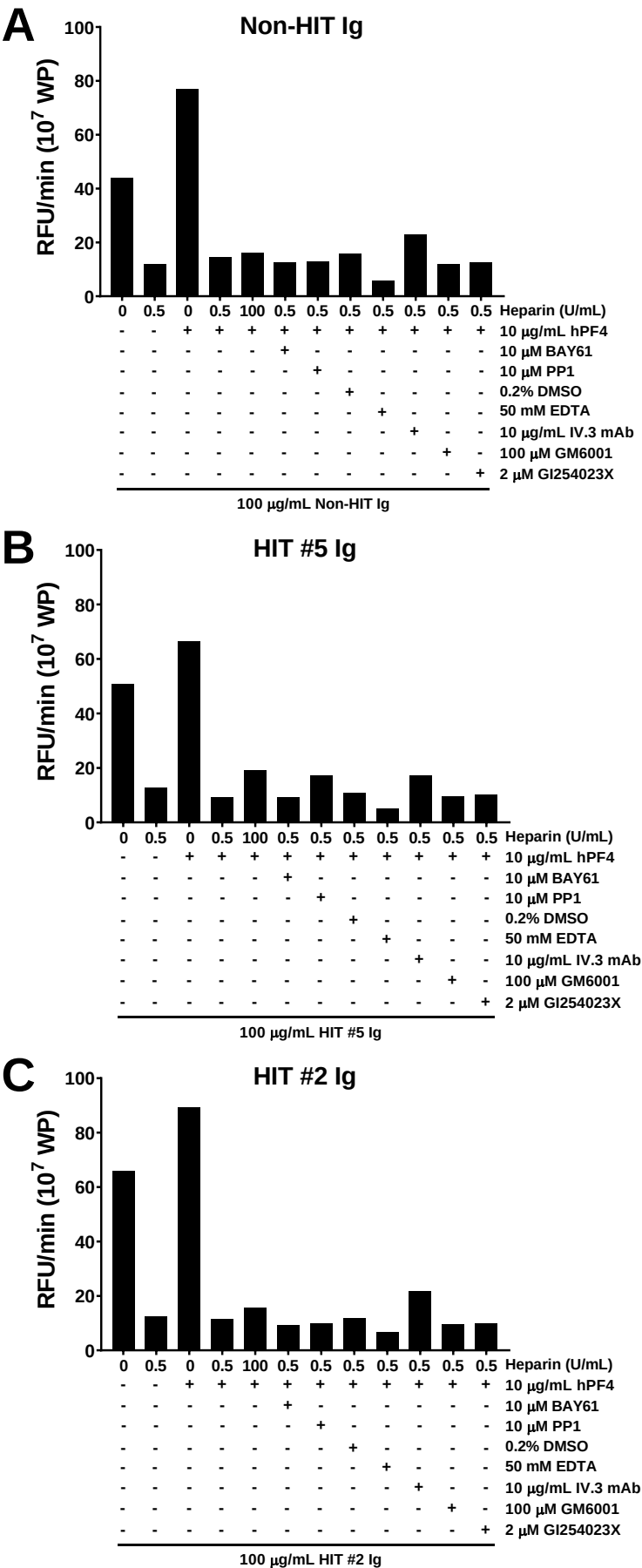


Figure 3.29 Rate of fluorescence activity due to cleavage of the ADAM10 sensor, GPVI-Cy3, on washed platelets exposed to human Ig in the presence of PF4 and heparin.

10⁷ washed platelets were mixed with 100 µg/mL of plasma Ig isolated from either non-HIT thrombocytopenic patients (A) or confirmed HIT patients (HIT #5 (B) or HIT #2 (C)), 0.5 U/mL or 100 U/mL heparin, and 10 µg/mL of PF4 in the presence or absence of metalloproteinase inhibitors (100 µM GM6001 or 2 µM GI54023X), signalling inhibitors (10 µM Bay 61-3606 or 10 µM PP1), divalent cation chelator (50 mM EDTA) or FcγRIIa function inhibitor (10 µg/mL IV.3 monoclonal antibody) as detailed in Section 2.2.11.2. 1 µM of the GPVI-Cy3 substrate was finally added to the platelet suspension and the fluorescence signal resulting from cleavage of the substrate was continuously monitored for 2 h at 37°C. Fluorescence activity (RFU) at 20 min was corrected for background fluorescence detected in wells containing the substrate and buffer alone. The rate of activity (RFU/min) was calculated by subtracting the RFU at time zero from the RFU at 20 min and dividing the difference by 20 min.

3.4 Discussion

New ways to evaluate the pathology of HIT antibodies are valuable tools both to begin to quantify the potency of an antibody, and to better understand the molecular pathways triggered in platelets exposed to HIT antibodies. Understanding and modulating these pathways may be important for the application of new or even existing therapeutics for the treatment of patients with clinical suspicion of or confirmed HIT. Using combinations of heparin, affinity-purified PF4 and affinity-purified Igs, a flow cytometric assay measuring upregulation of P-selectin on donor platelets was established. This assay was used to assess signalling pathways engaged in donor platelets exposed to anti-platelet autoantibodies. As HIT antibody-induced GPVI shedding has previously been demonstrated to occur in assays using donor platelets, this assay was extended to include evaluation of the change in GPVI levels and release of sGPVI.

The anti-PF4/heparin monoclonal antibody, KKO which was previously shown to activate platelets in a manner similar to HIT antibodies was first used to establish a set of experimental conditions to assess HIT antibody-induced platelet activation. Platelet degranulation, in the form of the SRA has been employed extensively to evaluate HIT antibody pathology, however data assessing KKO-induced degranulation by measuring P-selectin exposure is less prevalent. The KKO antibody was developed by Arepally and colleagues (2000) by immunising mice with recombinant human PF4 and heparin. In the presence of therapeutic heparin dose and PF4, KKO induced a seven-fold increase in P-selectin expression in one of the three donors tested, but only mildly (<2-fold increase) in the other two donors (Figure 3.7). This was a heparin-dependent effect and required functional FcγRIIa, as saturating heparin concentration and IV.3 antibody blockade returned P-selectin expression to baseline levels in all donors, an effect

that is similar to HIT antibodies and is in agreement with previous studies (Arepally et al., 2000, Rauova et al., 2006). The inhibitory effect conferred by saturating heparin dose is attributed to the disruption of the PF4/heparin antigenic complex at high heparin concentration as visualised by atomic force microscopy (Greinacher et al., 2006).

The differences in the extent of P-selectin upregulation between this study and the published literature may be attributed to differences between PF4 preparations. PF4 exists in monomers, dimers and tetramers in solution depending on the pH. Dimeric and tetrameric forms of PF4 are prevalent at pH>3.0 (Mayo and Chen, 1989). Mutational studies of PF4 revealed that tetrameric PF4 is important for the formation of stable, large antigenic complexes with heparin (Rauova et al., 2005) to enable efficient interaction of KKO with three of the four monomers within the tetrameric PF4 as demonstrated in a recent X-ray crystallography study (Cai et al., 2015, Cai et al., 2016). Hence, the proportions of PF4 oligomers may impact on the exposure of the neo-epitopes on PF4 in a stable conformation for KKO recognition and resulted in the observed differences in platelet activation.

More recently, a humanised form of the monoclonal anti-PF4/heparin antibody, 5B9 which carries a human Fc fragment was developed by Kizlik-Masson and colleagues (2017). 5B9 is an IgG1 antibody with anti-platelet properties that resemble a human HIT IgG. As the human FcγRIIIa binds mouse IgG subclasses poorly (Bruhns and Jonsson, 2015), this humanised antibody will facilitate future studies of IgG-FcγRIIIa interactions and extend our understanding in the pathophysiology of HIT.

In addition to assessing loss of GPVI from platelet surface by flow cytometry, the resulting change in sGPVI levels as a consequence of GPVI shedding was also measured by ELISA. On average, close to 50% of platelets had reduced GPVI levels and between 66-100 ng/mL of

sGPVI was released, when donor platelets were exposed to Ig purified from HIT patients' plasma, in the presence of low heparin dose and PF4. The extent of GPVI shedding varied between Ig from different HIT patients. Notably, total Ig from non-HIT-mediated thrombocytopenic patients did not cause GPVI shedding or release of sGPVI in donor platelets, suggesting that the loss of GPVI observed in this experimental setting was unique to the antibody present in HIT patients. As seen with the P-selectin expression induced by KKO antibody, GPVI shedding triggered by HIT Ig was diminished at high heparin dose and blockade of Fc γ RIIa. Taken together, binding of the PF4/heparin antibody complex to Fc γ RIIa triggered a cascade of signalling events which culminated in the irreversible cleavage of GPVI ectodomain to produce sGPVI in a heparin-dependent manner, in line with the findings of Gardiner and colleagues (2008b).

Attenuation of GPVI shedding by pre-treating donor platelets with divalent cation chelator (EDTA), Syk inhibitor (BAY61) and Src family kinase inhibitor (PP1) indicated that HIT Ig-mediated GPVI shedding required divalent cations and ITAM signalling. Although a metalloproteinase inhibitor was not utilised in this study, the most likely metalloproteinase involved is ADAM10 as it is the predominant metalloproteinase to cleave human GPVI (Gardiner et al., 2007, Facey et al., 2016). Taken together, the series of experiments in this chapter identified the key molecules, namely Fc γ RIIa, possibly ADAM10 and Syk, involved in GPVI shedding triggered by anti-PF4/heparin antibody isolated from patients with confirmed HIT.

Some loss of GPVI due to HIT Ig and increased P-selectin expression due to KKO antibody were observed in the absence of heparin or PF4. In this circumstance, the antigenic complex could be formed by residual PF4 on washed platelets binding to heparin, or in the absence of

heparin, physiological polyanionic molecules such as polyphosphates (Cines et al., 2016) and glycosaminoglycans (Padmanabhan et al., 2015, Rauova et al., 2006) on the platelet surface, which could substitute for heparin. The formation of this alternative antigenic complex may lead to exposure of neo-epitopes on PF4 that were recognised by HIT Ig or KKO antibody, consequently causing GPVI shedding and platelet activation. This finding is corroborated by Rauova and colleagues (2006) who demonstrated binding of KKO and platelet activation by HIT Ig were diminished but not abolished at low PF4 concentrations. Washed platelets contain PF4 and are likely to have membrane associated PF4, which together are sufficient to form a complex with heparin. In the current study, significant HIT Ig-mediated loss of GPVI could be achieved in suspensions containing heparin, however the greatest loss was demonstrated with addition of exogenous PF4 and low dose heparin.

Inter-donor variability in platelet responses to the same HIT Ig was observed and is in agreement with published literature (Warkentin et al., 1992, Salem and van der Weyden, 1983). These differences in platelet reactivity may be dependent on the capacity of donor platelets to release PF4 upon platelet activation or the levels of PF4 already present on the platelet surface. An increase in surface PF4 levels would contribute to more antigenic complex being formed on the platelet surface as proposed by Rauova and colleagues (2006). This may turn facilitate the crosslinking of Fc γ R1a receptors on platelets, potentially resulting in an increase in platelet recruitment, activation and GPVI shedding. In addition, platelet reactivity towards HIT Ig may be modulated by polymorphisms in the pathological HIT receptor, Fc γ R1a which will be explored in Chapter 5. This is in line with the observed consistency in donor-specific platelet reactivity towards the various HIT Ig tested. In other words, the donor who demonstrated the greatest loss of GPVI induced by HIT Ig from one patient, also had the greatest extent of GPVI shedding induced by Ig from other HIT patients.

Variability in the capacity of HIT Ig isolated from different HIT patients to induce GPVI shedding in donor platelets was observed (Figure 3.22) and is corroborated by previous studies which demonstrated variation in the activity of HIT Ig to cause platelet activation (Rauova et al., 2006, Padmanabhan et al., 2015). Padmanabhan and colleagues (2015) observed a 1.5-9-fold increase in P-selectin expression on donor platelets treated with 57 HIT Ig samples while 15-70% loss of GPVI was observed in donor platelets treated with HIT Ig samples from three patients in this study. The variation in platelet-activating capacity may be attributed to the levels of platelet-activating anti-PF4/heparin antibody present in the purified Ig preparation. Although equal concentrations of total Ig were added, the proportion of platelet-activating anti-PF4/heparin antibody is likely to vary between HIT patients. The inter-individual variability in the platelet-activating capacity of HIT Ig may be related to the differences in glycosylation levels between anti-platelet IgGs from individuals. Glycosylation patterns on IgG are reported to affect the stability, half-life and binding characteristics of the antibodies, and have been reported to impact on the extent of platelet destruction in autoimmune thrombocytopenia (Sonneveld et al., 2016, van de Bovenkamp et al., 2016). In addition, previous work demonstrated that the propensity to shed GPVI, resting ADAM10 activity and the extent to which ADAM10 activity can be elevated vary between donors (Facey et al., 2016) which may contribute to the donor-dependent responses observed here. Long-term storage of plasma may also impact on the potency of the affinity-purified Ig. A study by Krakow and colleagues (2007) showed that PF4/heparin antibodies were stable at 4°C for up to 4 days with no significant change detected in antibody titre measured by ELISA. However, the effect of long-term storage at -80°C for over a decade remains to be investigated.

Although similar conclusions can be drawn from both the sGPVI measurements by ELISA and surface GPVI levels by flow cytometry, the level of statistical significance achieved by

measuring sGPVI was stronger than by assessing intact GPVI levels via flow cytometry. This may be due to the differences in the sensitivity of each assay. The sGPVI ELISA is inherently cleaner with less background signal than flow cytometry due to the target enrichment step whereby sGPVI was initially captured by a polyclonal antibody before the addition of the monoclonal detection antibody. Hence, a gain in sGPVI was more readily detectable than a loss in surface GPVI. Another limitation of the flow cytometry analyses presented here is the semi-quantitative nature of the assay. A more quantitative comparison may be achieved by using a bead-based fluorescence flow cytometry to generate a standard curve from which a better estimation of the number of receptors present on each platelet can be interpolated.

EDTA was used to demonstrate the requirement for divalent cations due to its ability to chelate divalent cations such as Zn^{2+} , which is essential for metalloproteolytic activity. However, the depletion of other divalent cations like Ca^{2+} will also impact on $\alpha_{\text{IIb}}\beta_3$ integrin activity. Nevertheless, GPVI shedding has been demonstrated to not require active $\alpha_{\text{IIb}}\beta_3$ integrin whereby platelets treated with $\alpha_{\text{IIb}}\beta_3$ integrin inhibitors like eptifibatide (Montague et al., 2018) or GRGDSP peptide (Gardiner et al., 2004, Gardiner et al., 2008b) still undergo ligand-induced proteolytic cleavage of GPVI.

Proteolysis of GPVI by HIT Ig was consistently attenuated by blockade of $\text{Fc}\gamma\text{RIIa}$ and Syk kinase inhibition, making platelet $\text{Fc}\gamma\text{RIIa}$ and Syk kinase attractive target molecules for designing novel HIT therapy. Although $\text{Fc}\gamma\text{RIIa}$ is the platelet-activating receptor in HIT, this low affinity Fc receptor is important in mediating other cellular functions in myeloid cells and is the most widely expressed activating IgG receptor in humans (Bruhns, 2012). Hence, a platelet-specific approach in targeting $\text{Fc}\gamma\text{RIIa}$ would be ideal. One approach might be to combine the specificity of a non-activating GPVI antibody with the variable region of the IV.3 antibody to

localise the blockade of FcγRIIa to platelets, and thus minimise the thrombotic risk in HIT. Notably, two groups have successfully demonstrated the use of selective inhibitors of Syk, R406 on human platelets (Lhermusier et al., 2011); PRT-060318 on both human platelets and in transgenic mice (Reilly et al., 2011), in attenuating platelet activation triggered by anti-PF4/heparin antibodies. These reports support the findings presented in Figure 3.18 and Figure 3.26 where pre-treatment of platelets with a Syk inhibitor consistently attenuated HIT antibody-mediated GPVI shedding.

Because ADAM10 is the metalloproteinase responsible for cleavage of human GPVI, it was important to investigate the effect of HIT immune complex on platelet ADAM10 activity. Although the FRET-based assay was able to monitor metalloproteolytic activity on the platelet surface, the inclusion of heparin to the assay, severely diminished substrate cleavage. Hence, HIT antibody-induced changes to platelet ADAM10 activity could not be measured in this assay. Future studies aiming to measure ADAM10 activity may consider replacing heparin with other polyanionic molecules such as polyvinyl sulfonate which forms complexes with PF4 and has been used in an immunoassay for HIT testing (Immucor, 2017). Glycosaminoglycans like chondroitin sulphate and heparan sulphate, which have been shown to be substitutes for heparin, may also be considered (Visentin et al., 1994, Greinacher et al., 1994, Padmanabhan et al., 2015). While heparin diminished the fluorescence activity, PF4, a positively charged molecule, had the opposite effect. The mechanism by which heparin or PF4, and potentially other charged molecules, interfere with the cleavage of the GPVI-Cy3 ADAM10 sensor warrants further investigation.

In summary, Ig purified from confirmed HIT patients, but not non-HIT-mediated thrombocytopenic patients caused cleavage of GPVI ectodomain and the consequent release

of sGPVI that was dependent on heparin, FcγRIIa, divalent cations and ITAM signalling through Syk and Src family kinases, in a donor-dependent manner. Shedding of GPVI occurred through engagement of FcγRIIa by the Fc-portion of anti-PF4/heparin antibodies which recognised the antigenic complex formed by PF4 in the presence of low dose heparin. Collectively, these observations raised the possibility that plasma sGPVI levels may be a novel method to inform on the presence or absence of platelet-activating HIT antibodies. This will be investigated in Chapter 4.

Chapter 4 Evaluation of sGPVI in a prospective cohort suspected of HIT

4.1 Background

HIT is a potentially fatal complication of heparin therapy caused by antibodies against PF4 in complex with heparin. The clinical management of patients suspected of HIT is challenged by the limitations of laboratory tools to evaluate HIT pathology. Not all anti-PF4/heparin antibodies are pathogenic, and ‘real world’ laboratory testing largely cannot distinguish activating from non-activating anti-PF4/heparin antibodies. Due to the pro-thrombotic and potentially fatal nature of HIT, clinicians are trained to recognise the symptoms of HIT and initiate therapy promptly. What emerges however is a trend to over diagnose and alter therapy (switching anticoagulants) often unnecessarily, leaving patients at risk of significant bleeding (Cuker, 2011). Therefore, there is a significant unmet need for a simple, reliable and readily available assay that detects platelet-activating antibodies to confirm or exclude HIT in a timely manner.

4.1.1 Existing HIT diagnostic tools and their limitations

The diagnosis of HIT typically relies on the combination of clinical assessment and laboratory testing to detect anti-PF4/heparin antibodies followed by an evaluation of the platelet-activating capacity of the antibodies. The strengths and limitations of existing tools are discussed in Section 1.3.5 and summarised in Table 1.2.

The 4Ts score and the newer HEP score are pre-test scoring systems designed to assess a patient’s risk of HIT to guide initial clinical management and further testing (Cuker et al., 2010, Cuker et al., 2012, Pishko et al., 2018). The excellent negative predictive value of a low 4Ts

score allows clinicians to confidently exclude patients from HIT laboratory testing (Cuker et al., 2012) however scoring of a patient can vary significantly between clinicians with different levels of experience (Pishko et al., 2018). These scoring systems do not inform on the presence of HIT antibody nor an individual patient's platelet reactivity.

Samples from patients with intermediate/high 4Ts scores are commonly sent for PF4/heparin antibody testing. HIT immunoassays detect anti-PF4/heparin antibodies with high sensitivity (~97% to 99%) (Greinacher et al., 2007, Husseinzadeh et al., 2017) but with low specificity (82% to 87%) (Husseinzadeh et al., 2017) due to the relatively high incidence of anti-PF4/heparin antibodies in hospitalised patients who are exposed to unfractionated heparin. As the immunoassays do not discriminate between pathological and non-platelet-activating antibodies, the detection of anti-PF4/heparin antibodies in a thrombocytopenic patient does not equate to a diagnosis of HIT, consequently resulting in the high false positive rates associated with HIT immunoassays (Lo et al., 2007, Nazi et al., 2015). In addition, patient plasma or serum samples are batch analysed for PF4/heparin antibodies at specialist facilities which (in Australia) can take weeks. In reality, clinicians rely on the clinical scoring systems to make decisions around anticoagulant management of patients suspected of HIT and then re-evaluate anticoagulant usage once laboratory test results are returned.

To address this issue, functional assays such as HIPA and the SRA both using washed platelets from healthy donors, were developed to quantify the platelet-activating capacity of anti-PF4/heparin antibodies (Section 1.3.5.3). The SRA is regarded as a gold standard for HIT diagnosis, as it returns sensitivity and specificity values of 100% and 97%, respectively (Warkentin et al., 2005). However, the SRA is batch analysed, requires 'high responder' donor platelets and use of radioisotopes, and is performed only in specialist centres (1 in Australia).

These limitations mean data may take weeks to become accessible and so SRA results rarely feature in real-time HIT evaluations. There is also a lack of information on the patient's platelet status due to the use of donor platelets.

4.1.2 HIT overdiagnosis – a significant clinical consideration

HIT overdiagnosis and resultant treatment alterations can potentially impact on patient outcomes and wellbeing. An incorrect diagnosis of HIT can expose patients to increased bleeding risk and thrombotic complications (Smythe et al., 2011) due to unnecessary heparin cessation and initiation of costly alternative anticoagulants. Reports in the literature described cohorts of patients suspected of HIT, where 41% were prescribed with direct thrombin inhibitors despite a very low clinical probability of HIT when assessed retrospectively (Cuker et al., 2010). In particular, the HIT ELISA has been associated with potential overdiagnosis of HIT (Lo et al., 2007), attributed to low specificity of HIT immunoassays. There is an unmet need for simple, reliable and readily available assays that detect platelet-activating antibodies. Further, ideally such an assay would assess platelet activation status in the patient to help confirm or exclude HIT.

4.1.3 Rationale for considering sGPVI in evaluation of HIT

In Chapter 3, HIT antibodies were shown to cause a loss of intact GPVI from the platelet surface and liberation of sGPVI in donor platelets. Similar to the SRA, this loss of GPVI was evident at low heparin concentration (0.5 U/mL) but attenuated at high heparin dose (100 U/mL) and required functional FcγRIIa. The shedding of GPVI was metalloproteinase-dependent and required functional ITAM signalling. These observations imply that release of sGPVI may reflect functional engagement of FcγRIIa, and be a marker of pathological HIT antibody.

There are numerous reasons to consider measuring sGPVI for the detection of platelet-activating anti-PF4/heparin antibodies. Firstly, GPVI is a platelet-specific membrane protein and is stable on circulating, non-activated platelets (Gardiner et al., 2007). Secondly, the release of sGPVI occurs only under conditions of activation (ligand binding to GPVI or FcγRIIa as well as under shear stress and in acute coagulopathy), thus addressing the requirement for platelet-specific information in the setting of HIT. Thirdly, plasma sGPVI has been shown to be independent of age and gender (Al-Tamimi et al., 2012a). However, as plasma sGPVI can be increased in numerous conditions as discussed in Section 1.4.2, careful interpretation of sGPVI levels is necessary. Importantly, plasma sGPVI reports on a specific patient's platelet activity, an element missing from existing HIT diagnostic assays. The sGPVI ELISA has a similar procedure as HIT immunoassays and therefore could be integrated into existing HIT testing laboratories.

4.1.4 Overview of investigations

With the hypothesis that increased or low plasma sGPVI levels inform on the presence or absence of an activating/pathological anti-PF4/heparin antibody, this chapter aimed to evaluate the utility of sGPVI to predict HIT pathogenesis (Figure 4.1). Using a prospectively recruited cohort of patients suspected of HIT, standard clinical measures (4Ts score, polyspecific HIT ELISA) and plasma sGPVI were assessed and compared with SRA-positivity. The association between plasma sGPVI levels, HIT antibody levels or SRA-positivity and patient characteristics were explored. Finally, the diagnostic usefulness of plasma sGPVI considered in conjunction with 4Ts score and HIT antibody levels (ELISA OD values) was explored using a positive SRA to define HIT.

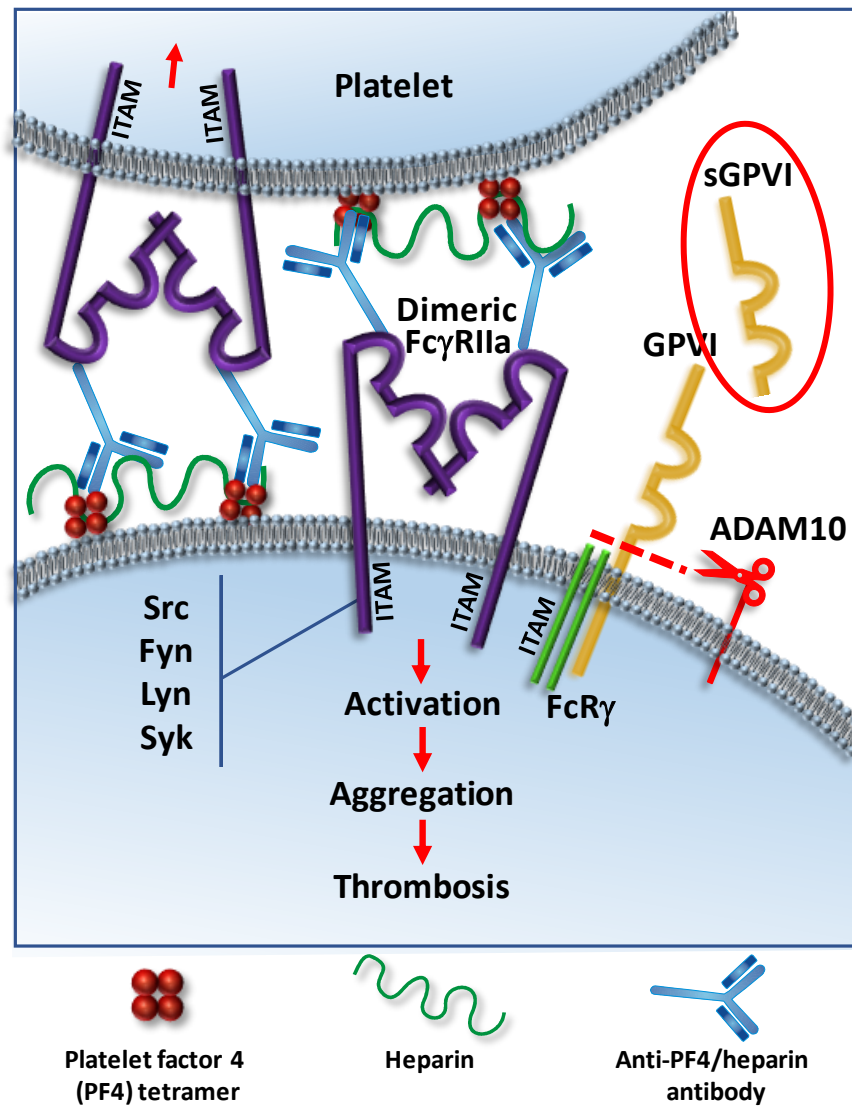


Figure 4.1 Evaluation of sGPVI in a prospective cohort suspected of HIT.

This chapter will focus on assessing the utility of plasma sGPVI (circled in red) to inform on platelet-activating antibodies in patients at risk of HIT.

4.2 Aims

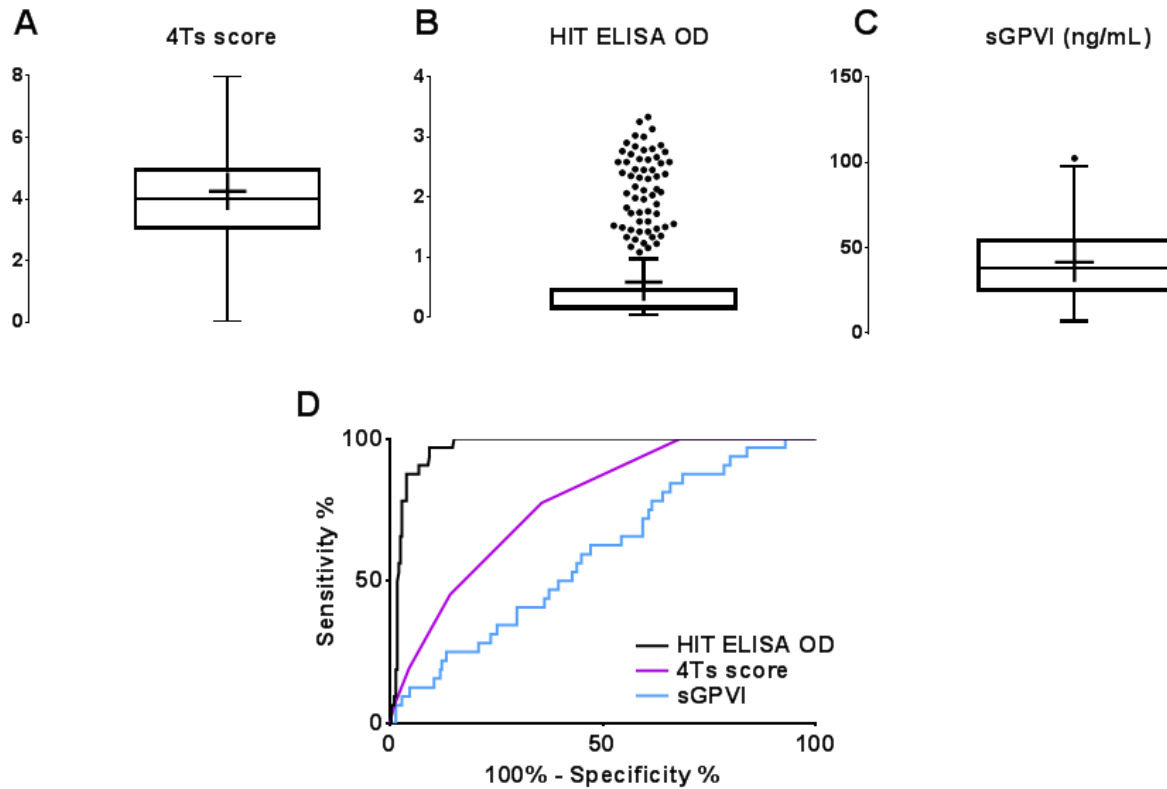
This chapter focused on assessing the value of plasma sGPVI to inform on the presence of pathological platelet-activating antibodies and the value of considering sGPVI in conjunction with current tools for HIT diagnosis when evaluating patients at risk of HIT.

4.3 Results

In collaboration with Dr Adam Cuker (Children's Hospital of Philadelphia, Pennsylvania, USA and a specialist HIT referral centre for north east America), 310 consecutive, hospitalised adult patients (≥ 18 years of age) were prospectively recruited between June 2012 and January 2015 from the Hospital of the University of Pennsylvania and an affiliated community hospital, Penn Presbyterian Medical Centre as described (Pishko et al., 2018). Demographic and clinical information was collected at the time of enrolment. 4Ts score was assigned by a haematology fellow or attending haematologist at enrolment. Blood collection and plasma isolation were performed as described in Section 2.2.3.2. HIT laboratory testing included a polyspecific HIT antibody ELISA and an SRA, and was performed on samples from all patients at the Hospital of the University of Pennsylvania. The SRA was conducted using patient plasma and donor PRP as described (Cines et al., 1980). Patients with a positive SRA were classified as having HIT (HIT+). A positive SRA was defined by less than 5% serotonin-release in the absence of heparin and greater than 20% release after addition of 0.1 or 0.5 U/mL heparin (Pishko et al., 2018). Plasma sGPVI levels were measured blinded to HIT status by Mrs Jing Jing (Melbourne, Australia) as described (Section 2.2.9.1).

4.3.1 Distribution and performance characteristics of 4Ts score, HIT ELISA and sGPVI

The distribution of 4Ts score, polyspecific HIT ELISA optical density (OD) and sGPVI levels for this cohort is summarised in Figure 4.2. The 4Ts score was 4.25 ± 1.42 (mean $\pm$ SD), while the mean polyspecific HIT ELISA OD was 0.58 ± 0.83 and ranged between 0.03-3.33. Mean plasma sGPVI level was 41.48 ± 21.02 ng/mL with a range of 7.00-102.3 ng/mL. Using a positive SRA result as the determinant of HIT, receiver operating characteristics (ROC) curves were constructed to determine the individual performance characteristics of 4Ts score, polyspecific HIT ELISA and sGPVI (Figure 4.2D). HIT ELISA was the best predictor of SRA outcome with an area under the curve (AUC) of 0.9689 ($p < 0.0001$) followed by 4Ts score (AUC = 0.7741, $p < 0.0001$). When considered on its own as a standalone tool, sGPVI (AUC = 0.5911, $p = 0.0916$) was a relatively poorer predictor of SRA outcome than the HIT ELISA or the 4Ts score.



	HIT ELISA OD	4Ts score	sGPVI
AUC	0.9689	0.7741	0.5911
Standard error	0.0096	0.0378	0.0495
95% CI	0.9502-0.9877	0.7001-0.8482	0.4940-0.6881
p-value	<0.0001	<0.0001	0.0916

Figure 4.2 Data distribution and operating characteristics of clinical and laboratory parameters.

Box-and-whisker plots summarising the distribution of (A) 4Ts score (n=292), (B) polyspecific HIT ELISA OD (n=310) and (C) plasma sGPVI levels (n=310) in the prospective cohort of consecutive adult inpatients. + denotes mean values. (D) Receiver operating characteristic (ROC) curves of polyspecific HIT ELISA, 4Ts score and sGPVI in predicting HIT defined by a positive serotonin-release assay result. OD, optical density; AUC, area under ROC curve; CI, confidence interval.

4.3.2 sGPVI was associated with patient type and thrombosis occurrence

Patient characteristics and their association with SRA outcome and plasma sGPVI levels were computed by Pearson's Chi-square analysis as summarised in Table 4.1. 32 of the 310 recruited patients gave a positive SRA outcome. SRA outcome was independent of age ($\chi^2=67.61$, $p=0.374$), gender ($\chi^2=0.60$, $p=0.502$), ethnicity ($\chi^2=2.86$, $p=0.502$), patient type ($\chi^2=15.68$, $p=0.546$) and admission into intensive care unit (ICU) ($\chi^2=2.06$, $p=0.302$), but weakly correlated with thrombosis ($\chi^2=11.49$, $p=0.059$). Expectedly, SRA outcome was significantly associated with 4Ts score ($\chi^2=29.93$, $p<0.0001$) and presence of anti-PF4/heparin antibody as measured by the polyspecific HIT ELISA ($\chi^2=172.40$, $p<0.0001$). None of the SRA-positive patients were assigned a 4Ts score of less than three while all SRA-positive patients had HIT ELISA OD values of greater than 0.50 OD units. 18 of 32 (56.3%) of the SRA-positive patients were admitted for cardiac-related diagnoses and 14 out of 32 (43.8%) SRA-positive patients experienced a thrombotic event.

A cut-off value of 50 ng/mL was set to define low or high plasma sGPVI level as this was the optimal cut-off value as shown in the list of specificity and sensitivity values for the sGPVI ROC curve in Appendix 7. As summarised in Table 4.1, plasma sGPVI level was not influenced by age ($\chi^2=54.92$, $p=0.710$), gender ($\chi^2=0.14$, $p=0.710$) and ethnicity ($\chi^2=6.51$, $p=0.178$). Unlike the SRA, sGPVI was not associated with 4Ts score ($\chi^2=12.87$, $p=0.269$) and HIT ELISA ($\chi^2=1.54$, $p=0.710$). However, sGPVI was significantly associated with the reason for hospital admission ($\chi^2=37.71$, $p=0.012$), ICU admission ($\chi^2=24.62$, $p<0.0001$) and thrombosis occurrence ($\chi^2=15.01$, $p=0.013$), consistent with its association with prothrombotic disease (Section 1.4.2). Of the patients who were not admitted to the ICU ($n=128$), 82% (105 of 128) demonstrated lower plasma sGPVI levels. In addition, lower sGPVI levels were observed in 73% (150 of 205)

sGPVI was associated with patient type and thrombosis occurrence

of patients without an apparent thrombotic event compared to patients with thrombosis. These observations suggest a potential utility of plasma sGPVI to reflect on platelet activity in the patient.

Table 4.1 Association between patient characteristics and SRA outcome, and plasma sGPVI levels.

Associations were assessed using Pearson's Chi-square analysis. The *p*-values reported were corrected for multiple testing using the Benjamini-Hochberg method (Benjamini and Hochberg, 1995). SRA, serotonin-release assay; ICU, intensive care unit; OD, optical density.

Patient characteristics	n	SRA				sGPVI			
		Negative	Positive	χ^2	<i>p</i> -value	≤50 ng/mL	>50 ng/mL	χ^2	<i>p</i> -value
Number of patients	310	278	32			205	105		
Age (years)	19-92	19-92	36-87	67.61	0.374	19-92	24-92	54.92	0.710
Gender				0.60	0.502			0.14	0.710
Male	164	145	19			110	54		
Female	146	133	13			95	51		
Ethnicity				2.86	0.502			6.51	0.178
Caucasian	226	199	27			151	75		
Hispanic	4	4	-			4	-		
African American	73	68	5			48	25		
Asian	7	7	-			2	5		
Patient type				15.68	0.546			37.71	0.012
Cardiac Surgery	132	119	13			67	65		
Cardiology	47	42	5			34	13		
Medicine	32	30	2			27	5		
MICU	24	23	1			16	8		
Oncology	17	17	-			16	1		
General Surgery	12	10	2			10	2		
Neurology	10	8	2			7	3		
Neurosurgery	7	6	1			7	-		
Trauma Surgery	6	5	1			4	2		
Thoracic Surgery	5	4	1			4	1		
Vascular Surgery	5	4	1			2	3		
Urology	3	2	1			3	-		
Cardiac ICU	2	2	-			1	1		
ENT	2	1	1			2	-		
Surgery	2	1	1			2	-		

sGPVI was associated with patient type and thrombosis occurrence

Patient characteristics	n	SRA		χ^2	p-value	sGPVI		χ^2	p-value
		Negative	Positive			≤50 ng/mL	>50 ng/mL		
Gastroenterology	2	2	-			1	1		
Obstetrics	1	1	-			1	-		
Orthopaedic Surgery	1	1	-			1	-		
ICU				2.06	0.302			24.62	<0.0001
Yes	182	167	15			100	82		
No	128	111	17			105	23		
4Ts score				29.93	<0.0001			12.87	0.269
Low (0-3)	83	83	0			61	22		
Intermediate (4-5)	158	141	17			102	56		
High (6-8)	51	37	14			34	17		
Not available	18	17	1			8	10		
HIT ELISA (OD)				172.40	<0.0001			1.54	0.710
<0.50	234	234	0			157	77		
0.50-0.99	14	13	1			10	4		
1.00-2.00	27	21	6			18	9		
>2.00	35	10	25			20	15		
Thrombosis				11.49	0.059			15.01	0.013
0	205	188	17			150	55		
1	27	26	1			14	13		
2	59	46	13			32	27		
3	1	1	-			1	-		
Not available	18	17	1			8	10		

4.3.3 High false positive rates associated with HIT ELISA

HIT immunoassays established to detect anti-PF4/heparin antibodies have high levels of false positive (Lo et al., 2007, Nazi et al., 2015) and this was true of data related to the polyspecific HIT ELISA presented here. This assay utilised a solid phase of PF4 in complex with polyvinyl sulfonate to capture all antibodies that recognise the exposed PF4 epitopes created when PF4 is complexed with a linear polyanionic molecule (Immucor, 2017). However, not all detectable anti-PF4 antibodies caused platelet activation and this lack of specificity to pathological antibodies contribute to its high false positive rate. To demonstrate the lack of specificity for platelet-activating antibodies, the associations between HIT ELISA OD values, 4Ts score and SRA outcome were evaluated.

Figure 4.3A depicts the distribution of HIT ELISA OD values observed across the three groups of patients with low, intermediate or high 4Ts scores. The HIT ELISA OD values were (mean $\pm$ SD) 0.318 ± 0.534 OD units (range 0.03 to 3.33) in low 4Ts score, 0.661 ± 0.884 OD units (range 0.03 to 3.25) in intermediate 4Ts score and 1.15 ± 1.18 (range 0.07 to 2.90) in high 4Ts score. As expected, higher HIT ELISA OD values were associated with patients who were scored as an intermediate or high 4Ts score compared with OD values in patients with a low 4Ts score (low versus intermediate, $p=0.010$; low versus high, $p=0.011$) (Figure 4.3A). No significant difference in OD values were detected between the intermediate and high 4Ts score ($p=0.398$). 10 of 83 patients with low 4Ts scores recorded antibody levels above 0.5 OD units and 10 of 18 patients with high 4Ts scores recorded OD values of less than 0.5. Among patients with high 4Ts scores, two distinct populations of low and high OD values were apparent.

Analysis of HIT ELISA OD values in SRA-negative ($n=278$) versus SRA-positive ($n=32$) patients indicates that amount of antibody is a strong positive predictor but a poor negative predictor

of SRA outcome (Figure 4.3B). As expected, HIT ELISA OD values were significantly higher ($p<0.0001$) in SRA-positive patients at (mean $\pm$ SD) 2.33 ± 0.565 OD units versus 0.377 ± 0.580 OD units in SRA-negative patients. Whilst the OD values ranged from 0.03 to 3.33 OD units in SRA-negative patients and 0.61 to 3.25 OD units in SRA-positive patients (Figure 4.3A), every patient who tested positive by SRA, had OD values above the threshold of 0.5 OD units. However, 43 of 278 patients had OD values exceeding the threshold value but produced a negative SRA result. This observation clearly illustrated a high level of false positivity associated with HIT ELISA which lacks specificity for platelet-activating HIT antibodies, consistent with published data (Lo et al., 2007, Nazi et al., 2015). As Section 4.3.2 demonstrated that plasma sGPVI can reflect HIT antibody anti-platelet activity, the associations between patient plasma sGPVI levels and existing HIT diagnostic parameters were subsequently evaluated in Section 4.3.4.

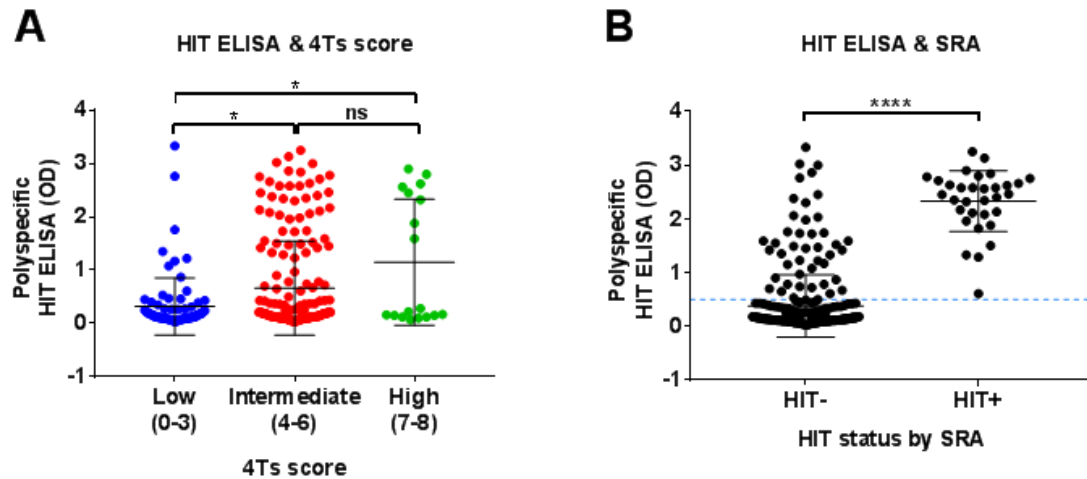


Figure 4.3 High false positive rates associated with polyspecific HIT ELISA.

(A) Association between HIT ELISA and 4Ts score. Differences in OD values between patients with low (0 to 3; n=83), intermediate (4 to 6; n=191) and high (7-8; n=18) 4Ts scores were evaluated by Kruskal-Wallis test with Dunn's multiple comparisons. (B) Relationship between HIT ELISA and SRA outcome in 278 SRA-positive and 32 SRA-negative patients assessed by Mann-Whitney test. Blue dashed line represents the cut-off value of 0.5 OD units which define a positive HIT ELISA. Error bars indicate mean $\pm$ SD. * $p < 0.05$, **** $p < 0.0001$, ns indicates not significant.

4.3.4 Increased plasma sGPVI levels associated with high clinical probability for HIT

Plasma sGPVI levels were significantly associated with patient type and thrombosis occurrence (Section 4.3.2), suggesting sGPVI may inform on platelet activity. Hence, the association between plasma sGPVI levels and existing HIT diagnostic parameters were evaluated here.

Figure 4.4A shows the variable plasma sGPVI levels among patients assigned with low (0-3), intermediate (4-6) or high (7-8) 4Ts scores. sGPVI levels were (mean $\pm$ SD) 39.30 ± 19.17 ng/mL (range 7.97 to 97.61 ng/mL) in the low 4Ts score group, 41.42 ± 21.56 ng/mL (range 7.00 to 102.3 ng/mL) in the intermediate 4Ts score group and 41.19 ± 21.70 ng/mL (range 13.19 to 84.76 ng/mL) in the high 4Ts score group. No significant differences in sGPVI levels were observed between the three groups.

Plasma sGPVI levels were not correlated with anti-PF4/heparin antibody levels (Figure 4.4B). In 310 patients, Spearman's correlation analysis revealed a weak correlation coefficient of $r_s=0.1933$ ($p=0.0006$ due to large sample size) between these parameters whereby sGPVI explained only 3.74% ($r_s^2=0.0374$) of the variation seen in HIT ELISA OD values. In this cohort, an increased sGPVI level was not specific to patients with positive SRA outcome (Figure 4.4C). Comparable levels of plasma sGPVI ($p=0.092$) were observed in SRA-positive and SRA-negative patients at (mean $\pm$ SD) 47.39 ± 21.34 ng/mL (range 14.38 to 93.65 ng/mL) versus 40.8 ± 20.91 ng/mL (range 7.00 to 102.3 ng/mL), respectively.

Given that clinical parameters are used in combination rather than as standalone tools to guide clinical management, the association between sGPVI and combinations of 4Ts, HIT ELISA OD values and SRA outcome were considered in Figure 4.4D. Patients were stratified into low-risk (4Ts score <4 , HIT ELISA OD <0.5 units and negative SRA results) and high-risk (4Ts score ≥ 4 , HIT ELISA OD ≥ 0.5 and a positive SRA outcome) groups. The 4Ts score and OD cut-off values

were based on established guidelines to determine clinical probability for HIT (Cuker et al., 2018). Despite the lack of association between sGPVI and these parameters when considered in isolation, a marginal but significant ($p=0.044$) increase in plasma sGPVI was detected in the high-risk group (mean $\pm$ SD) at 46.70 ± 21.33 ng/mL (range 14.38 to 93.65 ng/mL) compared to the low-risk group at 38.04 ± 19.06 ng/mL (range 7.97 to 97.61 ng/mL). This result suggests that sGPVI levels may help stratify patients based on their risk for developing HIT. Plasma sGPVI levels were associated with ICU admission, patient type and thrombosis occurrence (Section 4.3.2) as sGPVI may be increased in pathologies not directly related to HIT. Hence, the utility of sGPVI as a negative predictor of platelet activation to exclude HIT was evaluated in conjunction with established HIT diagnostic tools (Section 4.3.5).

