Molecular mechanisms that control platelet receptors in people with thrombocytopenia

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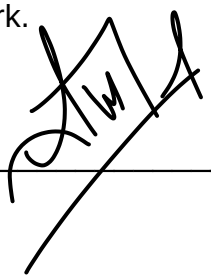
DECLARATION OF CANDIDATE

The work presented in this thesis is original and the result of a full-time study from August 2016 to October 2020 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. Attribution of work is stated in each of the figure legends in Chapter 3 where the work presented was the candidate's own work or where others contributed laboratory work. The candidate contributed to the drafting and edited the text associated with the paper Rabbolini et al, 2017 and also produced the figure that was published as part of this work. All work presented in Chapter 4 is the candidate's own work.

signed: 

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ETHICS DISCLOSURE

The Australian National University Human Research Ethics Committee provided ethics approval (2016/317 and 2017/924) for collecting human blood for the experiments described in this thesis.

DEDICATION

To my husband, SanaUllah, your love and support always encouraged me and made me what I am today. Every single day that I spend with you, I realise how lucky I am.
You made my life beautiful

"To ask questions, to search for answers, to research- I mean Re-search in nature what is already there, but has not been revealed, so far is the most fascinating and the most exciting things we can dream of doing and what we would like to continue doing."

-Rolf M. Zinkernagel-

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Publications and abstracts arising from this thesis

1. Rabbolini DJ, Gardiner EE, Morel-Kopp M-C, Dunkley S, **Jahangiri A**, Lee CS- M, Stevenson WS, Ward CM. Anti-Glycoprotein VI mediated ITP: An under-recognised and significant entity? *Res Prac Thromb Haemost.* (2017) 1: (2) 291- 295.
2. Hicks SM, **Jahangiri A**, Coupland LA, Choi PY, Gardiner EE. Novel scientific approaches and future research directions in understanding ITP. *Platelets* (2020) 31: 315-321.
3. **Jahangiri A,** Montague SJ, Hicks SM, Choi P, van der Wal DE and, Gardiner EE**.** Platelet receptor modulation in immune thrombocytopenia and thrombocytopenia. Poster presentation Gordon Research Conference *Cell Biology of Megakaryocytes and Platelets*, 2017 Galveston, TX, USA

Abstract

Platelet receptors are fundamental to the haemostatic properties of platelets. GPIbα binds VWF, and GPVI binds collagen, subsequently activating intracellular signaling cascades that culminate in platelet aggregation and clot formation. Pathological shedding of platelet receptors GPVI and GPIbα may contribute to platelet dysfunction and increased bleeding risk. Such changes are demonstrated in case reports of patients with acquired autoimmune diseases such as immune thrombocytopenia (ITP) where antibodies target platelets.

A single-center, observational analysis was conducted among patients presenting with primary ITP as outpatients (platelet count < 50 x 10^9/L) or as hospital in-patients with platelet counts less than 120 x 10^9/L (TCP). ITP diagnosis was determined by clinical assessment. All cohorts were compared to contemporaneously collected healthy controls. Samples were collected in anticoagulants and processed and analysed within 3 h of collection. Parameters measured included platelet count, and platelet receptor quantitation by flow cytometry, in a whole blood assay designed to maximize platelet evaluation. Samples from 17 primary ITP patients, or 19 TCP patients with non-autoimmune thrombocytopenia, and 23 healthy donors were obtained. Surface GPIbα, αIIb integrin and GPVI levels were notably lower in patients with low platelet count compared to healthy controls analysed on the same day. Levels of tetraspanin CD9 and ADAM10 remained within normal ranges for both ITP and TCP patient samples. Regression analysis showed no relationship between platelet GPIbα or GPVI and platelet count in healthy donors but correlation between GPIbα or CD9 and platelet count in ITP samples, and between GPVI and platelet count in TCP samples. This difference may signal differences in the mechanism of thrombocytopenia between groups. Using our in-house ELISA, sGPVI levels in plasma from HD, ITP and TCP patients show no significant differences. GPVI and GPIbα were both significantly reduced ($p<0.01$ or smaller) in patients with TCP or with ITP, relative to healthy controls. There was some preliminary evidence of dysregulated glycosylation of the platelets in samples from people with ITP. These data imply that platelets generated by patients with ITP or TCP have reduced levels of the key adhesion-signalling receptors that initiate platelet activation and haemostatic responses. This molecular

deficiency may underpin bleeding that is otherwise not explained by the platelet count. These sorts of measurements may have future utility to examine mechanisms of cell receptor shedding, the nature of the anti-platelet autoantibody and may predict bleeding in ITP patients. Platelet protein surface levels may stratify ITP patients and may help in the evaluation of bleeding risk. Reduction in platelet surface receptors may indicate platelet activation and signal the presence of antiplatelet autoantibodies in ITP. The whole blood assay is rapid and easy to perform, with minimal handling of the samples and may be suitable for future standardisation in a clinical haematology facility.