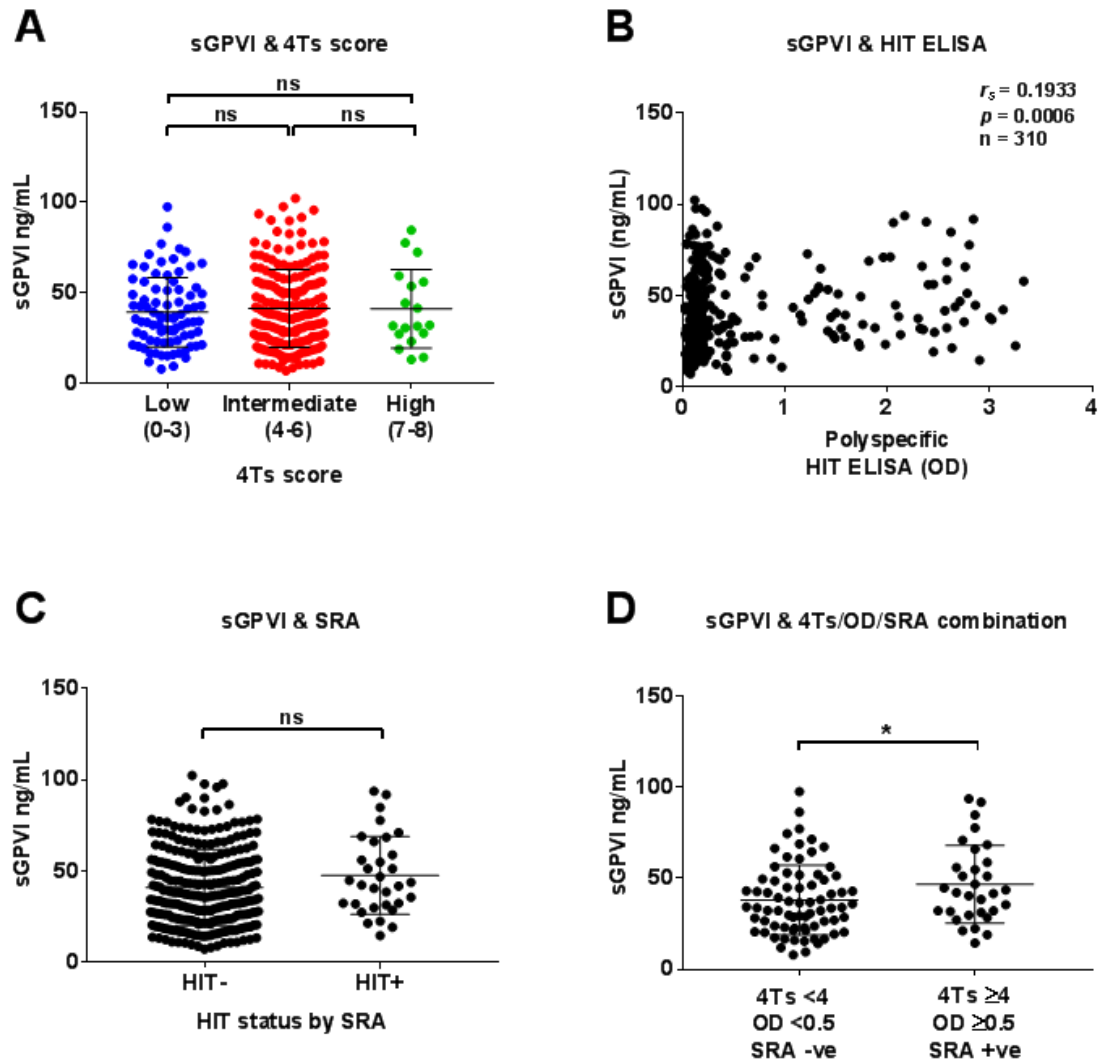


Figure 4.4 Relationship between plasma sGPVI levels and existing HIT diagnostic parameters.

The association between plasma sGPVI and 4Ts score (A), anti-PF4/heparin antibody levels as measured by HIT ELISA (B), SRA outcome (C) and combination established of HIT diagnostic tools (D). (A) Differences in plasma sGPVI levels between patients with low 4Ts score ($n=83$), intermediate 4Ts score ($n=191$) and high 4Ts score ($n=18$) were evaluated by Kruskal-Wallis test with Dunn's multiple comparisons. (B) Spearman's correlation (r_s) between sGPVI levels and HIT ELISA OD values in 310 patients prospectively evaluated for HIT. (C) Relationship between sGPVI levels and SRA outcome in 278 SRA-positive and 32 SRA-negative patients assessed by Mann-Whitney test. (D) Association between sGPVI levels and risk of HIT defined by a combination of 4Ts score, HIT ELISA OD value and SRA outcome. The low-risk group ($n=73$) was defined by 4Ts score <4 , HIT ELISA OD <0.5 units and a negative SRA outcome while patients in the high-risk group ($n=31$) were assigned 4Ts score ≥ 4 , HIT ELISA OD ≥ 0.5 and a positive SRA. Unpaired t -test was computed to compare differences in sGPVI levels between the two risk groups. Error bars indicate mean $\pm$ SD. * $p < 0.05$, ns indicates not significant.

4.3.5 Utility of including sGPVI measurements in HIT evaluation

Firstly, all 310 patients were considered in this assessment (Table 4.2). A threshold level of HIT antibodies of OD ≥ 0.5 units was used to identify patients likely to develop HIT by using the SRA outcome as the determinant. The performance of HIT ELISA alone was compared to the performance achieved by considering both HIT ELISA OD ≥ 0.5 units and sGPVI ≥ 50 ng/mL. The threshold value for HIT ELISA was based on published guidelines (Cuker et al., 2018) while the sGPVI cut-off was the optimal cut-off value based on data from this cohort as shown in the list of specificity and sensitivity values for the sGPVI ROC curve in Appendix 7. Inclusion of sGPVI improves the accuracy (86% to 89%), specificity (84% to 95%) and positive predictive value (PPV) (0.42 to 0.46) by reducing the number of false positives from 44 to 15 individuals. However, this was achieved at a cost of lower sensitivity (100% to 41%) and lower negative predictive value (NPV) (1.00 to 0.93).

Patients with intermediate 4Ts scores were often challenging to diagnose and manage clinically. For this reason, this patient group was also assessed separately from the cohort. When evaluating patients with intermediate 4Ts scores of 4-6, inclusion of sGPVI in tandem with HIT ELISA showed a similar outcome with improvements in the accuracy (84% to 87%), specificity (81% to 95%) and PPV (0.45 to 0.53) but a reduction in the sensitivity (100% to 40%) and NPV (1.00 to 0.91) (Table 4.2). These observations highlighted the potential of sGPVI as a negative predictor for HIT by reducing the number of false positives identified by HIT ELISA.

Table 4.2 Utility of plasma sGPVI levels considered in tandem with 4Ts score and HIT ELISA.

(A) All 310 patients at risk of developing HIT were considered. HIT ELISA OD ≥ 0.5 units was used to identify patients likely to develop HIT. This was then compared to the stratification outcome when considering both HIT ELISA and sGPVI (≥ 50 ng/mL). Positive SRA outcome was used as the determinant for HIT. (B) Similar stratification strategy as (A) applied to patients with intermediate 4Ts scores of 4-6. SRA, serotonin-release assay; PPV, positive predictive value; NPV, negative predictive value.

(A) All 4Ts score (n=310)		SRA+	SRA-	Accuracy	Sensitivity	Specificity	PPV	NPV
HIT ELISA				86%	100%	84%	0.42	1.00
	Positive	32	44					
	Negative	0	234					
HIT ELISA and sGPVI				89%	41%	95%	0.46	0.93
	Positive	13	15					
	Negative	19	263					

(B) 4Ts score: 4-6 (n=191)		SRA+	SRA-	Accuracy	Sensitivity	Specificity	PPV	NPV
HIT ELISA				84%	100%	81%	0.45	1.00
	Positive	25	31					
	Negative	0	135					
HIT ELISA and sGPVI				87%	40%	95%	0.53	0.91
	Positive	10	9					
	Negative	15	157					

In order to test the utility of sGPVI as a negative predictor, sGPVI was layered on as a second stratification step after stratifying by 4Ts score and HIT ELISA. 292 patients were included in this assessment, 18 patients were excluded due to unavailable 4Ts score. SRA outcome was used as the determinant of HIT. Firstly, patients were identified as likely to develop HIT by 4Ts score ≥ 4 and HIT ELISA OD ≥ 0.5 . From the pool of false positives identified based on SRA outcome, sGPVI < 50 ng/mL was used to identify patients who were unlikely to develop HIT (Table 4.3A). An overall improvement in accuracy (89% to 97%), specificity (87% to 96%) and PPV (0.48 to 0.76) conferred by the additional stratification by sGPVI was seen. A similar improvement in the overall diagnostic performance was observed when assessing patients with intermediate 4Ts scores of 4-6 (Table 4.3B, accuracy: 84% to 95%, specificity 81% to 95%, PPV: 0.45 to 0.74). These observations highlighted the potential of sGPVI as a negative predictor for HIT to improve on the accuracy of existing tools for HIT evaluation when considered in combination.

Table 4.3 Inclusion of plasma sGPVI levels for evaluation of HIT likelihood.

(A) 292 patients were included in this assessment. Firstly, patients were identified as likely to develop HIT by 4Ts score ≥ 4 and HIT ELISA OD ≥ 0.5 units. From the pool of false positives identified based on SRA outcome, sGPVI < 50 ng/mL was used to identify patients unlikely to develop HIT. (B) Patients with 4Ts scores of 4-6 were considered in this assessment. HIT ELISA OD ≥ 0.5 units was first applied to identify patients likely to develop HIT. Then, sGPVI < 50 ng/mL was used to identify patients unlikely to develop HIT from the pool of false positives generated in the first step. SRA, serotonin-release assay; PPV, positive predictive value; NPV, negative predictive value.

(A) All 4Ts score (n=292)		SRA+	SRA-	Accuracy	Sensitivity	Specificity	PPV	NPV
4Ts and HIT ELISA				89%	100%	87%	0.48	1.00
	Positive	31	33					
	Negative	0	228					
+sGPVI				97%	100%	96%	0.76	1.00
	Positive	31	10					
	Negative	0	251					

(B) 4Ts score: 4-6 (n=191)		SRA+	SRA-	Accuracy	Sensitivity	Specificity	PPV	NPV
HIT ELISA				84%	100%	81%	0.45	1.00
	Positive	25	31					
	Negative	0	135					
+sGPVI				95%	100%	95%	0.74	1.00
	Positive	25	9					
	Negative	0	157					

4.4 Discussion

HIT overdiagnosis is a significant clinical consideration, largely driven by a lack of readily available specific HIT diagnostic tests. Platelet-activation assays to confirm HIT generally provide retrospective data and so do not guide timely patient management. Hence, there is a significant unmet need for a simple, reliable and readily available assay that detects platelet-activating antibodies and informs on the patient's platelet activation status to confirm or exclude HIT. Here, the utility of plasma sGPVI levels to inform on platelet activity was explored in relation to established tools for HIT diagnosis using a prospective cohort of patients at risk of developing HIT.

The merits of existing HIT diagnostic tools are well established through significant association between 4Ts score, HIT ELISA and SRA outcome (Table 4.1), and the favourable performance characteristics of 4Ts score and HIT ELISA in predicting SRA positivity (Figure 4.2D). These established tools aid in identifying patients likely to develop HIT but do not provide patient platelet-specific information and run the risk of overdiagnosis (Cuker, 2011, Nazi et al., 2015). The only patient- and platelet-specific information available to the attending clinician is the platelet count embedded within the 4Ts score. The specificity of 4Ts score in this cohort was evident whereby no patients assigned 4Ts score of ≤ 3 was positive by SRA, supporting the utility of the 4Ts score to rule out further HIT testing.

The HIT ELISA has excellent sensitivity in predicting SRA outcome. However, not all detectable anti-PF4/heparin antibodies caused platelet activation, leading to the high false positive rate and low specificity associated with HIT immunoassays (Nazi et al., 2015). In the current dataset, a positive HIT ELISA produced a negative SRA outcome in 15% (43 of 278) of patients. The nature of the polyspecific HIT ELISA which detects all three IgG, IgA and IgM classes of

antibodies further reduces its specificity as reported by Warkentin and colleagues (2005) who described similar levels of IgA and IgM formation between non-HIT and HIT patients.

Early and correct identification of patients who will develop thrombosis and thrombocytopenia related to heparin therapy is vital, as this allows early cessation of heparin and the introduction of alternative anticoagulant therapy. It is equally important to avoid the unnecessary cessation of heparin due to the 10-20% increased bleeding risk associated with starting alternative anticoagulants like direct thrombin inhibitors (Cuker, 2011, Marler et al., 2015). Inappropriate heparin cessation may also result in fatal thrombotic complications (Smythe et al., 2011). Hence, there is value in being able to exclude HIT in light of a positive HIT immunoassay. While plasma sGPVI levels poorly predicted SRA outcome (Figure 4.2D, Figure 4.4C), lower sGPVI levels were observed in patients without a thrombosis occurrence (Table 4.1), suggesting increased sGPVI did reflect engagement of patient platelets by antibody. Similarly, lower sGPVI levels were observed in the low-risk group defined by 4Ts score <4, HIT ELISA OD <0.5 units and a negative SRA outcome (Figure 4.4D). The potential value of sGPVI may rest with its negative predictive value to exclude the presence of platelet activation in individuals with high antibody titres. When considered in conjunction with 4Ts score and HIT ELISA OD values, plasma sGPVI improved the overall accuracy, specificity and PPV (Table 4.2, Table 4.3).

There are many advantages of incorporating sGPVI measurements when evaluating a patient's risk of HIT. First, sGPVI is a platelet-specific biomarker that circulates at low levels in plasma from healthy individuals but is released from activated platelets (Al-Tamimi et al., 2012a). Second, sGPVI levels are independent of age, gender and ethnicity as reported here and in published literature (Al-Tamimi et al., 2011a, Pennings et al., 2014). Third, sGPVI provides

patient platelet-specific information and the turnaround time to complete sGPVI measurements is on par with the HIT ELISA and likely to be shorter than the SRA. In fact, sGPVI could be measured concurrently with anti-PF4/heparin immunoassays with the potential to combine both measurements in a multiplex assay in the future. Inclusion of sGPVI measurements may contribute to a more rapid risk stratification of patients with clinical suspicion of HIT, especially those with an intermediate 4Ts score (Table 4.2B, Table 4.3B).

These reasons provide a sound rationale to propose inclusion of plasma sGPVI measurements into the existing algorithm for HIT diagnosis as proposed in Figure 4.5 adapted from Qiao and colleagues (2015). The 4Ts score is first calculated for heparin-exposed patients with a sudden inexplicable drop in platelet count to assess HIT propensity. Plasma samples from patients with intermediate or high 4Ts score of ≥ 4 are simultaneously analysed for anti-PF4/heparin antibody by HIT immunoassay and sGPVI by ELISA using established thresholds. Patient plasma sGPVI level after heparin infusion is (ideally) compared to their baseline level pre-heparin exposure to obtain a fold-change (Δ) in sGPVI. The cut-off value of ≥ 2 -fold is based on the average sGPVI at (mean $\pm$ SD) 47.39 ± 21.34 ng/mL in this cohort of SRA-positive patients compared to the average in 45 healthy individuals (mean $\pm$ SD: 18.93 ± 16.52 ng/mL, Figure 5.8). The optimal cut-off value will need to be formally determined in another prospective cohort of query HIT patients. Patients who tested negative in both ELISAs (OD < 0.5 units and < 2 -fold increase in sGPVI) are unlikely to develop HIT while HIT is likely in patients with positive HIT ELISA (OD ≥ 0.5 units) and ≥ 2 -fold increase in sGPVI. Further functional assays should then be performed in the case of patients testing positive for either HIT ELISA or sGPVI. With an effect size of 0.314, the current study only has a 35% power to detect a significant increase in plasma sGPVI levels in SRA+ patients. To demonstrate with 80% power, a difference at $p < 0.05$,

future studies would require 88 SRA+ patients and 879 SRA- patients, assuming that 10% of query HIT patients are positive by SRA.

Circulating sGPVI levels varied widely among individuals studied here, which is to be expected in such a heterogeneous patient group (Table 4.1). It is likely to be more accurate to consider the change in sGPVI levels when assessing FcγRIIa-mediated platelet activation by comparing the levels after heparin infusion to baseline sGPVI prior to heparin exposure. This strategy to assess the change in sGPVI levels relative to baseline would serve to normalise the initial variation in sGPVI levels and potentially strengthen the argument that sGPVI can aid in identifying patients with platelet-activating antibodies. In addition, increased sGPVI levels not related to heparin exposure could also be factored in by assessing the change in sGPVI levels post-heparin infusion relative to pre-heparin infusion. Unfortunately, samples that could provide sGPVI levels pre-heparin infusion were not available in this cohort to test this hypothesis. Acquisition of these pre-heparin exposure samples whilst challenging, could be feasible at least in tertiary medical care units where blood samples are routinely stored for up to 7 days post analysis (personal communications with The Canberra Hospital Haematology laboratory).

Although sGPVI is a marker of platelet activation, some of the SRA-positive patients had sGPVI levels that were comparable to healthy individuals. This may be related to the timing of blood collection. Higher sGPVI levels would be expected during the acute phase of HIT when thrombocytopenia is evident, and platelets are activated. An evaluation of the temporal profile of sGPVI levels during the course of HIT would be of interest in future studies. The lack of correlation between sGPVI levels and SRA outcome may also relate to the stability of circulating sGPVI. Little is known about the half-life of plasma sGPVI in the human body. In

cynomolgus monkeys, sGPVI returned to baseline levels within 24 hours after lipopolysaccharide challenge (Naitoh et al., 2015). The same group also reported that intravenously-administered recombinant human GPVI ectodomain fragment remained in circulation at an elimination half-life of about 2.5 h in monkeys.

There is no clear explanation of why a proportion of SRA-positive patients had sGPVI levels in the normal range (Figure 4.4C). Whilst a number of groups have reported that dimeric sGPVI and GPVI fusion proteins specifically bind to collagen and other proteins (Massberg et al., 2004, Ungerer et al., 2011) high affinity binding partners of monomeric sGPVI remain to be discovered. It is highly likely that all of sGPVI circulate in a monomeric form. However, further investigation is necessary to fully assess the half-life of sGPVI in the human circulation, characterise binding partners of circulating sGPVI and consider an association between high sGPVI levels and thrombotic occurrence where high levels of fibrin and fibrin degradation products (potential GPVI binding partners) would be expected. Plasma sGPVI levels may also be regulated by the activity of metalloproteinase ADAM10 which will be explored in Chapter 6.

There are numerous factors governing the capability of anti-PF4/heparin antibodies to activate platelets. These include FcγRIIa receptor density, platelet PF4 content and FcγRIIa receptor polymorphism all of which may influence the ability of anti-PF4/heparin antibodies to trigger GPVI shedding. Indeed, Chong and colleagues (1993b) reported a dynamic control of platelet FcγRIIa which increased during the acute phase of HIT and returned to baseline one to three months later. Platelet FcγRIIa expression also increased with disease severity. The stoichiometry and concentration of PF4 oligomers are also likely to influence platelet

engagement (discussed in Section 3.4) while the contribution of receptor polymorphism will be investigated in Chapter 5.

In summary, plasma sGPVI is a valuable measure of platelet activation that improved the overall diagnostic performance of the HIT immunoassay. There is likely to be a complex interplay between engagement of Fc γ RIIa, activation of metalloproteinase and shedding of GPVI during platelet activation by anti-PF4/heparin antibodies. A combination of parameters will be required to delineate pathological from non-pathological HIT antibody to better predict HIT or HITT.

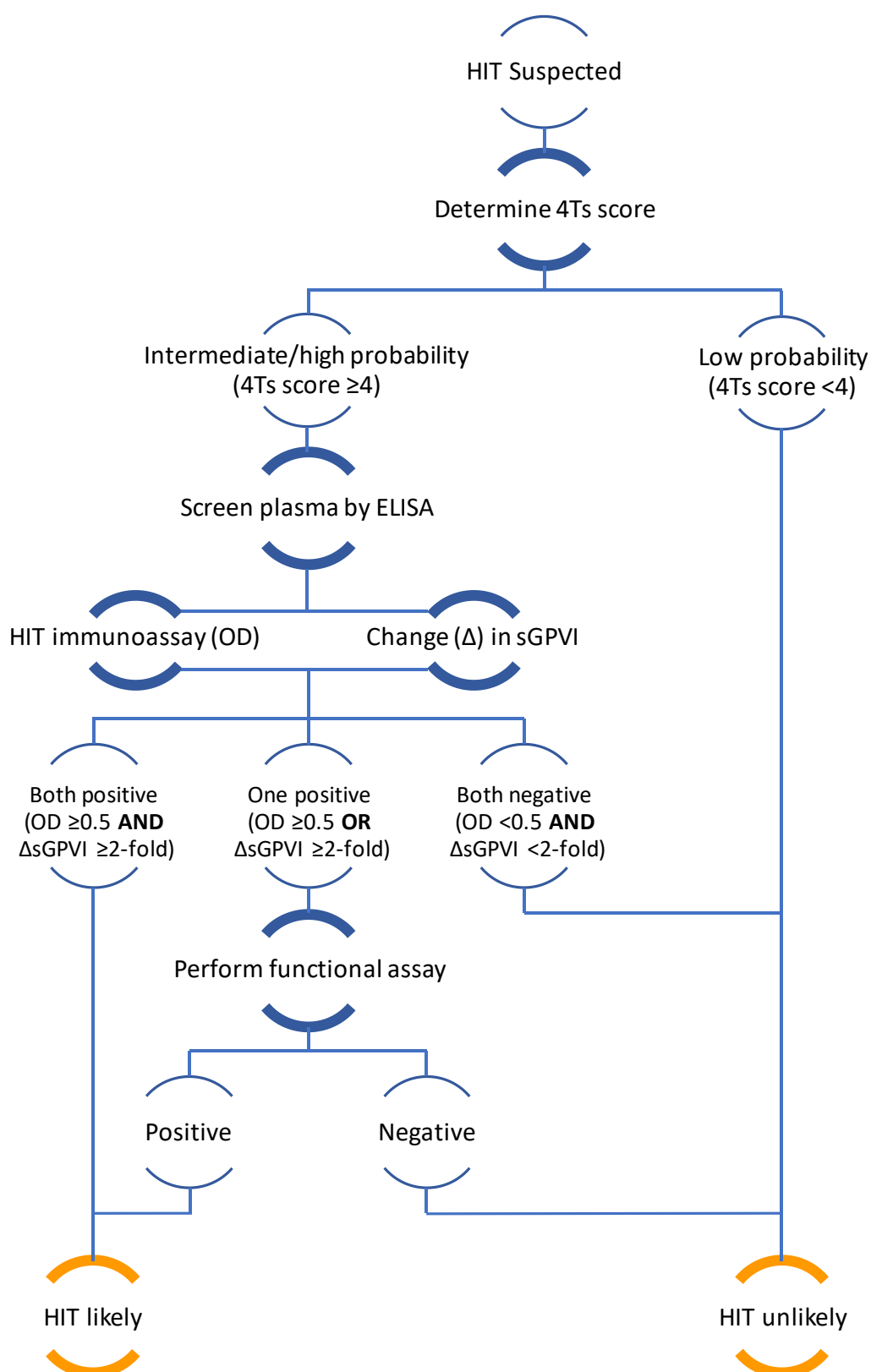


Figure 4.5 Proposed algorithm for HIT diagnosis.

Change in plasma sGPVI level relative to pre-heparin exposure is proposed to be assessed simultaneously with existing HIT immunoassay. Thick blue arcs indicate diagnostic tools, OD = optical density. Adapted from Qiao and colleagues (2015).

Chapter 5 Influence of *FCGR2A* polymorphisms on platelet reactivity and HIT susceptibility

5.1 Background

As discussed in earlier chapters, despite the extensive use of heparin and high morbidity associated with HIT, there remains a lack of diagnostic tools to calculate HIT risk. Identification of genetic biomarkers for HIT predisposition may be advantageous in acknowledging the need for risk stratification prior to heparin exposure and/or monitoring of heparinised patients. The pathological HIT receptor Fc γ RIIa is encoded by seven exons within the *FCGR2A* gene, located on the long arm of chromosome 1 at position 23.3 (1q23.3) (Grundy et al., 1989). Three polymorphic sites located at amino acids 27, 127 and 131 of Fc γ RIIa have been reported.

5.1.1 *FCGR2A* H131R polymorphism

The H131R polymorphism is the most widely studied non-synonymous SNP of *FCGR2A* at nucleotide 519 (519G>A, rs1801274) on exon 4 which results in the replacement of arginine (R) by histidine (H) at amino acid 131. Both alleles are co-dominantly expressed in the human population at a genotype frequency of 28%, 47.5% and 24.5% for 131HH, 131HR and 131RR genotypes, respectively (Rollin et al., 2015). H131R sits in the contact interface between the receptor and its ligand (the fragment constant region (Fc) of IgG) in the second Ig-like domain of Fc γ RIIa (Figure 1.3 in Chapter 1). This substitution of a weak base (H) for a strong base (R) does not impact on expression levels (Anderson et al., 1987, Brandt et al., 1995) but alters the ligand affinity of Fc γ RIIa. While the 131H allotype binds both human IgG1 and IgG2, the 131R

allotype binds IgG1 but has a poor affinity for IgG2 (Anderson et al., 1987, Warmerdam et al., 1991, Bruhns et al., 2009, Anania et al., 2018).

5.1.2 *FCGR2A* Q127K polymorphism

Norris and colleagues (1998) described an *FCGR2A* polymorphic site in exon 4 encoding amino acid residue 127 in a healthy female who was homozygous for the R allele at position 131. This individual carried a SNP (C>A, rs139202723) which resulted in a non-synonymous glutamine (Q) to lysine (K) substitution at position 127 in the ligand-binding domain of FcγRIIa, proximal to H131R. Q127K is a rare variant with a minor allele frequency of 0.0002-0.0005 (The Genomes Project, 2017). Interestingly, the presence of the positively-charged lysine residue three amino acids apart restored the binding affinity of R131 for IgG2 whereby the allele combination of K127 and R131 on FcγRIIa enabled binding of both IgG1 and IgG2 (Norris et al., 1998). This observation highlights the functional significance of a combinatorial effect across distinct polymorphic sites.

5.1.3 *FCGR2A* Q27W polymorphism

A dinucleotide substitution (CA>TG, rs201218628) on exon 3 of *FCGR2A* replaces a glutamine (Q) with tryptophan (W) at amino acid 27 which is located at the dimerising region in the first Ig-like domain situated at the interface between two FcγRIIa monomers (Figure 1.3 in Chapter 1). Nagelkerke and colleagues (2019) reported ethnicity differences in the allele frequency of 27W in extensive cohorts of 919 Europeans, 428 Chinese and 508 Africans at 0.12, 0.00 and 0.11, respectively, with a virtually homogenous expression of the 27Q allele among the Han-Chinese. Q27W is reported to have no effect on receptor function or IgG binding when studied in isolation using cell line models (Warmerdam et al., 1990). In human peripheral blood mononuclear cells, there was no apparent difference in FcγRIIa surface expression between

individuals carrying the polar 27Q or the non-polar 27W (Flinsenberg et al., 2014). However, Q27W was associated with common variable immunodeficiency, and abrogated calcium flux and loss of phosphorylation was detected in cultured cells overexpressing the 27W variant of FcγRIIIa (Flinsenberg et al., 2014).

5.1.4 *FCGR2A* polymorphism and incidence of HIT

The differential binding affinity between the 131H and 131R allotypes is important in immune cell regulation. Several studies have reported an association between the H131R functional variant and multiple autoimmune conditions (see Section 1.2.4) indicating a role for FcγRIIIa in immunity regulation. In the setting of HIT, the H131R polymorphism has yielded inconsistent conclusions regarding HIT propensity. Four studies have noted an overrepresentation of the H131 variant among patients with HIT (Burgess et al., 1995, Brandt et al., 1995, Denomme et al., 1997, Bachelot-Loza et al., 1998). In contrast, Carlsson and colleagues (1998) reported an association between the R131 variant and HIT in a large cohort of 389 patients who developed HIT. A further two studies found no association between the H131R polymorphism and HIT susceptibility (Arepally et al., 1997, Rollin et al., 2015). Nevertheless, the 131R allele has been implicated with increased risk of thrombosis in patients with HIT (Rollin et al., 2015). The variation in cohort sizes, definition of HIT-positivity and patient populations may account for the inconsistencies observed. In addition, these studies only considered the H131R polymorphism in isolation of the Q27W variant. In fact, to date, no studies have assessed combinations of each of these polymorphisms and incidence of HIT or any other immune-related syndrome.

The combined effect of H131R and Q27W *FCGR2A* polymorphisms may explain the donor-dependent capacity for platelet degranulation in the SRA, the confirmatory test for HIT which

requires high responding donor platelets (Section 1.3.5.3), and the high false positive rates associated with HIT immunoassays whereby patients carrying anti-PF4/heparin antibodies do not develop HIT (Section 4.3.3). These *FCGR2A* polymorphisms may also be the basis for the variation in loss of GPVI between donor platelets exposed to HIT immune complex (Sections 3.3.2 and 3.3.6.1).

5.1.5 Overview of investigations

Figure 5.1 highlights the FcγRIIa polymorphic sites to be considered in the context of HIT pathogenesis in this chapter. The work presented here explored the genetic influence of Q27W and H131R *FCGR2A* polymorphisms on circulating sGPVI levels, thrombotic propensity, levels of anti-PF4/heparin antibodies and HIT susceptibility in cohorts of healthy individuals, non-HIT thrombocytopenic patients and patients suspected of HIT. The relative and combined contributions of these polymorphic sites at the dimerising region (Q27W) and ligand-binding domain (H131R) of FcγRIIa have been investigated.

It is hypothesised that HIT susceptibility may be independent of *FCGR2A* Q27W and H131R polymorphisms in isolation but is associated with *FCGR2A* across both polymorphic sites when assessed in combination. Combination of these polymorphisms may influence platelet reactivity and potentially modulate the risk of HIT.

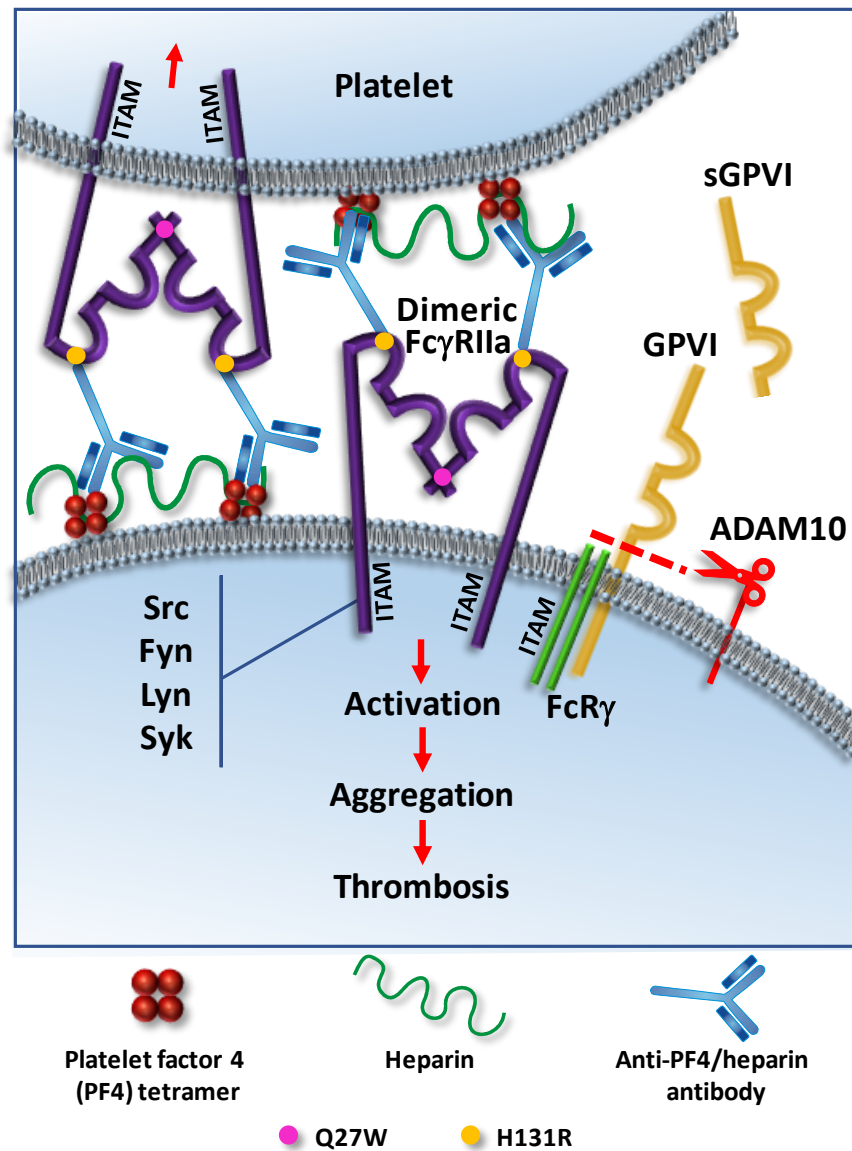


Figure 5.1 Evaluation of the influence of haplotype based Q27W and H131R *FCGR2A* polymorphisms on platelet reactivity and HIT susceptibility.

This chapter will focus on assessing the effect of the combination of *FCGR2A* polymorphisms (Q27W in pink, H131R in yellow) on platelet reactivity and risk of HIT in cohorts of healthy individuals, non-HIT thrombocytopenic patients and patients suspected of HIT.

5.2 Aims

To explore the influence of *FCGR2A* in-phase haplotype combination at Q27W and H131R on platelet reactivity (measured by plasma sGPVI levels and thrombotic propensity), levels of anti-PF4/heparin antibodies and HIT susceptibility in cohorts of healthy individuals, non-HIT thrombocytopenic patients and patients suspected of HIT.

5.3 Results

5.3.1 *FCGR2A* H131R genotype distributions in healthy and thrombocytopenic cohorts

In order to compare the genotype distribution of *FCGR2A* polymorphic site at amino acid 131 in healthy and thrombocytopenic cohorts, genomic DNA was isolated by phenol-chloroform extraction from whole blood collected from 45 healthy donors, 31 non-HIT thrombocytopenic (TCP) patients and 105 patients suspected of HIT (Section 2.2.10.1). A PCR-RFLP method was performed to assess the genotype at amino acid 131 of FcγRIIIa. The target region of the gene was amplified by PCR and the product was subjected to *Bst*UI restriction enzyme digestion as described (Section 2.2.10.3). The enzyme-digested products were electrophoresed on a 4% agarose gel (Section 2.2.10.2) to visualise the restriction digestion patterns for genotype determination. The genotype for every individual was validated by PacBio sequencing (Section 2.2.10.5). In the case of one individual with discrepant results yielded via PCR-RFLP and PacBio sequencing, Sanger sequencing was performed to confirm the genotype (Section 2.2.10.8).

Figure 5.2 depicts the three possible restriction digestion patterns for *FCGR2A* H131R genotyping. A single band of 343 bp indicates homozygosity for the H allele (HD0012 and HD0017) while a shorter product of 322 bp indicates homozygosity for the R allele (HD0013). Heterozygous individuals produced two bands comprising of 343 bp and 322 bp products as

shown by HD0011, HD0016 and HD0018 (Figure 5.2). The corresponding undigested PCR products (366 bp) were included for each individual for size comparison. Figure 5.3 shows the Sanger sequencing chromatogram for the individual (HD0029) with inconsistent genotype obtained by PCR-RFLP (heterozygous 131HR) versus PacBio sequencing (homozygous 131HH). The overlapping peaks for nucleotides T and C highlighted on the chromatogram indicated that HD0029 is heterozygous 131HR, consistent with the PCR-RFLP result.

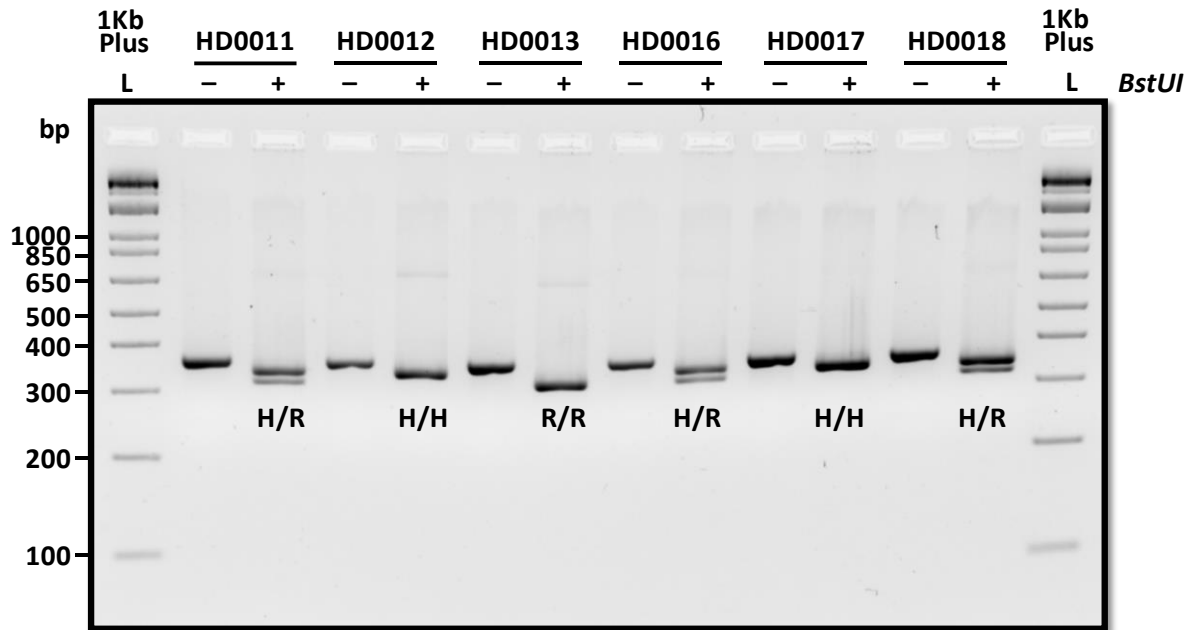


Figure 5.2 Restriction digestion patterns for *FCGR2A* H131R genotyping by PCR-RFLP.

Genomic DNA was isolated by phenol-chloroform extraction as described (Section 2.2.10.1) followed by PCR amplification of a region within exon 4 of *FCGR2A* harbouring the H131R polymorphic site (Section 2.2.10.3). The PCR products were subjected to *BstUI* restriction enzyme digestion and the products were electrophoresed on a 4% agarose gel (Section 2.2.10.2) to visualise the restriction digestion patterns for genotype determination. The three possible restriction digestion patterns for *FCGR2A* H131R genotyping are shown. A single band of 343 bp indicates homozygosity for the H allele (HD0012 and HD0017) while a shorter product of 322 bp indicates homozygosity for the R allele (HD0013). Heterozygous individuals produced two bands comprising of 343 bp and 322 bp products as shown (HD0011, HD0016 and HD0018). The corresponding undigested PCR products (366 bp) were included for each individual for size comparison. L, DNA size marker; -, undigested PCR product; +, *BstUI*-digested product.

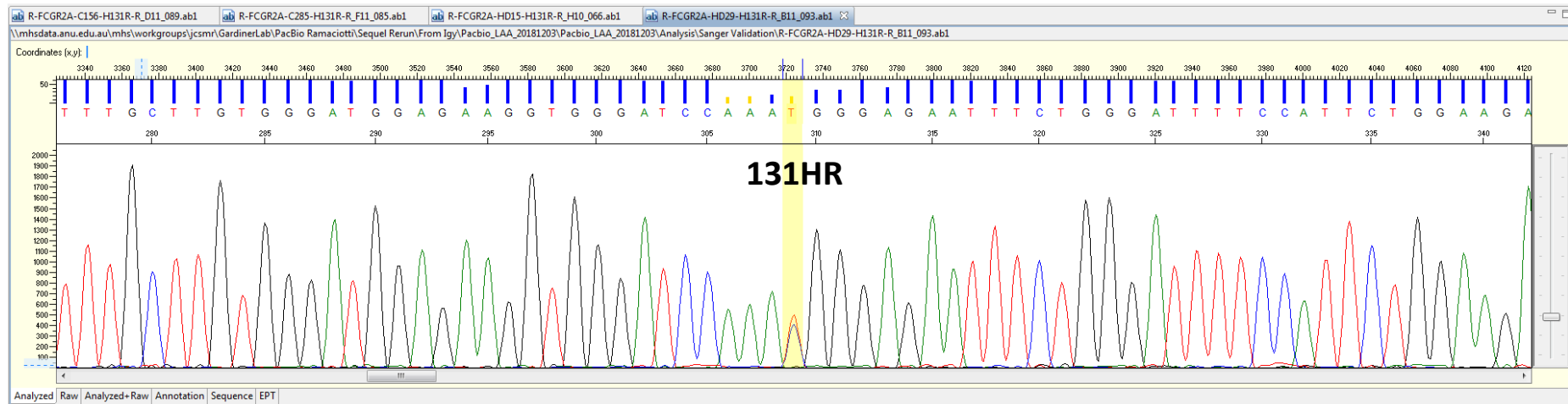


Figure 5.3 Sanger sequencing chromatogram to verify the *FCGR2A* H131R genotype for HD0029.

PCR-RFLP and PacBio sequencing generated inconsistent outcomes for HD0029 for the *FCGR2A* H131R polymorphism. Sanger sequencing was performed on the PCR product from this individual as described (Section 2.2.10.8). The overlapping peaks for nucleotides T (red) and C (blue) highlighted on the chromatogram indicated that HD0029 is a 131HR heterozygote, consistent with the PCR-RFLP result.

Figure 5.4A compares the *FCGR2A* H131R genotype frequencies between 45 healthy donors, 31 non-HIT thrombocytopenic patients and 105 patients at risk of HIT. In all three cohorts, the most common genotype was heterozygous 131HR at 44%, 45% and 57% for healthy donors, non-HIT thrombocytopenic patients and query HIT patients, respectively. Homozygous 131HH was the least common in the query HIT cohort (19%), while homozygous 131RR had the lowest frequency among the healthy donors (18%) and non-HIT thrombocytopenic patients (26%). Chi-square analysis revealed an absence of a significant difference in genotype distribution between the cohorts although the difference between healthy donors and query HIT patients almost reached statistical significance at $p=0.051$ (Figure 5.4A).

In Figure 5.4B, query HIT patients were stratified into non-HIT or HIT-positive (HIT/HITT) defined by SRA positivity. HIT-positive patients were further stratified into presence (HITT) or absence (HIT) of thrombosis based on 4Ts score. An increase in the proportion of 131HR but a decrease in 131HH homozygosity were observed among patients who developed HIT compared to non-HIT patients (Figure 5.4B). However, there were no differences in the genotype distributions. Of note, all seven patients who developed HITT carried at least one R allele at amino acid 131 of FcγRIIa with an under-representation of 131HH among this group, although there was no significant difference in the genotype frequency between patients with HIT and those with HITT ($p=0.331$, Figure 5.4B).

The genotype and allele frequencies for the three cohorts are summarised in Table 5.1. The R allele was almost as common as the H allele with an overall allelic frequency of 0.49. The frequencies of the 131R allele among the healthy donors, non-HIT thrombocytopenic patients and query HIT patients were comparable at 0.40, 0.48 and 0.52, respectively (Fisher's exact test $p>0.05$). As shown in Table 5.1 and Figure 5.4B, the highest frequency of the R allele at

0.57 was observed among patients with HITT although it was not significantly higher than the minor allele frequency among HIT patients at 0.50 (Fisher's exact test $p=0.7345$).

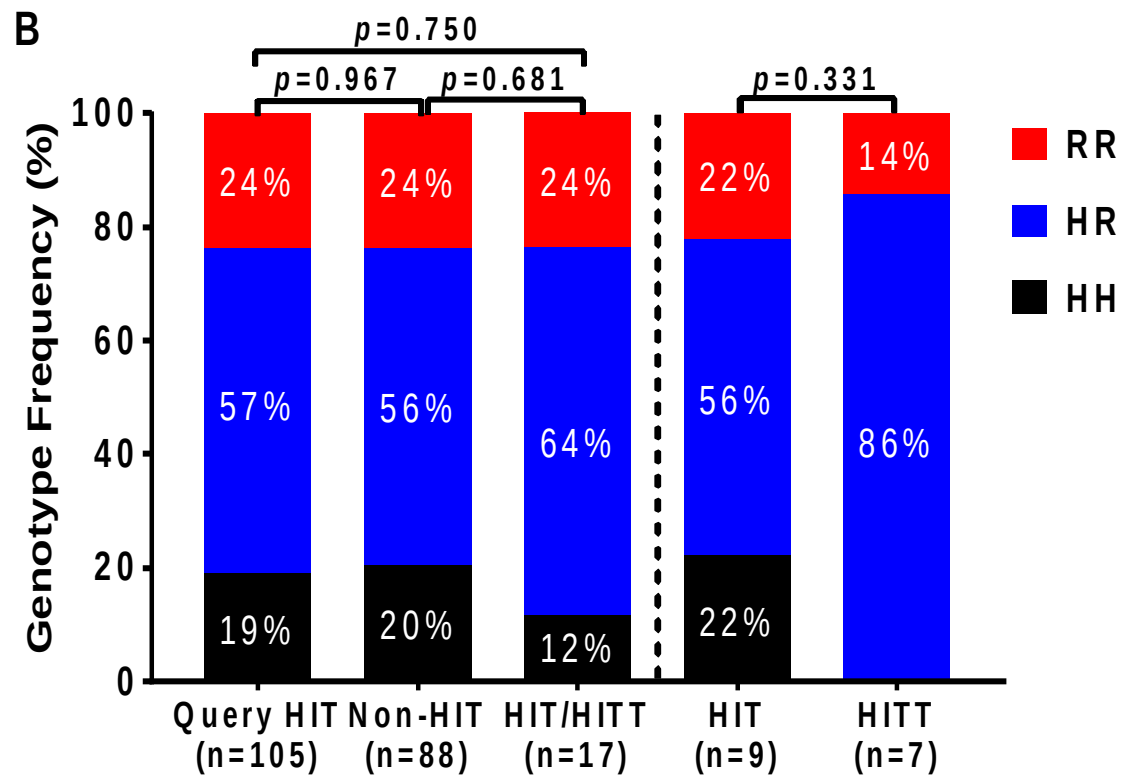
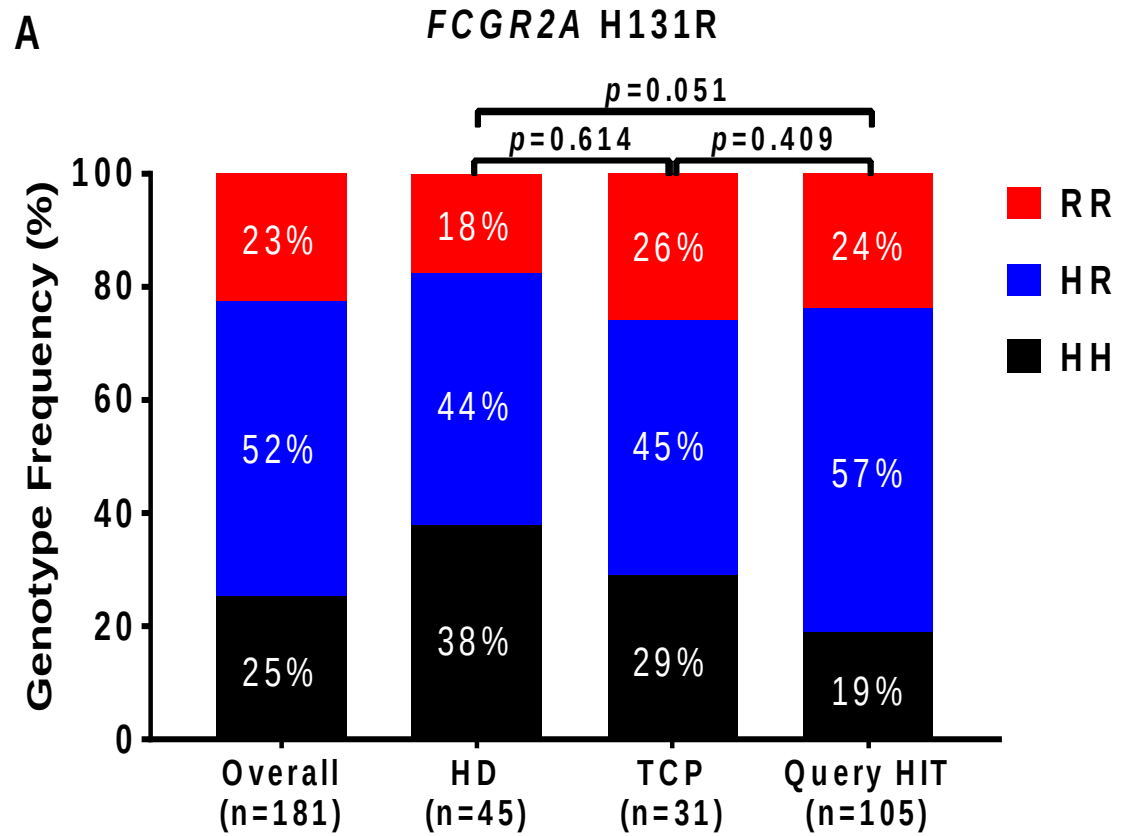


Figure 5.4 *FCGR2A* H131R genotype distributions in healthy individuals and thrombocytopenic patients.

Genomic DNA was isolated from 181 individuals by phenol-chloroform extraction as described (Section 2.2.10.1) followed by PCR amplification of a region within exon 4 of *FCGR2A* harbouring the H131R polymorphic site (Section 2.2.10.3). Genotyping was performed by PCR restriction fragment length polymorphism analysis using the *Bst*UI restriction enzyme. Panel A compares the H131R genotype distribution between 45 healthy donors, 31 non-HIT thrombocytopenic patients (TCP) and 105 patients at risk of HIT. In panel B, query HIT patients were stratified into non-HIT or HIT-positive (HIT/HITT) defined by SRA positivity. HIT-positive patients were further stratified into presence (HITT) or absence (HIT) of thrombotic complications. Chi-square analysis was performed to determine the association between genotype frequency and cohort type.

Table 5.1 Genotype and allele frequencies of FCGR2A H131R polymorphism.

Analysis of the three groups of individuals, healthy donors (HD), non-HIT thrombocytopenic patients (TCP) and query HIT patients. Query HIT patients were stratified into non-HIT or HIT-positive (HIT/HITT) defined by SRA positivity. HIT-positive patients were further stratified into presence (HITT) or absence (HIT) of thrombosis. One HIT-positive patient was excluded from the final analysis due to missing data on thrombosis occurrence.

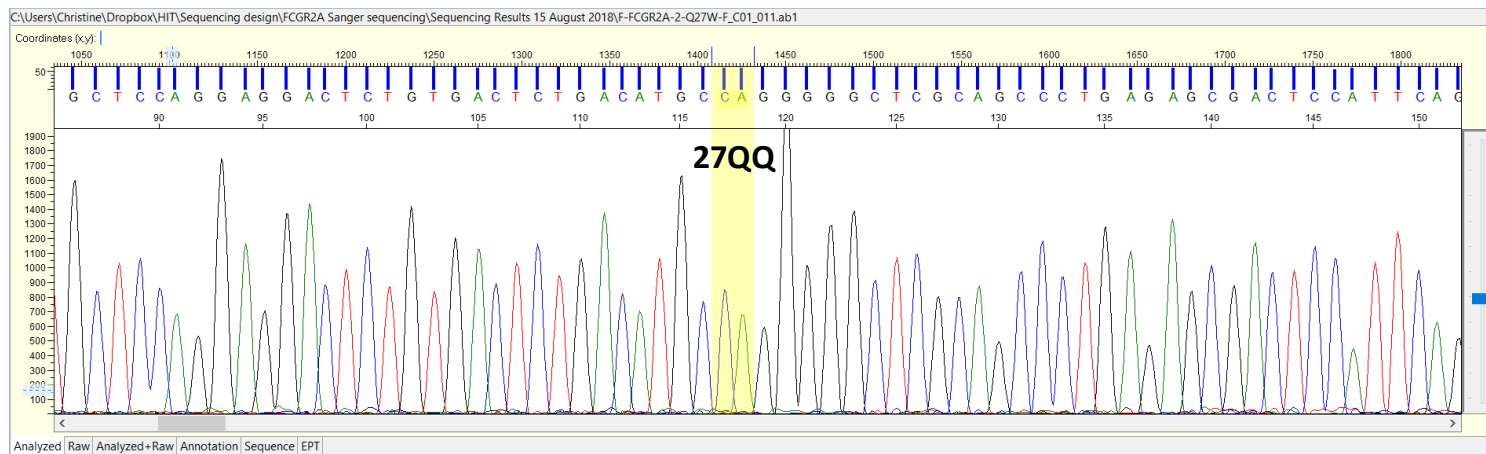
	Overall (%)	HD (%)	TCP (%)	Query HIT (%)	Non-HIT (%)	HIT/HITT (%)	HIT (%)	HITT (%)
n	181	45	31	105	88	17	9	7
Genotype frequency								
HH	46 (25.4)	17 (37.8)	9 (29.0)	20 (19.0)	18 (20.4)	2 (11.8)	2 (22.2)	0 (0.0)
HR	94 (51.9)	20 (44.4)	14 (45.2)	60 (57.1)	49 (55.7)	11 (64.7)	5 (55.6)	6 (85.7)
RR	41 (22.7)	8 (17.8)	8 (25.8)	25 (23.8)	21 (23.9)	4 (23.5)	2 (22.2)	1 (14.3)
Minor allele frequency (131R)	0.49	0.40	0.48	0.52	0.52	0.56	0.50	0.57

5.3.2 *FCGR2A* Q27W genotype distributions in healthy and thrombocytopenic cohorts

In order to compare the genotype distribution of *FCGR2A* polymorphic site at amino acid 27 within the dimerising region of FcγRIIa in healthy and thrombocytopenic individuals, genomic DNA was isolated by phenol-chloroform extraction from whole blood collected from 45 healthy donors, 31 non-HIT thrombocytopenic (TCP) patients and 105 patients suspected of HIT (Section 2.2.10.1). The target region of the gene was amplified by PCR and prepared for sequencing on the PacBio Sequel platform as described (Section 2.2.10.5). The genotype at position 27 of FcγRIIa was validated by Sanger sequencing (Section 2.2.10.8) in six individuals. The representative chromatogram traces for the *FCGR2A* Q27W polymorphism are shown in Figure 5.5. The CA dinucleotide highlighted in Figure 5.5A indicates the homozygous wildtype 27QQ. The dinucleotide substitution of CA for TG in Figure 5.5B results in the homozygous variant allele 27WW.

Unexpectedly, a single 27QR genotype was observed in one individual with query HIT, identified as C285. This SNP was confirmed by Sanger sequencing as illustrated in the chromatogram trace in Figure 5.6 which shows a CA>CG single nucleotide substitution. This individual is heterozygous for the Q and R alleles as indicated by the overlapping peaks for nucleotides A (green) and G (black). This individual was excluded in the subsequent analyses of genotype and haplotype distributions.

HD0002



C156

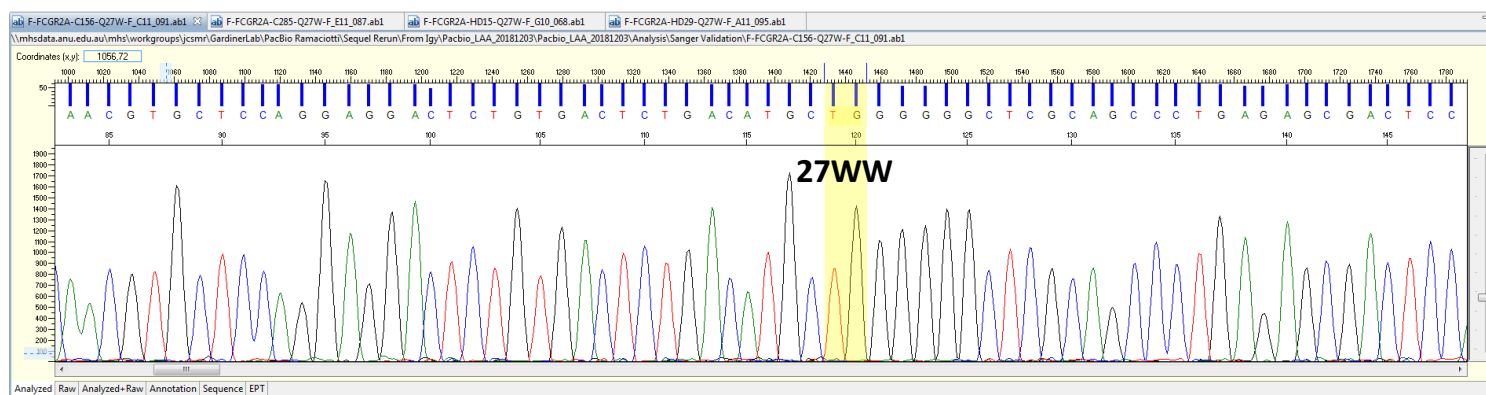


Figure 5.5 Sanger sequencing chromatogram traces depicting the *FCGR2A* Q27W polymorphism.

Sanger sequencing was performed on the PCR product amplified from the gDNA of six individuals to validate the genotype determined by PacBio sequencing for *FCGR2A* Q27W polymorphism as described in Section 2.2.10.8. (A) The CA dinucleotide (highlighted) indicates the homozygous wildtype 27QQ. (B) The dinucleotide substitution of CA for TG (highlighted) results in the variant allele, 27WW.

C285

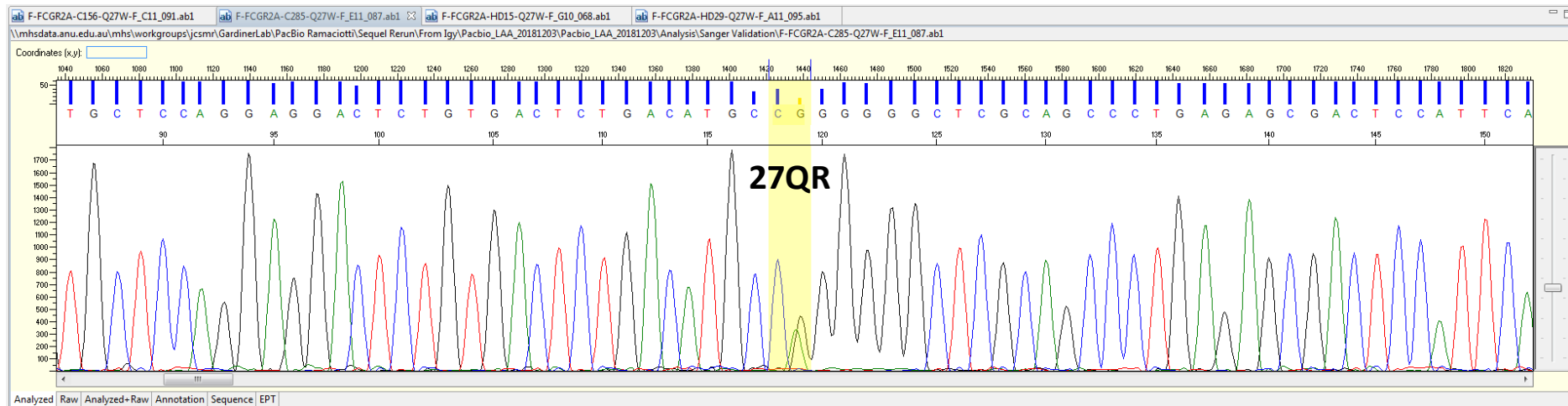


Figure 5.6 Sanger sequencing chromatogram of the rare *FCGR2A* Q27R polymorphism observed in one individual.

The 27QR genotype was observed in a query HIT individual, C285, and was confirmed by Sanger sequencing performed as described (Section 2.2.10.8). The chromatogram shows a CA>CG single nucleotide substitution. This individual is heterozygous for the Q and R alleles as indicated by the overlapping peaks for nucleotides A (green) and G (black).

Figure 5.7A compares the *FCGR2A* Q27W genotype frequencies between 45 healthy donors, 31 non-HIT thrombocytopenic patients and 104 patients at risk of HIT. The alternative allele (27W) resulting from a dinucleotide substitution was considerably uncommon. Of the 180 individuals, only three individuals were homozygous for 27WW. The genotype distributions were comparable between the three cohorts (Chi-square analysis $p>0.05$) with homozygous 27QQ being the most common genotype at 76%, 71% and 80% in healthy donors, non-HIT thrombocytopenic patients and query HIT patients, respectively (Figure 5.7A). The homozygous variant allele 27WW was the least common at 2%, 3% and 1%, in healthy donors, non-HIT thrombocytopenic patients and query HIT patients, respectively (Figure 5.7A).

In Figure 5.7B, patients suspected of HIT were stratified into non-HIT or HIT-positive (HIT/HITT) defined by SRA positivity. HIT-positive patients were further stratified into presence (HITT) or absence (HIT) of thrombotic complications. Chi-square analysis revealed that there was no statistically significant association between the genotype distribution of Q27W and HIT susceptibility or occurrence of HITT. It is worth noting that there was a lack of homozygous 27WW among patients who developed HIT most likely due to the low allelic frequency compounded by the small sample size of HIT-positive patients.

The genotype and allele frequencies for the three cohorts are summarised in Table 5.2. The variant allele W was remarkably rare with an overall allelic frequency of 0.12. The frequencies of this allele among the healthy donors, non-HIT thrombocytopenic patients and query HIT patients were comparable at 0.13, 0.16 and 0.11, respectively (Fisher's exact test $p>0.05$). As observed in Table 5.2 and Figure 5.7B, there was no statistically significant difference in the 27W allelic frequency between patients with HIT/HITT (allelic frequency of 0.06) compared to non-HIT patients (allelic frequency of 0.11) (Fisher's exact test $p=0.5414$).

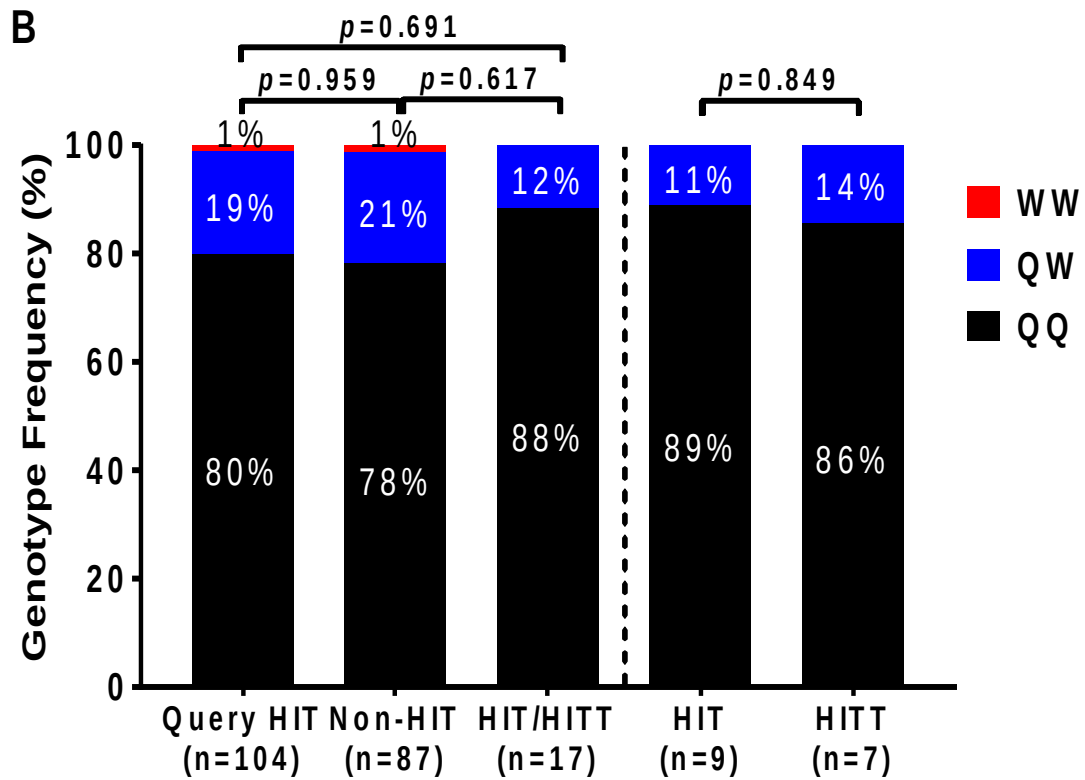
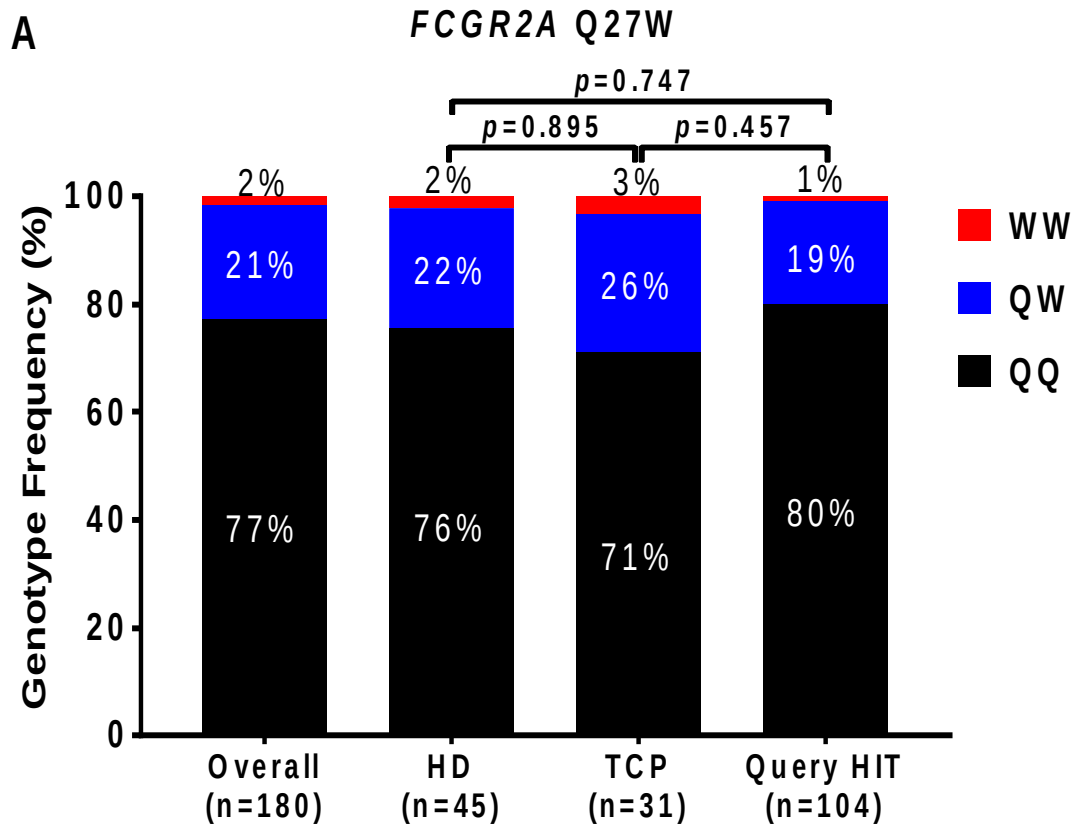


Figure 5.7 *FCGR2A* Q27W genotype distributions in healthy individuals and thrombocytopenic patients.

Genomic DNA was isolated by phenol-chloroform extraction from whole blood collected from 181 individuals (Section 2.2.10.1). The target region of the gene was amplified by PCR and prepared for sequencing on the PacBio Sequel platform as described (Section 2.2.10.5). One query HIT individual with the 27QR genotype was excluded from the analysis. Panel A compares the Q27W genotype distribution between 45 healthy donors, 31 non-HIT thrombocytopenic patients (TCP) and 104 patients at risk of HIT. In panel B, query HIT patients were stratified into non-HIT or HIT-positive (HIT/HITT) defined by SRA positivity. HIT-positive patients were further stratified into presence (HITT) or absence (HIT) of thrombosis. Chi-square analyses were performed to determine the association between genotype frequency and cohort type.

Table 5.2 Genotype and allele frequencies of *FCGR2A* Q27W polymorphism.

Analysis of the three groups of individuals, healthy donors (HD), non-HIT thrombocytopenic patients (TCP) and query HIT patients. Query HIT patients were stratified into non-HIT or HIT-positive (HIT/HITT) defined by SRA positivity. HIT-positive patients were further stratified into presence (HITT) or absence (HIT) of thrombosis. One HIT-positive patient was excluded from the final stratification due to missing data on thrombosis occurrence.

	Overall (%)	HD (%)	TCP (%)	Query HIT (%)	Non-HIT (%)	HIT/HITT (%)	HIT (%)	HITT (%)
n	180	45	31	104	87	17	9	7
Genotype frequency								
QQ	139 (77.2)	34 (75.6)	22 (71.0)	83 (79.8)	68 (78.2)	15 (88.2)	8 (88.9)	6 (85.7)
QW	38 (21.1)	10 (22.2)	8 (25.8)	20 (19.2)	18 (20.7)	2 (11.8)	1 (11.1)	1 (14.3)
WW	3 (1.7)	1 (2.2)	1 (3.2)	1 (1.0)	1 (1.1)	0 (0.0)	0 (0.0)	0 (0.0)
Minor allele frequency (27W)	0.12	0.13	0.16	0.11	0.11	0.06	0.06	0.07

5.3.3 sGPVI levels in thrombocytopenic patients

Irreversible cleavage of GPVI ectodomain on platelets exposed to GPVI ligands, pathophysiological shear stress, anti-platelet antibodies or active coagulation factors results in release of sGPVI (Section 1.4.1). Plasma sGPVI has emerged as a platelet-specific activation marker in various clinical settings. Here, levels of sGPVI in double-spun plasma samples (Section 2.2.3) were measured using an established sandwich ELISA in the three cohorts as described (Section 2.2.9.1).

Figure 5.8 compares plasma sGPVI levels between 45 healthy individuals, 29 non-HIT thrombocytopenic patients, 88 HIT- patients defined by a negative SRA and 18 HIT+ patients with positive SRA outcomes. One-way ANOVA with Tukey's multiple comparison was performed to determine significant differences in plasma sGPVI levels between the groups. Comparable sGPVI levels ($p=0.4433$) were observed between healthy individuals at (mean $\pm$ SD) 18.93 ± 16.52 ng/mL and non-HIT thrombocytopenic patients at 12.07 ± 12.66 ng/mL. Among the patients who were suspected of HIT (HIT- and HIT+), plasma sGPVI levels were comparable in SRA-positive patients (HIT+) compared to those who were negative (HIT-) at 47.96 ± 22.32 ng/mL versus 40.85 ± 21.52 ng/mL, respectively ($p=0.4849$). Nevertheless, sGPVI levels in both the HIT- and HIT+ groups were significantly increased when compared to healthy individuals ($p<0.0001$) or non-HIT thrombocytopenic patients ($p<0.0001$) (Figure 5.8).

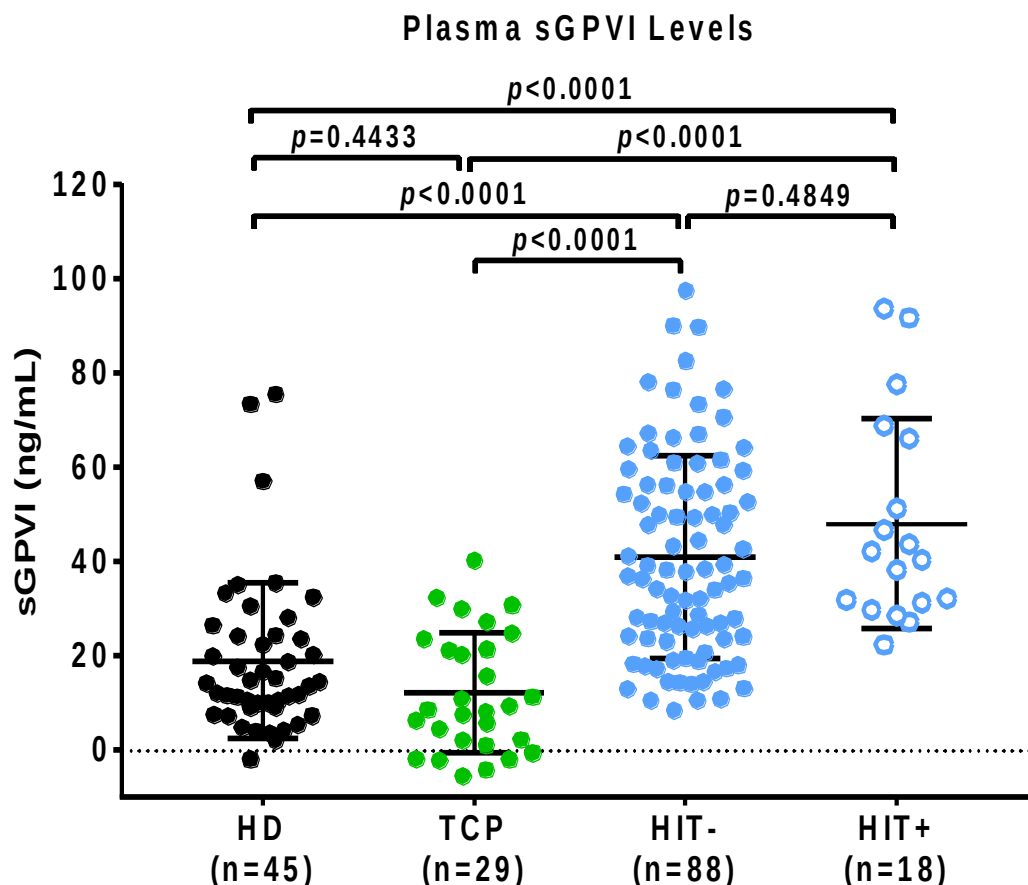


Figure 5.8 Plasma sGPVI levels in healthy and thrombocytopenic individuals.

Levels of circulating sGPVI in double-spun plasma samples (Section 2.2.3) was measured by an established sandwich ELISA as described (Section 2.2.9.1) in 45 healthy individuals, 29 non-HIT thrombocytopenic patients (TCP), 88 HIT- patients defined by a negative SRA and 18 HIT+ patients with positive SRA outcomes. One-way ANOVA with Tukey's multiple comparisons was performed to determine significant differences in plasma sGPVI levels between the groups. Error bars indicate mean $\pm$ SD.

5.3.4 Association between FCGR2A H131R polymorphism and sGPVI levels

The H131R polymorphism located at the ligand binding region of FcγRIIa has been shown to influence receptor affinity for IgG subclasses (see Section 1.2.4). Data presented in Chapter 3 demonstrated the ability of FcγRIIa-mediated anti-platelet antibodies to release sGPVI to extents that were donor-dependent. Using plasma sGPVI as a measure of platelet reactivity, the influence of FCGR2A H131R polymorphism on platelet activity was assessed.

Figure 5.9 shows the relationship between an individual's genotype at amino acid 131 of FcγRIIa and circulating plasma sGPVI levels. When the three cohorts were analysed separately, no significant differences in plasma sGPVI levels were observed between homozygous 131HH, heterozygous 131HR and homozygous 131RR individuals in the healthy donor cohort (Figure 5.9A), non-HIT thrombocytopenic patients (Figure 5.9B) and query HIT patients (Figure 5.9C). Interestingly, higher plasma sGPVI levels ($p=0.0081$) were observed among 131RR homozygotes at (mean $\pm$ SD) 36.79 ± 21.09 ng/mL compared to individuals with 131HH at 24.44 ± 21.17 ng/mL, when the three cohorts were analysed together in Figure 5.9D. The average plasma sGPVI levels was influenced by the number of R alleles. For instance, heterozygote 131HR individuals carrying an R allele showed higher sGPVI levels compared to 131HH (32.60 ± 24.29 ng/mL versus 24.44 ± 21.17 ng/mL) but lower sGPVI levels than individuals with two R alleles (36.79 ± 21.09 ng/mL in 131RR), although the observed differences did not achieve statistical significance ($p=0.1365$ and $p=0.3778$, respectively).

Similar results were observed when individuals were stratified by the presence or absence of an R allele (131HH vs 131HR/RR) as depicted in Figure 5.10. The R allele was not associated with plasma sGPVI levels when the cohorts were analysed separately as shown in healthy donors ($p=0.7370$, Figure 5.10A), non-HIT thrombocytopenic patients ($p=0.1500$, Figure 5.10B)

and query HIT cohort ($p=0.1129$, Figure 5.10C). However, the presence of an R allele was significantly associated with increased plasma sGPVI level (33.85 ± 23.38 ng/mL versus 24.44 ± 21.17 ng/mL in 131HH, $p=0.0088$) when the cohorts were considered together (Figure 5.10D).

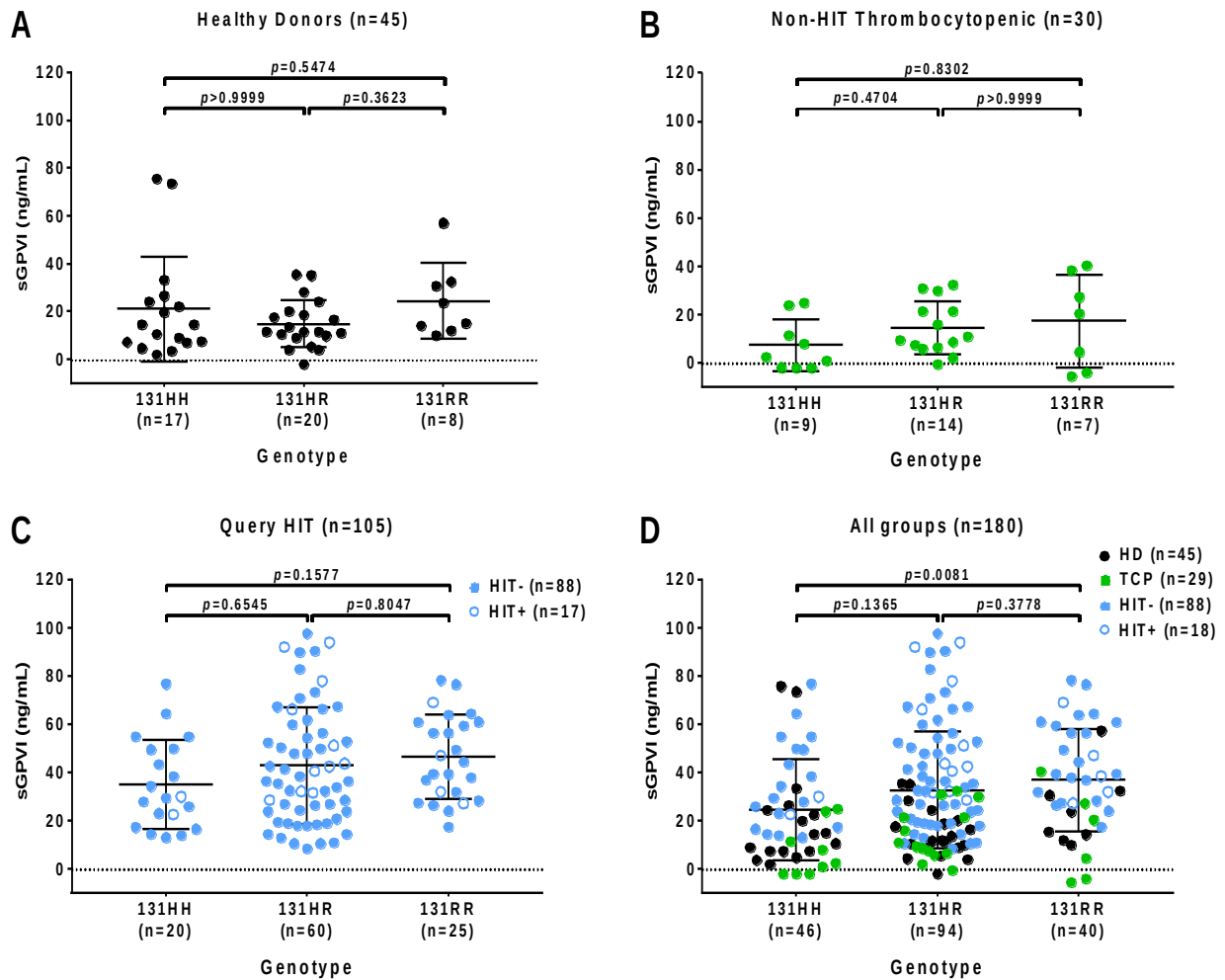


Figure 5.9 Association between *FCGR2A* H131R polymorphism and circulating sGPVI levels.

Levels of circulating sGPVI in double-spun plasma samples (Section 2.2.3) were measured by an established sandwich ELISA as described (Section 2.2.9.1) in (A) 45 healthy individuals, (B) 30 non-HIT thrombocytopenic patients (TCP), (C) 88 HIT- patients defined by a negative SRA and 17 HIT+ patients with positive SRA outcomes. Genomic DNA was isolated from these individuals by phenol-chloroform extraction as described (Section 2.2.10.1) followed by PCR amplification of a region within exon 4 of *FCGR2A* harbouring the H131R polymorphic site (Section 2.2.10.3). Genotyping was performed by PCR restriction fragment length polymorphism analysis using the *Bst*UI restriction enzyme. One-way ANOVA (Kruskal-Wallis) with Dunn's multiple comparisons tests were performed to determine the influence of H131R genotype on plasma sGPVI levels in (A) healthy donors, (B) non-HIT thrombocytopenic patients (TCP), (C) query HIT patients and (D) combination of the three cohorts. Error bars indicate mean $\pm$ SD.

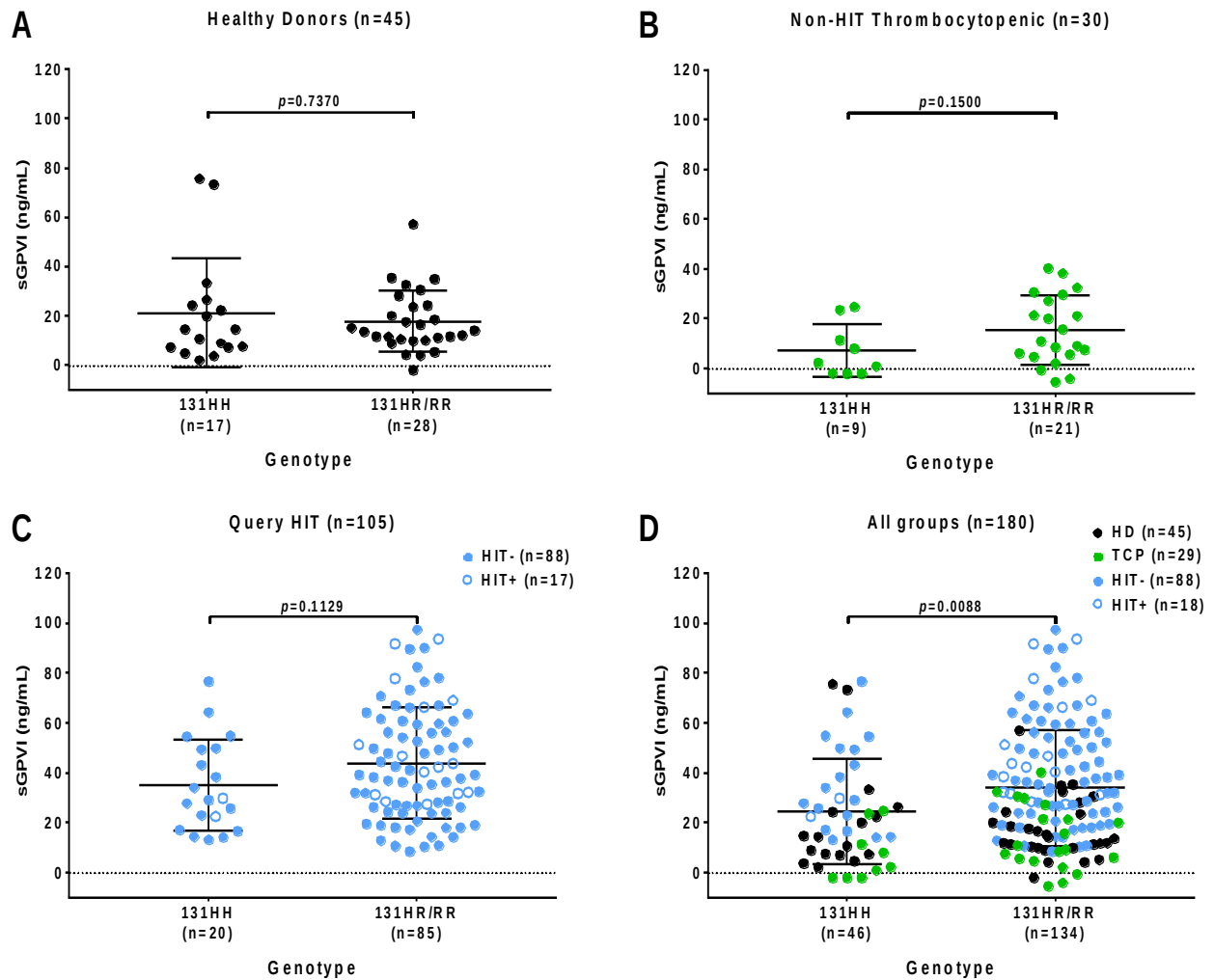


Figure 5.10 Association between *FCGR2A* 131R allele and circulating sGPVI levels.

Levels of circulating sGPVI in double-spun plasma samples (Section 2.2.3) were measured by an established sandwich ELISA as described (Section 2.2.9.1) in (A) 45 healthy individuals, (B) 30 non-HIT thrombocytopenic patients (TCP), (C) 88 HIT- patients defined by a negative SRA and 17 HIT+ patients with positive SRA outcomes. Genomic DNA was isolated from these individuals by phenol-chloroform extraction as described (Section 2.2.10.1) followed by PCR amplification of a region within exon 4 of *FCGR2A* harbouring the H131R polymorphic site (Section 2.2.10.3). Genotyping was performed by PCR restriction fragment length polymorphism analysis using the *Bst*UI restriction enzyme. Mann-Whitney tests were performed to determine the influence of the 131R allele on plasma sGPVI levels in (A) healthy donors, (B) non-HIT thrombocytopenic patients (TCP), (C) query HIT patients and (D) combination of the three cohorts. Error bars indicate mean $\pm$ SD.

5.3.5 Association between *FCGR2A* Q27W polymorphism and sGPVI levels

Data presented in Chapter 3 demonstrated the ability of FcγRIIIa-mediated anti-platelet antibodies to release sGPVI to various extents in a donor-dependent manner. In addition, the Q27W polymorphism located within the dimerising region of FcγRIIIa has been shown to impact on signal transduction (see Section 1.2.4) which may affect platelet activity. Using plasma sGPVI as a measure of platelet reactivity, the influence of *FCGR2A* Q27W polymorphism on platelet activity was assessed as presented in Figure 5.11. The association between an individual's genotype at amino acid 27 and circulating levels of sGPVI was assessed in 45 healthy donors (Figure 5.11A), 30 non-HIT thrombocytopenic patients (Figure 5.11B) and 104 query HIT patients (Figure 5.11C). *FCGR2A* Q27W polymorphism did not influence the levels of circulating plasma sGPVI in all three cohorts as individuals expressing the 27QQ genotype had comparable sGPVI levels as those carrying the variant allele (27QW or 27WW) ($p>0.05$). This lack of association was also observed in the three cohorts combined ($p=0.3581$, Figure 5.11D).

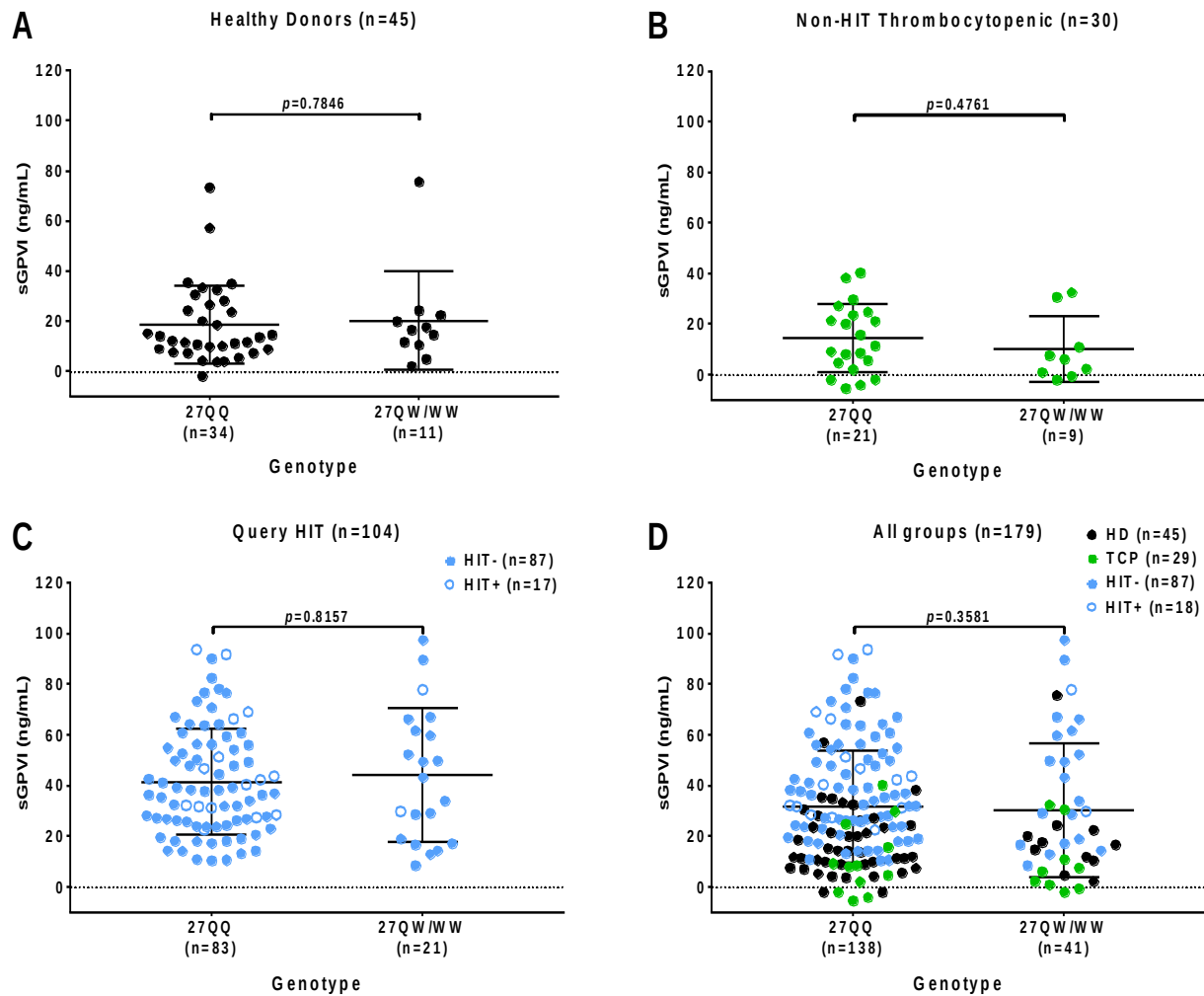


Figure 5.11 Association between *FCGR2A* Q27W polymorphism and circulating sGPVI levels.

Levels of circulating sGPVI in double-spun plasma samples (Section 2.2.3) were measured by an established sandwich ELISA as described (Section 2.2.9.1) in (A) 45 healthy individuals, (B) 30 non-HIT thrombocytopenic patients (TCP), (C) 87 HIT- patients defined by a negative SRA and 17 HIT+ patients with positive SRA outcomes. Genomic DNA was isolated by phenol-chloroform extraction from whole blood collected from these individuals (Section 2.2.10.1). The target region of the gene was amplified by PCR and prepared for sequencing on the PacBio Sequel platform as described (Section 2.2.10.5). One query HIT individual with the 27QR genotype was excluded from the analysis. Mann-Whitney tests were performed to determine the influence of the 27W allele on plasma sGPVI levels in (A) healthy donors, (B) non-HIT thrombocytopenic patients (TCP), (C) query HIT patients and (D) combination of the three cohorts. Error bars indicate mean $\pm$ SD.

5.3.6 *FCGR2A* polymorphisms and anti-PF4/heparin antibody levels

Figure 5.12 shows the relationship between *FCGR2A* polymorphisms and anti-PF4/heparin antibody levels, measured by a polyspecific HIT ELISA in optical density units (OD), among 105 patients suspected of HIT. There was no significant association between levels of anti-PF4/heparin antibodies and the three possible genotypes for each polymorphism, Q27W ($p>0.05$, Figure 5.12A) and H131R ($p>0.05$, Figure 5.12B). Figure 5.12C assessed the effect of the minor allele 27W compared to the major allele 27Q by comparing the antibody levels in 27QQ homozygotes with 27QW and 27WW. The presence of the variant allele did not influence the level of anti-PF4/heparin antibodies (mean $\pm$ SD: 0.7611 ± 0.9605 OD units versus 0.6471 ± 0.8573 OD units, $p=0.7768$). Of note, HIT+ patients, represented by open circles, who are homozygous for the 131H allele showed higher levels of the HIT antibody (>2.0 OD units) compared to HIT+ patients who carry at least one R allele at position 131 (Figure 5.12D). However, among the query HIT cohort, there was no difference in anti-PF4/heparin antibody levels between the two alleles (mean $\pm$ SD: 0.5690 ± 0.9146 OD units versus 0.7713 ± 0.9406 OD units, $p=0.2543$, Figure 5.12D).

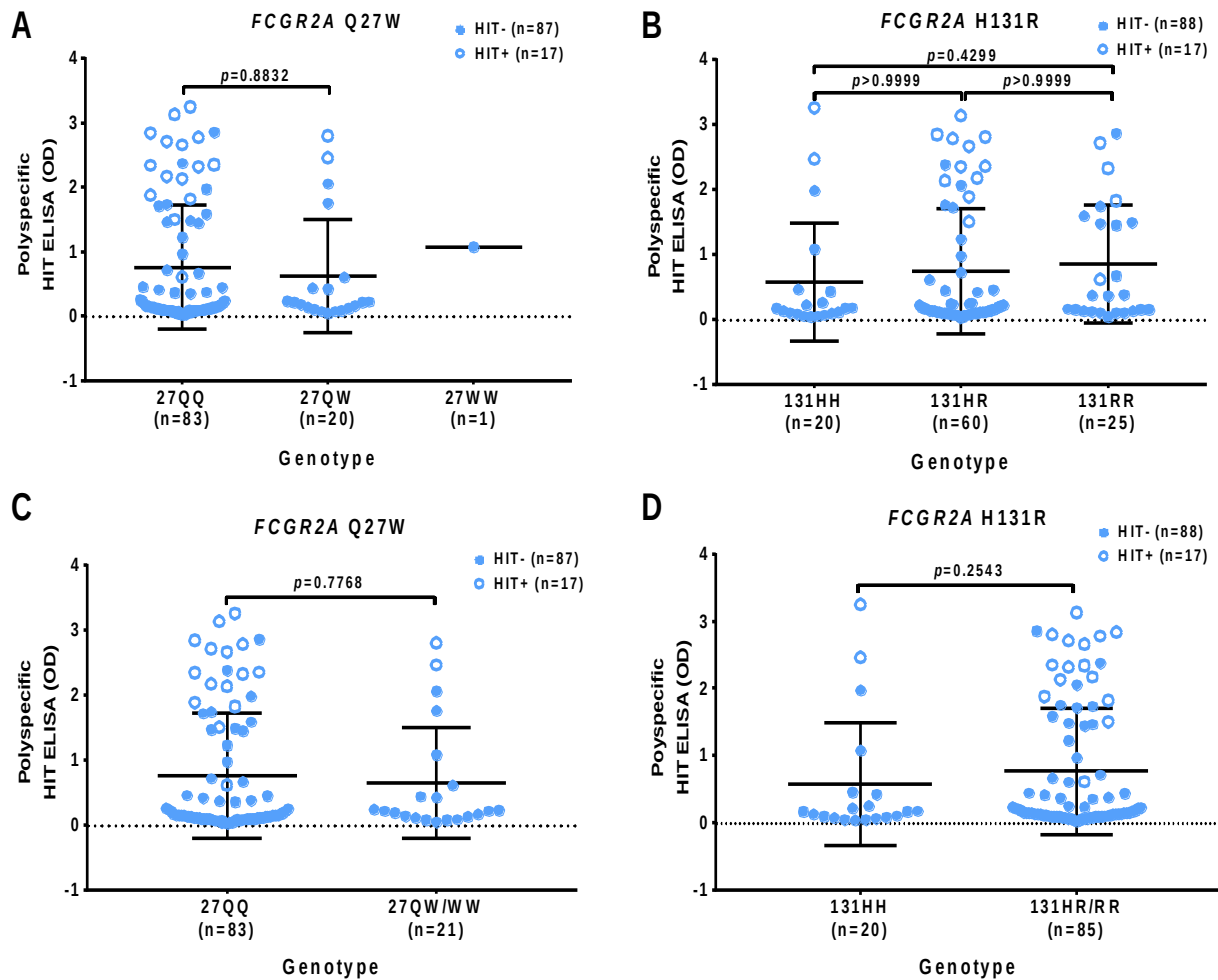


Figure 5.12 *FCGR2A* polymorphisms and anti-PF4/heparin antibody levels in patients suspected of HIT.