The relative contributions of platelet consumption and platelet production to the clinical observation of thrombocytopenia are an ongoing clinical problem. These studies have developed a new molecular analysis of the GPIbα receptor and established new clinical tools for monitoring platelet receptors relevant to platelet function.

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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
BSA	Bovine serum albumin
CRP-xL	Crosslinked collagen-related peptide
DTT	Dithiothreitol
dTTP	Deoxythymidine triphosphate
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
Fc	Fragment crystallisable
FcRγ-chain	Fc receptor gamma-chain
FRET	Fluorescent resonance energy transfer
gDNA	Genomic DNA
GI	GI254023X
GM	GM6001
GP	Glycoprotein
HIT	Heparin-induced thrombocytopenia
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase

Ig	Immunoglobulin
ITAM	Immunoreceptor tyrosine-based activation motif
ITP	Immune thrombocytopenic purpura
kDa	Kilo Dalton
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
PLCγ2	Phospholipase Cγ2
PMA	Phorbol myristate acetate
PPP	Platelet-poor plasma
PRP	Platelet-rich plasma
PVDF	Polyvinylidene difluoride
RBC	Red blood cell
RT	Room temperature
SD	Standard deviation

SDS	Sodium dodecyl sulphate
SFK	Src family kinase
sGPVI	Soluble GPVI
SH2	Src homology 2
SLP-76	SH2 domain-containing leukocyte phosphoprotein of 76 kDa
Syk	Spleen tyrosine kinase
Taq	*Thermus aquaticus*
TBS	Tris buffered saline
TBS-T	Tris buffered saline with Tween-20
TEMED	*N*,*N*,*N'*,*N'*-Tetramethylethylenediamine
VWF	Von Willebrand factor
WBC	White blood cell

Chapter 1 Introduction

1.1 Introductory remarks

Blood is an essential fluid of living organisms and contains red cells, a range of white cells and, platelets (Brecher and Cronkite, 1950). Platelets (also known as thrombocytes historically) were one of the last cells to be described, mainly because of their size (1-2 microns), making them difficult to discern using standard light microscopy (Kuter, 1998). Initially, platelets were thought to be bacterial particles or red blood cell (RBC) fragments in the early studies (Spaet, 1980, Kuter, 1998). Later on, it emerged that platelets could aggregate in response to a range of agonists and that this activity occurred as part of a normal haemostatic response (Weiss and Lages, 1997). The platelet count for healthy individuals ranges between 150 to 450 x 10^9/L. Platelets are produced by the billions daily by the parental cell called the megakaryocyte in bone marrow (Noetzli et al., 2019, Machlus and Italiano, 2013) and possibly in the lung (Lefrancais et al., 2017). Platelet production is controlled by a hormone produced in the liver called thrombopoietin (TPO), which binds to Mpl receptors present on platelets and megakaryocytes (Kaushansky, 1998). TPO regulates the rate of maturation of megakaryocytes however there are clearly a number of other mechanisms that contribute to maintenance of a normal peripheral blood platelet count (Grozovsky, Giannini, Falet, & Hoffmeister, 2015). Mature megakaryocytes produce proplatelets which are released into the bloodstream and eventually break apart to form platelets (Nagata et al., 1997). Platelet destruction occurs in a coordinated fashion, mediated by resident macrophages (Kuppfer cells) in the liver and in the spleen. Contributing roles for the glycan landscape of platelets are known to trigger removal of aged or exhausted platelets from circulation (Hoffmeister et al., 2003; Rivadeneyra, Falet, & Hoffmeister, 2021). However, there remains much to learn about the molecular mechanisms that underpin both a platelet's evolution and lifespan.

1.2 Thrombocytopenia

Thrombocytopenia is the term used to describe a low platelet count, often defined as the 2.5th lower percentile platelet count than that of an average platelet count (Cheng et al., 2004)(D. J. Kuter, 2021). It is unclear whether individuals with a platelet count less than 150 x 10^9/L should be classified as borderline thrombocytopenic (Zimmer et al., 2006, Stasi et al., 2006).