Genomic DNA was isolated from whole blood collected from 105 patients suspected of HIT (Section 2.2.10.1). The genomic region spanning exons 3 and 4 of *FCGR2A*, encompassing the Q27W and H131R polymorphic sites, was amplified as described in Section 2.2.10.4 and prepared for sequencing on the PacBio Sequel platform (Section 2.2.10.5). Anti-PF4/heparin antibody levels in plasma samples of these patients were measured by a polyspecific HIT ELISA in optical density units (OD). Panels A and B show the anti-PF4/heparin antibody levels based on genotypes at Q27W (A) and H131R (B). Panels C and D evaluate the influence of the variant alleles, 27W and 131R, respectively, on anti-PF4/heparin antibody levels. Between group differences were computed using Mann-Whitney tests in panels A, C and D while one-way ANOVA (Kruskal-Wallis) with Dunn's multiple comparisons was performed in panel B. Error bars indicate mean $\pm$ SD.

5.3.7 Long-read sequencing of exons 3 and 4 of *FCGR2A* on PacBio Sequel

To obtain in-phase haplotype information of the two FcγRIIIa polymorphic sites, Q27W and H131R, PCR products from the genomic DNA (Section 2.2.10.1) of 181 individuals were subjected to long-read sequencing on the PacBio Sequel platform. The genomic region spanning exons 3 and 4 of *FCGR2A*, encompassing the Q27W and H131R polymorphic sites which are 3540 bp apart, was amplified as described in Section 2.2.10.4. An example DNA agarose gel (Section 2.2.10.2) of the PCR products with an expected size of 4084 bp is shown in Figure 5.13A. The PCR products were subsequently tagged with unique barcodes for the 181 individuals (Section 2.2.10.5) and pooled into two batches consisting of 92 individuals in pool 1 at 87 ng of PCR product per individual and 93 individuals in pool 2 at 86 ng of PCR product per individual (Section 2.2.10.5.4). The agarose gel in Figure 5.13B represents the purified pooled amplicons containing the unique barcodes for long-read sequencing.

As detailed in Section 2.2.10.6, the two pools were sequenced on the PacBio Sequel using a Sequel Sequencing Kit v2.1 with two SMRT cells containing a pool of 92 individuals on one SMRT cell and 93 individuals on the other. Raw sequencing reads were processed using the SMRT Link LAA pipeline to generate consensus sequences and obtain phased haplotype information across the two polymorphic sites for every individual as described in Section 2.2.10.7. For each allele per individual, 250 reads with about 200x coverage was obtained. With 92 individuals pooled on one SMRT cell, a read depth of 18,400x was achieved for this region spanning exons 3 and 4 of *FCGR2A*.

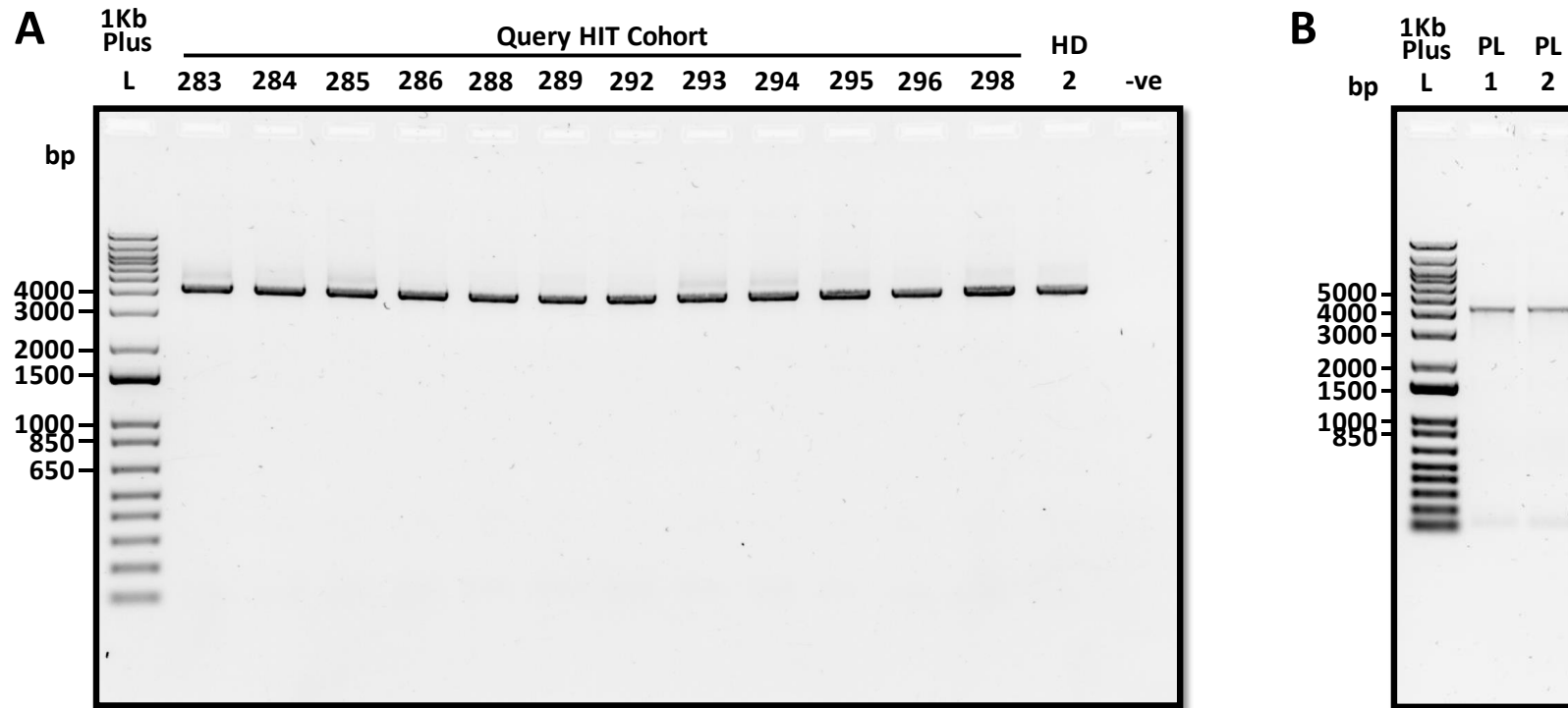


Figure 5.13 PCR amplification of *FCGR2A* encompassing exons 3 and 4.

The genomic region spanning exons 3 and 4 of *FCGR2A*, encompassing the Q27W and H131R polymorphic sites which are 3540 bp apart, was amplified as described in Section 2.2.10.4. Panel A is an example DNA agarose gel (1.2%, Section 2.2.10.2) of the PCR products (expected size 4084 bp) amplified from genomic DNA of query HIT patients and a healthy donor (HD2). -ve represents the no DNA template control sample. The PCR products were subsequently tagged with unique barcodes for the 181 individuals and pooled into two batches consisting of 92 individuals in pool 1 (PL1) at 87 ng of PCR product per individual and 93 individuals in pool 2 (PL2) at 86 ng of PCR product per individual (Section 2.2.10.5). Panel B shows a 0.7% agarose gel of purified pooled amplicons containing the unique barcodes (expected size 4116 bp), PL1 and PL2, for long-read sequencing. L, DNA size marker.

5.3.8 *FCGR2A* Q27W and H131R haplotype distributions

The ten possible haplotype combinations of the Q27W and H131R polymorphisms are listed in Figure 5.14 and Table 5.3 which compare the frequency of each haplotype combination between the three cohorts of 45 healthy donors, 31 non-HIT thrombocytopenic patients and 104 patients suspected of HIT. One query HIT patient, C285, who carries the 27QR genotype (QHRH haplotype) as described in Section 5.3.2 was excluded from the analysis.

The most common haplotype among the healthy individuals and query HIT patients is QHQR/QRQH which occurred at a frequency of 35.56% and 44.23%, respectively (Figure 5.14, Table 5.3). In the non-HIT thrombocytopenic cohort, QHQR/QRQH and QRQR were the two most common haplotype at 25.81%. One individual from each group expressed the WHWH haplotype which was the least common across all three cohorts. Chi-square analysis of the haplotype frequency showed no association between *FCGR2A* haplotype at the two polymorphic sites and cohort type, suggesting a comparable haplotype distribution between all three cohorts ($\chi^2=10.35$, d.f.=10, $p=0.4102$). Strikingly, the minor allele 27W and 131R did not co-occur on the same allele as evidenced by the absence of the QHWR/WHQR, QRWR/WRQR, WHWR/WRWH and WRWR haplotype combinations among the 181 individuals (Table 5.3).

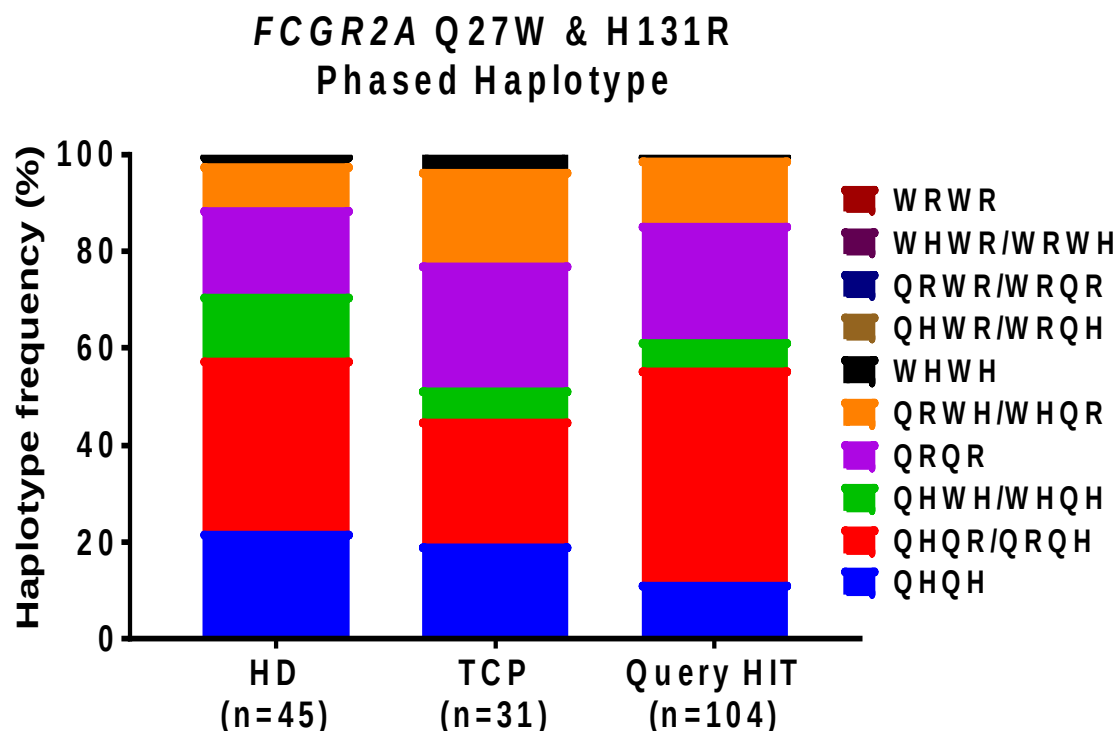


Figure 5.14 Frequency distribution of phased haplotype combinations across the Q27W and H131R polymorphisms in *FCGR2A*.

Genomic DNA was extracted from 45 healthy donors, 31 non-HIT thrombocytopenic patients (TCP) and 105 query HIT patients as described (Section 2.2.10.1). To obtain in-phase haplotype information of the two FcγRIIa polymorphic sites, Q27W and H131R, PCR products from the genomic DNA of 181 individuals (Section 2.2.10.4) were subjected to long-read sequencing on the PacBio Sequel platform using two SMRT cells containing a pool of 92 individuals on one SMRT cell and 93 individuals on the other (Section 2.2.10.6). Raw sequencing reads were processed using the SMRT Link Long Amplicon Analysis pipeline to generate consensus sequences and obtain phased haplotype information across the two polymorphic sites for every individual as described in Section 2.2.10.7. Chi-square analysis comparing the haplotype distribution across the three cohorts revealed no significant association ($\chi^2=10.35$, d.f.=10, $p=0.4102$). One query HIT patient with the 27QR genotype (QHRH haplotype) was excluded from the analysis. d.f., degrees of freedom.

Table 5.3 FCGR2A Q27W and H131R phased haplotype frequency.

Frequency distribution of the ten possible haplotype combinations across the Q27W and H131R polymorphisms in *FCGR2A* in 180 individuals comprising of healthy donors, non-HIT thrombocytopenic patients and query HIT patients. Chi-square analysis comparing the haplotype distribution across the three cohorts revealed no significant association ($\chi^2=10.35$, d.f.=10, $p=0.4102$). d.f., degrees of freedom. #A query HIT patient with the 27QR genotype (QHRH haplotype) was excluded.

Amino acid 27	Amino acid 131	Healthy donor (%) n=45	Non-HIT Thrombocytopenia (%) n=31	Query HIT (%) n=104 [#]
Q	H	10 (22.22)	6 (19.35)	12 (11.54)
Q	H			
Q	H	16 (35.56)	8 (25.81)	46 (44.23)
Q	R			
Q	H	6 (13.33)	2 (6.45)	6 (5.77)
W	H			
Q	R	8 (17.78)	8 (25.81)	25 (24.04)
Q	R			
Q	R	4 (8.89)	6 (19.35)	14 (13.46)
W	H			
W	H	1 (2.22)	1 (3.23)	1 (0.96)
W	H			
Q	H	-	-	-
W	R			
Q	R	-	-	-
W	R			
W	H	-	-	-
W	R			
W	R	-	-	-
W	R			

5.3.9 *FCGR2A* Q27W and H131R haplotype association with HIT susceptibility

The H131R polymorphism influences receptor affinity for IgG subclasses (Anderson et al., 1987, Warmerdam et al., 1991, Bruhns et al., 2009, Anania et al., 2018) (see Section 1.2.4) but the Q27W polymorphism did not confer preferential IgG subclass binding when studied in isolation (Warmerdam et al., 1990). However, Q27W influences onset of signal transduction induced by immune complex binding (Flinsenbergh et al., 2014) (Section 1.2.4), which may affect platelet activity. The haplotype combination at positions 27 and 131 may impact on receptor function. Simultaneous evaluation of the Q27W and H131R polymorphic sites was performed in-phase to assess the contribution of *FcγR1a* genotype to HIT susceptibility.

Figure 5.15 and Table 5.4 summarise the distribution of *FCGR2A* haplotype at Q27W and H131R among patients who were suspected of HIT. These query HIT patients were first grouped as non-HIT (n=87) or HIT-positive (HIT/HITT, n=17) defined by SRA positivity. The haplotype frequency followed the same trend as observed in Figure 5.14 whereby QHQR/QRQH was the most common haplotype combination in all patients suspected of HIT regardless of SRA outcome (41.38% in non-HIT, 58.82% in HIT-positive, Table 5.4). The only query HIT patient carrying the relatively uncommon WHWH combination was SRA-negative. The haplotype distribution was independent of HIT susceptibility ($\chi^2=2.607$, d.f.=5, $p=0.7603$). HIT-positive patients were further grouped according to presence (HITT, n=7) or absence (HIT, n=9) of thrombosis. There was a lack of HITT patients with the QHQH and QHWH haplotypes while the QRWH/WHQR combination was unrepresented among patients who did not experience a thrombotic event (Figure 5.15 and Table 5.4). The observed differences in haplotype proportions did not achieve statistical significance due to the limited sample sizes ($\chi^2=3.132$, d.f.=4, $p=0.5359$).

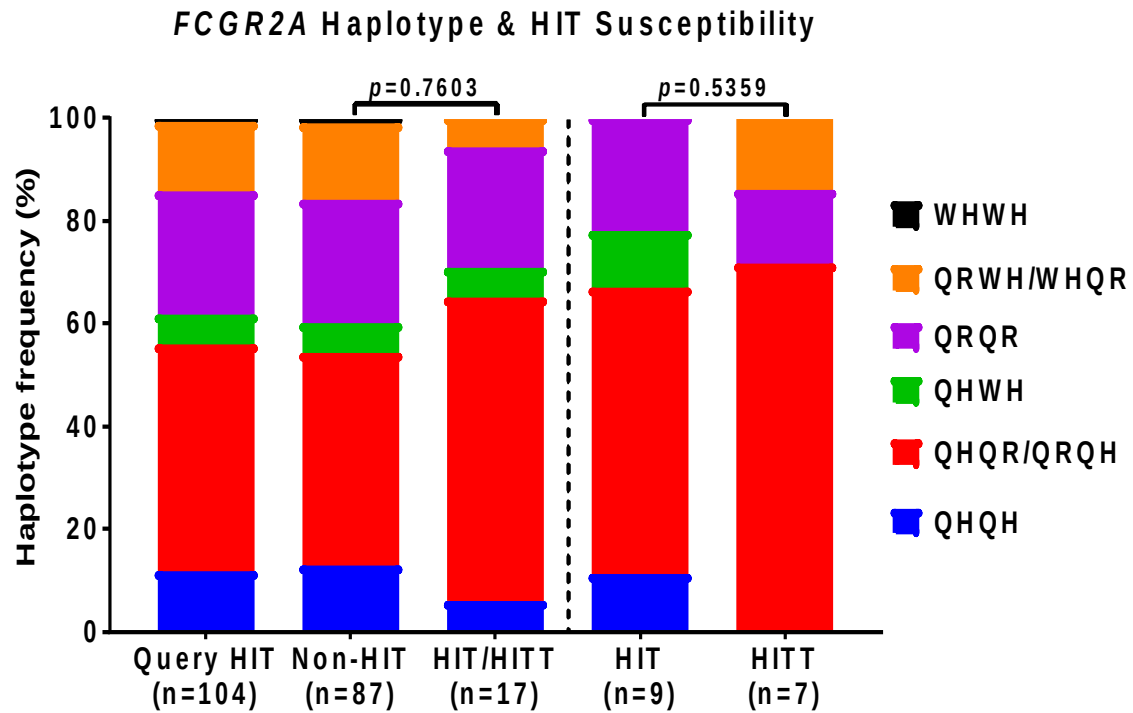


Figure 5.15 Association between *FCGR2A* Q27W and H131R haplotype distribution and HIT susceptibility.

Genomic DNA was extracted from 105 query HIT patients as described (Section 2.2.10.1). To obtain in-phase haplotype information of the two *FcγRIIa* polymorphic sites, Q27W and H131R, PCR products amplified from the genomic DNA (Section 2.2.10.4) were subjected to long-read sequencing on the PacBio Sequel platform (Section 2.2.10.6). Raw sequencing reads were processed using the SMRT Link Long Amplicon Analysis pipeline to generate consensus sequences and obtain phased haplotype information across the two polymorphic sites for every individual as described in Section 2.2.10.7. Chi-square analyses comparing the haplotype distribution between non-HIT and HIT/HITT ($\chi^2=2.607$, d.f.=5, $p=0.7603$) and between HIT and HITT ($\chi^2=3.132$, d.f.=4, $p=0.5359$) were performed. One query HIT patient with the 27QR genotype (QHRH haplotype) was excluded from the analysis. d.f., degrees of freedom.

Table 5.4 FCGR2A Q27W and H131R haplotype distribution in patients suspected of HIT.

Frequency distribution of the haplotype combinations across the Q27W and H131R polymorphisms in *FCGR2A* in 105 query HIT patients of which 17 individuals tested positive with the SRA. Among the SRA-positive patients, 7 patients experienced a thrombotic event. Chi-square analyses were performed to compare the haplotype distribution between non-HIT and HIT/HITT ($\chi^2=2.607$, d.f.=5, $p=0.7603$) and between HIT and HITT ($\chi^2=3.132$, d.f.=4, $p=0.5359$). d.f., degrees of freedom. #A query HIT patient with the 27QR genotype (QHRH haplotype) was excluded. ##Thrombosis data was missing for one patient and therefore was excluded in subsequent analysis comparing HIT and HITT.

Amino acid 27	Amino acid 131	Query HIT (%) n=104 [#]	Non-HIT (%) n=87	HIT/HITT (%) n=17 ^{##}	HIT (%) n=9	HITT (%) n=7
Q	H	12 (11.54)	11 (12.64)	1 (5.88)	1 (11.11)	0 (0.00)
Q	H					
Q	H	46 (44.23)	36 (41.38)	10 (58.82)	5 (55.56)	5 (71.43)
Q	R					
Q	H	6 (5.77)	5 (5.75)	1 (5.88)	1 (11.11)	0 (0.00)
W	H					
Q	R	25 (24.04)	21 (24.14)	4 (23.53)	2 (22.22)	1 (14.29)
Q	R					
Q	R	14 (13.46)	13 (14.94)	1 (5.88)	0 (0.00)	1 (14.29)
W	H					
W	H	1 (0.96)	1 (1.15)	0 (0.00)	0 (0.00)	0 (0.00)
W	H					

In Figure 5.15, the association between *FCGR2A* haplotype and HIT susceptibility was assessed among patients suspected of HIT in the query HIT cohort. Here, the analysis was repeated in a combination of the three cohorts comprising of healthy donors, non-HIT thrombocytopenic patients and query HIT patients to determine whether an overrepresentation of a particular haplotype combination exist among patients who developed HIT. Table 5.5 describes the frequency distribution of the haplotype combinations across the Q27W and H131R polymorphisms in 180 individuals of which 18 were HIT-positive patients. The 162 non-HIT individuals comprise of 45 healthy donors, 30 non-HIT thrombocytopenic patients and 87 non-HIT patients who were suspected of HIT. As observed previously, QHQR was the most common haplotype combination in both non-HIT (37.04%) and HIT-positive (55.56%) groups. While 3 of the non-HIT patients express the WHWH combination (1.85%), none of the patients with HIT carried this haplotype combination. However, Chi-square analysis revealed no significant association between *FCGR2A* haplotype and HIT susceptibility ($\chi^2=4.302$, d.f.=5, $p=0.5069$).

In Table 5.6, the haplotype frequency was calculated by counting the number of times each haplotype occurred on either the maternal and/or paternal alleles (diploid) and assessed for an association with HIT development. In both non-HIT and HIT groups, the Q|R haplotype occurred most frequently at 47.84% and 58.33%, respectively, followed by Q|H (39.20% and 36.11%, respectively) while W|H was the least common at 12.96% and 5.56%, respectively. The W|R haplotype was not represented among the 181 individuals sequenced, indicating that the 27W and 131R variants never co-occurred as stated in Section 5.3.8 and Table 5.3. The haplotype frequencies were comparable between the two groups suggesting that HIT development is independent of the haplotype at Q27W and H131R ($\chi^2=2.264$, d.f.=2, $p=0.3224$).

Table 5.5 FCGR2A Q27W and H131R haplotype distribution and HIT susceptibility.

Frequency distribution of the haplotype combinations across the Q27W and H131R polymorphisms in *FCGR2A* in 180 individuals of which 18 were HIT-positive patients. The 162 non-HIT individuals comprise of 45 healthy donors, 30 non-HIT thrombocytopenic patients and 87 non-HIT patients who were suspected of HIT (query HIT cohort). Chi-square analysis was performed to assess the association between haplotype distribution and HIT susceptibility ($\chi^2=4.302$, d.f.=5, $p=0.5069$). d.f., degrees of freedom. [#]A query HIT patient (non-HIT) with the 27QR genotype (QHRH haplotype) was excluded.

Amino acid 27	Amino acid 131	Not HIT (%) n=162 [#]	HIT (%) n=18
Q	H	27 (16.67)	1 (5.56)
Q	H		
Q	H	60 (37.04)	10 (55.56)
Q	R		
Q	H	13 (8.02)	1 (5.56)
W	H		
Q	R	36 (22.22)	5 (27.78)
Q	R		
Q	R	23 (14.20)	1 (5.56)
W	H		
W	H	3 (1.85)	-
W	H		
Q	H	-	-
W	R		
Q	R	-	-
W	R		
W	H	-	-
W	R		
W	R	-	-
W	R		

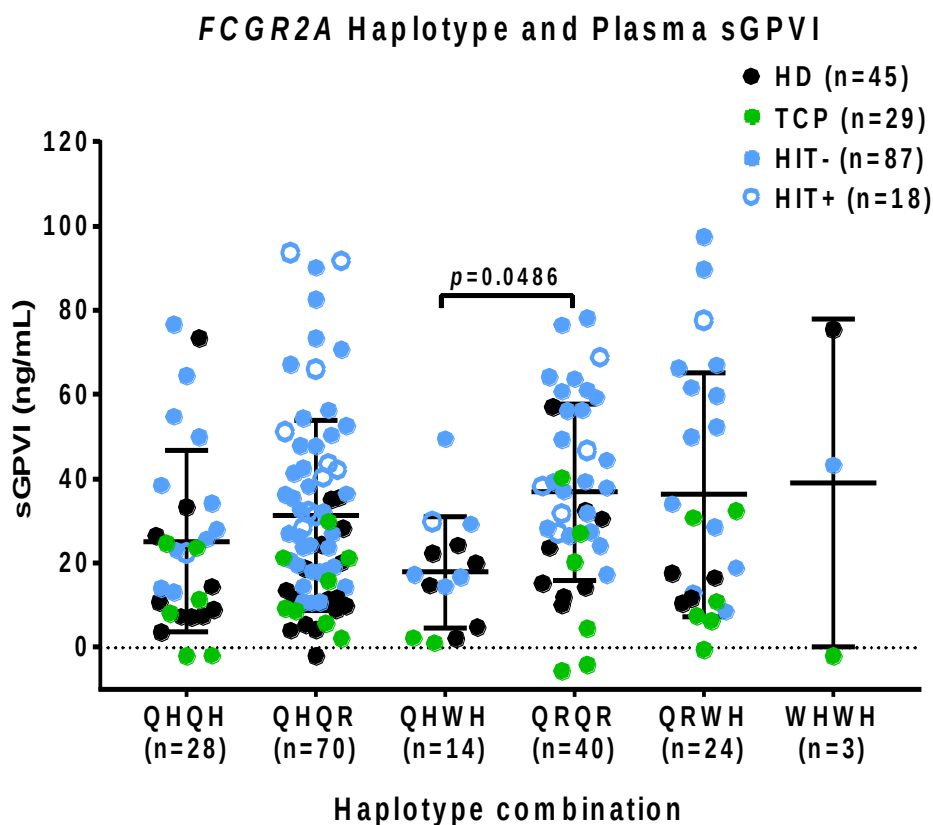
Table 5.6 Frequency of haplotype at Q27W and H131R between non-HIT individuals and patients who developed HIT.

Haplotype frequency was calculated by taking into account the number of occurrences on either the maternal and/or paternal alleles (2n, diploid) in 180 individuals of which 18 were HIT-positive patients. The 162 non-HIT individuals comprised of 45 healthy donors, 30 non-HIT thrombocytopenic patients and 87 non-HIT patients who were suspected of HIT (query HIT cohort). Chi-square analysis was performed to determine the association between haplotype frequency and HIT susceptibility ($\chi^2=2.264$, d.f.=2, $p=0.3224$). d.f., degrees of freedom. #A query HIT patient (non-HIT) with the 27QR genotype (QHRH haplotype) was excluded.

Haplotype	Not HIT (%) n=162# 2n=324	HIT (%) n=18 2n=36
Q H	127 (39.20)	13 (36.11)
W H	42 (12.96)	2 (5.56)
Q R	155 (47.84)	21 (58.33)
W R	0 (0.00)	0 (0.00)

5.3.10 *FCGR2A* haplotype and plasma sGPVI levels

Data presented in Chapter 3 demonstrated that engagement of platelet FcγRIIa can release sGPVI to various extents in a donor-dependent manner. Using plasma sGPVI as a measure of platelet reactivity, the influence of *FCGR2A* Q27W and H131R haplotype on platelet activity was assessed. Figure 5.16 compares the circulating sGPVI levels between individuals expressing the six haplotype combinations of Q27W and H131R polymorphisms. Plasma sGPVI levels were measured by ELISA as described (Section 2.2.9.1) in 45 healthy individuals, 29 non-HIT thrombocytopenic patients (TCP), 87 query HIT patients with negative SRA results and 18 SRA-positive HIT patients. Plasma sGPVI levels were highly variable even among individuals of the same *FCGR2A* haplotype combination. Interestingly, individuals of the QHWH haplotype had relatively lower average circulating sGPVI levels at 17.79 ± 13.33 ng/mL (mean $\pm$ SD). One-way ANOVA (Kruskal-Wallis) test with Dunn's multiple comparisons indicated a lower sGPVI level among QHWH individuals compared to the QRQR haplotype (36.79 ± 21.09 ng/mL, $p=0.0486$). None of the other pairwise comparisons between each haplotype combination achieved statistical significance ($p>0.05$). This observation is consistent with the findings in Section 5.3.4 whereby individuals carrying a 131R allele were associated with higher sGPVI levels. Here, the difference in sGPVI levels between QHWH and QRQR individuals was largely governed by the genotype at H131R.



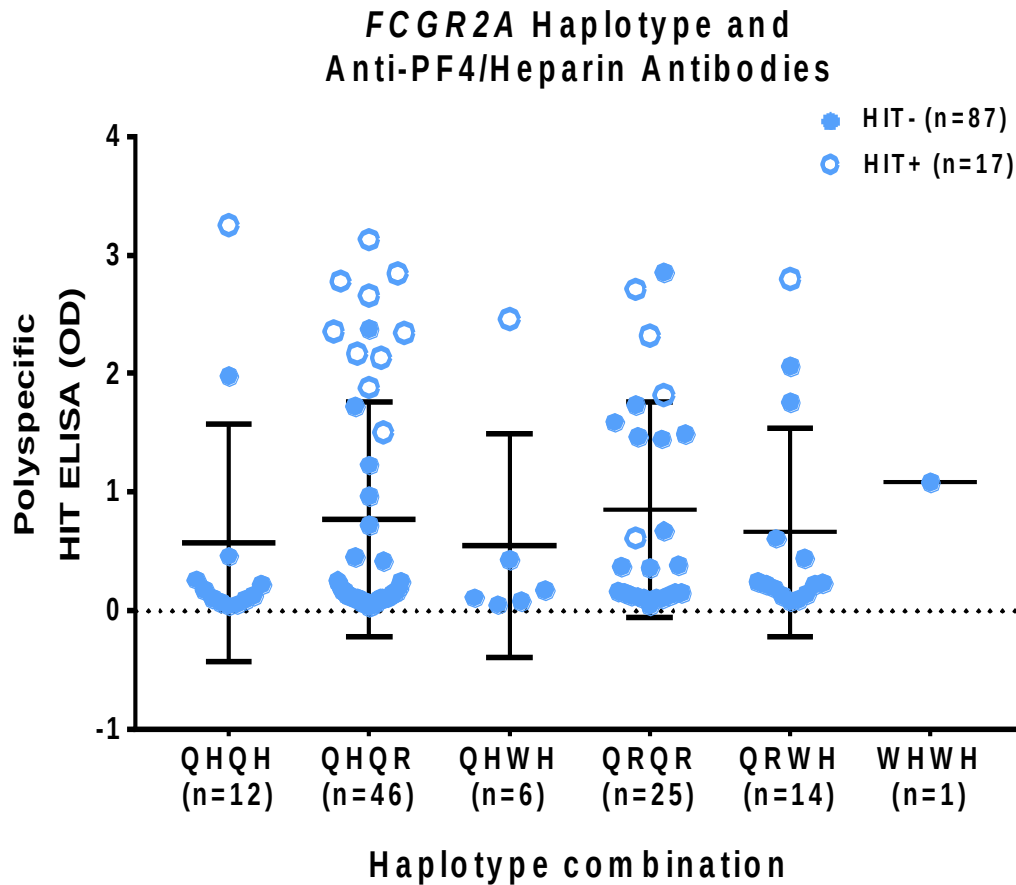
Dunn's multiple comparisons test	Mean rank diff.	Summary	Adjusted <i>p</i> -value
QHQH vs. QHQR	-16.44	ns	>0.9999
QHQH vs. QHWH	14.82	ns	>0.9999
QHQH vs. QRQR	-32.55	ns	0.1620
QHQH vs. QRWH	-20.99	ns	>0.9999
QHQH vs. WHWH	-27.82	ns	>0.9999
QHQR vs. QHWH	31.26	ns	0.5904
QHQR vs. QRQR	-16.11	ns	>0.9999
QHQR vs. QRWH	-4.552	ns	>0.9999
QHQR vs. WHWH	-11.39	ns	>0.9999
QHWH vs. QRQR	-47.37	*	0.0486
QHWH vs. QRWH	-35.81	ns	0.5982
QHWH vs. WHWH	-42.64	ns	>0.9999
QRQR vs. QRWH	11.56	ns	>0.9999
QRQR vs. WHWH	4.725	ns	>0.9999
QRWH vs. WHWH	-6.833	ns	>0.9999

Figure 5.16 Association between *FCGR2A* haplotype and plasma sGPVI levels.

Plasma sGPVI levels were measured by ELISA as described (Section 2.2.9.1) in 45 healthy individuals, 29 non-HIT thrombocytopenic patients (TCP), 87 query HIT patients with negative SRA results and 18 SRA-positive HIT patients. PCR products were amplified from the genomic DNA of these individuals (Section 2.2.10.4) and subjected to long-read sequencing on the PacBio Sequel platform (Section 2.2.10.6) to obtain in-phase haplotype information. One-way ANOVA (Kruskal-Wallis) test with Dunn's multiple comparisons was performed to evaluate the influence of *FCGR2A* haplotype combination on circulating sGPVI levels. Error bars indicate mean $\pm$ SD.

5.3.11 *FCGR2A* haplotype and anti-PF4/heparin antibody levels

Figure 5.17 compares the levels of anti-PF4/heparin antibodies, measured by a polyspecific HIT ELISA in OD units, in 104 query HIT patients with different *FCGR2A* Q27W and H131R haplotype combinations. Of the 104 patients suspected of HIT, 17 were SRA-positive. The average anti-PF4/heparin antibody levels for each haplotype combination ranged between 0.550 to 0.847 OD units. Confirmed HIT patients (HIT+) had antibody levels greater than the group average for their respective haplotype combinations except for one HIT+ patient with the QRQR haplotype. One-way ANOVA with Tukey's multiple comparisons test showed that the levels of anti-PF4/heparin antibodies were independent of an individual's haplotype at *FCGR2A* Q27W and H131R ($p=0.9441$).



Tukey's multiple comparisons test	Mean diff.	Summary	Adjusted <i>p</i> -value
QHQH vs. QHQR	-0.1964	ns	0.9881
QHQH vs. QHWH	0.01833	ns	>0.9999
QHQH vs. QRQR	-0.2785	ns	0.9613
QHQH vs. QRWH	-0.08952	ns	0.9999
QHQH vs. WHWH	-0.5117	ns	0.9955
QHQR vs. QHWH	0.2148	ns	0.9954
QHQR vs. QRQR	-0.08202	ns	0.9993
QHQR vs. QRWH	0.1069	ns	0.9991
QHQR vs. WHWH	-0.3152	ns	0.9995
QHWH vs. QRQR	-0.2968	ns	0.9834
QHWH vs. QRWH	-0.1079	ns	>0.9999
QHWH vs. WHWH	-0.5300	ns	0.9955
QRQR vs. QRWH	0.1889	ns	0.9913
QRQR vs. WHWH	-0.2332	ns	0.9999
QRWH vs. WHWH	-0.4221	ns	0.9981

Figure 5.17 The distribution of anti-PF4/heparin antibody levels by *FCGR2A* Q27W and H131R haplotype in patients suspected of HIT.

Levels of anti-PF4/heparin antibodies were measured by a polyspecific HIT ELISA in optical density (OD) units, in 104 patients suspected of HIT, of which 17 were SRA-positive. PCR products were amplified from the genomic DNA of these individuals (Section 2.2.10.4) and subjected to long-read sequencing on the PacBio Sequel platform (Section 2.2.10.6) to obtain in-phase haplotype information. One-way ANOVA with Tukey's multiple comparisons test was performed to evaluate the influence of *FCGR2A* haplotype combination on anti-PF4/heparin antibody levels. Error bars indicate mean $\pm$ SD.

5.3.12 *FCGR2A* haplotype and thrombosis score

Figure 5.18 and Table 5.7 compare the proportion of query HIT patients who experienced a thrombotic event based on their haplotype combinations at *FCGR2A* Q27W and H131R. A thrombosis score of 0 indicates absence of thrombotic event while a score of 1 indicates progressive or recurrent thrombosis or a suspected thrombosis (Cuker et al., 2012). A score of 2 was given to patients with new thrombotic events or in the presence of skin necrosis. A thrombotic event was present in each haplotype group except for the rare WHWH haplotype (Figure 5.18, Table 5.7). Chi-square analysis showed a lack of association between *FCGR2A* Q27W and H131R haplotype combination and thrombosis occurrence ($\chi^2=6.55$, d.f.=10, $p=0.7671$).

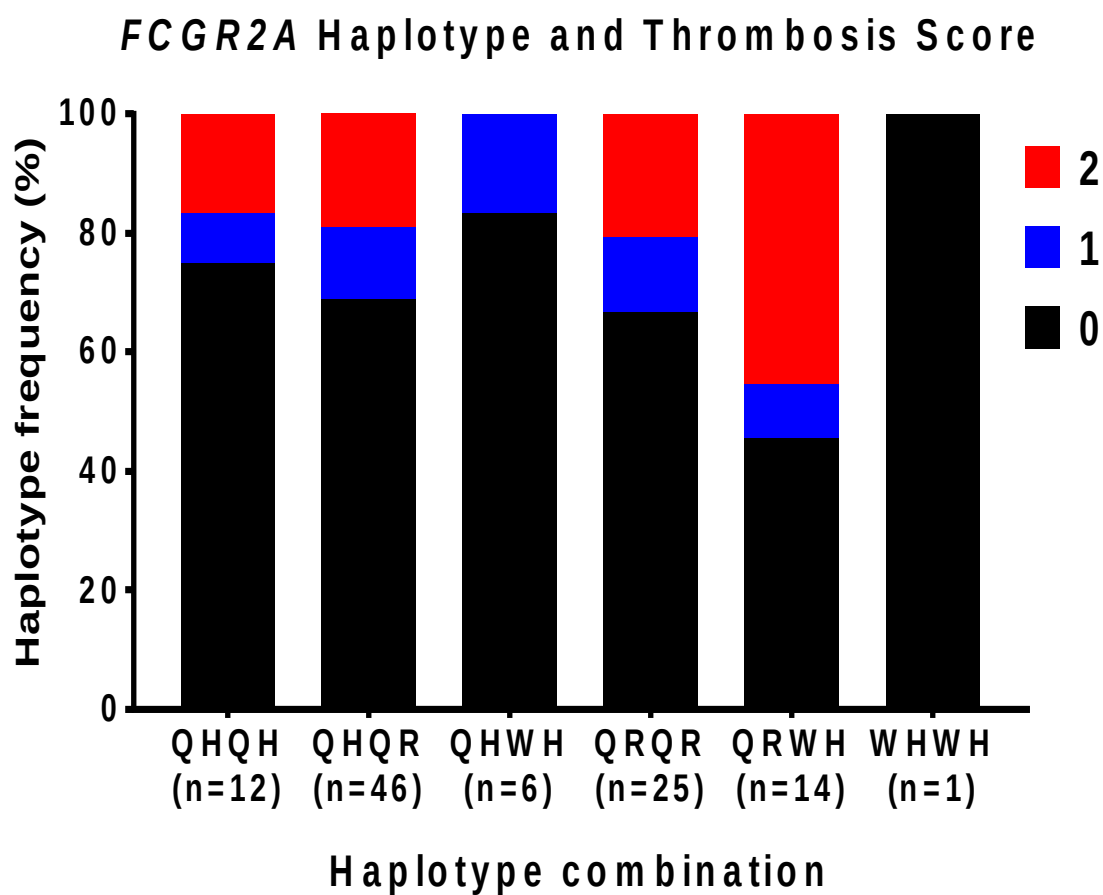


Figure 5.18 The proportion of patients who experienced a thrombotic event based on their *FCGR2A* Q27W and H131R haplotype combination in a cohort of patients suspected of HIT.

A thrombosis score of 0 indicates absence of thrombotic event while a score of 1 indicates progressive or recurrent thrombosis or a suspected thrombosis (Cuker et al., 2012) in a cohort of 96 query HIT patients. A score of 2 was given to patients with new thrombotic events or in the presence of skin necrosis. PCR products were amplified from the genomic DNA of these patients (Section 2.2.10.4) and subjected to long-read sequencing on the PacBio Sequel platform (Section 2.2.10.6) to obtain in-phase haplotype information. Chi-square analysis was performed to evaluate the relationship between thrombosis occurrence and *FCGR2A* haplotype combination ($\chi^2=6.55$, d.f.=10, $p=0.7671$).

Table 5.7 FCGR2A haplotype combination and thrombosis occurrence in query HIT patients.

Frequency distribution of thrombosis occurrence stratified by FCGR2A Q27W and H131R haplotype combination in 96 patients suspected of HIT. A thrombosis score of 0 indicates absence of thrombotic event while a score of 1 indicates progressive or recurrent thrombosis or a suspected thrombosis. A score of 2 was given to patients with new thrombotic events or in the presence of skin necrosis. Chi-square analysis was performed to evaluate the relationship between thrombosis occurrence and FCGR2A haplotype combination ($\chi^2=6.55$, d.f.=10, $p=0.7671$).

Amino acid 27	Amino acid 131	Thrombosis score		
		0 (%) n=65	1 (%) n=11	2 (%) n=20
Q	H	9 (13.85)	1 (9.09)	2 (10.00)
Q	H			
Q	H	29 (44.62)	5 (45.45)	8 (40.00)
Q	R			
Q	H	5 (7.69)	1 (9.09)	0 (0.00)
W	H			
Q	R	16 (24.62)	3 (27.27)	5 (25.00)
Q	R			
Q	R	5 (7.69)	1 (9.09)	5 (25.00)
W	H			
W	H	1 (1.54)	0 (0.00)	0 (0.00)
W	H			

5.4 Discussion

Despite the extensive use of heparin and high morbidity of HIT, there is a lack of clinical tools to evaluate HIT risk prior to heparin administration. The inability to predict HIT remains a liability associated with heparin therapy. Identification of genetic factors which influence HIT susceptibility may be valuable for risk stratification prior to heparin treatment and/or monitoring of heparinised patients. Here, the genetic influence of *FCGR2A* polymorphisms at Q27W and H131R on circulating sGPVI levels, thrombotic risk, levels of anti-PF4/heparin antibodies and HIT susceptibility were investigated. The contributions of these polymorphic sites at the dimerising region and ligand-binding domain of FcγRIIa, respectively, were first investigated in isolation and then in combination.

The minor allele frequency of 27W was 0.12 among the 180 individuals investigated here, consistent with the frequency of 27W reported in 919 Europeans (Nagelkerke et al., 2019), which is the only published population study of the *FCGR2A* Q27W polymorphism, to date. The rarity of the variant allele is expected considering its requirement for a dinucleotide substitution. Conversely, the 131R variant occurred at an almost similar rate as the 131H allele, at a frequency of 0.49 in 181 individuals. This is comparable to the 131R allele frequency reported by Rollin and colleagues (2015) in 206 healthy individuals at 0.48 although a lower rate at 0.41 was reported by Clancy and colleagues (2019) while Brandt and colleagues (1995) observed a higher frequency at 0.57. There are several reports which support ethnic differences in the distribution of the alleles among healthy subjects. For example, lower rates of the 131R allele were observed in the Chinese and Japanese cohorts compared to the African and European cohorts (Moriya et al., 2014, Nagelkerke et al., 2019).

In agreement with previous studies (Arepally et al., 1997, Rollin et al., 2015), HIT susceptibility was independent of the *FCGR2A* H131R genotype, as shown by the data presented here. However, other studies found a significant association between the H131R polymorphism and HIT development (Burgess et al., 1995, Brandt et al., 1995, Bachelot-Loza et al., 1998, Carlsson et al., 1998). The lack of association observed here may be due to the fact that the control group was not matched for ethnicity with patients who developed HIT. A lack of association was also observed between the Q27W polymorphism and HIT development although the low allele frequency of 27W may hamper the detection of any potential effect unlike the two variants of H131R which have almost equal allelic frequencies. Based on this cohort, the difference in the proportions of H131 versus R131 allele between non-HIT and HIT patients has an effect size of 0.33, which would require 2041 non-HIT and 204 HIT patients to demonstrate with 80% power, a difference at $p < 0.05$ in the allele frequency. On the other hand, the effect size for the difference in the proportions of Q27 versus W27 allele between non-HIT and HIT patients was 0.40, which would require 1393 non-HIT and 139 HIT patients to demonstrate with 80% power, a difference at $p < 0.05$.

Nevertheless, a higher frequency of the 131R allele was observed among patients with HIT compared to HIT patients. This observation corroborates the findings of Rollin and colleagues (2015) who demonstrated an increased risk of thrombosis among 131RR patients with HIT. The authors postulated that the differential IgG subclass binding affinity between FcγRIIa H and R allotypes may account for the over-representation of the 131R allele in HIT patients. In this scenario, endogenous IgG1 and IgG2 compete with HIT immune complexes for FcγRIIa binding, thereby reducing platelet activation and risk of thrombosis in patients expressing the 131H allele. Due to the lack of IgG2 binding, 131RR patients rely on IgG1 to modulate HIT immune complex-mediated platelet activation, thereby increasing the extent of receptor

engagement. The authors also demonstrated an increased platelet reactivity measured by platelet aggregation in response to HIT antibodies in 131RR donor PRP compared to that in 131HH or 131HR donors. This hyperreactive phenotype of FcγRIIa R allotype is consistent with reports by Clancy and colleagues (2019) of hyperresponsive platelets in patients with systemic lupus erythematosus and Zhou and colleagues (2015) in healthy donors.

The 131R allele was also associated with higher circulating sGPVI levels (Figure 5.10) which supports the increase in platelet reactivity observed in homozygous 131RR individuals. When stimulated with HIT antibodies, procoagulant activity (measured by plasma clotting time) and tissue factor mRNA levels in whole blood from 131RR donors were significantly higher than 131HR or 131HH individuals (Rollin et al., 2015). In line with increased plasma sGPVI levels and tissue factor synthesis in 131RR individuals, active Factor X, common to the intrinsic and tissue factor-mediated coagulation pathway, triggers GPVI release (Al-Tamimi et al., 2011b). These findings provide further evidence which suggests an enhanced platelet reactivity phenotype contributed by the 131R allele.

An association between FcγRIIa polymorphism and anti-PF4/heparin antibody levels was not evident in this cohort of query HIT patients, consistent with another study (Rollin et al., 2015). However, two HIT patients with the homozygous 131HH genotype presented with higher circulating levels of anti-PF4/heparin antibody compared to HIT patients carrying the 131R allele. Although the 131H allele may confer a protective effect against platelet reactivity as explained above, Rollin and colleagues (2015) demonstrated that the FcγRIIa H131R polymorphism effect was no longer evident at higher concentrations of HIT antibodies. In other words, an excessive level of anti-PF4/heparin antibodies overcame the protective effect

of the FcγRIIIa 131H allotype which may explain the higher antibody levels present in HIT patients expressing the 131HH genotype.

Interestingly, the variant alleles 27W and 131R never co-occur on the same allele when assessed in 181 individuals. This may suggest a deleterious consequence of co-expression of these alleles and suggests a potential functional link between the two seemingly spacially distant amino acids. A 19-amino acid insertion within the cytoplasmic tail of FcγRIIIa was recently reported to confer a longer retention of the activated receptor on the membrane after ligand engagement (Anania et al., 2018). This variant only exists in the 131R allotype which does not bind IgG2. Impaired internalisation of ligand-bound receptor has been postulated to enhance activating signals which causes increased calcium mobilisation resulting in increased platelet activation and degranulation as observed by Anania and colleagues (2018). The co-expression of 27W and 131R on the same allele may impact on receptor internalisation and thereby confer a deleterious effect which remains to be investigated.

There was no apparent over-representation of a particular haplotype combination of *FCGR2A* at positions 27 and 131 among HIT patients compared to non-HIT individuals. However, the present study is limited by the small number of HIT patients. There may be a small effect size associated with *FCGR2A* haplotype combination which would require a larger cohort to reach statistical significance. To detect a significant overrepresentation of a particular haplotype combination across the H131R and Q27W polymorphic sites, 1596 non-HIT and 160 HIT patients are required to demonstrate with 80% power, a difference at $p < 0.05$ due to the small effect size of 0.38 based on this cohort. Further, the functional implications of the ten possible

FCGR2A haplotype combinations on antibody binding and FcγRIIa signalling warrant further investigations.

This is the first study to explore the influence of *FCGR2A* haplotype across two polymorphic sites on HIT susceptibility. The work presented here demonstrated an association between the 131R allele and circulating sGPVI levels, suggesting a role for the variant allele in platelet reactivity. HIT susceptibility was not influenced by FcγRIIa Q27W and H131R genotype in isolation, or in combination. Although *FCGR2A* polymorphisms were not associated with HIT antibody levels, higher levels of circulating anti-PF4/heparin antibodies were present in 131HH patients who developed HIT. HIT susceptibility is probably contributed by a complex interplay of factors and polymorphisms within *FCGR2A* is unlikely to be the sole factor in HIT pathology. Combinations of other genetic variants identified in a recent HIT GWAS study should be assessed in the future (Karnes et al., 2015). In addition to a larger cohort size, ethnicity-matched controls should be considered for future genetic association studies. Genetic biomarkers have the potential to be used prior to heparin administration as a screening tool to avoid or reduce the length of heparin administration in patients at risk of HIT. Hence, future efforts in exploring the genetic predisposition of HIT in larger cohorts should account for ethnicity differences and should consider the multifactorial aspects of HIT pathology.

Chapter 6 Regulation of platelet ADAM10 activity

6.1 Background

As a result of vascular injury or change in biorheological properties of blood, circulating platelets become activated through an interplay between platelet surface molecules and extracellular matrix proteins (detailed in Section 1.1.2). Platelet activation leads to release of bioactive molecules from intracellular storage organelles. In addition to platelet secretion, metalloproteinase-mediated receptor shedding is an important consequence of platelet activation. GPVI is one of several adhesive receptors which contribute to thrombus initiation and therefore, play key roles in the function of platelets in thrombosis. The recent findings that GPVI also binds fibrinogen (Mangin et al., 2018) and fibrin (Mammadova-Bach et al., 2015, Alshehri et al., 2015) hint at an important role for GPVI in thrombus stabilisation. Considering the importance of GPVI in thrombus formation, understanding the regulation of GPVI shedding may provide insights into modulating thrombosis occurrence by targeting platelet metalloproteinases.

6.1.1 Metalloproteolysis of GPVI on activated platelets

On human platelets, GPVI is predominantly cleaved by ADAM10 to release sGPVI (Gardiner et al., 2007) while ADAM17 also contributes to GPVI shedding in mice (Bender et al., 2010). Shedding of GPVI can be induced under a variety of conditions including engagement of platelet FcγRIIa by anti-platelet antibodies as presented in Chapters 3 and 4, or exposure to GPVI ligands and pathophysiological shear stress as discussed in Section 1.4.1. The irreversible proteolysis of the ligand-binding ectodomain of GPVI has been postulated to be a mechanism

for downregulating platelet reactivity by altering receptor density. To date, there is no known biological function for the 55 kDa sGPVI fragment.

6.1.2 ADAM10 catalytic activity versus sGPVI levels

ADAM10 is ubiquitously expressed in nucleated cells as a zymogen whereby the pro-domain keeps ADAM10 in an inactive form and aids in intracellular trafficking (Peron et al., 2018). While ADAM10 metalloproteolytic activity does not require its cytoplasmic domain nor post-translational processing of its pro-domain (Maretzky et al., 2015), studies have found evidence which supports an important role for the ADAM10 transmembrane domain in proteolytic activity and substrate selectivity, potentially via interactions with tetraspanin membrane proteins (Haining et al., 2012, Noy et al., 2016, Matthews et al., 2017). ADAM10 activity can be inhibited using a broad spectrum metalloproteinase inhibitor GM6001, an ADAM10 selective inhibitor GI254023X (Ludwig et al., 2005), a divalent cation chelator EDTA or the ADAM10 prodomain (Moss et al., 2007).

ADAM10-mediated shedding of GPVI occurs rapidly within seconds of exposure to agonists, suggesting the presence of active ADAM10 on circulating platelets. Indeed, work by Facey and colleagues (2016) detected active ADAM10 on the surface of untreated platelets which supports the idea that platelets express mature ADAM10 (no prodomain). Despite the presence of active ADAM10 on the platelet surface, GPVI levels remain stable on circulating platelets from healthy individuals with minimal evidence of shedding (Gardiner et al., 2007). There are also apparent differences in the mechanism governing shedding of GPVI induced by different agonists. Although sGPVI levels were increased when platelets were stimulated with GPVI ligands such as collagen, CRP-xL or convulxin, the ADAM10 activity was comparable to resting conditions (Facey et al., 2016). However, exposure to ligand-independent stimuli like

pathological shear stress at $10,000\text{ s}^{-1}$ or a generic activator of ADAM10, NEM, markedly increased platelet ADAM10 activity with a corresponding elevation in sGPVI levels. Taken together, the stability of GPVI on circulating platelets despite the presence of active ADAM10 suggests that there is a mechanism that protects GPVI from being cleaved under resting conditions. Upon platelet activation, ADAM10-mediated shedding of GPVI occurs with or without an increase in ADAM10 activity, depending on the type of activator used. The mechanisms that regulate of platelet ADAM10 remain to be fully characterised.

6.1.3 Inhibitory roles of TIMPs on ADAM10

The four TIMP isoforms are endogenous inhibitors of ADAMs and MMPs, and possess some redundancy in their abilities to inhibit specific metalloproteinases (Section 1.5.3 and summarised in Table 1.3) (Gresele et al., 2017, Jackson et al., 2017). Crystallographic studies revealed that the N-terminal region of human TIMPs interact with the catalytic domain of metalloproteinases in a 1:1 stoichiometry and inhibit proteolytic activity by disrupting the coordination of Zn^{2+} within the enzyme active site (Murphy, 2011, Khokha et al., 2013). Previous work in nucleated cells revealed that ADAM10 is inhibited by TIMP1 (Schelter et al., 2011) and TIMP3 (Scilabra et al., 2018) while studies *in vitro* using rhADAM10 demonstrated that TIMP1 exhibited a markedly lower inhibition constant than TIMP3 (Amour et al., 2000). TIMPs have been detected within sub-membranous structures of platelets and are released upon platelet activation (Villeneuve et al., 2009, Fong et al., 2011, Khokha et al., 2013). However, a functional role for TIMPs in regulating platelet ADAMs activity remain to be explored.

6.1.4 Overview of investigations

Figure 6.1 highlights the aspect of TIMPs and platelet interaction with a focus on the influence of TIMPs on platelet ADAM10 activity that is addressed in this chapter. Firstly, the expression of TIMPs on the surface of resting and activated platelets from healthy donors was investigated by flow cytometry. The relationship between platelet surface TIMPs, plasma TIMPs and circulating sGPVI levels were explored. Subsequently, a FRET assay utilising a fluorogenic peptide substrate containing the GPVI amino sequence cleaved by ADAM10 was optimised and utilised to assess the effect of TIMPs on platelet ADAM10 activity under resting and activated conditions. Findings from this approach motivated the study on whether TIMPs modulate shedding of GPVI upon platelet activation. It is hypothesised that TIMPs are present on the platelet surface and are able to modulate platelet ADAM10 activity.

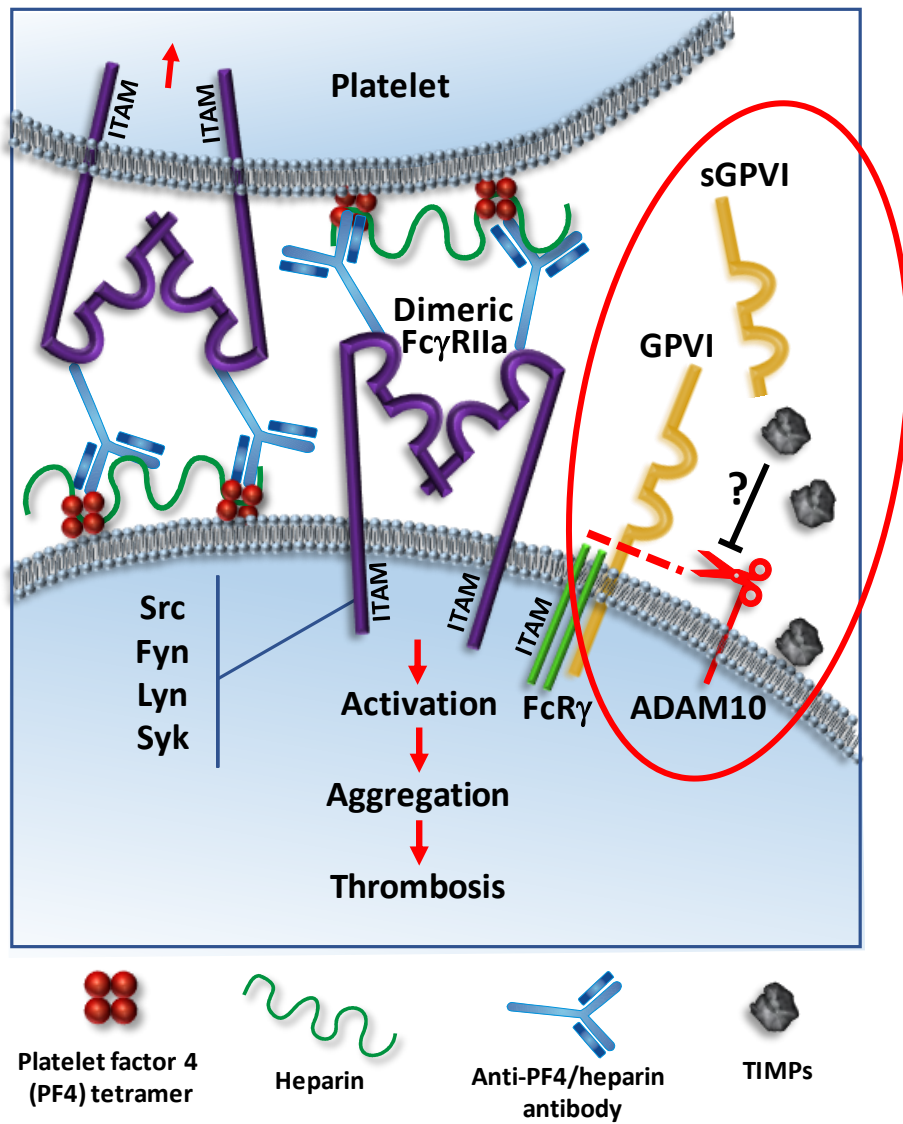


Figure 6.1 Regulation of platelet ADAM10 activity

This chapter will explore the interaction between tissue inhibitors of metalloproteinase (TIMPs) and platelets, and assess the role of TIMPs, represented by rocks, in regulating the activity of platelet ADAM10, represented as scissors.

6.2 Aims

This chapter aimed to explore the role for TIMPs in regulating platelet ADAM10 activity by evaluating the levels of TIMPs on the surface of resting and activated washed platelets and PRP, and in the plasma of healthy donors. This chapter also aimed to assess the effect of exogenous TIMPs on platelet ADAM10 activity and GPVI shedding in response to platelet activation.

6.3 Results

6.3.1 Specificity of anti-human TIMPs antibodies used in detection of platelet surface TIMPs

Commercially-obtained rabbit polyclonal anti-human TIMPs antibodies listed in Table 2.3 (Section 2.1.2) were used for flow cytometric detection of TIMPs on platelets. To assess cross-reactivity between anti-TIMPs antibodies, antibody recognition of recombinant human (rh) TIMPs was tested by Western blot. rhTIMP1-4 (0.2 µg per lane, Table 2.4) were electrophoresed on a 12% reducing SDS-PAGE and electroeluted onto a PVDF membrane (Section 2.2.4.4). Each membrane containing the four TIMP isoforms were separately probed with saturating concentrations of each of the polyclonal anti-human TIMPs. Bound antibody was detected with an HRP-conjugated secondary antibody and chemiluminescence using a ChemiDoc imaging system (Section 2.2.4.4).

Figure 6.2 shows that there was no apparent detection of any rhTIMPs by the anti-hTIMP1 polyclonal antibody. This may be due to loss or absence of the major epitope within the denatured form of rhTIMP1 despite the antibody being polyclonal. Anti-hTIMP2 antibody specifically recognised a sharp band at ~25 kDa corresponding to rhTIMP2 with minimal cross-reactivity with rhTIMP4 (faint band at ~25 kDa). Likewise, anti-hTIMP3 antibody also specifically detected rhTIMP3 (strong band of approximately 22-25 kDa) with minor cross-reactivity with rhTIMP4 (faint band at ~22 kDa). A light band between 46-58 kDa was also recognised by anti-hTIMP3 antibody. Anti-hTIMP4 detected an ~22 kDa band and another band between 32-46 kDa of rhTIMP4. Some cross-reactivity with rhTIMP1 and rhTIMP3 may also be present (light band at ~22 kDa). On balance, apart from anti-hTIMP1, all of the antibodies recognised the appropriate recombinant protein. Because of the reduced utility of anti-hTIMP1 antibody in Western blots and because this antibody has been demonstrated to

perform well in flow cytometry assays (Clark et al., 2011), it was decided to evaluate levels of platelet TIMP proteins by flow cytometry.

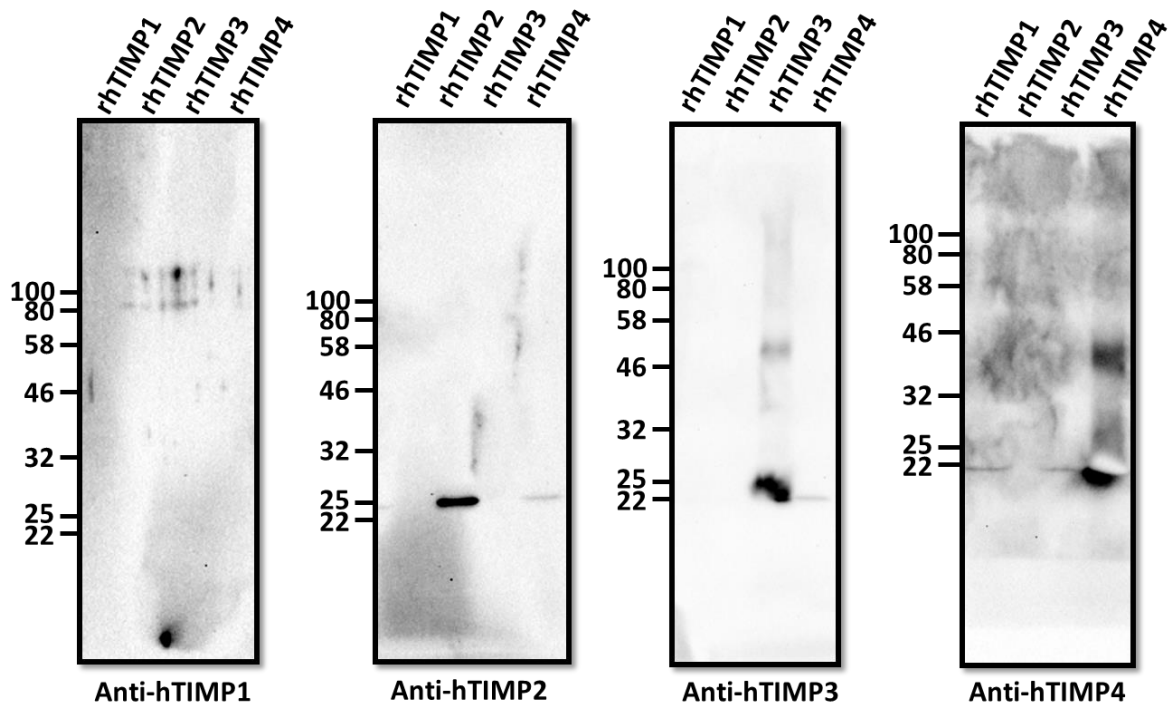


Figure 6.2 Specificity of anti-human TIMPs antibodies used in flow cytometric detection of platelet surface TIMPs.

Recombinant human (rh) TIMP1-4 (0.2 μ g per lane, Table 2.4) were electrophoresed on a 12% reducing SDS-PAGE and electroeluted onto PVDF membranes as described (Section 2.2.4.4). After blocking with skim milk, each membrane containing the four TIMP isoforms were separately probed with 2 μ g/mL of polyclonal anti-human TIMPs antibodies as indicated (Table 2.3) for 4 h at RT. Following a further 1 h incubation with an HRP-conjugated secondary antibody, chemiluminescence substrate was added to the membranes and chemiluminescence signals were detected using a ChemiDoc imaging system (Section 2.2.4.4).

6.3.2 All four TIMPs were detectable to various extents on resting platelets

Levels of TIMPs on the surface of resting platelets were evaluated by flow cytometry using the antibodies presented in Section 6.3.1 and Figure 6.2. Washed platelets and PRP from six healthy individuals were prepared as described (Section 2.2.2.2 and Section 2.2.2.1, respectively). Platelets were incubated with either one of the anti-hTIMP polyclonal antibodies followed by subsequent labelling with an Alexa Fluor 488-conjugated secondary antibody and surface-associated antibodies were quantified by flow cytometry (Section 2.2.8).

Example histogram traces from one healthy donor illustrating the shift in fluorescence contributed by anti-hTIMP antibody binding relative to background fluorescence (Alexa Fluor 488-conjugated secondary antibody) in washed platelet preparation and PRP are shown in Figure 6.3. Washed platelets from this individual produced a rightward shift in histogram peak for each TIMP isoform relative to control Ab, suggesting detectable levels of all four TIMPs. An increase in fluorescence was recorded for all TIMP isoforms in PRP but to a lesser extent than the level observed in washed platelet preparation (Figure 6.3).

In Figure 6.4, the level of platelet surface TIMPs in six donors were evaluated either as percent positive relative to the Alexa Fluor 488-conjugated secondary antibody (control Ab) (panels A(i) and B(i)) or Alexa Fluor 488 fluorescence geomean (panels A(ii) and B(ii)). On resting washed platelets, all four TIMPs were detectable to different extents with considerable variation between donors (Figure 6.4A(i) and A(ii)). The percentage of TIMP2-positive platelets was significantly greater than the control Ab alone when evaluated using one-way ANOVA with Dunnett's multiple comparisons ($p=0.0425$, Figure 6.4A(i)). The average geomeans for each TIMP antibody were comparable to the control Ab ($p>0.05$, Figure 6.4A(ii)).

The variability in TIMP surface levels between donors were diminished in PRP compared to washed platelets (Figure 6.4B(i) and B(ii)). Consistent with washed platelets, all four TIMPs were detectable on the surface of PRP whereby the percentage of TIMP-positive platelets were above the control Ab for all four TIMPs (TIMP1 $p=0.0175$, TIMP2 $p=0.0068$, TIMP3 $p=0.0455$, TIMP4 $p=0.0354$) as depicted in Figure 6.4B(i). Higher levels of TIMP1 and TIMP2 were detected compared to TIMP3 and TIMP4 as observed in washed platelet preparations (Figure 6.4B(i)). However, differences in the respective geomeans detected for each TIMP did not achieve statistical significance ($p>0.05$, Figure 6.4B(ii)) relative to control Ab.

All four TIMPs were detectable to various extents on resting platelets

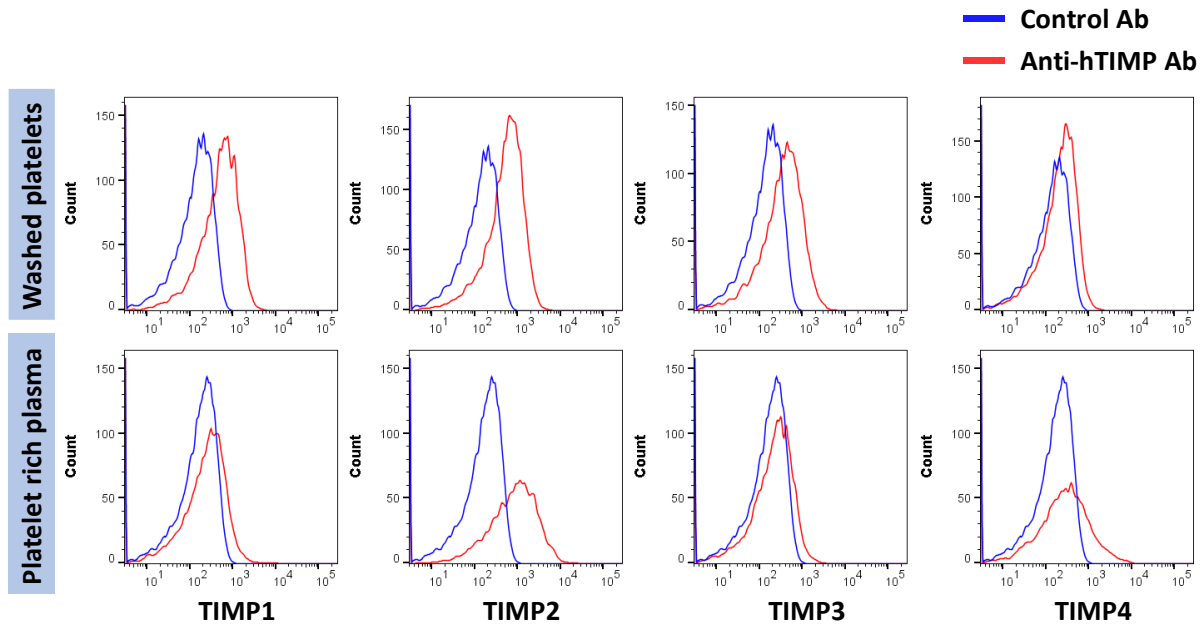


Figure 6.3 Histogram traces of TIMP antibody binding on resting platelets.

Washed platelets and platelet rich plasma were prepared as described (Section 2.2.2.2 and Section 2.2.2.1, respectively). Platelets were incubated with either one of the anti-hTIMP polyclonal antibodies followed by subsequent labelling with an Alexa Fluor 488-conjugated secondary antibody and acquired on a flow cytometer as described (Section 2.2.8). Levels of TIMP isoforms on the surface of resting platelets in washed platelet preparation (top panel) and platelet rich plasma (bottom panel) obtained from one healthy individual are shown. Fluorescence contributed by anti-hTIMP antibodies (red traces) were compared to the fluorescence contributed by control antibody (blue traces, Alexa Fluor 488-conjugated secondary antibody).

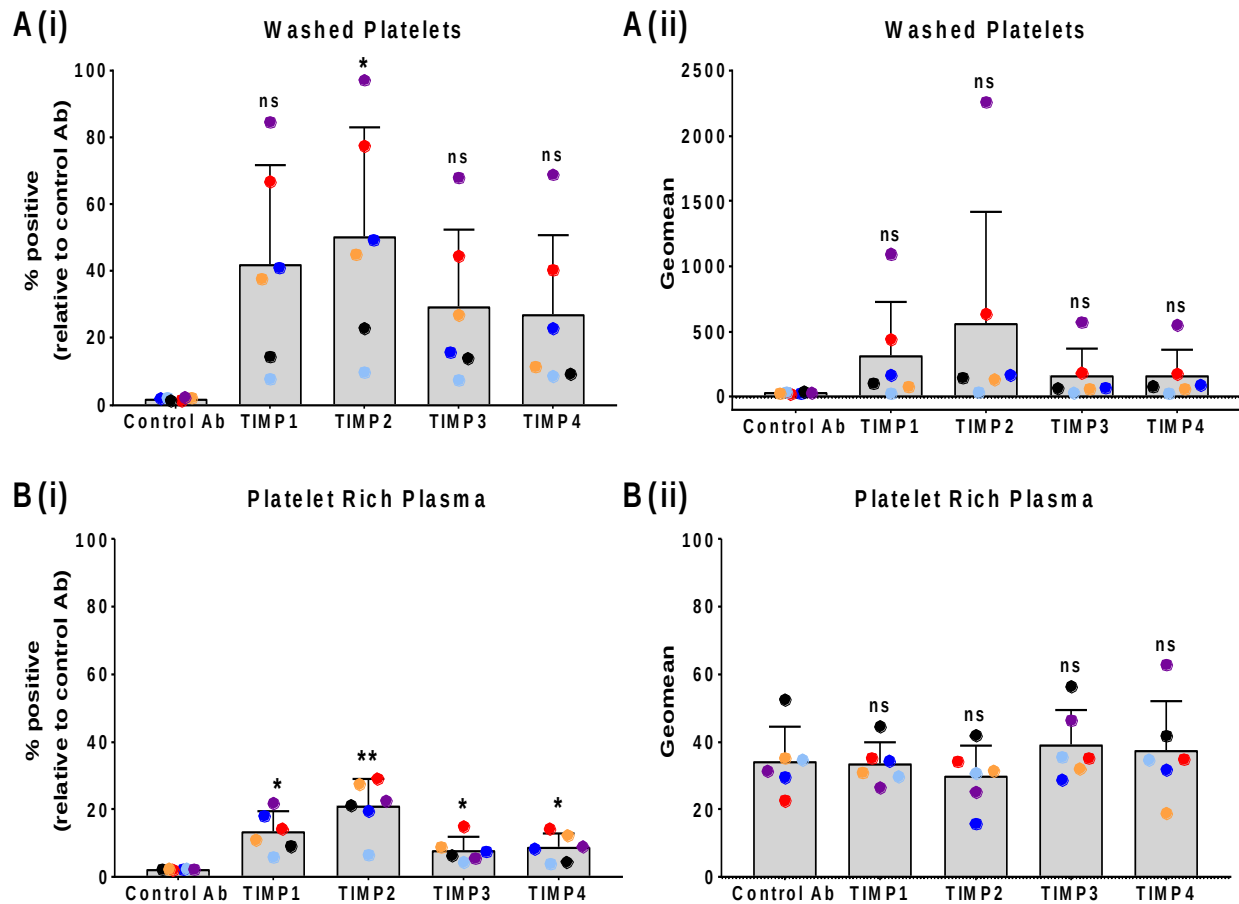


Figure 6.4 Levels of TIMPs on resting platelet surface.

Levels of TIMPs on the surface of resting platelets were evaluated by flow cytometry using the antibodies presented in Section 6.3.1 and Figure 6.4. Washed platelets and PRP from six unique healthy individuals were prepared as described (Section 2.2.2.2 and Section 2.2.2.1, respectively). Platelets were incubated with either one of the anti-hTIMP polyclonal antibodies followed by subsequent labelling with an Alexa Fluor 488-conjugated secondary antibody and acquired on a flow cytometer as described (Section 2.2.8). Panel A shows washed platelet data while panel B shows data in platelet rich plasma. The level of platelet surface TIMPs were evaluated by Alexa Fluor 488 fluorescence represented as percent positive relative to the Alexa Fluor 488-conjugated secondary antibody (control Ab) (left panels A(i) and B(i)) or Alexa Fluor 488 fluorescence geomean (right panels A(ii) and B(ii)). Asterisks refer to significance relative to control Ab. ns, not significant. * $p < 0.05$, ** $p < 0.01$, one-way ANOVA with Dunnett's multiple comparisons. Error bars indicate mean $\pm$ SD.

6.3.3 Levels of TIMPs increased on the surface of activated/degranulated platelets

As variable levels of TIMPs were detected on resting platelets (Section 6.3.2) and TIMPs have been previously detected within intracellular platelet compartments (Villeneuve et al., 2009, Fong et al., 2011, Burkhart et al., 2012), the effect of platelet activation on the surface levels of TIMPs was evaluated in washed platelets and PRP. Washed platelets and PRP from six healthy individuals were prepared as described (Section 2.2.2.2 and Section 2.2.2.1, respectively) and treated with 30 µg/mL collagen or 10 µM A23187 calcium ionophore then made to 50 mM EDTA (final concentration) to stop the reactions. An aliquot of platelet suspension from each reaction tube was assessed for P-selectin upregulation as a marker for platelet degranulation and to confirm platelet activation. Suspensions of platelets were mixed with anti-hTIMP polyclonal antibodies and Alexa Fluor 488-conjugated secondary antibody and acquired on a flow cytometer as described (Section 2.2.8).

Figure 6.5A shows example histogram traces depicting an increase in fluorescence due to P-selectin upregulation on the surface of collagen- or calcium ionophore-activated platelets (blue and grey traces, respectively) compared to resting platelets. Under these conditions, collagen- or calcium ionophore-treated washed platelets and PRP demonstrated variable but increased levels of P-selectin for all six donors (Figure 6.5B and C). Activated washed platelets exhibited at least a 4-fold increase in mean P-selectin expression ($p=0.0018$ for collagen treatment, $p=0.0013$ for A23187 treatment, Figure 6.5B) while a greater than 7-fold increase in P-selectin expression was observed using PRP ($p=0.0348$ for collagen treatment, $p=0.0056$ for A23187 treatment, Figure 6.5C).

Using these conditions of activation, the binding of TIMP antibodies to resting (blue traces) and collagen- or calcium ionophore-activated platelets (red traces) are illustrated in the

example histogram traces from one healthy donor in Figure 6.6. The levels of each TIMP isoform present on the surface of resting and activated washed platelets are evaluated as percent positive relative to the secondary antibody (control Ab) (left panels of Figure 6.7) or fluorescence geomean (right panels of Figure 6.7). Collagen-treated washed platelets showed no significant increase relative to resting platelets for all four TIMP isoforms ($p>0.05$, one-way ANOVA with Dunn's multiple comparisons test) when evaluated by percent positive (Figure 6.7A(i), B(i), C(i) and D(i)) and geomean (Figure 6.7A(ii), B(ii), C(ii) and D(ii)). To variable extents across donors, significant increases in surface levels of all four TIMP isoforms were detected upon treatment with calcium ionophore ($p=0.0011$ to $p=0.0187$). Only the percent positive increase in TIMP3 with calcium ionophore treatment did not achieve statistical significance ($p=0.1665$, Figure 6.7C(i)).

Consistent with data in Figure 6.4, the increase in TIMP surface levels upon activation was less pronounced in PRP (Figure 6.8). Surface levels of all four TIMP isoforms on collagen-treated PRP were comparable to resting platelets ($p>0.05$, one-way ANOVA with Dunn's multiple comparisons test) with the exception for TIMP3 when assessed by fluorescence geomean ($p=0.0418$, Figure 6.8C(ii)). In contrast, calcium ionophore treatment significantly increased levels of TIMP1 ($p=0.0187$, Figure 6.8A(i)), TIMP3 ($p=0.0078$, Figure 6.8C(ii)) and TIMP4 ($p=0.0418$, Figure 6.8D(i) and $p=0.0030$, Figure 6.8D(ii)) but not TIMP2 ($p>0.05$, Figure 6.8B(i) and B(ii)) when evaluated by either percent positive or fluorescence geomean. Taken together, these data indicate increased levels of TIMPs were achieved under conditions of platelet degranulation, and this was more pronounced in washed platelets compared to PRP. The data indicate platelets store TIMPs 1, 2 and 4 which can be brought to the surface under conditions of strong activation and may suggest a role for plasma components in influencing either detection of, or dynamic levels of TIMPs on the platelet surface.

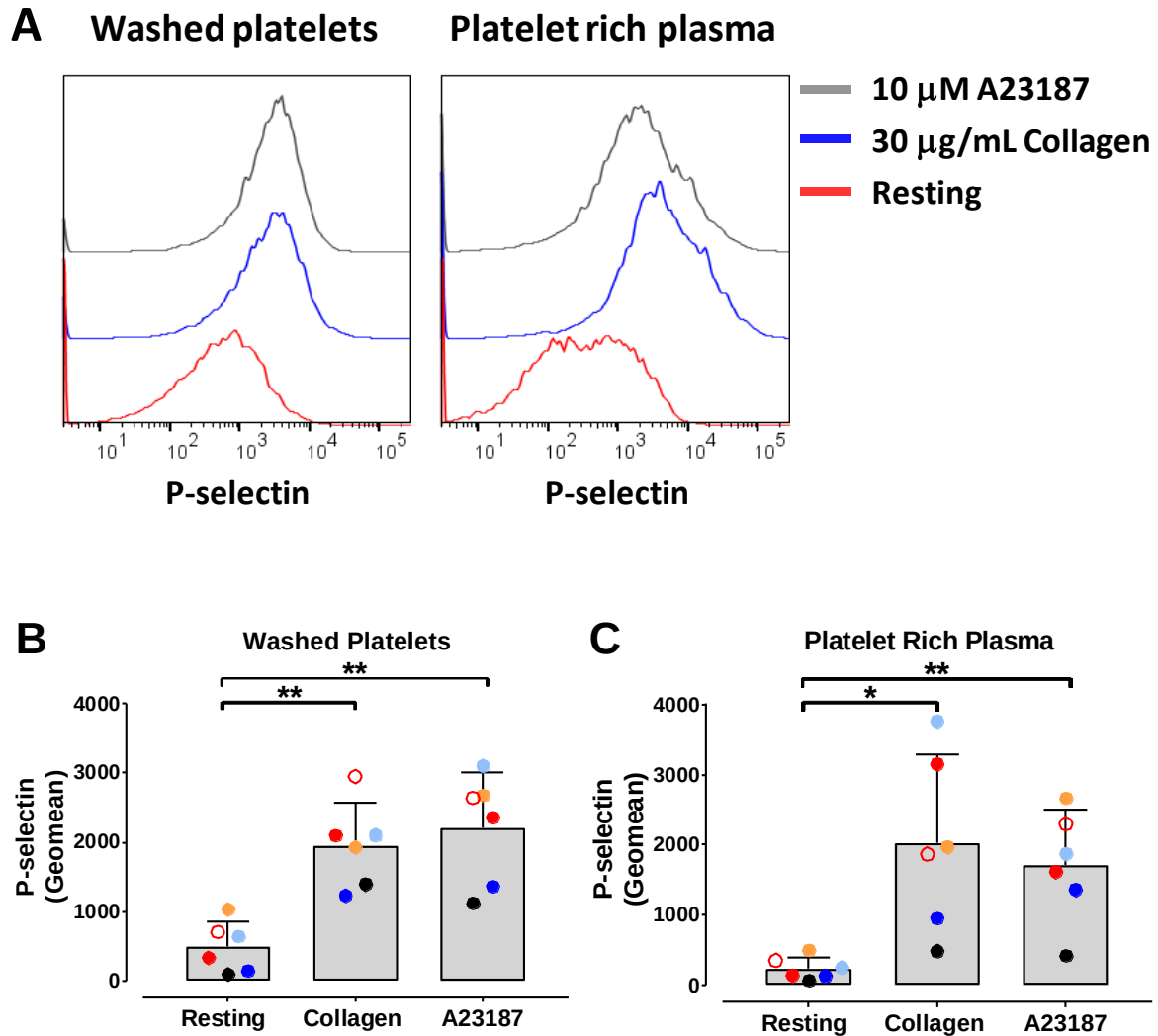


Figure 6.5 Increased P-selectin expression on platelets treated with collagen or calcium ionophore.

Washed platelets from six unique healthy individuals were prepared as described (Section 2.2.2.2) and treated with 30 μ g/mL collagen or 10 μ M A23187 calcium ionophore for 2 h at RT. Platelets were then labelled with PE-conjugated anti-CD62P monoclonal antibody (AK4) or PE-conjugated IgG1 isotype control to assess for platelet degranulation, and acquired on a flow cytometer as described (Section 2.2.8). Panel A shows example histogram traces of fluorescence contributed by P-selectin antibody binding observed in one donor. Level of P-selectin expression in washed platelet preparation (panel B) and platelet rich plasma (panel C) were evaluated by fluorescence geomean. Asterisks refer to significance relative to resting platelets. * p <0.05, ** p <0.01, one-way ANOVA with Tukey's multiple comparisons. Error bars indicate mean $\pm$ SD.

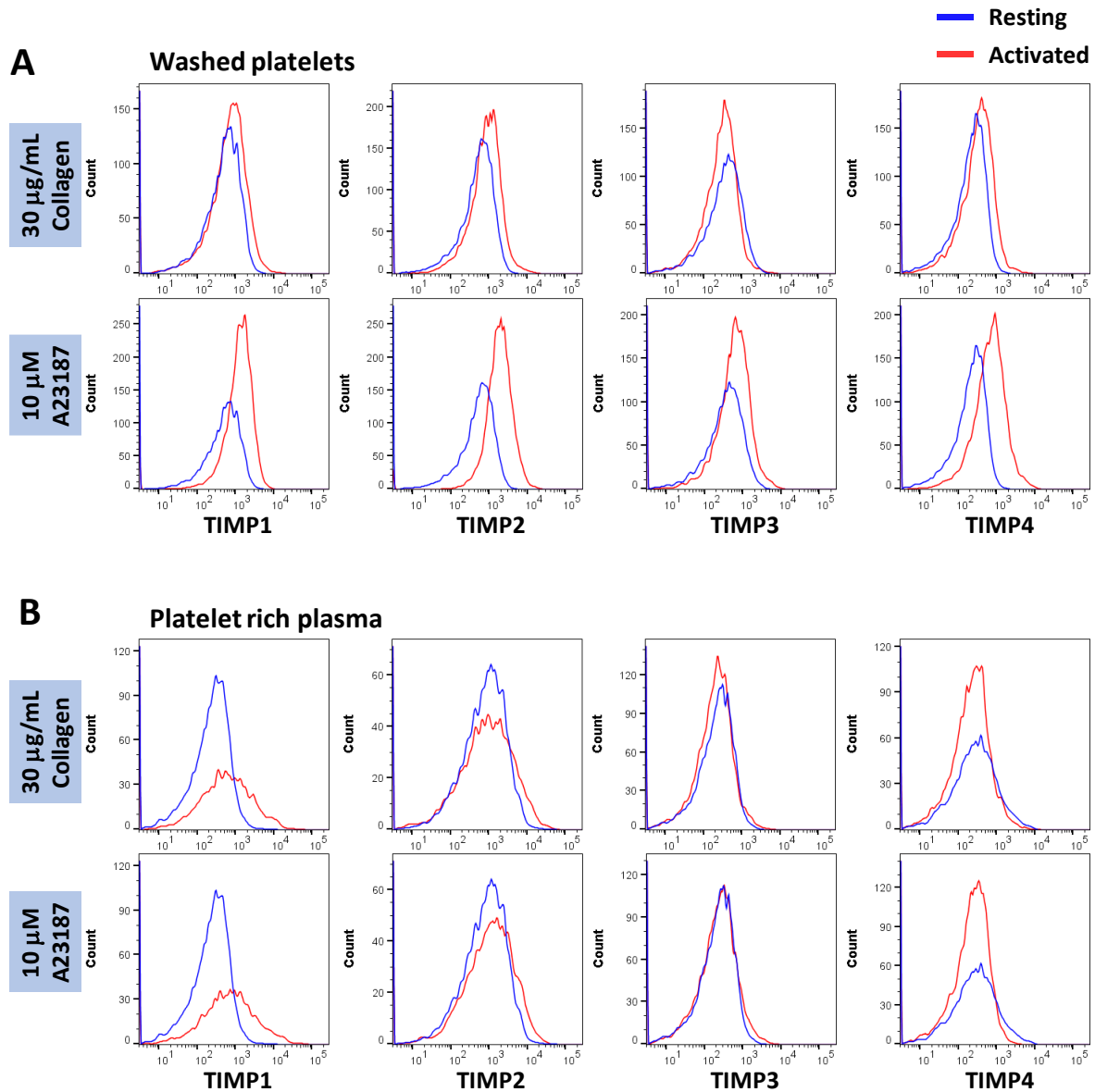


Figure 6.6 Histogram traces of TIMP isoforms on the surface of resting and activated platelets.

Washed platelets from healthy individuals were prepared as described (Section 2.2.2.2) and treated with 30 µg/mL collagen or 10 µM A23187 calcium ionophore for 2 h at RT. Platelet suspensions were made to 50 mM EDTA (final concentration) to stop the reactions at the end of the incubation period. Platelets were then incubated with either one of the anti-hTIMP polyclonal antibodies followed by subsequent labelling with an Alexa Fluor 488-conjugated secondary antibody and acquired on a flow cytometer as described (Section 2.2.8). Levels of TIMP isoforms on the surface of resting and activated platelets in washed platelet preparation (panel A) and platelet rich plasma (panel B) obtained from one healthy individual are shown. Level of TIMPs on either collagen- or calcium ionophore-activated platelets (red traces) were compared to the levels on resting platelets (blue traces).

Washed Platelets

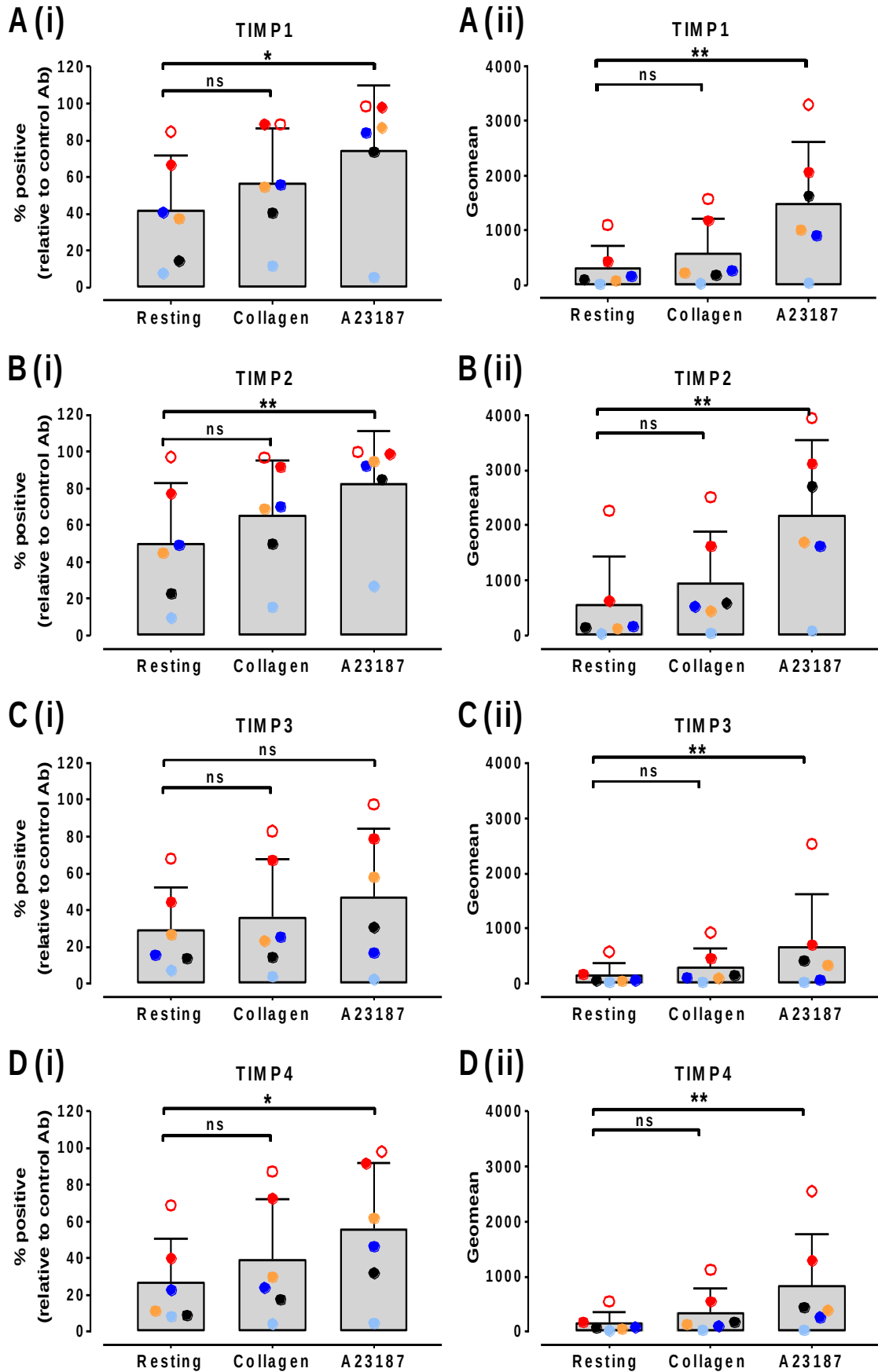


Figure 6.7 Levels of TIMPs on the surface of activated washed platelets.

Washed platelets from six unique healthy individuals were prepared as described (Section 2.2.2.2) and treated with 30 µg/mL collagen or 10 µM A23187 calcium ionophore for 2 h at RT. Platelet suspensions were made to 50 mM EDTA (final concentration) to stop the reactions at the end of the incubation period. Platelets were then incubated with either one of the anti-hTIMP polyclonal antibodies followed by subsequent labelling with an Alexa Fluor 488-conjugated secondary antibody and acquired on a flow cytometer as described (Section 2.2.8). The level of platelet surface TIMPs were evaluated by Alexa Fluor 488 fluorescence represented as percent positive relative to the Alexa Fluor 488-conjugated secondary antibody (control Ab) (left panels) or Alexa Fluor 488 fluorescence geomean (right panels). Asterisks refer to significance relative to resting platelets. ns, not significant. * $p < 0.05$, ** $p < 0.01$, one-way ANOVA with Dunn's multiple comparisons. Error bars indicate mean $\pm$ SD.

Platelet Rich Plasma

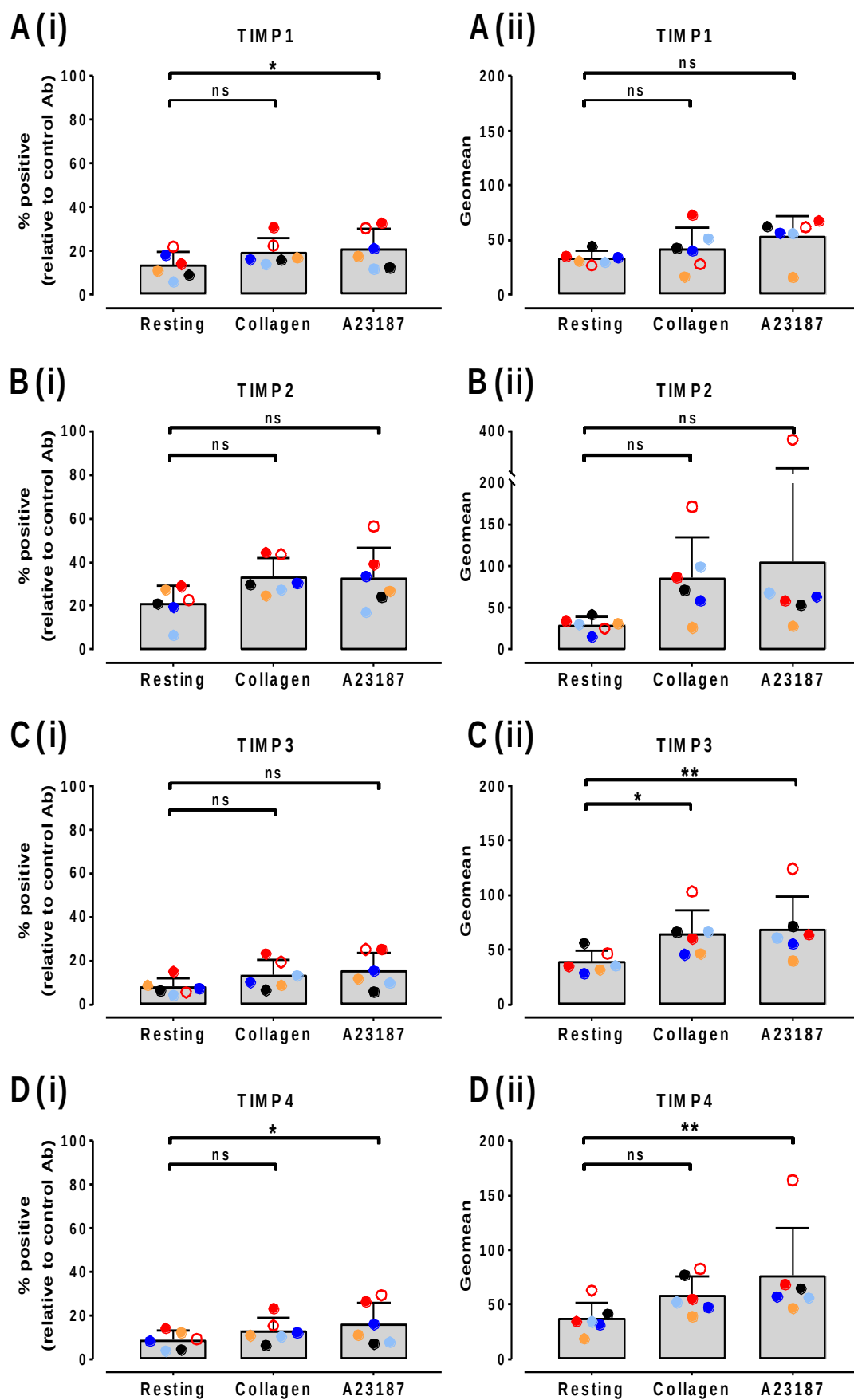


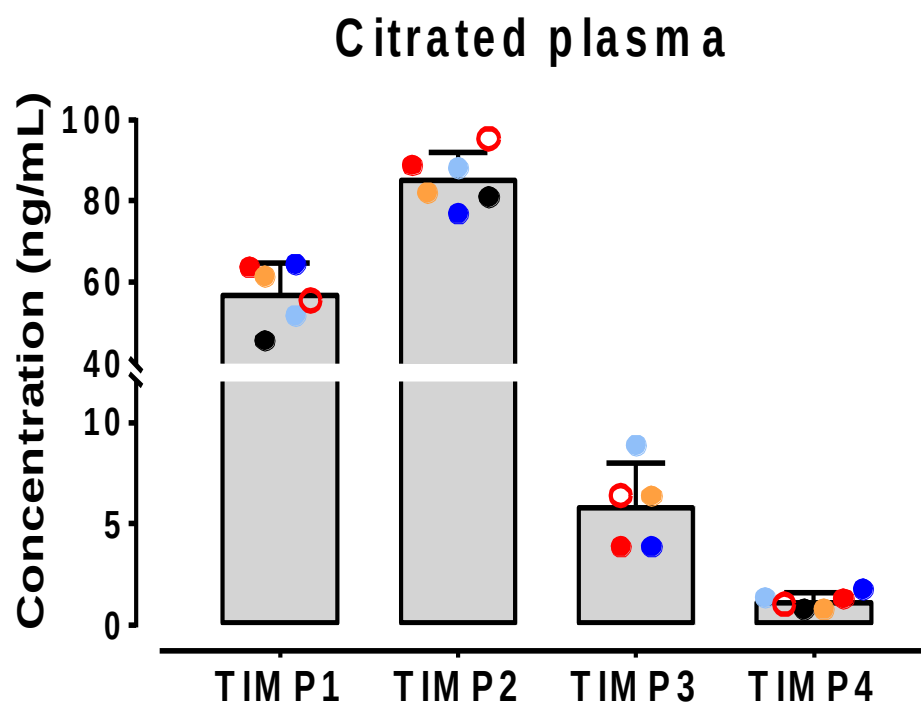
Figure 6.8 Levels of TIMPs on the surface of activated platelets in platelet rich plasma.