Mechanisms by which platelet counts lower include increased destruction of the platelets by the liver and spleen, which is part of a haemostatic response, or the reduced production in the bone marrow (Grodzielski et al., 2018). Classic examples of bone marrow failure syndromes are aplastic anemia, myelodysplastic syndromes, and chemotherapy-induced thrombocytopenia (Dokal and Vulliamy, 2010). Similarly, in the conditions like disseminated intravascular coagulation (DIC) and thrombotic microangiopathies, increased destruction of bone marrow can occur (Aster, 1966). In cases like primary immune thrombocytopenia (ITP) and viral infection, several mechanisms, including raising autoantibodies that target proteins on the platelet surface or molecular mimicry, can cause a low platelet count (Stasi, 2012).

Thrombocytopenia is significant clinically because it can lead to elevated bleeding risk and, in the worst scenarios, haemorrhage due to compromised platelet functions (Hui et al., 2011). A platelet count of 20 x 10^9/L usually results in mucocutaneous bleeding, and below 10 x 10^9/L, the likelihood of severe bleeding such as intracranial haemorrhages has more significant potential (Psaila et al., 2009, Izak and Bussel, 2014). For patients afflicted with severe acute respiratory syndrome or infected with coronaviruses, thrombocytopenia is significant comorbidity (Liu et al., 2020, Zulfiqar et al., 2020, Al-Samkari et al., 2020). Recent studies have shown that immune thrombocytopenia (ITP) is associated with COVID-19 infection (Bomhof et al., 2020, Zulfiqar et al., 2020). The following section will explain the detailed structure and function of the platelets.

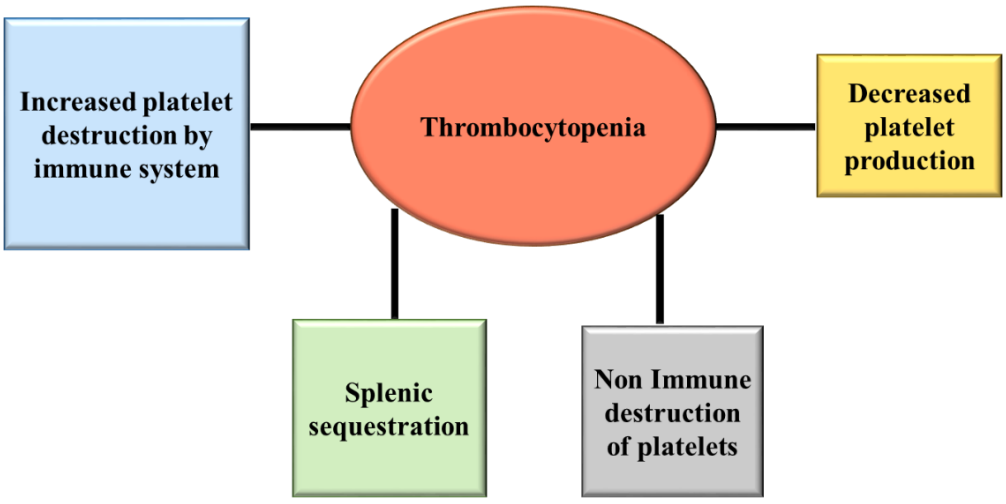

Figure 1: Causes of thrombocytopenia.

Increased platelet destruction can cause thrombocytopenia in the spleen or liver or

reduced platelet production by megakaryocytes (Aster, 1966). The initial triggers of ITP are unknown, but a few diseases contribute to immune dysregulation. Because of the lack of standards for a diagnosis of ITP, isolated thrombocytopenia only explained it.

1.3 Platelet structure and function

Platelets are disc shape fragments released from megakaryocytes in the bone marrow. They play a significant role in haemostasis and have emerging roles in innate immunity (Marcoux et al., 2020, Koupenova et al., 2018). The diameter of a platelet is about 1 to 2 μm, and a healthy individual could have a platelet count ranging from 150 to 400 $x10^9$/L (Schmitt et al., 2001). During circulation in the vascular system, platelets are resting and remain unreactive (Gresele et al., 2017). At the site of vascular injury, platelets become interactive with the blood vessel wall and start the pathways that lead to platelet plug formation (Min and Abrams, 2013). On the surface of the platelet, a range of glycoprotein (GP) receptors are present, along with granules contents current within the platelet and signaling proteins, platelets are activated and become reactive to the matrix protein of sub-endothelial, which is exposed upon vascular injury (Li et al., 2010, McFadyen and Kaplan, 2015). Within the platelet there are a host of alpha- and dense granules as well as lysosomes (Chen, Yuan, & Li, 2018). Whilst platelets are anucleate and do not carry genomic deoxyribonucleic acid (DNA), platelets contain a significant amount of ribonucleic acid (RNA) as well as all the molecular machinery (ribosomes, golgi apparatus, endoplastic reticulum and mitochondria) required to translate RNA to protein (De Wispelaere & Freson, 2022).