Platelet rich plasma from six unique healthy individuals were prepared as described (Section 2.2.2.1) and treated with 30 $\mu\text{g/mL}$ collagen or 10 μM A23187 calcium ionophore for 2 h at RT. Platelet suspensions were made to 50 mM EDTA (final concentration) to stop the reactions at the end of the incubation period. Platelets were then incubated with either one of the anti-hTIMP polyclonal antibodies followed by subsequent labelling with an Alexa Fluor 488-conjugated secondary antibody and acquired on a flow cytometer as described (Section 2.2.8). The level of platelet surface TIMPs were evaluated by Alexa Fluor 488 fluorescence represented as percent positive relative to the Alexa Fluor 488-conjugated secondary antibody (control Ab) (left panels) or Alexa Fluor 488 fluorescence geomean (right panels). Asterisks refer to significance relative to resting platelets. ns, not significant. * $p < 0.05$, ** $p < 0.01$, one-way ANOVA with Dunn's multiple comparisons. Error bars indicate mean $\pm$ SD.

6.3.4 Relationship between circulating TIMPs, platelet surface TIMPs and plasma sGPVI levels

In order to investigate the relationship between platelet-bound and free TIMPs, levels of TIMPs in the plasma of the six healthy individuals presented in Section 6.3.2 and Section 6.3.3 were quantified by ELISA (Section 2.2.9.2). Citrate-anticoagulated plasma was evaluated for levels of the four TIMP isoforms simultaneously in a multiplex format using magnetic microparticles pre-coated with antibodies specific for the four human TIMP isoforms. The emission of luminescence signal was proportional to the amount of TIMP (analyte) bound to the microparticles and quantification was performed by interpolating the luminescence signal against a range of standards with known concentrations of each TIMP isoform.

Human plasma contained higher levels of TIMP1 and TIMP2 relative to TIMP3 and TIMP4 (Figure 6.9). Among the isoforms, levels of TIMP2 was the highest at 85.39 ± 6.592 ng/mL (mean $\pm$ SD) followed by TIMP1 at 57.05 ± 7.497 ng/mL as depicted on Figure 6.9. In contrast, over 10-fold lower concentrations of TIMP3 (5.90 ± 2.087 ng/mL) and TIMP4 (1.20 ± 0.382 ng/mL) were present in the plasma of six healthy donors.



Tukey's multiple comparisons test	Mean diff.	Summary	Adjusted <i>p</i> -value
TIMP1 vs. TIMP2	-28.34	**	0.0048
TIMP1 vs. TIMP3	51.15	***	0.0004
TIMP1 vs. TIMP4	55.85	****	<0.0001
TIMP2 vs. TIMP3	79.49	****	<0.0001
TIMP2 vs. TIMP4	84.19	****	<0.0001
TIMP3 vs. TIMP4	4.703	*	0.0276

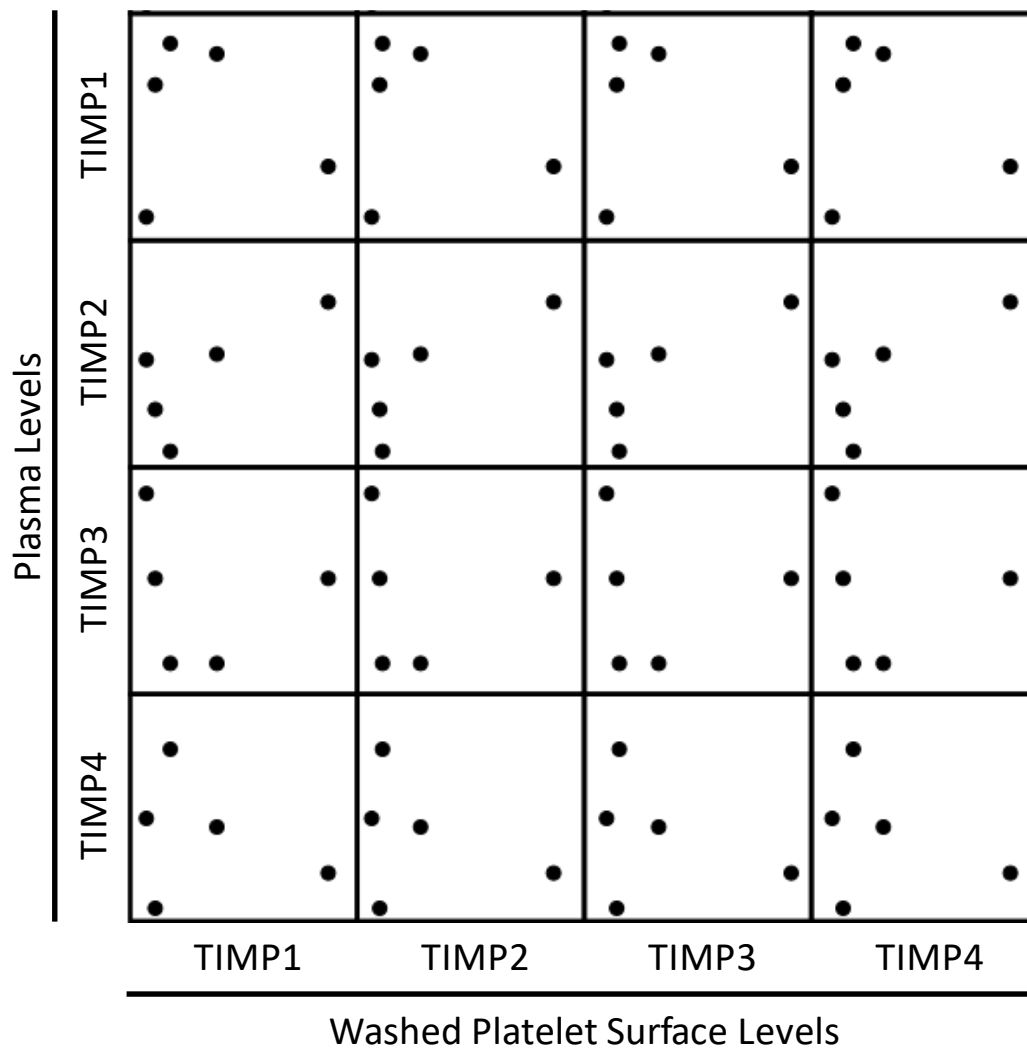
Figure 6.9 Levels of plasma TIMPs in six healthy donors.

Plasma TIMPs were quantified by a magnetic bead-based ELISA approach described in Section 2.2.9.2 using citrate-anticoagulated plasma obtained from six healthy donors as described (Section 2.2.3.1). The levels of the four TIMP isoforms were measured simultaneously in a multiplex format using magnetic microparticles which were pre-coated with antibodies specific for the four human TIMP isoforms. The emission of luminescence signal is proportional to the amount of analyte bound to the microparticles. Quantification of plasma TIMP levels was performed by interpolating the luminescence signal against a range of standards with known concentrations of each TIMP isoform. Error bars indicate mean $\pm$ SD.

This data showed a striking consistency in a trend where TIMP isoform levels on platelets and in plasma matched whereby higher levels of TIMP1 and TIMP2 were detected compared to TIMP3 and TIMP4. The relationship between plasma and surface levels of TIMPs was thus evaluated.

The scatterplot matrix (Figure 6.10) describes the relationships between levels of each TIMP isoform in plasma (y-axis) versus surface-bound TIMPs on resting washed platelets (x-axis) in five healthy donors. The accompanying table summarises the relationship between plasma and surface TIMP isoforms as assessed by Pearson correlation. There was no correlation ($p>0.05$, Figure 6.10) observed between levels of TIMPs in plasma and on the surface of resting washed platelets as assessed in five healthy individuals.

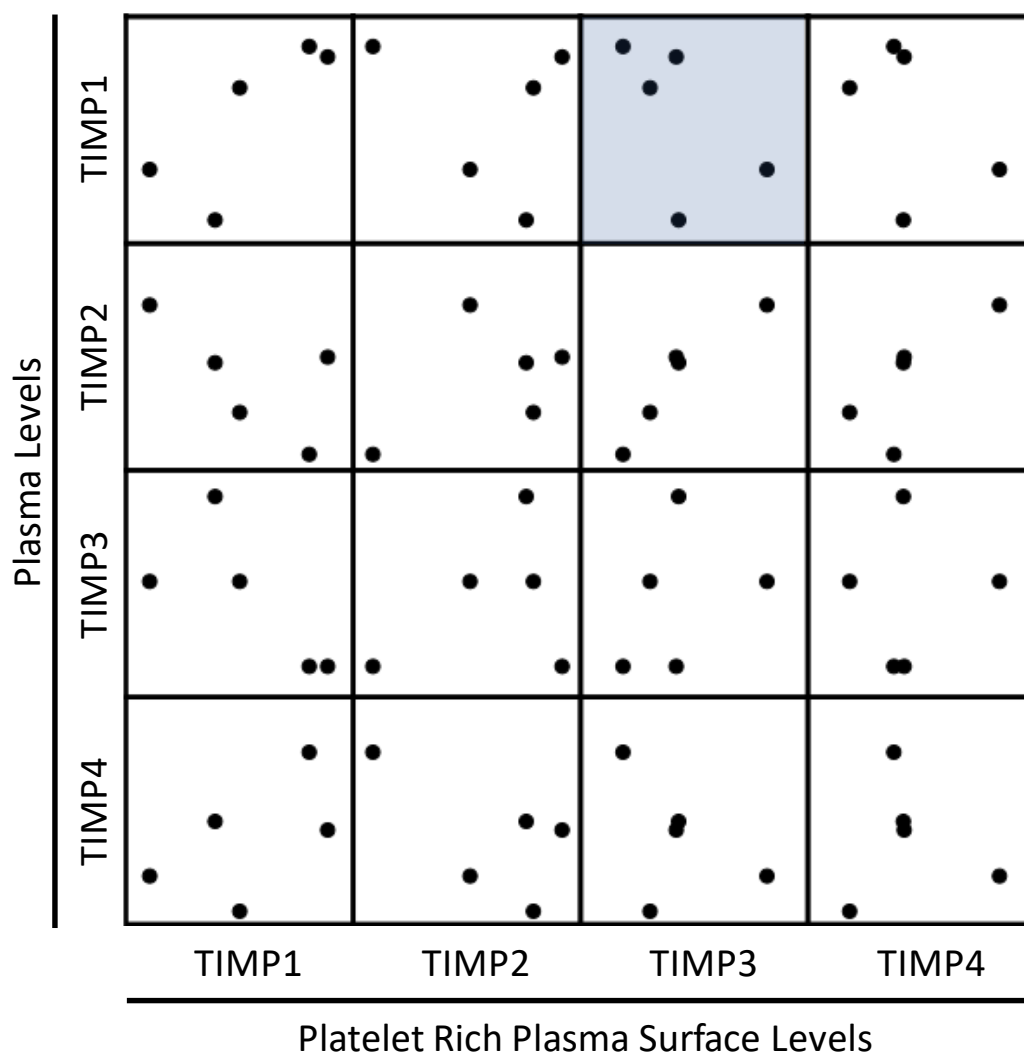
In contrast, a significant negative correlation was apparent (Figure 6.11) between plasma level of TIMP1 and surface-bound TIMP3 in PRP (Pearson correlation = -0.836, $p=0.038$) as highlighted by the shaded plot in the scatterplot matrix. No other correlations between plasma and PRP surface levels achieved statistical significance ($p>0.05$, Figure 6.11).



		Washed platelet surface levels			
Plasma levels		TIMP1	TIMP2	TIMP3	TIMP4
TIMP1	Pearson correlation	0.093	0.016	0.020	0.019
	<i>p</i> -value	0.860	0.976	0.970	0.971
TIMP2	Pearson correlation	0.763	0.779	0.775	0.745
	<i>p</i> -value	0.077	0.068	0.070	0.090
TIMP3	Pearson correlation	-0.149	-0.039	-0.050	-0.082
	<i>p</i> -value	0.810	0.950	0.936	0.896
TIMP4	Pearson correlation	-0.095	-0.160	-0.163	-0.149
	<i>p</i> -value	0.858	0.763	0.757	0.778

Figure 6.10 Relationship between plasma TIMPs and levels of TIMPs on the surface of resting washed platelets.

Plasma TIMPs were quantified by a magnetic bead-based ELISA approach described in Section 2.2.9.2 using citrate-anticoagulated plasma obtained from five healthy donors as described (Section 2.2.3.1). Levels of TIMPs on resting washed platelets from the same individuals were evaluated by flow cytometry as presented in Figure 6.4. Washed platelets were prepared as described (Section 2.2.2.2) and incubated with either one of the anti-hTIMP polyclonal antibodies followed by subsequent labelling with an Alexa Fluor 488-conjugated secondary antibody. Platelets were then acquired on a flow cytometer as described (Section 2.2.8). The level of platelet surface TIMPs were represented as fluorescence geomean. The scatterplot matrix describes the correlations between levels of each TIMP isoform in plasma (y-axis) and on the surface of resting washed platelets (x-axis). The table of correlation values summarises the relationship between plasma and surface TIMP isoforms as assessed by Pearson correlation.



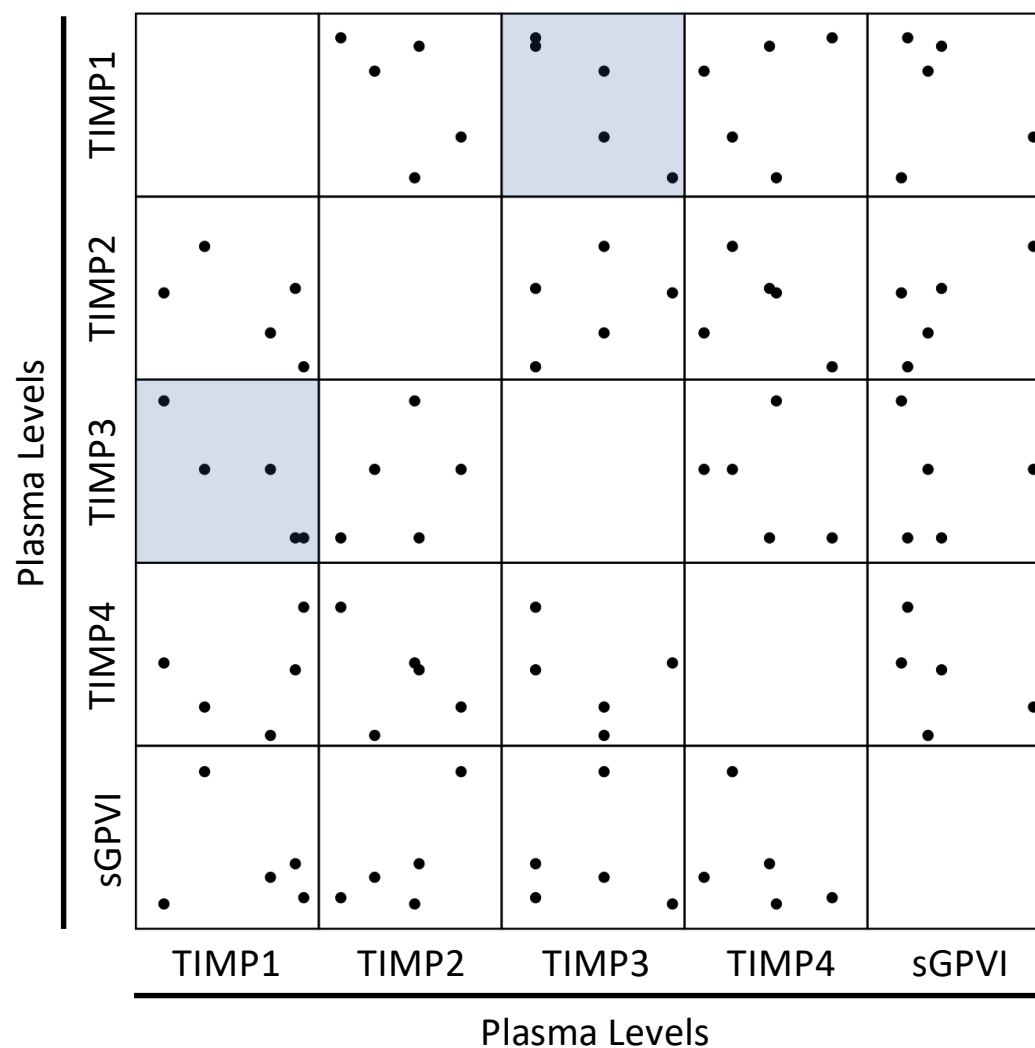
		Platelet rich plasma surface levels			
Plasma levels		TIMP1	TIMP2	TIMP3	TIMP4
TIMP1	Pearson correlation	-0.380	-0.618	-0.836*	-0.396
	p-value	0.458	0.191	0.038	0.437
TIMP2	Pearson correlation	-0.581	0.116	0.244	0.674
	p-value	0.227	0.827	0.642	0.142
TIMP3	Pearson correlation	-0.717	0.336	0.322	0.085
	p-value	0.173	0.580	0.597	0.892
TIMP4	Pearson correlation	-0.193	-0.739	-0.644	-0.107
	p-value	0.714	0.093	0.167	0.840

Figure 6.11 Relationship between plasma TIMPs and levels of TIMPs on the surface of platelets in platelet rich plasma.

Plasma TIMPs were quantified by a magnetic bead-based ELISA approach described in Section 2.2.9.2 using citrate-anticoagulated plasma obtained from five healthy donors as described (Section 2.2.3.1). Levels of TIMPs on resting platelets from the same individuals were evaluated by flow cytometry as presented in Figure 6.4. Platelet rich plasma was prepared as described (Section 2.2.2.1) and incubated with either one of the anti-hTIMP polyclonal antibodies followed by subsequent labelling with an Alexa Fluor 488-conjugated secondary antibody. Platelets were then acquired on a flow cytometer as described (Section 2.2.8). The level of platelet surface TIMPs were represented as fluorescence geomean. The scatterplot matrix describes the correlations between levels of each TIMP isoform in plasma (y-axis) and on the surface of resting platelets (x-axis). Shaded plot represents a significant correlation. The table of correlation values summarises the relationship between plasma and surface TIMP isoforms as assessed by Pearson correlation. Asterisk represents a significant correlation.

As TIMPs are endogenous inhibitors of ADAMs (Section 1.5.3) and human GPVI is predominantly cleaved by ADAM10, the relationship between platelet and plasma TIMPs and circulating sGPVI levels was explored.

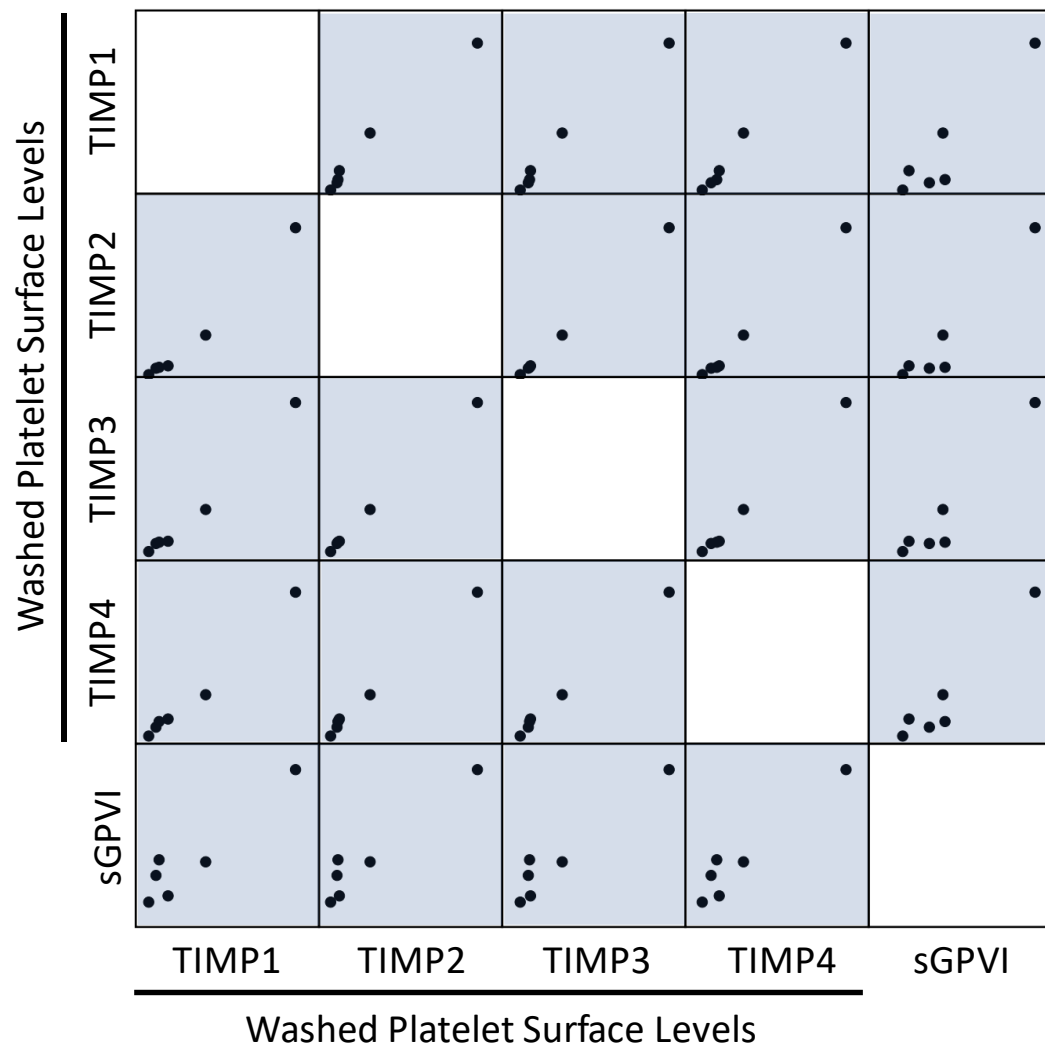
Figure 6.12 shows that plasma concentrations of TIMP1 and TIMP3 were negatively correlated (Pearson correlation = -0.921, $p=0.026$) in samples from five healthy donors. There was no correlation observed between the plasma levels of other TIMP isoforms. Circulating sGPVI levels were also observed to be independent of plasma concentrations of TIMPs (Figure 6.12). Strikingly, a positive linear correlation was found between the surface levels of TIMP isoforms on resting washed platelets whereby the pairwise Pearson correlation values ranged between 0.992 and 1.000 ($p<0.001$) as shown in Figure 6.13. Despite the small sample size of six healthy individuals, levels of TIMPs on the surface of resting platelets were positively correlated with circulating sGPVI levels at Pearson correlation values of 0.936 to 0.958 ($p<0.01$) (Figure 6.13). However, the significant correlations observed in resting washed platelets were not apparent in PRP. As depicted in Figure 6.14, levels of TIMP isoforms were independent of one another on the surface of platelets in PRP. Also, the surface levels of TIMPs were not correlated with plasma sGPVI levels except for TIMP4 (Pearson correlation = 0.836, $p=0.038$) (Figure 6.14). Taken together, the surface levels of TIMP isoforms in the absence of plasma components appear to be positively correlated with levels of circulating sGPVI in a small group of healthy individuals.



		Plasma levels				
Plasma levels		TIMP1	TIMP2	TIMP3	TIMP4	sGPVI
TIMP1	Pearson correlation	1	-0.141	-0.921*	0.527	-0.150
	<i>p</i> -value		0.789	0.026	0.283	0.776
TIMP2	Pearson correlation	-0.141	1	0.370	-0.216	0.708
	<i>p</i> -value	0.789		0.540	0.681	0.115
TIMP3	Pearson correlation	-0.921*	0.370	1	-0.384	-0.030
	<i>p</i> -value	0.026	0.540		0.523	0.962
TIMP4	Pearson correlation	0.527	-0.216	-0.384	1	-0.413
	<i>p</i> -value	0.283	0.681	0.523		0.416
sGPVI	Pearson correlation	-0.150	0.708	-0.030	-0.413	1
	<i>p</i> -value	0.776	0.115	0.962	0.416	

Figure 6.12 Relationship between concentration of TIMPs in plasma and circulating sGPVI levels in healthy individuals.

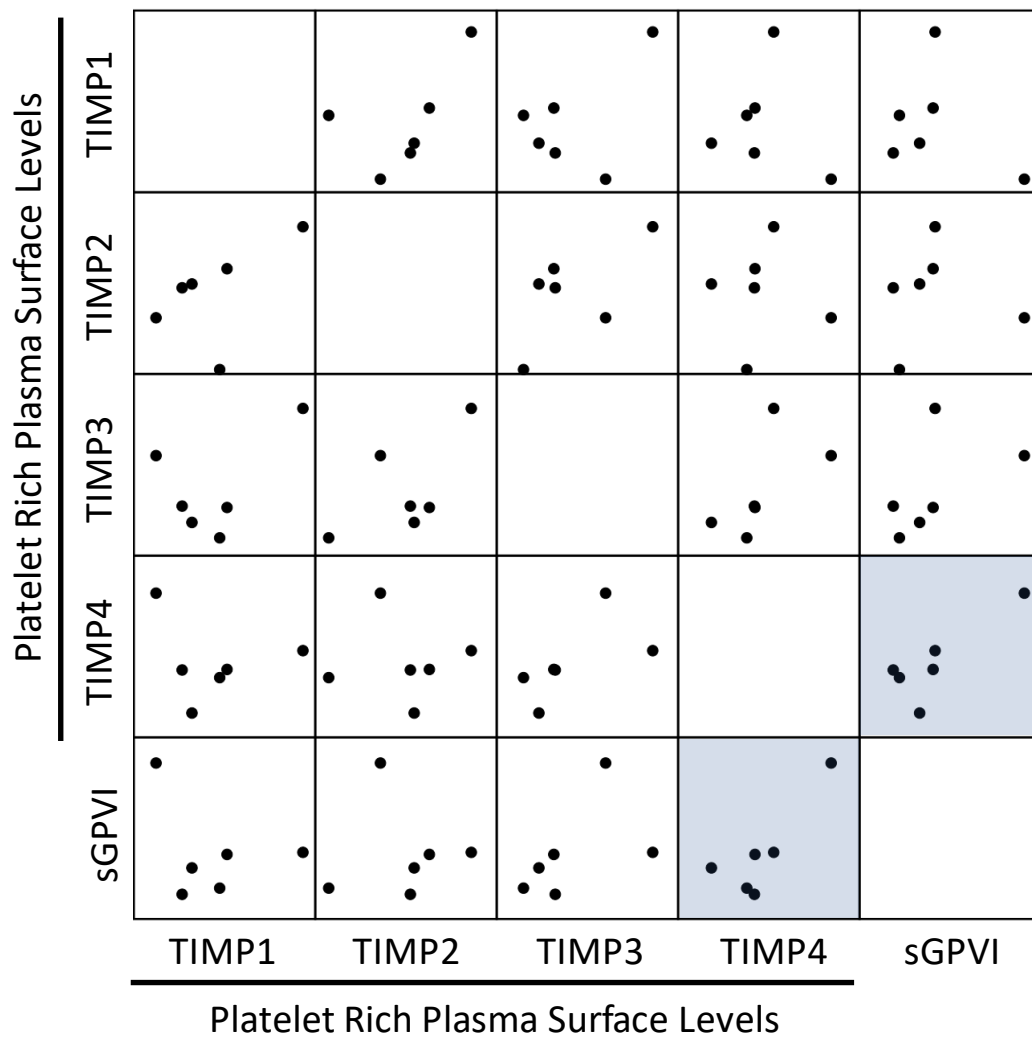
Plasma TIMPs were quantified by a magnetic bead-based ELISA approach described in Section 2.2.9.2 using citrate-anticoagulated plasma obtained from five healthy donors as described (Section 2.2.3.1). Circulating sGPVI levels were also measured in citrate-anticoagulated plasma by sandwich ELISA as described in Section 2.2.9.1. The scatterplot matrix describes the correlations between plasma concentration of each TIMP isoform and circulating sGPVI levels. Shaded plot represents a significant correlation. The table of correlation values summarises the relationship between concentration of TIMPs in plasma and circulating sGPVI levels as assessed by Pearson correlation. Asterisk represents a significant correlation.



		Washed platelet surface levels				Plasma
Washed platelet surface levels		TIMP1	TIMP2	TIMP3	TIMP4	sGPVI
TIMP1	Pearson correlation	1	0.992**	0.993**	0.993**	0.936**
	<i>p</i> -value		0.000	0.000	0.000	0.006
TIMP2	Pearson correlation	0.992**	1	1.000**	0.998**	0.955**
	<i>p</i> -value	0.000		0.000	0.000	0.003
TIMP3	Pearson correlation	0.993**	1.000**	1	0.999**	0.957**
	<i>p</i> -value	0.000	0.000		0.000	0.003
TIMP4	Pearson correlation	0.993**	0.998**	0.999**	1	0.958**
	<i>p</i> -value	0.000	0.000	0.000		0.003
Plasma sGPVI	Pearson correlation	0.936**	0.955**	0.957**	0.958**	1
	<i>p</i> -value	0.006	0.003	0.003	0.003	

Figure 6.13 Relationship between levels of TIMPs on the surface of resting washed platelets and circulating sGPVI levels in healthy individuals.

Levels of TIMPs on resting washed platelets from six healthy donors were evaluated by flow cytometry as presented in Figure 6.4. Washed platelets were prepared as described (Section 2.2.2.2) and incubated with either one of the anti-hTIMP polyclonal antibodies followed by subsequent labelling with an Alexa Fluor 488-conjugated secondary antibody. Platelets were then acquired on a flow cytometer as described (Section 2.2.8). The levels of platelet surface TIMPs were represented as fluorescence geomean. Circulating sGPVI levels in citrate-anticoagulated plasma (Section 2.2.3.1) were measured by sandwich ELISA as described in Section 2.2.9.1. The scatterplot matrix describes the correlations between resting platelet surface levels of each TIMP isoform and circulating sGPVI levels. Shaded plot represents a significant correlation. The table of correlation values summarises the relationship between levels of TIMPs on platelets and circulating sGPVI levels as assessed by Pearson correlation. Asterisk represents a significant correlation.



		Platelet rich plasma surface levels				Plasma
Platelet rich plasma surface levels		TIMP1	TIMP2	TIMP3	TIMP4	sGPVI
TIMP1	Pearson correlation	1	0.559	0.485	-0.177	-0.309
	<i>p</i> -value		0.249	0.329	0.737	0.551
TIMP2	Pearson correlation	0.559	1	0.636	-0.046	0.010
	<i>p</i> -value	0.249		0.174	0.931	0.984
TIMP3	Pearson correlation	0.485	0.636	1	0.621	0.522
	<i>p</i> -value	0.329	0.174		0.188	0.288
TIMP4	Pearson correlation	-0.177	-0.046	0.621	1	0.836*
	<i>p</i> -value	0.737	0.931	0.188		0.038
Plasma sGPVI	Pearson correlation	-0.309	0.010	0.522	0.836*	1
	<i>p</i> -value	0.551	0.984	0.288	0.038	

Figure 6.14 Relationship between levels of TIMPs on the surface of resting platelets in platelet rich plasma and circulating sGPVI levels in healthy individuals.

Levels of TIMPs on resting platelets from six healthy donors were evaluated by flow cytometry as presented in Figure 6.4. Platelet rich plasma was prepared as described (Section 2.2.2.1) and incubated with either one of the anti-hTIMP polyclonal antibodies followed by subsequent labelling with an Alexa Fluor 488-conjugated secondary antibody. Platelets were then acquired on a flow cytometer as described (Section 2.2.8). The levels of platelet surface TIMPs were represented as fluorescence geomean. Circulating sGPVI levels in citrate-anticoagulated plasma (Section 2.2.3.1) were measured by sandwich ELISA as described in Section 2.2.9.1. The scatterplot matrix describes the correlations between resting platelet surface levels of each TIMP isoform and circulating sGPVI levels. Shaded plot represents a significant correlation. The table of correlation values summarises the relationship between levels of TIMPs on platelets and circulating sGPVI levels as assessed by Pearson correlation. Asterisk represents a significant correlation.

6.3.5 ADAM10 activity measurements by FRET assay

The positive correlation observed between levels of TIMPs on the surface of resting washed platelets and plasma sGPVI levels (Figure 6.13, Section 6.3.4) may suggest a role for TIMPs in modulating sGPVI levels through interactions with ADAM10 on platelets. A FRET assay established by Facey and colleagues (2016) was modified to evaluate the effect of TIMPs on ADAM10 activity (Section 2.2.11). This assay utilised a GPVI-Cy3 substrate consisting of a peptide with sequence matching the amino acid residues 236-248 of GPVI that is cleaved by ADAM10 (Figure 2.3, Section 2.2.11). Cleavage of the peptide sequence releases the BHQ2 quencher from the Cy3 fluorophore, thus enabling the detection of fluorescence signal expressed as RFU. Considering that human platelets express both ADAM10 and ADAM17, the specificity of this assay was assessed using rhADAM10 and rhADAM17 (Section 2.2.11.1).

Figure 6.15 tracks the increase in fluorescence over time as GPVI-Cy3 is cleaved by rhADAM10 (Figure 6.15A) or rhADAM17 (Figure 6.15B). Cleavage of GPVI-Cy3 increased with time and enzyme concentrations of either rhADAM10 or rhADAM17. At equimolar concentrations of enzyme and GPVI-Cy3 substrate, rhADAM10 effected 5- to 10-fold more cleavage of substrate above rhADAM17 after 120 min (Figure 6.15C). Pre-treatment of rhADAM10 with a divalent cation chelator (50 mM EDTA), an ADAM10-specific inhibitor (2 μ M GI254023X) or a broad spectrum metalloproteinase inhibitor (100 μ M GM6001) essentially abolished the catalytic ability of rhADAM10 to hydrolyse GPVI-Cy3 substrate as demonstrated in Figure 6.15D. Taken together, cleavage of the GPVI-Cy3 FRET substrate is predominantly mediated by ADAM10, requires divalent cations and is sensitive to metalloproteinase inhibitors. These observations support the use of this assay to monitor ADAM10 activity.

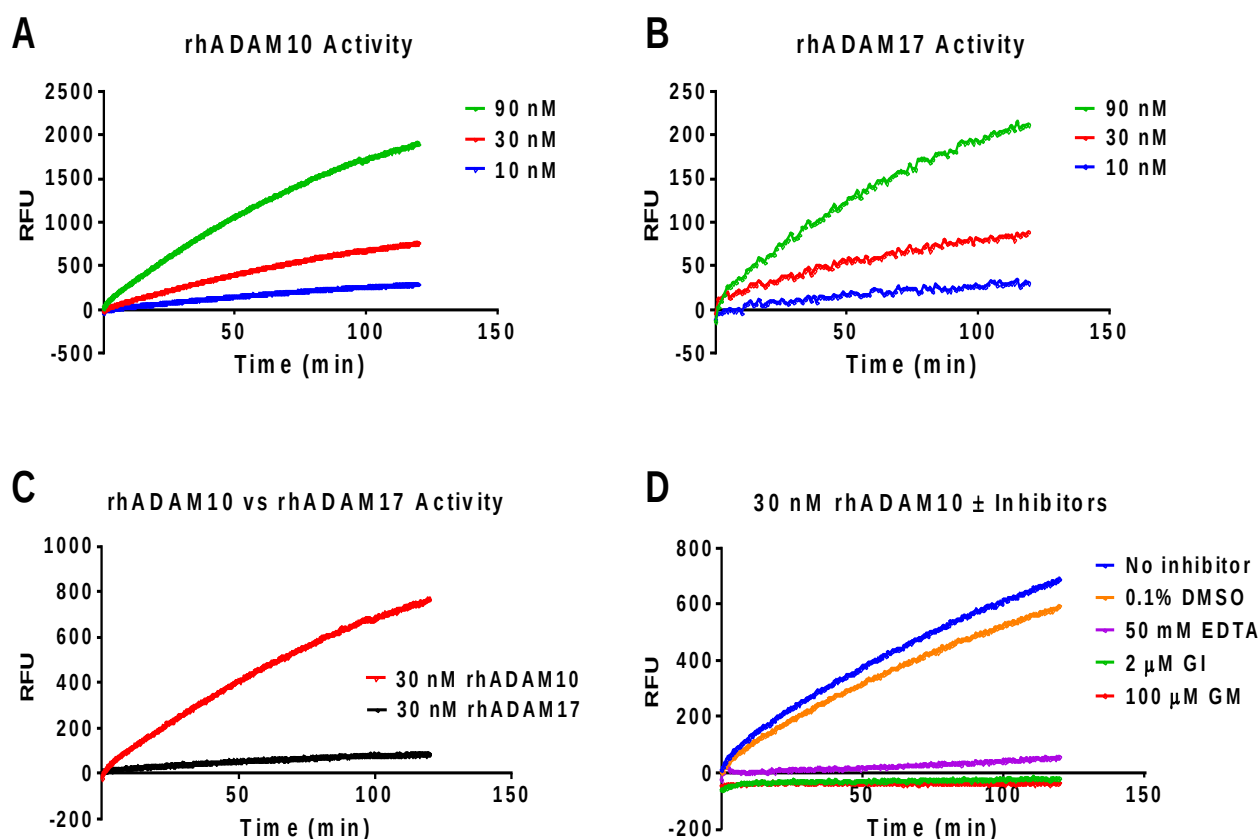


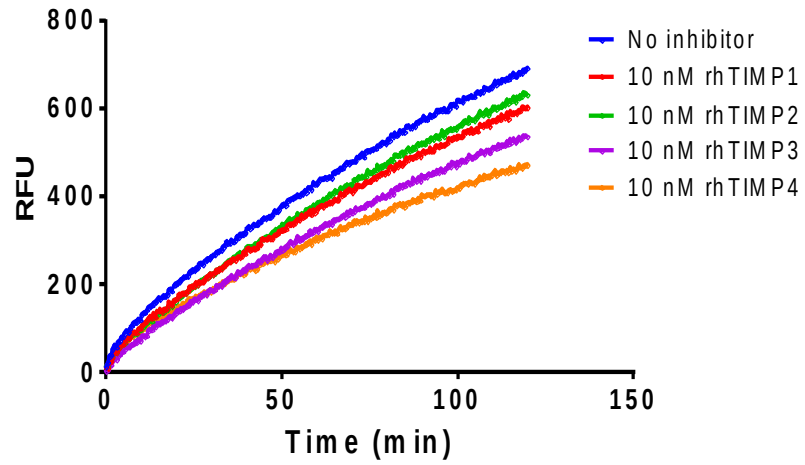
Figure 6.15 GPVI-Cy3 FRET substrate is predominantly cleaved by rhADAM10.

A fluorescence resonance energy transfer assay (FRET) was modified from Facey and colleagues (2016) to evaluate ADAM10 activity. This assay utilised a GPVI-Cy3 substrate consisting of a peptide with sequence matching the amino acid residues 236-248 of GPVI that is cleaved by ADAM10 (Figure 2.3, Section 2.2.11). Cleavage of the peptide sequence releases the BHQ2 quencher from the Cy3 fluorophore, thus enabling the detection of fluorescence signal expressed as relative fluorescence units (RFU) which was corrected for background fluorescence measured in wells containing the buffer and substrate alone. The specificity of this assay was assessed using recombinant human (rh) ADAM10 and ADAM17 as described in Section 2.2.11.1. Panels A and B track the increase in fluorescence over time due to cleavage of GPVI-Cy3 by various concentrations of rhADAM10 and rhADAM17, respectively. Panel C compares the GPVI-Cy3 cleavage activity between rhADAM10 and rhADAM17 at equimolar concentrations. The effect of pre-treatment of rhADAM10 with metalloproteinase inhibitors (50 mM EDTA, 2 μM GI254023X or 100 μM GM6001) or 0.1% DMSO vehicle control on GPVI-Cy3 cleavage activity are presented in panel D. GI, GI254023X (ADAM10-specific inhibitor); GM, GM6001 (broad metalloproteinase inhibitor).

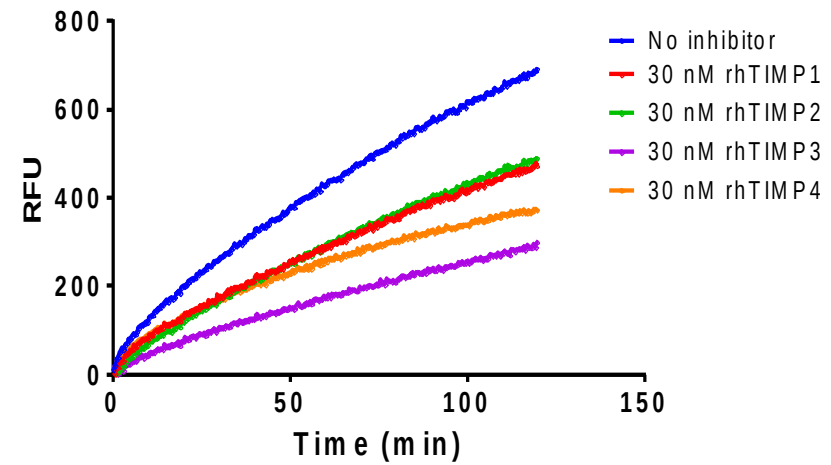
6.3.6 Effect of rhTIMPs on rhADAM10 activity

The ADAMs FRET assay described in Section 6.3.5 was used to investigate the effect of rhTIMPs on rhADAM10 activity on GPVI-Cy3 substrate. 30 nM rhADAM10 was pre-incubated with 0-90 nM of each rhTIMP isoform for 15 min at RT before exposure to the GPVI-Cy3 substrate as described (Section 2.2.11.1). Figure 6.16 shows the fluorescence signals produced by rhADAM10 in the presence of 10 nM (Figure 6.16A), 30 nM (Figure 6.16B) and 90 nM (Figure 6.16C) of rhTIMPs. All four rhTIMP isoforms inhibited rhADAM10 activity to some extent, consistent with published data showing redundancy between TIMP isoforms (Murphy, 2011), and the extent of inhibition was dose-dependent. Figure 6.16D compares the rate of increase in fluorescence (RFU/min) at 20 min calculated by subtracting the RFU at time zero from the RFU at 20 min and dividing the difference by 20 min. The most apparent differences in the rate of activity between the rhTIMP isoforms occurred at the highest concentration of 90 nM. At 90 nM, the extent of inhibition was 2.4-fold with rhTIMP1, 1.4-fold with rhTIMP2, 4.3-fold with rhTIMP3 and 2.0-fold with rhTIMP4, relative to the rate of rhADAM10 activity at 20 min in the absence of inhibitors (Figure 6.16D). rhTIMP3 showed the greatest inhibition of rhADAM10 activity rate at all the concentrations tested, consistent with the literature which shows that TIMP3 preferentially inhibits ADAM10 (Scilabra et al., 2018).

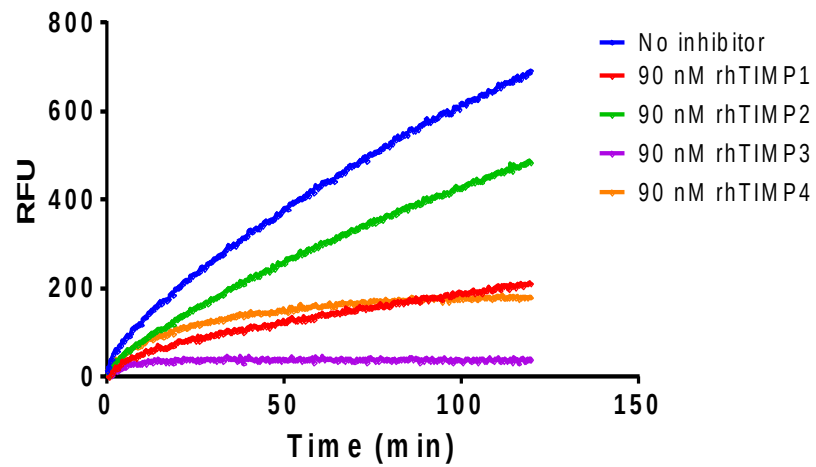
A 10 nM rhTIMPs



B 30 nM rhTIMPs



C 90 nM rhTIMPs



D Rate at 20 min

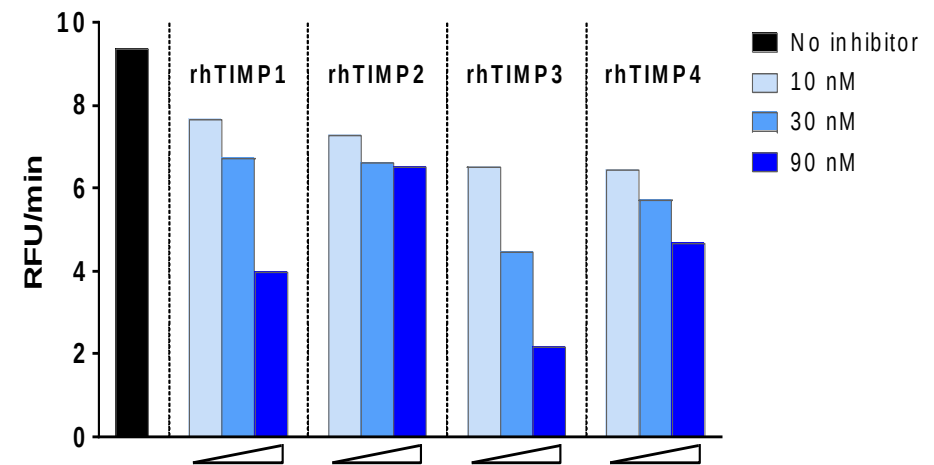


Figure 6.16 All four rhTIMP isoforms inhibited rhADAM10 activity in a dose-dependent manner.

A fluorescence resonance energy transfer assay (FRET) was modified from Facey and colleagues (2016) to evaluate the effect of rhTIMPs on rhADAM10 activity. This assay utilised a GPVI-Cy3 substrate consisting of a peptide with sequence matching the amino acid residues 236-248 of GPVI that is cleaved by ADAM10 (Figure 2.3, Section 2.2.11). Cleavage of the peptide sequence releases the BHQ2 quencher from the Cy3 fluorophore, thus enabling the detection of fluorescence signal expressed as relative fluorescence units (RFU) which was corrected for background fluorescence measured in wells containing the buffer and substrate alone. 30 nM rhADAM10 was pre-incubated with 10 nM, 30 nM or 90 nM of each rhTIMP isoform for 15 min at RT before exposure to the GPVI-Cy3 substrate as described (Section 2.2.11.1). The fluorescence signals produced by rhADAM10 in the presence of 10 nM, 30 nM or 90 nM rhTIMPs are depicted in panels A, B and C, respectively. Panel D compares the rate of increase in fluorescence (RFU/min) at 20 min calculated by subtracting the RFU at time zero from the RFU at 20 min and dividing the difference by 20 min.

6.3.7 Effect of rhTIMPs on native platelet ADAM10 activity under resting conditions

As all four rhTIMP isoforms inhibited rhADAM10 activity to various extents, the experimental design in Section 6.3.6 was modified to assess the effect of rhTIMPs on native platelet ADAM10 activity in washed platelets. In place of rhADAM10, washed platelets (Section 2.2.2.2), were used as the source of enzyme in the FRET assay. Washed platelets (10^7) were pre-treated with 1 $\mu\text{g}/\text{mL}$ of rhTIMPs, 100 μM GM6001, 2 μM GI254023X, 50 mM EDTA or 0.1% DMSO vehicle control for 30 min at RT before exposure to 1 μM GPVI-Cy3 substrate as described (Section 2.2.11.2). Fluorescence signal was continuously monitored for 1.5 h at 37°C. The fluorescence signal released from the cleavage of GPVI-Cy3 was expressed in RFU and corrected for background fluorescence measured in wells containing the buffer and substrate alone.

Figure 6.17A illustrates the cleavage of GPVI-Cy3 mediated by 10^7 washed platelets from one healthy donor. The blue curve (untreated sample) shows ADAM10 activity on resting washed platelets. Among the rhTIMP isoforms, rhTIMP3 showed the greatest inhibition of platelet ADAM10 activity. As expected, pre-treatment of platelets with an ADAM10-specific inhibitor (2 μM GI254023X), a broad spectrum metalloproteinase inhibitor (100 μM GM6001) or a divalent cation chelator (50 mM EDTA) markedly diminished platelet ADAM10 activity (Figure 6.17A).

The effect of these inhibitors on platelet ADAM10 rate of activity in three to six unique healthy individuals was evaluated (Figure 6.17B). The rate of activity (RFU/min) is calculated by subtracting the RFU at time zero from the RFU at 20 min and dividing the difference by 20 min. The rate of ADAM10 activity on resting platelets were variable among six healthy donors with a mean $\pm$ SD of 88.78 ± 37.23 RFU/min. Consistent with the effect on rhADAM10, rhTIMP3

significantly reduced resting platelet ADAM10 activity rate by 2.3-fold at 38.76 ± 22.66 RFU/min ($p=0.0054$). Platelet ADAM10 activity was essentially abolished ($p<0.0001$) in the presence of metalloproteinase inhibitors whereby a 24.6-fold, 9.4-fold and 16.6-fold reduction in rate of activity was observed with 100 μ M GM6001, 2 μ M GI254023X and 50 mM EDTA, respectively (Figure 6.17B). The inhibition of platelet ADAM activity by rhTIMP3 represented 63% of that obtained using the ADAM10-specific inhibitor GI254023X. Pre-treatment of platelets with rhTIMP1 did not significantly reduce ADAM10 activity rate ($p=0.2192$). In contrast, the mean rate of platelet ADAM10 activity remained stable in the presence of rhTIMP2 (89.90 ± 33.34 RFU/min, $p=0.9999$) or rhTIMP4 (94.09 ± 19.49 RFU/min, $p=0.9994$) (Figure 6.17B).

The relationship between ADAM10 activity on resting washed platelets and circulating sGPVI levels was explored in a limited group of six healthy individuals. The rate of hydrolysis of the GPVI-Cy3 substrate was used as a measure of the catalytic activity of active ADAM10 on donor platelets. sGPVI levels were quantified in plasma samples of these six donors as previously described (Section 2.2.9.1). Pearson correlation analysis (Figure 6.17C) revealed a correlation score of $r=0.0952$ which was not statistically significant ($p=0.8576$) and thus, suggests that ADAM10 activity rate quantified here may not be directly proportional to circulating sGPVI levels in a small sample size of six donors, and that other factors influence sGPVI levels.

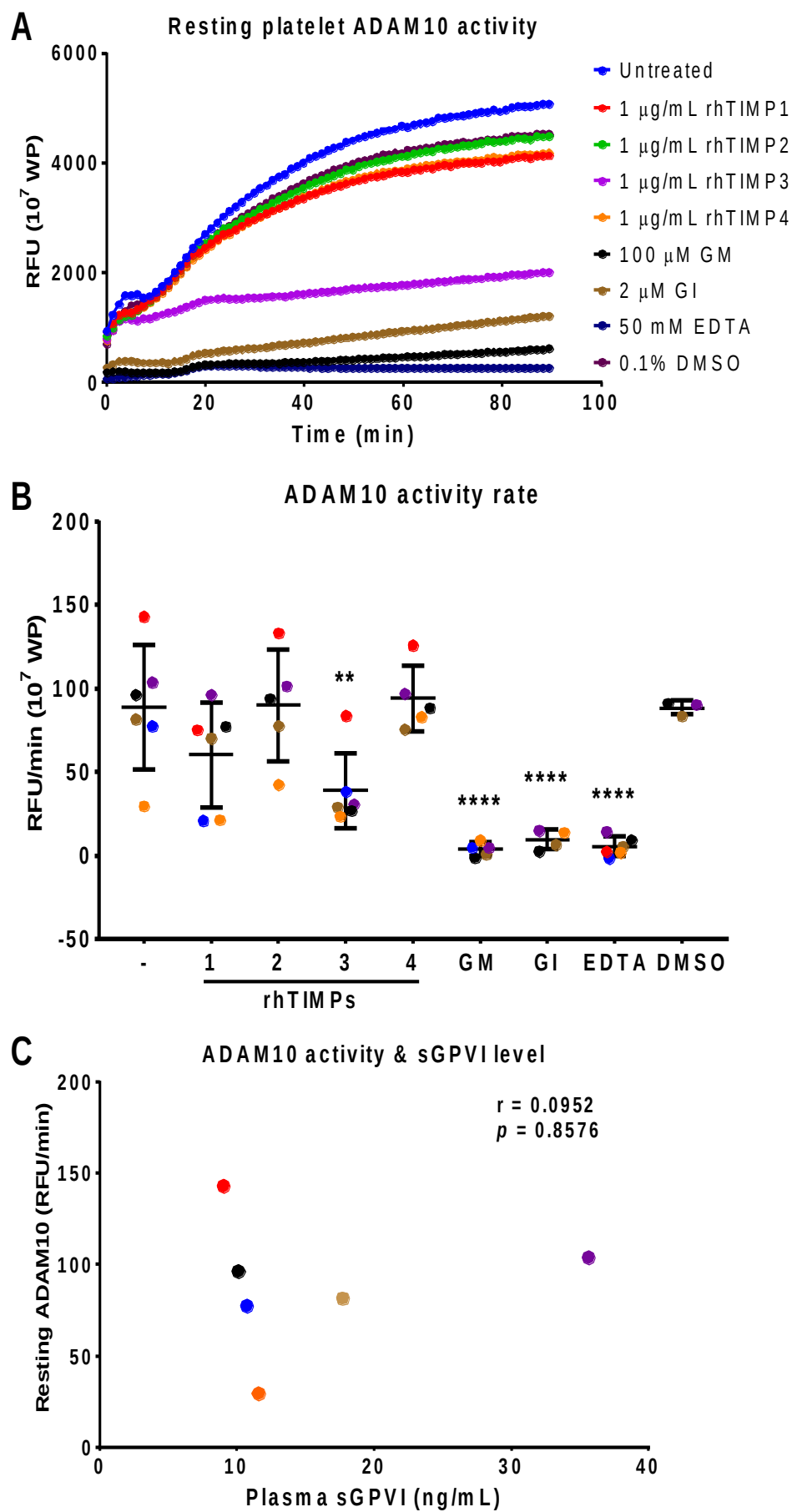


Figure 6.17 Effect of rhTIMP isoforms on resting platelet ADAM10 activity.

A fluorescence resonance energy transfer assay (FRET) was modified from Facey and colleagues (2016) to evaluate the effect of rhTIMPs on platelet ADAM10 activity. This assay utilised a GPVI-Cy3 substrate consisting of a peptide with sequence matching the amino acid residues 236-248 of GPVI that is cleaved by ADAM10 (Figure 2.3, Section 2.2.11). Cleavage of the peptide sequence releases the BHQ2 quencher from the Cy3 fluorophore, thus enabling the detection of fluorescence signal expressed as relative fluorescence units (RFU) which was corrected for background fluorescence measured in wells containing the buffer and substrate alone. 10^7 washed platelets from healthy individuals were prepared as described (Section 2.2.2.2). To evaluate the effect of metalloproteinase inhibitors, 10^7 washed platelets were pre-treated with 1 $\mu\text{g/mL}$ of rhTIMPs, 100 μM GM6001, 2 μM GI254023X, 50 mM EDTA or 0.1% DMSO vehicle control for 30 min at RT before exposure to 1 μM GPVI-Cy3 substrate as described (Section 2.2.11.2). Fluorescence signal was continuously monitored for 1.5 h at 37°C. Panel A illustrates the kinetic curves observed in 10^7 washed platelets from one healthy donor. Panel B compares the effect of the metalloproteinase inhibitors on the rate of ADAM10 activity in three to six healthy donors. The rate of activity was calculated by subtracting the RFU at time zero from the RFU at 20 min and dividing the difference by 20 min. GI, GI254023X (ADAM10-specific inhibitor); GM, GM6001 (broad metalloproteinase inhibitor). ** $p < 0.01$, **** $p < 0.0001$, one-way ANOVA with Dunnett's multiple comparisons. Error bars indicate mean $\pm$ SD. Panel C shows the relationship between rate of platelet ADAM10 activity on resting washed platelets and sGPVI levels in plasma samples of six healthy donors (Section 2.2.3.1) quantified using an established sandwich ELISA approach as previously described (Section 2.2.9.1). Pearson correlation analysis was performed to evaluate the influence of ADAM10 rate of activity on circulating sGPVI levels.

6.3.8 Effect of rhTIMPs on activated platelet ADAM10 activity

As rhTIMP3 reduced the rate of ADAM10 activity on resting platelets (Figure 6.17), the effect of rhTIMPs on platelet ADAM10 activity was explored under conditions of platelet activation. 10^7 washed platelets from healthy donors (Section 2.2.2.2) were pre-treated with 1 $\mu\text{g/mL}$ of rhTIMPs, 2 μM GI254023X or 50 mM EDTA for 30 min at RT before exposure to 1 μM GPVI-Cy3 substrate in the presence of 10 $\mu\text{g/mL}$ CRP-xL, 10 $\mu\text{g/mL}$ collagen, calcium ionophore (10 μM A23187) or a potent activator of ADAM10 (5 mM NEM) as described (Section 2.2.11.2). Fluorescence signal was continuously monitored for 1.5 h at 37°C. The fluorescence signal released from the hydrolysis of GPVI-Cy3 was expressed in RFU and corrected for background fluorescence measured in wells containing the buffer and substrate alone.

Among all the four rhTIMP isoforms tested, only rhTIMP3 achieved a statistically significant reduction in ADAM10 activity rate in activated platelets (Figure 6.18). On exposure to GPVI agonists, calcium ionophore or a potent activator of ADAM10, pre-treatment of platelets with rhTIMP3 significantly lowered the rate of ADAM10 activity by 1.9- to 3.2-fold compared to untreated platelets ($p < 0.05$, Figure 6.18C), consistent with the effect observed on resting platelets (Figure 6.17B). However, the extent of ADAM10 inhibition by rhTIMP3 was inferior to the ADAM10-specific inhibitor GI254023X (Figure 6.18E) or EDTA (Figure 6.18F). Platelets treated with GI254023X (Figure 6.18E) showed a 7.2- to 12.0-fold lower rate of ADAM10 activity on activated platelets while the presence of EDTA (Figure 6.18F) reduced the rate of activity by 6.9- to 15.9-fold.

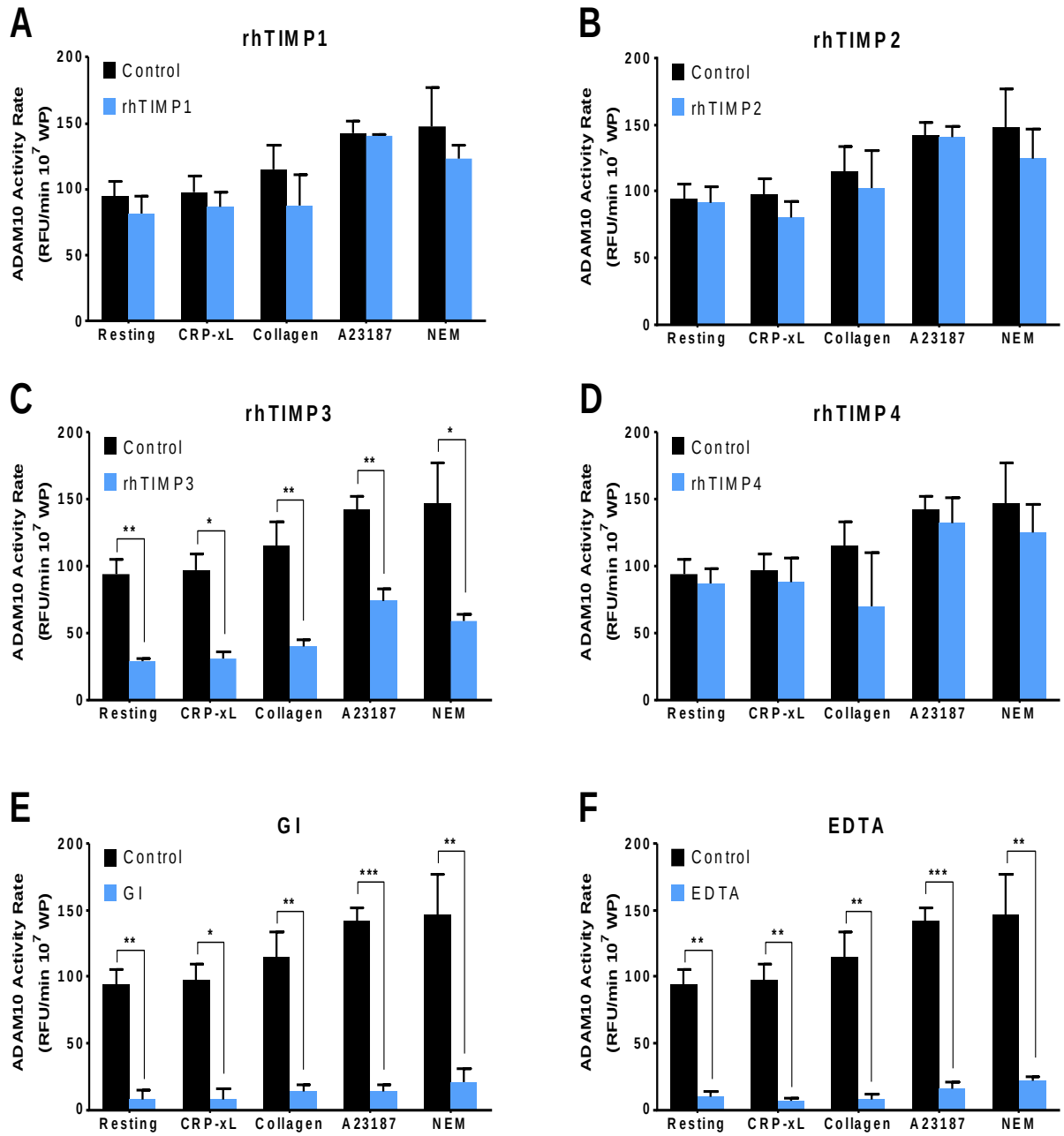


Figure 6.18 Effect of rhTIMP isoforms on activated platelet ADAM10 activity.

A fluorescence resonance energy transfer assay (FRET) was performed to evaluate the effect of rhTIMPs on platelet ADAM10 activity. This assay utilised a GPVI-Cy3 substrate consisting of a peptide with sequence matching the amino acid residues 236-248 of GPVI that is cleaved by ADAM10 (Figure 2.3, Section 2.2.11). Cleavage of the peptide sequence releases the BHQ2 quencher from the Cy3 fluorophore, thus enabling the detection of fluorescence signal expressed as relative fluorescence units (RFU) which was corrected for background fluorescence measured in wells containing the buffer and substrate alone. 10^7 washed platelets from healthy donors (Section 2.2.2.2) were pre-treated with 1 $\mu\text{g/mL}$ of rhTIMPs (A to D), 2 μM GI254023X (E) or 50 mM EDTA (F) for 30 min at RT before exposure to 1 μM GPVI-Cy3 substrate in the presence of GPVI agonists (10 $\mu\text{g/mL}$ CRP-xL or 10 $\mu\text{g/mL}$ collagen), calcium ionophore (10 μM A23187) or a potent activator of ADAM10 (5 mM NEM) as described (Section 2.2.11.2). Fluorescence signal was continuously monitored for 1.5 h at 37°C. The rate of activity was calculated by subtracting the RFU at time zero from the RFU at 20 min and dividing the difference by 20 min. GI, GI254023X (ADAM10-specific inhibitor); CRP-xL, crosslinked collagen-related peptide; NEM, N-ethyl maleimide. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, statistical significance between pairs were determined using the Holm-Sidak method. Error bars indicate mean $\pm$ SD.

6.3.9 Effect of rhTIMPs on GPVI release upon platelet activation

Among the rhTIMP isoforms, inhibition of platelet ADAM10 activity under resting and activated conditions was unique to rhTIMP3 as presented in Figure 6.17 and Figure 6.18. The interaction between rhTIMP3 and platelet ADAM10, and its implication for GPVI shedding was further explored. Washed platelets from three unique healthy donors, prepared as described (Section 2.2.2.2), were pre-treated with 1 µg/mL of rhTIMPs, 2 µM GI254023X (ADAM10-specific inhibitor) or 50 mM EDTA for 30 min at RT. Platelets were subsequently stimulated with 10 ng/mL convulxin, 30 µg/mL CRP-xL, 10 µg/mL collagen or calcium ionophore (10 µM A23187) for 2 h at RT. At the end of the incubation period, platelet suspensions were made to 50 mM EDTA and levels of sGPVI in the platelet supernatant was quantified by ELISA (Section 2.2.9.1) to assess the effect of rhTIMPs on GPVI shedding in activated platelets.

Activation of washed platelets increased sGPVI levels from 12.09 ± 0.88 ng/mL (mean $\pm$ SD) in resting platelets to 46.93 ± 4.84 ng/mL with convulxin, 35.09 ± 8.26 ng/mL with CRP-xL, 23.06 ± 5.57 ng/mL with collagen and 137.88 ± 4.01 ng/mL with calcium ionophore treatments (Figure 6.19). Surprisingly, pre-treatment with rhTIMP3 did not alter release of sGPVI on activated platelets. A 1.3-fold reduction in convulxin-triggered sGPVI release was achieved by rhTIMP2 ($p=0.0059$, Figure 6.19B) while rhTIMP4 enhanced the release of sGPVI triggered by calcium ionophore by 1.1-fold ($p=0.0322$, Figure 6.19D).

As shown in Figure 6.19E, ADAM10 was responsible for shedding of GPVI induced by GPVI agonists or calcium ionophore whereby pre-treatment of platelets with the ADAM10-specific inhibitor GI254023X diminished the levels of sGPVI released by 1.5- to 4.7-fold ($p<0.05$), depending on the agonists used. EDTA markedly reduced the levels of sGPVI liberated by greater than 2-fold in all conditions of platelet activation (Figure 6.19F).

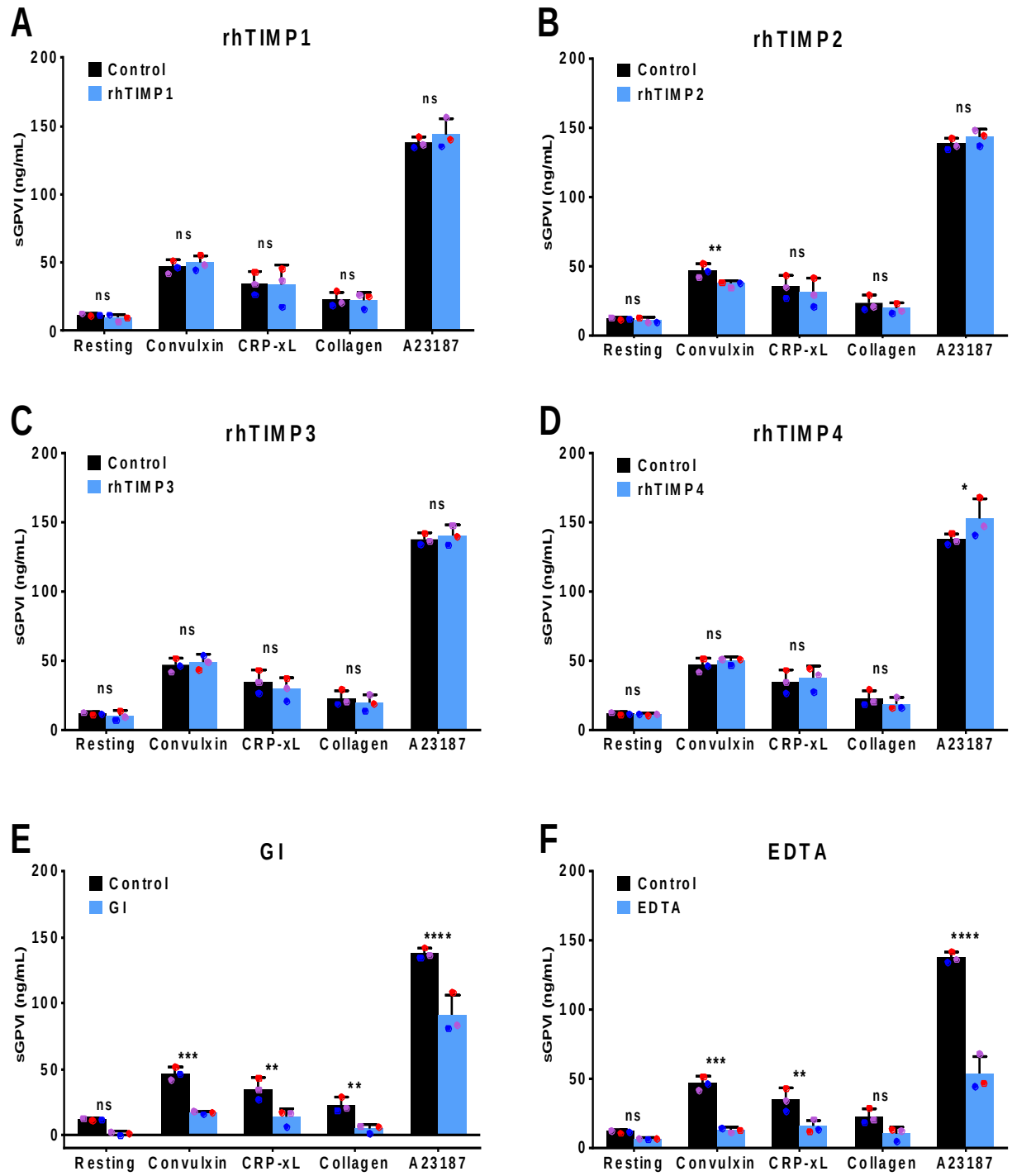


Figure 6.19 Effect of rhTIMP isoforms on release of sGPVI in activated platelets.

Washed platelets from three unique healthy donors, prepared as described (Section 2.2.2.2), were pre-treated with 1 µg/mL of rhTIMPs (A to D), 2 µM GI254023X (E) or 50 mM EDTA (F) for 30 min at RT. Platelets were subsequently stimulated with GPVI agonists (10 ng/mL convulxin, 30 µg/mL CRP-xL or 10 µg/mL collagen) or calcium ionophore (10 µM A23187) for 2 h at RT. At the end of the incubation period, platelet suspensions were made to 50 mM EDTA final concentration to stop the reactions. Levels of sGPVI in the platelet supernatant was quantified by a sandwich ELISA approach as described in Section 2.2.9.1. Two-way ANOVA with Sidak's multiple comparison tests were performed to assess the effect of rhTIMPs on GPVI shedding in activated platelets. GI, GI254023X (ADAM10-specific inhibitor); CRP-xL, crosslinked collagen-related peptide; ns, not significant. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Error bars indicate mean $\pm$ SD.

6.4 Discussion

Published studies have reported expression of TIMPs in platelet lysates using techniques such as Western blotting and mass spectrometry as summarised in Table 1.3 (Section 1.5.3) while Villeneuve and colleagues (2009) observed sub-cellular localisation of TIMPs in platelets by immunofluorescence. However, platelet surface expression of TIMPs has not yet been explored. This chapter assessed the platelet surface expression of TIMPs and the role of TIMPs in regulating platelet ADAM10 activity. Firstly, levels of the four TIMP isoforms on the surface of resting and activated platelets were evaluated by flow cytometry in healthy individuals. Then, the relationship between plasma concentration of TIMPs, levels of TIMPs on the platelet surface and circulating sGPVI levels were explored. The effect of rhTIMPs on healthy donor platelet ADAM10 activity was assessed by a FRET-based approach under resting and activated conditions. Finally, the implication of rhTIMPs and platelet ADAM10 interactions on GPVI shedding was investigated.

All four TIMP isoforms were detectable by flow cytometry on washed platelets and PRP under resting conditions. This observation is supported by the detection of TIMP1, TIMP2 and TIMP3 in platelet lysates by mass spectrometry (Burkhart et al., 2012). Although TIMP4 was detected at low levels by flow cytometry, this isoform was not detected in the platelet proteome (Burkhart et al., 2012) but was strongly detected via Western blot analysis of platelet lysates (Radomski et al., 2002). The lack of concordance in TIMP4 detection in previous studies may be related to differences in the detection technique or antibody used. In line with the relative abundance of TIMP transcripts in platelets (TIMP1 > TIMP2 > TIMP3 > TIMP4) reported by Cecchetti and colleagues (2011), higher levels of surface TIMP1 and TIMP2 were observed compared to surface TIMP3 and TIMP4 on washed platelets and PRP. Intriguingly, TIMP4

mRNA was absent in the platelet transcriptome despite low levels of TIMP4 transcript detected in megakaryocytes (Cecchetti et al., 2011). Detection of TIMP4 on the platelet surface, within sub-cellular platelet compartments (Villeneuve et al., 2009) and in platelet lysate (Radomski et al., 2002) may suggest the ability of platelets to take up TIMPs from plasma and/or transfer of TIMPs from megakaryocyte progenitors.

The plasma concentrations of TIMP1 (57.05 ± 7.497 ng/mL), TIMP2 (85.39 ± 6.592 ng/mL), TIMP3 (5.90 ± 2.087 ng/mL) and TIMP4 (1.20 ± 0.382 ng/mL) in healthy individuals reported here are comparable to previous reports (Palei et al., 2008, Su et al., 2017, Hendrickson et al., 2018, Cavusoglu et al., 2017). All four TIMP isoforms were more readily detectable on washed platelets compared to PRP. This may be due to specific or non-specific steric hindrance by plasma components which may reduce antibody binding consistent with higher levels of antibody binding detected in washed platelet preparations compared to PRP. Of note, the variation in platelet associated TIMPs between donors were smaller in the PRP system compared to washed platelets. This may be attributed to a relatively consistent level of non-membrane associated TIMPs between individuals and may reflect binding properties of the free form of each TIMP relative to the membrane-bound TIMP, serving to reduce the variability in surface TIMP levels observed in PRP.

Levels of surface TIMPs were increased on activated platelets compared to resting platelets notably in response to calcium ionophore (A23187) treatment. Villeneuve and colleagues (2009) observed an upregulation of all four TIMP isoforms in the supernatant of thrombin-activated platelets, while Fong and colleagues (2011) reported the release of TIMP1, TIMP2 and TIMP3 by PMA-activated platelets. These findings suggest that platelet degranulation may mobilise an intracellular store of TIMPs to the membrane surface. Equally possible, platelet

activation/degranulation results in changes to membrane properties, for example, exposure of P-selectin and phosphatidylserine, which may favour localisation of plasma TIMPs to the platelet membrane. CD63, a glycoprotein found in dense granules which is released upon platelet activation, is a surface receptor for TIMP1 (Jung et al., 2006). Increase in the levels of surface TIMPs could presumably occur through interactions with CD63 and other surface receptors which are exposed upon platelet activation.

The levels of plasma sGPVI were positively correlated with the surface levels of all four TIMP isoforms on resting washed platelets. In line with the increase of TIMPs observed on activated platelets, TIMPs may behave as markers of platelet activation. Increased plasma levels of TIMP1 have been reported in patients with hypertension, and is associated with congestive heart failure (Lieb et al., 2019), consistent with reports that platelets are activated in heart failure (Chung and Lip, 2006). Increased plasma levels of TIMP1 and TIMP2 were also observed in patients with acute coronary syndrome (Derosa et al., 2007). In addition, plasma TIMP3 levels were increased in patients with abdominal aortic aneurysms (Lamblin et al., 2010). Of note, the authors also found a significant positive correlation between plasma levels of TIMP3 and soluble glycoprotein V, a platelet activation marker, in these patients. Radomski and colleagues (2002) reported the release of TIMP4 upon platelet aggregation induced by collagen and thrombin. Taken together, these findings support a potential role for TIMPs as platelet activation markers. However, the data presented here is limited by the small sample size of healthy donors and therefore, warrants further investigation in a larger cohort to assess changes in the levels of TIMPs upon platelet activation. Nevertheless, the lack of correlation between levels of plasma TIMPs and surface TIMPs may suggest that a level of control exist in TIMP localisation, which prevents the respective TIMP levels from reaching an equilibrium.

rhTIMP1 and rhTIMP3 reduced resting platelet ADAM10 activity while only rhTIMP3 reduced ADAM10 rate of activity when platelets were stimulated. This observation is supported by the inhibitory effect of rhTIMP1 and rhTIMP3 on rhADAM10 activity presented here and elsewhere (Amour et al., 2000). The unique inhibitory effect of rhTIMP3 on activated platelet ADAM10 activity may reflect molecular differences in conformation of the TIMP isoforms. Crystallography studies by Wisniewska and colleagues (2008) revealed that the N-terminal domain of TIMP3 is more tightly packed in the catalytic site of ADAM17 which may allow favourable interactions and confer greater binding efficiency. This finding concurs with the data presented here whereby TIMP3 appeared to be the most effective TIMP isoform in inhibiting platelet ADAM10 activity.

Although rhTIMP3 exhibited an inhibitory effect on ADAM10 activity, the lower ADAM10 activity did not translate to a lower sGPVI release in a shedding assay *in vitro*. In healthy donor washed platelets, rhTIMP3 as well as other TIMPs did not protect GPVI from shedding upon stimulation with convulxin, collagen, CRP-xL or calcium ionophore despite a reduction in platelet ADAM10 activity by rhTIMP3 in the presence of these agonists. There was also a lack of correlation between resting platelet ADAM10 activity, TIMP levels and circulating sGPVI levels. As the rate of activity was based on the cleavage of the GPVI-Cy3 substrate which was readily accessible in the platelet suspension at a concentration that was in excess of the membrane bound GPVI on platelets, these physical differences may account for the lack of translation between ADAM10 activity and levels of sGPVI released. Considering that shedding of GPVI on activated platelets was protected in the presence of an ADAM10 small molecule inhibitor, GI254023X (391.5 Da) or EDTA (292.2 Da), the relatively larger size of rhTIMP3 (24 kDa) may restrict its access to the GPVI cleavage site of ADAM10. This discordance between ADAM10 activity and its product (sGPVI) is reminiscent of the findings of Facey and colleagues

(2016) who demonstrated that an increase in the levels of sGPVI released can occur without an increase in the catalytic activity of platelet ADAM10 upon activation with GPVI ligands. However, an increase in ADAM10 activity driven by exposure to non-ligand platelet agonists such as fluid shear stress, calmodulin inhibitors or NEM, resulted in a corresponding increase in sGPVI (Facey et al., 2016). Hence, it would be interesting to investigate the effect of rhTIMP3 on ADAM10 activity and sGPVI release in response to these ligand-independent agonists.

The data presented here suggests new mechanisms for regulation of platelet ADAM10 activity. The dynamic platelet-associated levels of TIMPs may modulate ADAM10 activity and maintain platelet patency by stabilising receptor levels in response to platelet activation. Considering the role of GPVI in thrombus initiation (Sections 1.1.2 and 1.2.2), regulation of platelet ADAM10 by TIMPs may have implications for thrombus formation. Understanding the mechanistic basis of ADAM10 regulation may open therapeutic avenues in managing thrombotic and bleeding conditions. Future work should assess how TIMPs impact ADAM10 activity and GPVI surface levels in models of thrombus formation.

Chapter 7 Concluding remarks

Platelet receptors are the first blood entities to initiate responses to changes in the vascular environment, and they achieve this rapid reaction through engagement of a specialised suite of receptors and intricately managed intracellular molecular pathways. Understanding the molecular basis of platelet receptor regulation is important, both for the ability to identify and predict inappropriate platelet responses as well as identify and define new targets for therapy to control platelet function in pathological settings.

Work described in this thesis:

- (i) evaluated the platelet-activating ability of Ig isolated from HIT patient plasma samples, and the requirement for ITAM signalling and platelet ADAM10 activity in HIT Ig-induced GPVI shedding using platelets from healthy donors;
- (ii) assessed the value of plasma sGPVI to inform on pathological platelet-activating antibodies and the utility of considering sGPVI simultaneously with current clinical tools for HIT diagnosis when evaluating patients at risk of HIT;
- (iii) explored the influence of *FCGR2A* in-phase haplotype combination at Q27W and H131R on platelet reactivity, levels of anti-PF4/heparin antibodies and HIT susceptibility; and
- (iv) assessed the levels of TIMPs on resting and activated platelets, and the effect of exogenous TIMPs on platelet ADAM10 activity and GPVI shedding in response to platelet activation.

The challenges around achieving a correct diagnosis of HIT as outlined in Section 1.3.5 and in the introduction to Chapters 3 and 4 suggest that novel approaches to evaluate the pathology

of HIT antibodies are needed. In particular, definition of platelet molecular pathways leading to platelet activation and metalloproteolysis of GPVI that are activated when FcγRIIa is engaged by a pathological HIT antibody will help evaluate the potency of an anti-PF4/heparin antibody and to fully understand the molecular pathways activated in platelets exposed to HIT antibodies. Using combinations of heparin, affinity-purified PF4 and Ig preparations affinity-purified from HIT patient blood samples, a new flow cytometric assay to evaluate the modulation of GPVI levels was established. This assay was used to assess signalling pathways engaged in donor platelets exposed to anti-PF4/heparin autoantibodies (Chapter 3). True HIT antibodies induced shedding of up to 60% of GPVI from donor platelets when mixed with low dose heparin and PF4. HIT Ig-induced loss of GPVI was abolished at high heparin concentration and required functional FcγRIIa and ITAM signalling pathways (Syk and Src family kinases). Shedding of GPVI was metalloproteinase-dependent and a role for platelet-membrane localised ADAM10 was identified. This data is consistent with proposed models of PF4/heparin antibody activation of platelets via engagement of FcγRIIa by the Fc portion of the antibody (Brandt et al., 1995, Reilly et al., 2001, Gardiner et al., 2008b) and extends our knowledge of how FcγRIIa activation results in activation of ADAM10-mediated shedding processes. Further, this data provides additional rationale to consider whether the loss of GPVI was significant and if occurring *in vivo*.

Understanding and modulating these molecular pathways may be important for the application of new or existing therapeutics for the treatment of patients with clinical suspicion or confirmed HIT. In addition, the assay described here may be useful to assess heparin-dependency of the autoantibody or modified to evaluate the presence of other anti-platelet autoantibodies such as in patients with ITP. However, the need for viable and responding healthy donor platelets may impede the utility of the assay. The use of a B cell line expressing

FcγRIIIa to measure cellular activation by anti-PF4/heparin antibodies for HIT diagnosis has been reported (Cuker et al., 2013). A similar approach using a cell line which expresses both FcγRIIIa and GPVI may be developed to detect pathological anti-PF4/heparin antibodies. Further, the advent of platelet production *in vitro* (Thon et al., 2015, Ito et al., 2018) may pave the way to genetically engineer designer platelets with high responding characteristics potentially by modifying the receptor genotype and density, thereby circumventing the need for donor platelets.

The clinical management of patients suspected of HIT is challenged by the limitations of laboratory tools to evaluate HIT pathology. Established laboratory testing largely cannot distinguish activating from non-activating anti-PF4/heparin antibodies and the SRA which is able to identify activating antibodies, albeit on donor platelets, is not universally available. As the findings of Chapter 3 indicated that pathological HIT antibodies could trigger the release of GPVI from platelets, an evaluation of sGPVI levels in a prospective cohort of patients suspected of HIT was performed. The advantages of directly measuring a patient- and platelet-specific outcome in a rapid, benchtop assay that requires only standard laboratory equipment are clear. The data obtained from the prospective cohort indicated that measurement of sGPVI indeed helped in stratification of patients with and without platelet-activating anti-PF4/heparin antibodies and when considered together with other standard HIT diagnostic tests, improved the proportion of false negative testing. Findings in Chapter 4 also highlighted the potential of sGPVI as a negative predictor for HIT which improved the overall diagnostic performance of existing tools for HIT evaluation when considered in combination. It is conceivable that the negative predictive value of sGPVI would be useful in situations where the 4Ts score is intermediate (3-4) and the HIT antibody OD is mildly positive. Measurement of plasma sGPVI may address the need for a simple, reliable and readily available assay to

inform on the patient's platelet activation status to confirm or exclude HIT in a timely manner to minimise overdiagnosis and avoid unnecessary use of alternative anticoagulants.

The consequences of release of GPVI from platelets remains a key research question, however as GPVI is a major platelet receptor for collagen and fibrin, it is conceivable that proteolytic loss of this receptor could contribute to, or even precipitate bleeding events. Several reports have identified major bleeding as a common and important outcome (Kuter et al., 2017), with risk of major bleeding in patients with intermediate 4T score almost as high as the thrombotic risk (Pishko et al., 2019). Levels of sGPVI have been linked to both thrombosis and bleeding outcomes in patients with heart failure who received mechanocirculatory support (Muthiah et al., 2016). It will be of interest to evaluate sGPVI and bleeding events in cohorts of patients with suspicion of HIT as levels of sGPVI may also inform on bleeding propensity in this clinical setting where acute loss of GPVI can be triggered.

Identification of genetic factors which influence HIT susceptibility may be valuable for risk stratification prior to heparin treatment. The work presented in Chapter 5 demonstrated that the 131R allele of FcγRIIa is associated with higher circulating sGPVI levels and thrombosis occurrence among HIT patients, suggesting a role for the variant allele in platelet reactivity. Combining the *FCGR2A* genotype information with plasma sGPVI level may help predict thrombotic propensity, particularly in HIT patients with high HIT antibody titres. Blood samples required for *FCGR2A* genotyping and sGPVI measurements can be obtained with relative ease as the gDNA and plasma samples can be isolated from the same citrate-anticoagulated blood tube. However, the utility of sGPVI and *FCGR2A* genotype information remains to be validated in a cohort of prospectively recruited patients suspected of HIT. In addition, the feasibility of obtaining baseline measurements of plasma sGPVI before heparin

infusion as a strategy to normalize the initial variation in sGPVI levels should be investigated to potentially strengthen the argument that sGPVI can aid in identifying patients with platelet-activating antibodies and account for increased sGPVI levels not related to heparin exposure. A return of plasma sGPVI to baseline levels may also be useful to monitor the resolution of anti-platelet activity in confirmed HIT patients.

HIT is a complex, multifactorial syndrome and as such, other factors that are likely to influence HIT susceptibility may contribute to HIT risk. To demonstrate with 80% power a difference at $p < 0.05$, studies would need to evaluate cohorts of at least 1000 individuals suspected of HIT, a requisite sample size that easily exceeds the number of individuals in all query HIT cohorts reported to date. Nonetheless, a multivariate analysis of plasma sGPVI levels, *FCGR2A* genotype, plasma fibronectin levels and signalling capacity, for example phosphatase CD148 (*PTPRJ*) genotype may be beneficial to determine new contributing factors to HIT predisposition. It may be of value to measure plasma proteins such as fibronectin which have the potential to influence HIT Ig-mediated engagement of FcγRIIa (Chapter 3). Fibronectin has been demonstrated to inhibit platelet activation by anti-PF4/heparin antibodies *in vitro* and lower plasma fibronectin levels were observed in confirmed HIT patients (Krauel et al., 2018). A close collaboration encompassing multiple HIT referral centres and research laboratories to enable recruitment of a cohort of sufficient size, with annotated clinical data and measurement of risk factors would be required.

New mechanisms for regulation of platelet ADAM10 activity presented in Chapter 6 hint at the level of control of platelet receptor stability and may provide additional therapeutic avenues to modulate platelet reactivity by changing receptor density through TIMPs. Preliminary findings showed that exogenous TIMP3 reduced platelet ADAM10 activity on

resting and activated platelets, suggesting a potential utility for TIMP3 to control platelet sheddase activity. Synthetic analogues or recombinant fragments of TIMP3 linked to antibodies that target the platelet surface could be evaluated for platelet ADAM10 inhibitory properties and explored for utility as therapeutic tools to manage thrombotic or bleeding conditions. The impact of depleting endogenous TIMPs on ADAM10 activity and GPVI surface levels in models of thrombus formation also remains to be investigated. Increased levels of TIMPs on activated platelets may suggest a potential value for evaluating surface TIMPs as markers of platelet activation although a better understanding of the dynamics between surface-bound and circulating plasma TIMPs is necessary. A significant correlation between levels of platelet surface TIMPs and circulating sGPVI levels suggests that the variation in surface-bound TIMPs may explain some of the variability in plasma sGPVI levels among healthy donors. Increased levels of surface TIMPs may contribute to the lower sGPVI levels in some confirmed HIT patients. However, levels of platelet surface TIMPs among HIT patients or in the presence of anti-platelet antibodies remain to be evaluated.

In conclusion, the findings in this thesis extend our knowledge of the molecular pathway involved in HIT Ig-mediated GPVI shedding and presented a new mechanism for regulating platelet ADAM10 activity. This is also the first study to investigate the *FCGR2A* polymorphisms in-phase in a prospective cohort of patients suspected of HIT and to show that the two minor variants, 27W and 131R, do not co-occur on the same allele.

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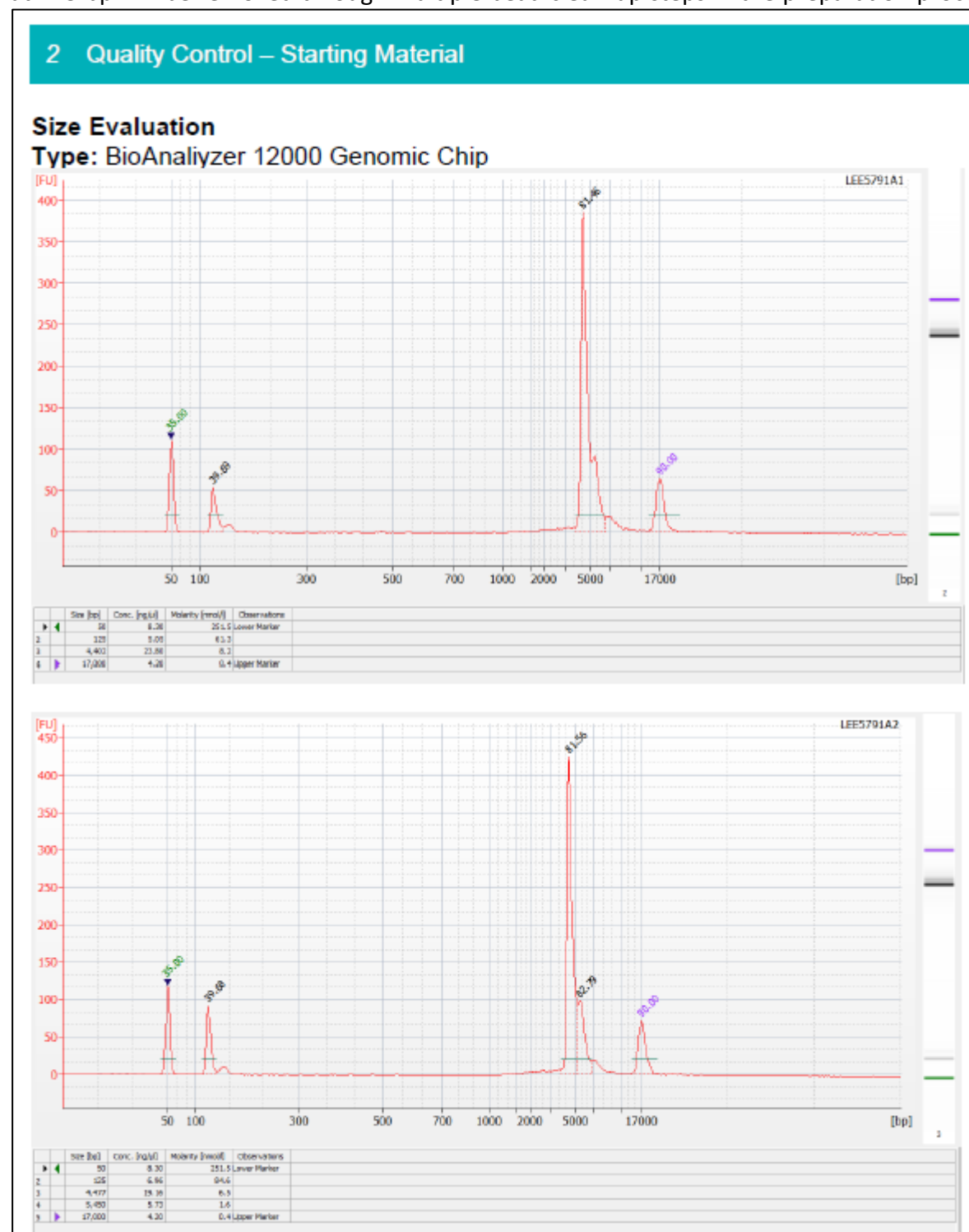
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Appendices

Appendix 1 Quality control check of the two pooled amplicons for long-read sequencing. The amount of pooled PCR amplicon submitted was 7563 ng in pool 1 and 7326 ng in pool 2. The Bioanalyzer trace showed a sharp, clean peak, 4-5 kb in size for both pools. The minor peaks at 125 bp will be removed through multiple bead clean-up steps in the preparation process.