Aggregation of platelets enables the formation of a platelet plug at the injury site within a blood vessel, which controls bleeding. Still, the uncontrolled activation of platelets under pathological conditions can cause various thrombotic conditions such as stroke, myocardial infarction, and sepsis (Claushuis et al., 2016). Platelets also play an essential role in inflammation (Engelmann and Massberg, 2013). The following sections will discuss a detailed description of platelet production, structure, contents, surface receptors, activation, and role in different diseases.

1.3.1 Formation of platelets

The average life span of platelets in healthy individuals is 7 to 10 days. If not activated during this time, the liver and spleen (Fritz et al., 1986, Grozovsky et al., 2010). When the circulating platelet count decreases, the megakaryocytes produce more (Jackson et al., 1984). To maintain a platelet count of 150-400 x 10^9/L, approximately 10^{11} platelets are

created every day by megakaryocytes (Avecilla et al., 2004)(Bianchi, Norfo, Pennucci, Zini, & Manfredini, 2016). Thrombopoietin (TPO) has a major role in regulating megakaryocyte maturation and platelet production in the bone marrow (Larson and Watson, 2006) but TPO is not the only driver of platelet production (Kile, 2014; David J. Kuter, 2014).

1.3.2 Platelet intracellular contents

Whilst platelets are anucleate and do not carry genomic deoxyribonucleic acid (DNA), platelets contain a significant amount of ribonucleic acid (RNA) as well as all the molecular machinery (ribosomes, golgi apparatus, endoplastic reticulum and mitochondria) required to translate RNA to protein (De Wispelaere & Freson, 2022)(Penington et al., 1976). Hence, platelets contain all the machinery required to synthesise proteins de novo, and several studies have now shown this capacity of platelets (Qiao et al., 2018, Davizon-Castillo et al., 2020). The invagination of the platelet surface membrane helps form an open canalicular system that is essential for an increase in surface area as platelets adhere and is also crucial for the release of granule contents (Sorrentino et al., 2015). Platelets can take up plasma proteins such as serotonin, von Willebrand factor (VWF), and fibrinogen (Javors et al., 2000). The actin cytoskeleton of the platelets maintains the plasma membrane's integrity and controls platelet shape change during activation (Calaminus et al., 2008). Within the platelet there are a host of alpha- and dense granules as well as lysosomes (Chen et al., 2018). Platelets include three types of secretory vesicles: dense granules, α- granules, and lysosomes. Within α-granules, the most abundant proteins are (VWF) and fibrinogen (Golebiewska et al., 2015). The platelet surface is populated with various surface receptors that play an essential role in the platelet’s life span and function, discussed in Section 1.3.4.

1.3.3 Platelet activation and thrombus formation

Hemostasis is the primary physiological function of platelets. Thrombi formation prevents blood loss and sustains vascular integrity (Clemetson, 2012). The platelet activation signaling process is induced, divided into three steps, one platelet’s receptor interaction with their respective agonists, two signaling events, and three activations of integrin αIIbβ3 (Leslie, 2010). When platelets are attached to the damaged endothelium, signaling is induced, which activates it, and a thrombus is formed. Upon injury, collage of endothelium is exposed, platelets adhere to it via GPVI and recruit more platelets to the injury site. In this way, they form platelet plugs or thrombus, which stops blood loss (Li et al., 2010). The process of platelet adhesion activation and thrombus formation is described in figure 1.2.

Under the condition of low shear stress, platelet surface receptor integrin αIIbβ3 binds to the collagen (Ni and Freedman, 2003). The exposed collagen is covered by VWF, an important ligand of the platelet receptor GPIb-IX-V complex, upon vascular injury. The interaction is known as tethering (Offermanns, 2006). Thrombus is only formed when platelet adhesion is stable. Platelet tethering permits collagen fiber to become close to the glycoprotein VI. During this interaction, the receptor will induce signaling that will cause the activation of the platelets (Alderton et al., 2001). Activation of integrin αIIbβ3 leads to the interaction with VWF and other platelets, resulting in firm adhesion (Li et al., 2010).