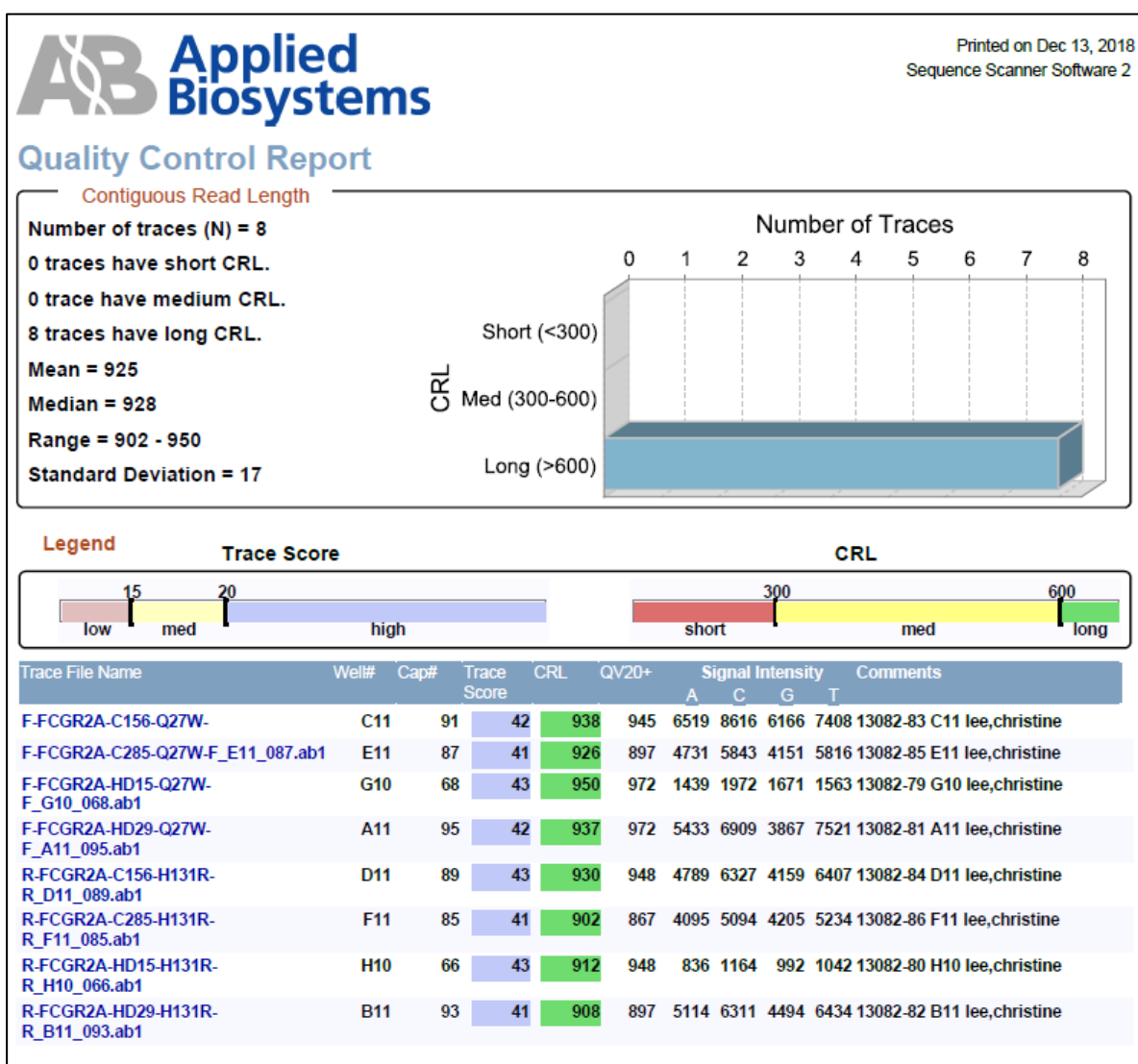
Appendix 2 Long amplicon analysis report for pool 1.

11/30/2018	SMRT Link
Long Amplicon Analysis (LAA) Report	
Analysis	LEE5791A1_LAA_301118
Analysis Id	597
Status	SUCCESSFUL: 34/34 total tasks completed.
Created By	z2251141
Date Created	11/30/2018, 11:50:24 AM
Date Updated	11/30/2018, 12:26:01 PM
Application	Long Amplicon Analysis (LAA)
SMRT Link Version	6.0.0.47841
Inputs	BAM Data: Auto-merged subreads @ 1543539013489
Path	/srv/smr/smrlink/userdata/jobs_root/000/000597
► Analysis Parameters	
Chimera Filter	true
Clustering	true
Filter Input Reads by Presence of Both Flanking Barcodes	false
Ignore End-Bases	71
Maximum Clustering Reads	400
Maximum Subread Length	0
Maximum Phasing Reads	500
Maximum Reads	2,000
Minimum Barcode Score	26
Minimum Subread Length	3,000
Minimum Predicted Accuracy	0.95
Minimum Allele/Haplotype Read Fraction	0.1
Minimum Allele/Haplotype Reads	20
Phasing	true
Random Number Generator Seed	42
Take Top N Sequences	0
Trim Sequence Ends	0
https://portal.ramaciotti.unsw.edu.au:8243/sl/#/analysis/job/597;show=all	
1/10	

Appendix 3 Long amplicon analysis report for pool 2.

11/28/2018	SMRT Link
Long Amplicon Analysis (LAA) Report	
Analysis	LEE_5791A2_LAA_subsets_all
Analysis Id	579
Status	SUCCESSFUL: 34/34 total tasks completed.
Created By	z2251141
Date Created	11/28/2018, 12:55:44 PM
Date Updated	11/28/2018, 1:31:41 PM
Application	Long Amplicon Analysis (LAA)
SMRT Link Version	6.0.0.47841
Inputs	BAM Data: Auto-merged subreads @ 1543370132948
Path	/srv/smr/smrlink/userdata/jobs_root/000/000579
► Analysis Parameters	
Chimera Filter	true
Clustering	true
Filter Input Reads by Presence of Both Flanking Barcodes	false
Ignore End-Bases	71
Maximum Clustering Reads	400
Maximum Subread Length	0
Maximum Phasing Reads	500
Maximum Reads	2,000
Minimum Barcode Score	26
Minimum Subread Length	3,000
Minimum Predicted Accuracy	0.95
Minimum Allele/Haplotype Read Fraction	0.1
Minimum Allele/Haplotype Reads	20
Phasing	true
Random Number Generator Seed	42
Take Top N Sequences	0
Trim Sequence Ends	0
https://portal.ramaciotti.unsw.edu.au:8243/sl/#/analysis/job/579;show=all	
1/10	

Appendix 4 Quality control report for Sanger sequencing.



Appendix 5 List of sensitivity and specificity values for 4Ts scores in a cohort of 292 prospectively recruited patients at risk of developing HIT.

4Ts Cutoff	Sensitivity (%)	95% CI	Specificity (%)	95% CI	Likelihood ratio
> 0.5000	100	88.78% to 100.0%	0.7663	0.09294% to 2.741%	1.008
> 1.500	100	88.78% to 100.0%	1.916	0.6249% to 4.414%	1.02
> 2.500	100	88.78% to 100.0%	11.11	7.569% to 15.57%	1.125
> 3.500	100	88.78% to 100.0%	31.8	26.20% to 37.83%	1.466
> 4.500	77.42	58.90% to 90.41%	64.37	58.23% to 70.18%	2.173
> 5.500	45.16	27.32% to 63.97%	85.82	80.99% to 89.82%	3.186
> 6.500	19.35	7.452% to 37.47%	95.4	92.11% to 97.60%	4.21
> 7.500	6.452	0.7911% to 21.42%	98.85	96.68% to 99.76%	5.613

Appendix 6 List of sensitivity and specificity values for HIT ELISA in a cohort of 310 prospectively recruited patients at risk of developing HIT.

HIT ELISA OD Cutoff	Sensitivity (%)	95% CI	Specificity (%)	95% CI	Likelihood ratio
> 0.03500	100	89.11% to 100.0%	1.439	0.3934% to 3.643%	1.015
> 0.04500	100	89.11% to 100.0%	2.878	1.250% to 5.591%	1.03
> 0.05500	100	89.11% to 100.0%	5.755	3.325% to 9.178%	1.061
> 0.06500	100	89.11% to 100.0%	8.273	5.317% to 12.16%	1.09
> 0.07500	100	89.11% to 100.0%	11.87	8.314% to 16.27%	1.135
> 0.08500	100	89.11% to 100.0%	14.39	10.48% to 19.07%	1.168
> 0.09500	100	89.11% to 100.0%	19.42	14.94% to 24.57%	1.241
> 0.1050	100	89.11% to 100.0%	26.26	21.18% to 31.85%	1.356
> 0.1150	100	89.11% to 100.0%	31.65	26.23% to 37.48%	1.463
> 0.1250	100	89.11% to 100.0%	37.41	31.70% to 43.39%	1.598
> 0.1350	100	89.11% to 100.0%	41.73	35.87% to 47.77%	1.716
> 0.1450	100	89.11% to 100.0%	45.68	39.72% to 51.74%	1.841
> 0.1550	100	89.11% to 100.0%	48.56	42.55% to 54.60%	1.944
> 0.1650	100	89.11% to 100.0%	52.52	46.47% to 58.51%	2.106
> 0.1750	100	89.11% to 100.0%	54.68	48.62% to 60.63%	2.206
> 0.1850	100	89.11% to 100.0%	57.55	51.51% to 63.44%	2.356
> 0.1950	100	89.11% to 100.0%	60.79	54.78% to 66.57%	2.55
> 0.2050	100	89.11% to 100.0%	63.31	57.35% to 68.99%	2.725
> 0.2150	100	89.11% to 100.0%	66.19	60.30% to 71.73%	2.957
> 0.2250	100	89.11% to 100.0%	68.71	62.90% to 74.11%	3.195
> 0.2350	100	89.11% to 100.0%	69.78	64.02% to 75.13%	3.31
> 0.2450	100	89.11% to 100.0%	71.22	65.51% to 76.47%	3.475
> 0.2550	100	89.11% to 100.0%	72.3	66.64% to 77.48%	3.61
> 0.2650	100	89.11% to 100.0%	73.74	68.15% to 78.82%	3.808
> 0.2750	100	89.11% to 100.0%	74.46	68.91% to 79.48%	3.915
> 0.2850	100	89.11% to 100.0%	74.82	69.29% to 79.81%	3.971
> 0.3000	100	89.11% to 100.0%	75.18	69.67% to 80.14%	4.029
> 0.3200	100	89.11% to 100.0%	75.54	70.05% to 80.48%	4.088
> 0.3350	100	89.11% to 100.0%	75.9	70.43% to 80.81%	4.149
> 0.3450	100	89.11% to 100.0%	76.98	71.58% to 81.80%	4.344
> 0.3550	100	89.11% to 100.0%	77.34	71.96% to 82.12%	4.413
> 0.3650	100	89.11% to 100.0%	78.06	72.73% to 82.78%	4.557
> 0.3750	100	89.11% to 100.0%	79.14	73.88% to 83.76%	4.793
> 0.3850	100	89.11% to 100.0%	79.5	74.27% to 84.09%	4.877

HIT ELISA OD Cutoff	Sensitivity (%)	95% CI	Specificity (%)	95% CI	Likelihood ratio
> 0.3950	100	89.11% to 100.0%	79.86	74.65% to 84.41%	4.964
> 0.4100	100	89.11% to 100.0%	80.22	75.04% to 84.73%	5.055
> 0.4250	100	89.11% to 100.0%	81.29	76.21% to 85.70%	5.346
> 0.4350	100	89.11% to 100.0%	82.37	77.38% to 86.67%	5.673
> 0.4450	100	89.11% to 100.0%	82.73	77.77% to 86.99%	5.792
> 0.4550	100	89.11% to 100.0%	83.09	78.16% to 87.31%	5.915
> 0.4650	100	89.11% to 100.0%	83.45	78.55% to 87.62%	6.043
> 0.4800	100	89.11% to 100.0%	83.81	78.95% to 87.94%	6.178
> 0.4950	100	89.11% to 100.0%	84.17	79.34% to 88.26%	6.318
> 0.5150	100	89.11% to 100.0%	84.53	79.74% to 88.57%	6.465
> 0.5700	100	89.11% to 100.0%	84.89	80.13% to 88.89%	6.619
> 0.6300	96.88	83.78% to 99.92%	85.25	80.53% to 89.20%	6.569
> 0.6600	96.88	83.78% to 99.92%	85.61	80.93% to 89.52%	6.733
> 0.6850	96.88	83.78% to 99.92%	85.97	81.32% to 89.83%	6.905
> 0.7100	96.88	83.78% to 99.92%	86.33	81.72% to 90.14%	7.087
> 0.7300	96.88	83.78% to 99.92%	86.69	82.12% to 90.45%	7.279
> 0.7600	96.88	83.78% to 99.92%	87.05	82.53% to 90.76%	7.481
> 0.8250	96.88	83.78% to 99.92%	87.77	83.33% to 91.38%	7.921
> 0.8850	96.88	83.78% to 99.92%	88.13	83.73% to 91.69%	8.161
> 0.9350	96.88	83.78% to 99.92%	88.49	84.14% to 91.99%	8.416
> 1.025	96.88	83.78% to 99.92%	88.85	84.55% to 92.30%	8.688
> 1.115	96.88	83.78% to 99.92%	89.21	84.95% to 92.60%	8.977
> 1.160	96.88	83.78% to 99.92%	89.57	85.36% to 92.90%	9.287
> 1.195	96.88	83.78% to 99.92%	89.93	85.77% to 93.20%	9.618
> 1.225	96.88	83.78% to 99.92%	90.29	86.18% to 93.50%	9.975
> 1.260	96.88	83.78% to 99.92%	90.65	86.60% to 93.80%	10.36
> 1.310	93.75	79.19% to 99.23%	90.65	86.60% to 93.80%	10.02
> 1.340	90.63	74.98% to 98.02%	91.01	87.01% to 94.10%	10.08
> 1.385	90.63	74.98% to 98.02%	91.37	87.43% to 94.39%	10.5
> 1.435	90.63	74.98% to 98.02%	92.09	88.26% to 94.97%	11.45
> 1.460	90.63	74.98% to 98.02%	92.45	88.68% to 95.26%	12
> 1.480	90.63	74.98% to 98.02%	92.81	89.11% to 95.55%	12.6
> 1.495	90.63	74.98% to 98.02%	93.17	89.53% to 95.84%	13.26
> 1.510	87.5	71.01% to 96.49%	93.17	89.53% to 95.84%	12.8
> 1.535	87.5	71.01% to 96.49%	93.53	89.96% to 96.12%	13.51
> 1.570	87.5	71.01% to 96.49%	93.88	90.39% to 96.40%	14.31

HIT ELISA OD Cutoff	Sensitivity (%)	95% CI	Specificity (%)	95% CI	Likelihood ratio
> 1.655	87.5	71.01% to 96.49%	94.6	91.26% to 96.95%	16.22
> 1.725	87.5	71.01% to 96.49%	94.96	91.69% to 97.22%	17.38
> 1.735	87.5	71.01% to 96.49%	95.32	92.14% to 97.49%	18.71
> 1.750	87.5	71.01% to 96.49%	95.68	92.58% to 97.75%	20.27
> 1.790	87.5	71.01% to 96.49%	96.04	93.03% to 98.01%	22.11
> 1.850	84.38	67.21% to 94.72%	96.04	93.03% to 98.01%	21.32
> 1.920	81.25	63.56% to 92.79%	96.04	93.03% to 98.01%	20.53
> 1.970	78.13	60.03% to 90.72%	96.04	93.03% to 98.01%	19.74
> 2.005	78.13	60.03% to 90.72%	96.4	93.48% to 98.26%	21.72
> 2.045	78.13	60.03% to 90.72%	96.76	93.94% to 98.51%	24.13
> 2.070	78.13	60.03% to 90.72%	97.12	94.41% to 98.75%	27.15
> 2.095	75	56.60% to 88.54%	97.12	94.41% to 98.75%	26.06
> 2.120	71.88	53.25% to 86.25%	97.12	94.41% to 98.75%	24.98
> 2.150	68.75	49.99% to 83.88%	97.12	94.41% to 98.75%	23.89
> 2.235	65.63	46.81% to 81.43%	97.12	94.41% to 98.75%	22.8
> 2.310	65.63	46.81% to 81.43%	97.48	94.88% to 98.98%	26.06
> 2.330	62.5	43.69% to 78.90%	97.48	94.88% to 98.98%	24.82
> 2.345	59.38	40.64% to 76.30%	97.48	94.88% to 98.98%	23.58
> 2.365	56.25	37.66% to 73.64%	97.48	94.88% to 98.98%	22.34
> 2.390	56.25	37.66% to 73.64%	97.84	95.36% to 99.20%	26.06
> 2.425	53.13	34.74% to 70.91%	97.84	95.36% to 99.20%	24.61
> 2.455	50	31.89% to 68.11%	98.2	95.85% to 99.41%	27.8
> 2.510	46.88	29.09% to 65.26%	98.2	95.85% to 99.41%	26.06
> 2.570	43.75	26.36% to 62.34%	98.2	95.85% to 99.41%	24.33
> 2.600	34.38	18.57% to 53.19%	98.2	95.85% to 99.41%	19.11
> 2.625	31.25	16.12% to 50.01%	98.2	95.85% to 99.41%	17.38
> 2.645	28.13	13.75% to 46.75%	98.2	95.85% to 99.41%	15.64
> 2.685	25	11.46% to 43.40%	98.2	95.85% to 99.41%	13.9
> 2.730	21.88	9.277% to 39.97%	98.2	95.85% to 99.41%	12.16
> 2.755	18.75	7.208% to 36.44%	98.2	95.85% to 99.41%	10.43
> 2.770	18.75	7.208% to 36.44%	98.56	96.36% to 99.61%	13.03
> 2.790	15.63	5.275% to 32.79%	98.56	96.36% to 99.61%	10.86
> 2.820	12.5	3.513% to 28.99%	98.56	96.36% to 99.61%	8.688
> 2.850	9.375	1.977% to 25.02%	98.56	96.36% to 99.61%	6.516
> 2.880	9.375	1.977% to 25.02%	98.92	96.88% to 99.78%	8.688
> 2.950	6.25	0.7661% to 20.81%	98.92	96.88% to 99.78%	5.792

HIT ELISA OD Cutoff	Sensitivity (%)	95% CI	Specificity (%)	95% CI	Likelihood ratio
> 3.010	6.25	0.7661% to 20.81%	99.28	97.43% to 99.91%	8.688
> 3.075	6.25	0.7661% to 20.81%	99.64	98.01% to 99.99%	17.38
> 3.190	3.125	0.07909% to 16.22%	99.64	98.01% to 99.99%	8.688
> 3.290	0	0.000% to 10.89%	99.64	98.01% to 99.99%	0

Appendix 7 List of sensitivity and specificity values for sGPVI levels in a cohort of 310 prospectively recruited patients at risk of developing HIT.

sGPVI Cutoff (ng/mL)	Sensitivity (%)	95% CI	Specificity (%)	95% CI	Likelihood ratio
> 7.485	100	89.11% to 100.0%	0.3597	0.009107% to 1.988%	1.004
> 8.280	100	89.11% to 100.0%	0.7194	0.08724% to 2.575%	1.007
> 8.730	100	89.11% to 100.0%	1.079	0.2231% to 3.121%	1.011
> 9.220	100	89.11% to 100.0%	1.439	0.3934% to 3.643%	1.015
> 9.965	100	89.11% to 100.0%	1.799	0.5865% to 4.147%	1.018
> 10.42	100	89.11% to 100.0%	2.158	0.7961% to 4.638%	1.022
> 10.56	100	89.11% to 100.0%	2.518	1.018% to 5.119%	1.026
> 10.68	100	89.11% to 100.0%	2.878	1.250% to 5.591%	1.03
> 10.79	100	89.11% to 100.0%	3.237	1.491% to 6.056%	1.033
> 10.96	100	89.11% to 100.0%	3.597	1.738% to 6.516%	1.037
> 11.47	100	89.11% to 100.0%	3.957	1.992% to 6.969%	1.041
> 12.07	100	89.11% to 100.0%	4.317	2.250% to 7.419%	1.045
> 12.33	100	89.11% to 100.0%	4.676	2.513% to 7.864%	1.049
> 12.74	100	89.11% to 100.0%	5.036	2.780% to 8.305%	1.053
> 13.14	100	89.11% to 100.0%	5.396	3.051% to 8.743%	1.057
> 13.39	100	89.11% to 100.0%	5.755	3.325% to 9.178%	1.061
> 13.85	100	89.11% to 100.0%	6.115	3.602% to 9.611%	1.065
> 14.16	100	89.11% to 100.0%	6.475	3.882% to 10.04%	1.069
> 14.29	100	89.11% to 100.0%	6.835	4.165% to 10.47%	1.073
> 14.38	100	89.11% to 100.0%	7.194	4.450% to 10.89%	1.078
> 14.46	96.88	83.78% to 99.92%	7.194	4.450% to 10.89%	1.044
> 14.54	96.88	83.78% to 99.92%	7.554	4.737% to 11.32%	1.048
> 14.55	96.88	83.78% to 99.92%	7.914	5.026% to 11.74%	1.052
> 14.92	96.88	83.78% to 99.92%	8.273	5.317% to 12.16%	1.056
> 15.33	96.88	83.78% to 99.92%	8.633	5.610% to 12.57%	1.06
> 15.40	96.88	83.78% to 99.92%	8.993	5.904% to 12.99%	1.064
> 15.47	96.88	83.78% to 99.92%	9.353	6.201% to 13.40%	1.069
> 15.72	96.88	83.78% to 99.92%	9.712	6.498% to 13.82%	1.073
> 16.06	96.88	83.78% to 99.92%	10.07	6.798% to 14.23%	1.077
> 16.34	96.88	83.78% to 99.92%	10.43	7.098% to 14.64%	1.082
> 16.54	96.88	83.78% to 99.92%	10.79	7.400% to 15.05%	1.086
> 16.62	96.88	83.78% to 99.92%	11.15	7.704% to 15.45%	1.09
> 16.69	96.88	83.78% to 99.92%	11.51	8.008% to 15.86%	1.095
> 16.97	96.88	83.78% to 99.92%	11.87	8.314% to 16.27%	1.099

sGPVI Cutoff (ng/mL)	Sensitivity (%)	95% CI	Specificity (%)	95% CI	Likelihood ratio
> 17.25	96.88	83.78% to 99.92%	12.23	8.620% to 16.67%	1.104
> 17.31	96.88	83.78% to 99.92%	12.59	8.928% to 17.07%	1.108
> 17.35	96.88	83.78% to 99.92%	13.31	9.547% to 17.88%	1.117
> 17.56	96.88	83.78% to 99.92%	13.67	9.858% to 18.28%	1.122
> 17.84	96.88	83.78% to 99.92%	14.03	10.17% to 18.68%	1.127
> 18.02	96.88	83.78% to 99.92%	14.39	10.48% to 19.07%	1.132
> 18.23	96.88	83.78% to 99.92%	14.75	10.80% to 19.47%	1.136
> 18.60	96.88	83.78% to 99.92%	15.47	11.43% to 20.26%	1.146
> 18.89	96.88	83.78% to 99.92%	15.83	11.74% to 20.66%	1.151
> 18.97	96.88	83.78% to 99.92%	16.19	12.06% to 21.05%	1.156
> 19.05	93.75	79.19% to 99.23%	16.19	12.06% to 21.05%	1.119
> 19.21	93.75	79.19% to 99.23%	16.55	12.38% to 21.45%	1.123
> 19.35	93.75	79.19% to 99.23%	16.91	12.69% to 21.84%	1.128
> 19.48	93.75	79.19% to 99.23%	17.27	13.01% to 22.23%	1.133
> 19.92	93.75	79.19% to 99.23%	17.63	13.33% to 22.62%	1.138
> 20.26	93.75	79.19% to 99.23%	17.99	13.65% to 23.01%	1.143
> 20.38	93.75	79.19% to 99.23%	18.35	13.98% to 23.40%	1.148
> 20.55	93.75	79.19% to 99.23%	18.71	14.30% to 23.79%	1.153
> 20.71	93.75	79.19% to 99.23%	19.06	14.62% to 24.18%	1.158
> 20.98	93.75	79.19% to 99.23%	19.42	14.94% to 24.57%	1.164
> 21.19	93.75	79.19% to 99.23%	19.78	15.27% to 24.96%	1.169
> 21.21	93.75	79.19% to 99.23%	20.14	15.59% to 25.35%	1.174
> 21.26	90.63	74.98% to 98.02%	20.14	15.59% to 25.35%	1.135
> 21.35	90.63	74.98% to 98.02%	20.5	15.91% to 25.73%	1.14
> 21.71	90.63	74.98% to 98.02%	20.86	16.24% to 26.12%	1.145
> 22.04	90.63	74.98% to 98.02%	21.22	16.57% to 26.50%	1.15
> 22.18	90.63	74.98% to 98.02%	21.58	16.89% to 26.89%	1.156
> 22.41	87.5	71.01% to 96.49%	21.58	16.89% to 26.89%	1.116
> 22.60	87.5	71.01% to 96.49%	21.94	17.22% to 27.27%	1.121
> 22.80	87.5	71.01% to 96.49%	22.3	17.55% to 27.66%	1.126
> 22.94	87.5	71.01% to 96.49%	22.66	17.88% to 28.04%	1.131
> 23.01	87.5	71.01% to 96.49%	23.02	18.20% to 28.42%	1.137
> 23.12	87.5	71.01% to 96.49%	23.38	18.53% to 28.81%	1.142
> 23.18	87.5	71.01% to 96.49%	23.74	18.86% to 29.19%	1.147
> 23.21	87.5	71.01% to 96.49%	24.1	19.19% to 29.57%	1.153
> 23.31	87.5	71.01% to 96.49%	24.46	19.52% to 29.95%	1.158

sGPVI Cutoff (ng/mL)	Sensitivity (%)	95% CI	Specificity (%)	95% CI	Likelihood ratio
> 23.56	87.5	71.01% to 96.49%	24.82	19.86% to 30.33%	1.164
> 23.76	87.5	71.01% to 96.49%	25.18	20.19% to 30.71%	1.169
> 23.79	87.5	71.01% to 96.49%	25.54	20.52% to 31.09%	1.175
> 23.96	87.5	71.01% to 96.49%	25.9	20.85% to 31.47%	1.181
> 24.16	87.5	71.01% to 96.49%	26.26	21.18% to 31.85%	1.187
> 24.28	87.5	71.01% to 96.49%	26.62	21.52% to 32.23%	1.192
> 25.00	87.5	71.01% to 96.49%	26.98	21.85% to 32.60%	1.198
> 25.74	87.5	71.01% to 96.49%	27.34	22.19% to 32.98%	1.204
> 25.86	87.5	71.01% to 96.49%	27.7	22.52% to 33.36%	1.21
> 25.90	87.5	71.01% to 96.49%	28.06	22.86% to 33.73%	1.216
> 25.94	87.5	71.01% to 96.49%	28.42	23.19% to 34.11%	1.222
> 26.03	87.5	71.01% to 96.49%	28.78	23.53% to 34.49%	1.229
> 26.11	87.5	71.01% to 96.49%	29.14	23.86% to 34.86%	1.235
> 26.26	87.5	71.01% to 96.49%	29.5	24.20% to 35.24%	1.241
> 26.44	87.5	71.01% to 96.49%	29.86	24.54% to 35.61%	1.247
> 26.56	87.5	71.01% to 96.49%	30.22	24.87% to 35.98%	1.254
> 26.84	87.5	71.01% to 96.49%	30.58	25.21% to 36.36%	1.26
> 27.03	87.5	71.01% to 96.49%	30.94	25.55% to 36.73%	1.267
> 27.11	87.5	71.01% to 96.49%	31.29	25.89% to 37.10%	1.274
> 27.24	84.38	67.21% to 94.72%	31.29	25.89% to 37.10%	1.228
> 27.34	84.38	67.21% to 94.72%	31.65	26.23% to 37.48%	1.235
> 27.59	84.38	67.21% to 94.72%	32.01	26.57% to 37.85%	1.241
> 27.86	84.38	67.21% to 94.72%	32.37	26.91% to 38.22%	1.248
> 27.96	84.38	67.21% to 94.72%	32.73	27.25% to 38.59%	1.254
> 28.07	84.38	67.21% to 94.72%	33.09	27.59% to 38.96%	1.261
> 28.20	84.38	67.21% to 94.72%	33.45	27.93% to 39.33%	1.268
> 28.36	84.38	67.21% to 94.72%	33.81	28.27% to 39.70%	1.275
> 28.46	84.38	67.21% to 94.72%	34.17	28.61% to 40.07%	1.282
> 28.56	81.25	63.56% to 92.79%	34.17	28.61% to 40.07%	1.234
> 28.67	81.25	63.56% to 92.79%	34.53	28.95% to 40.44%	1.241
> 28.83	81.25	63.56% to 92.79%	34.89	29.30% to 40.81%	1.248
> 29.17	81.25	63.56% to 92.79%	35.25	29.64% to 41.18%	1.255
> 29.39	81.25	63.56% to 92.79%	35.61	29.98% to 41.55%	1.262
> 29.57	81.25	63.56% to 92.79%	35.97	30.33% to 41.92%	1.269
> 29.74	78.13	60.03% to 90.72%	35.97	30.33% to 41.92%	1.22
> 29.77	78.13	60.03% to 90.72%	36.33	30.67% to 42.29%	1.227

sGPVI Cutoff (ng/mL)	Sensitivity (%)	95% CI	Specificity (%)	95% CI	Likelihood ratio
> 30.07	78.13	60.03% to 90.72%	36.69	31.01% to 42.65%	1.234
> 30.45	78.13	60.03% to 90.72%	37.05	31.36% to 43.02%	1.241
> 30.62	78.13	60.03% to 90.72%	37.41	31.70% to 43.39%	1.248
> 30.73	78.13	60.03% to 90.72%	38.13	32.39% to 44.12%	1.263
> 31.02	78.13	60.03% to 90.72%	38.49	32.74% to 44.49%	1.27
> 31.48	75	56.60% to 88.54%	38.49	32.74% to 44.49%	1.219
> 31.71	75	56.60% to 88.54%	38.85	33.09% to 44.85%	1.226
> 31.76	75	56.60% to 88.54%	39.21	33.43% to 45.22%	1.234
> 31.85	71.88	53.25% to 86.25%	39.21	33.43% to 45.22%	1.182
> 31.94	71.88	53.25% to 86.25%	39.57	33.78% to 45.58%	1.189
> 31.97	71.88	53.25% to 86.25%	39.93	34.13% to 45.95%	1.196
> 32.04	71.88	53.25% to 86.25%	40.29	34.47% to 46.31%	1.204
> 32.13	71.88	53.25% to 86.25%	40.65	34.82% to 46.68%	1.211
> 32.19	68.75	49.99% to 83.88%	40.65	34.82% to 46.68%	1.158
> 32.21	65.63	46.81% to 81.43%	40.65	34.82% to 46.68%	1.106
> 32.22	65.63	46.81% to 81.43%	41.01	35.17% to 47.04%	1.112
> 32.26	65.63	46.81% to 81.43%	41.37	35.52% to 47.40%	1.119
> 32.49	65.63	46.81% to 81.43%	41.73	35.87% to 47.77%	1.126
> 32.70	65.63	46.81% to 81.43%	42.09	36.22% to 48.13%	1.133
> 32.86	65.63	46.81% to 81.43%	42.45	36.56% to 48.49%	1.14
> 33.26	65.63	46.81% to 81.43%	42.81	36.91% to 48.85%	1.147
> 33.55	65.63	46.81% to 81.43%	43.17	37.26% to 49.21%	1.155
> 33.59	65.63	46.81% to 81.43%	43.53	37.61% to 49.58%	1.162
> 33.69	65.63	46.81% to 81.43%	43.88	37.96% to 49.94%	1.169
> 33.88	65.63	46.81% to 81.43%	44.24	38.32% to 50.30%	1.177
> 34.04	65.63	46.81% to 81.43%	44.6	38.67% to 50.66%	1.185
> 34.16	65.63	46.81% to 81.43%	44.96	39.02% to 51.02%	1.192
> 34.51	65.63	46.81% to 81.43%	45.32	39.37% to 51.38%	1.2
> 35.13	65.63	46.81% to 81.43%	45.68	39.72% to 51.74%	1.208
> 35.48	62.5	43.69% to 78.90%	45.68	39.72% to 51.74%	1.151
> 35.60	62.5	43.69% to 78.90%	46.04	40.07% to 52.10%	1.158
> 35.83	62.5	43.69% to 78.90%	46.4	40.43% to 52.46%	1.166
> 35.99	62.5	43.69% to 78.90%	46.76	40.78% to 52.82%	1.174
> 36.21	62.5	43.69% to 78.90%	47.12	41.13% to 53.17%	1.182
> 36.46	62.5	43.69% to 78.90%	47.48	41.49% to 53.53%	1.19
> 36.54	62.5	43.69% to 78.90%	47.84	41.84% to 53.89%	1.198

sGPVI Cutoff (ng/mL)	Sensitivity (%)	95% CI	Specificity (%)	95% CI	Likelihood ratio
> 36.58	62.5	43.69% to 78.90%	48.2	42.19% to 54.25%	1.207
> 36.65	62.5	43.69% to 78.90%	48.56	42.55% to 54.60%	1.215
> 36.73	62.5	43.69% to 78.90%	48.92	42.90% to 54.96%	1.224
> 36.92	62.5	43.69% to 78.90%	49.28	43.26% to 55.32%	1.232
> 37.21	62.5	43.69% to 78.90%	49.64	43.61% to 55.67%	1.241
> 37.36	62.5	43.69% to 78.90%	50	43.97% to 56.03%	1.25
> 37.61	62.5	43.69% to 78.90%	50.36	44.33% to 56.39%	1.259
> 37.91	62.5	43.69% to 78.90%	50.72	44.68% to 56.74%	1.268
> 37.99	62.5	43.69% to 78.90%	51.08	45.04% to 57.10%	1.278
> 38.03	62.5	43.69% to 78.90%	51.44	45.40% to 57.45%	1.287
> 38.11	62.5	43.69% to 78.90%	51.8	45.75% to 57.81%	1.297
> 38.19	62.5	43.69% to 78.90%	52.16	46.11% to 58.16%	1.306
> 38.30	62.5	43.69% to 78.90%	52.52	46.47% to 58.51%	1.316
> 38.40	62.5	43.69% to 78.90%	52.88	46.83% to 58.87%	1.326
> 38.50	59.38	40.64% to 76.30%	52.88	46.83% to 58.87%	1.26
> 38.92	59.38	40.64% to 76.30%	53.24	47.18% to 59.22%	1.27
> 39.28	59.38	40.64% to 76.30%	53.6	47.54% to 59.57%	1.28
> 39.31	59.38	40.64% to 76.30%	53.96	47.90% to 59.93%	1.29
> 39.35	59.38	40.64% to 76.30%	54.32	48.26% to 60.28%	1.3
> 39.43	59.38	40.64% to 76.30%	54.68	48.62% to 60.63%	1.31
> 39.91	59.38	40.64% to 76.30%	55.04	48.98% to 60.98%	1.321
> 40.72	56.25	37.66% to 73.64%	55.04	48.98% to 60.98%	1.251
> 41.23	56.25	37.66% to 73.64%	55.4	49.34% to 61.33%	1.261
> 41.41	56.25	37.66% to 73.64%	55.76	49.70% to 61.68%	1.271
> 41.54	56.25	37.66% to 73.64%	56.12	50.06% to 62.04%	1.282
> 41.79	53.13	34.74% to 70.91%	56.12	50.06% to 62.04%	1.211
> 42.02	53.13	34.74% to 70.91%	56.47	50.42% to 62.39%	1.221
> 42.08	53.13	34.74% to 70.91%	56.83	50.79% to 62.74%	1.231
> 42.13	53.13	34.74% to 70.91%	57.19	51.15% to 63.09%	1.241
> 42.20	50	31.89% to 68.11%	57.19	51.15% to 63.09%	1.168
> 42.46	50	31.89% to 68.11%	57.55	51.51% to 63.44%	1.178
> 42.69	50	31.89% to 68.11%	57.91	51.87% to 63.78%	1.188
> 42.74	50	31.89% to 68.11%	58.27	52.23% to 64.13%	1.198
> 42.86	50	31.89% to 68.11%	58.63	52.60% to 64.48%	1.209
> 43.00	50	31.89% to 68.11%	58.99	52.96% to 64.83%	1.219
> 43.07	50	31.89% to 68.11%	59.35	53.32% to 65.18%	1.23

sGPVI Cutoff (ng/mL)	Sensitivity (%)	95% CI	Specificity (%)	95% CI	Likelihood ratio
> 43.21	50	31.89% to 68.11%	59.71	53.69% to 65.53%	1.241
> 43.35	50	31.89% to 68.11%	60.07	54.05% to 65.87%	1.252
> 43.50	50	31.89% to 68.11%	60.43	54.42% to 66.22%	1.264
> 43.74	46.88	29.09% to 65.26%	60.43	54.42% to 66.22%	1.185
> 44.11	46.88	29.09% to 65.26%	60.79	54.78% to 66.57%	1.196
> 44.42	46.88	29.09% to 65.26%	61.15	55.15% to 66.91%	1.207
> 44.48	46.88	29.09% to 65.26%	61.51	55.51% to 67.26%	1.218
> 44.54	46.88	29.09% to 65.26%	61.87	55.88% to 67.61%	1.229
> 44.58	46.88	29.09% to 65.26%	62.23	56.25% to 67.95%	1.241
> 44.63	46.88	29.09% to 65.26%	62.59	56.61% to 68.30%	1.253
> 44.83	43.75	26.36% to 62.34%	62.59	56.61% to 68.30%	1.169
> 45.58	43.75	26.36% to 62.34%	62.95	56.98% to 68.64%	1.181
> 46.24	43.75	26.36% to 62.34%	63.31	57.35% to 68.99%	1.192
> 46.53	43.75	26.36% to 62.34%	63.67	57.71% to 69.33%	1.204
> 47.20	40.63	23.70% to 59.36%	63.67	57.71% to 69.33%	1.118
> 47.76	40.63	23.70% to 59.36%	64.03	58.08% to 69.67%	1.129
> 47.89	40.63	23.70% to 59.36%	64.39	58.45% to 70.02%	1.141
> 48.23	40.63	23.70% to 59.36%	64.75	58.82% to 70.36%	1.152
> 48.82	40.63	23.70% to 59.36%	65.11	59.19% to 70.70%	1.164
> 49.26	40.63	23.70% to 59.36%	65.47	59.56% to 71.05%	1.176
> 49.49	40.63	23.70% to 59.36%	65.83	59.93% to 71.39%	1.189
> 49.69	40.63	23.70% to 59.36%	66.19	60.30% to 71.73%	1.201
> 49.91	40.63	23.70% to 59.36%	66.55	60.67% to 72.07%	1.214
> 50.01	40.63	23.70% to 59.36%	66.91	61.04% to 72.41%	1.228
> 50.03	40.63	23.70% to 59.36%	67.27	61.41% to 72.75%	1.241
> 50.11	40.63	23.70% to 59.36%	67.63	61.78% to 73.09%	1.255
> 50.22	40.63	23.70% to 59.36%	67.99	62.15% to 73.43%	1.269
> 50.31	40.63	23.70% to 59.36%	68.35	62.52% to 73.77%	1.283
> 50.41	40.63	23.70% to 59.36%	68.71	62.90% to 74.11%	1.298
> 50.50	40.63	23.70% to 59.36%	69.06	63.27% to 74.45%	1.313
> 50.66	40.63	23.70% to 59.36%	69.42	63.64% to 74.79%	1.329
> 50.93	40.63	23.70% to 59.36%	69.78	64.02% to 75.13%	1.344
> 51.11	40.63	23.70% to 59.36%	70.14	64.39% to 75.46%	1.361
> 51.15	37.5	21.10% to 56.31%	70.14	64.39% to 75.46%	1.256
> 51.26	34.38	18.57% to 53.19%	70.14	64.39% to 75.46%	1.151
> 51.61	34.38	18.57% to 53.19%	70.5	64.76% to 75.80%	1.165

sGPVI Cutoff (ng/mL)	Sensitivity (%)	95% CI	Specificity (%)	95% CI	Likelihood ratio
> 51.96	34.38	18.57% to 53.19%	70.86	65.14% to 76.14%	1.18
> 52.28	34.38	18.57% to 53.19%	71.22	65.51% to 76.47%	1.195
> 52.62	34.38	18.57% to 53.19%	71.58	65.89% to 76.81%	1.21
> 52.88	34.38	18.57% to 53.19%	71.94	66.27% to 77.14%	1.225
> 53.08	34.38	18.57% to 53.19%	72.3	66.64% to 77.48%	1.241
> 53.45	34.38	18.57% to 53.19%	72.66	67.02% to 77.81%	1.257
> 53.79	34.38	18.57% to 53.19%	73.02	67.40% to 78.15%	1.274
> 54.03	34.38	18.57% to 53.19%	73.38	67.77% to 78.48%	1.291
> 54.28	34.38	18.57% to 53.19%	73.74	68.15% to 78.82%	1.309
> 54.38	34.38	18.57% to 53.19%	74.1	68.53% to 79.15%	1.327
> 54.53	34.38	18.57% to 53.19%	74.46	68.91% to 79.48%	1.346
> 54.67	34.38	18.57% to 53.19%	74.82	69.29% to 79.81%	1.365
> 54.79	31.25	16.12% to 50.01%	74.82	69.29% to 79.81%	1.241
> 54.87	31.25	16.12% to 50.01%	75.18	69.67% to 80.14%	1.259
> 54.90	31.25	16.12% to 50.01%	75.54	70.05% to 80.48%	1.278
> 55.38	31.25	16.12% to 50.01%	75.9	70.43% to 80.81%	1.297
> 55.86	31.25	16.12% to 50.01%	76.26	70.81% to 81.14%	1.316
> 55.97	28.13	13.75% to 46.75%	76.26	70.81% to 81.14%	1.185
> 56.09	28.13	13.75% to 46.75%	76.62	71.19% to 81.47%	1.203
> 56.21	28.13	13.75% to 46.75%	76.98	71.58% to 81.80%	1.222
> 56.33	28.13	13.75% to 46.75%	77.34	71.96% to 82.12%	1.241
> 56.38	28.13	13.75% to 46.75%	77.7	72.34% to 82.45%	1.261
> 57.06	28.13	13.75% to 46.75%	78.06	72.73% to 82.78%	1.282
> 57.75	28.13	13.75% to 46.75%	78.42	73.11% to 83.11%	1.303
> 57.83	28.13	13.75% to 46.75%	78.78	73.50% to 83.43%	1.325
> 58.30	28.13	13.75% to 46.75%	79.14	73.88% to 83.76%	1.348
> 58.82	25	11.46% to 43.40%	79.14	73.88% to 83.76%	1.198
> 59.05	25	11.46% to 43.40%	79.5	74.27% to 84.09%	1.219
> 59.31	25	11.46% to 43.40%	79.86	74.65% to 84.41%	1.241
> 59.61	25	11.46% to 43.40%	80.22	75.04% to 84.73%	1.264
> 60.22	25	11.46% to 43.40%	80.58	75.43% to 85.06%	1.287
> 60.76	25	11.46% to 43.40%	80.94	75.82% to 85.38%	1.311
> 60.98	25	11.46% to 43.40%	81.29	76.21% to 85.70%	1.337
> 61.41	25	11.46% to 43.40%	81.65	76.60% to 86.02%	1.363
> 61.97	25	11.46% to 43.40%	82.01	76.99% to 86.35%	1.39
> 62.32	25	11.46% to 43.40%	82.37	77.38% to 86.67%	1.418

sGPVI Cutoff (ng/mL)	Sensitivity (%)	95% CI	Specificity (%)	95% CI	Likelihood ratio
> 63.10	25	11.46% to 43.40%	82.73	77.77% to 86.99%	1.448
> 63.88	25	11.46% to 43.40%	83.09	78.16% to 87.31%	1.479
> 64.13	25	11.46% to 43.40%	83.45	78.55% to 87.62%	1.511
> 64.37	25	11.46% to 43.40%	83.81	78.95% to 87.94%	1.544
> 64.49	25	11.46% to 43.40%	84.17	79.34% to 88.26%	1.58
> 64.59	25	11.46% to 43.40%	84.53	79.74% to 88.57%	1.616
> 64.68	25	11.46% to 43.40%	84.89	80.13% to 88.89%	1.655
> 64.95	25	11.46% to 43.40%	85.25	80.53% to 89.20%	1.695
> 65.46	25	11.46% to 43.40%	85.61	80.93% to 89.52%	1.738
> 65.73	25	11.46% to 43.40%	85.97	81.32% to 89.83%	1.782
> 65.77	25	11.46% to 43.40%	86.33	81.72% to 90.14%	1.829
> 65.93	25	11.46% to 43.40%	86.69	82.12% to 90.45%	1.878
> 66.25	21.88	9.277% to 39.97%	86.69	82.12% to 90.45%	1.644
> 66.80	21.88	9.277% to 39.97%	87.05	82.53% to 90.76%	1.689
> 67.23	21.88	9.277% to 39.97%	87.41	82.93% to 91.07%	1.738
> 67.85	21.88	9.277% to 39.97%	87.77	83.33% to 91.38%	1.789
> 68.61	18.75	7.208% to 36.44%	87.77	83.33% to 91.38%	1.533
> 68.85	18.75	7.208% to 36.44%	88.13	83.73% to 91.69%	1.58
> 69.14	15.63	5.275% to 32.79%	88.13	83.73% to 91.69%	1.316
> 69.47	15.63	5.275% to 32.79%	88.49	84.14% to 91.99%	1.357
> 70.01	15.63	5.275% to 32.79%	88.85	84.55% to 92.30%	1.401
> 70.64	15.63	5.275% to 32.79%	89.21	84.95% to 92.60%	1.448
> 70.84	15.63	5.275% to 32.79%	89.57	85.36% to 92.90%	1.498
> 70.91	12.5	3.513% to 28.99%	89.57	85.36% to 92.90%	1.198
> 71.01	12.5	3.513% to 28.99%	89.93	85.77% to 93.20%	1.241
> 71.24	12.5	3.513% to 28.99%	90.29	86.18% to 93.50%	1.287
> 71.72	12.5	3.513% to 28.99%	90.65	86.60% to 93.80%	1.337
> 72.29	12.5	3.513% to 28.99%	91.01	87.01% to 94.10%	1.39
> 72.65	12.5	3.513% to 28.99%	91.37	87.43% to 94.39%	1.448
> 73.01	12.5	3.513% to 28.99%	91.73	87.84% to 94.68%	1.511
> 73.40	12.5	3.513% to 28.99%	92.09	88.26% to 94.97%	1.58
> 73.66	12.5	3.513% to 28.99%	92.45	88.68% to 95.26%	1.655
> 74.06	12.5	3.513% to 28.99%	92.81	89.11% to 95.55%	1.738
> 74.43	12.5	3.513% to 28.99%	93.17	89.53% to 95.84%	1.829
> 75.55	12.5	3.513% to 28.99%	93.53	89.96% to 96.12%	1.931
> 76.65	12.5	3.513% to 28.99%	93.88	90.39% to 96.40%	2.044

sGPVI Cutoff (ng/mL)	Sensitivity (%)	95% CI	Specificity (%)	95% CI	Likelihood ratio
> 76.71	12.5	3.513% to 28.99%	94.24	90.82% to 96.67%	2.172
> 76.90	12.5	3.513% to 28.99%	94.6	91.26% to 96.95%	2.317
> 77.27	12.5	3.513% to 28.99%	94.96	91.69% to 97.22%	2.482
> 77.58	12.5	3.513% to 28.99%	95.32	92.14% to 97.49%	2.673
> 77.94	9.375	1.977% to 25.02%	95.32	92.14% to 97.49%	2.005
> 78.26	9.375	1.977% to 25.02%	95.68	92.58% to 97.75%	2.172
> 80.51	9.375	1.977% to 25.02%	96.04	93.03% to 98.01%	2.369
> 83.11	9.375	1.977% to 25.02%	96.4	93.48% to 98.26%	2.606
> 83.78	9.375	1.977% to 25.02%	96.76	93.94% to 98.51%	2.896
> 84.39	9.375	1.977% to 25.02%	97.12	94.41% to 98.75%	3.258
> 85.55	6.25	0.7661% to 20.81%	97.12	94.41% to 98.75%	2.172
> 87.13	6.25	0.7661% to 20.81%	97.48	94.88% to 98.98%	2.482
> 88.93	6.25	0.7661% to 20.81%	97.84	95.36% to 99.20%	2.896
> 90.09	6.25	0.7661% to 20.81%	98.2	95.85% to 99.41%	3.475
> 91.01	6.25	0.7661% to 20.81%	98.56	96.36% to 99.61%	4.344
> 92.71	3.125	0.07909% to 16.22%	98.56	96.36% to 99.61%	2.172
> 94.73	0	0.000% to 10.89%	98.56	96.36% to 99.61%	0
> 96.71	0	0.000% to 10.89%	98.92	96.88% to 99.78%	0
> 97.64	0	0.000% to 10.89%	99.28	97.43% to 99.91%	0
> 100.0	0	0.000% to 10.89%	99.64	98.01% to 99.99%	0