On activation, platelets spread, ultimately increasing the surface area. Their morphology changes, actin forms filopodia and lamellipodia, which helps in the resilient attachment of the platelets to the collagen VWF (Calaminus et al., 2008). Binding of GPVI to the collagen and activation of integrin results in the secretion of the contents of α and dense granules, including adenosine diphosphate (ADP) and thromboxane (TxA_2) which start additional activation and aggregation of the platelets (Briggs et al., 2007).

Thrombus growth is promoted as the circulating platelets are recruited at the damage site (Mammadova-Bach et al., 2015). Through coagulation cascade activation, further thrombus formation is strengthened. Thrombin generated during platelet activation transforms fibrinogen to fibrin, forming a mesh that stabilises the thrombus and successfully stops blood loss (Renné et al., 2005). Platelets are also involved in many other biological processes in the body, including repairing tissues, inflammation, and host defense against bacteria (Semple et al., 2011, Koupenova et al., 2018).

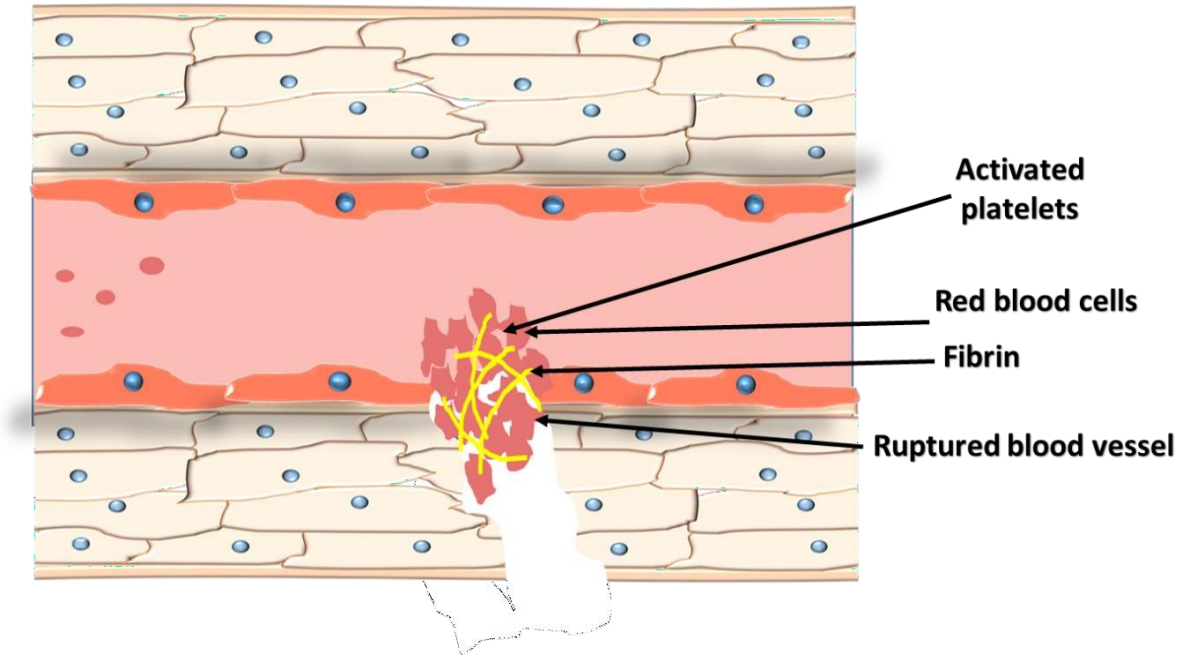

Figure 1.2: Platelet activation and roles in haemostasis

Exposure of subendothelial matrix proteins such as VWF and collagen provides the ligands that enable the adhesion of platelets to a vessel wall. After bonding, activation spreading occurs along with the release of soluble agonists (ADP, thromboxane), which help recruit platelets to the injury site and form a platelet plug. The coagulation process produces thrombin that interacts with platelet receptors and activates them, which triggers more platelets. Activated platelets adhere and spread, forming lamellipodia and filopodia and release granule contents. The coagulation cascade is triggered and harnessed to the activated platelet surface resulting in the generation of thrombin, formation of fibrin, and eventually a haemostatic plug is formed (Arce and Li, 2019).

1.3.4 Platelet Receptors

The platelet surface has receptors, which play significant roles in platelet function and determine platelet life span. Despite being anuclear, platelets can carry out protein synthesis as they contain mitochondria, ribosomes, and messenger RNA (mRNA) inherited during platelet production from megakaryocytes (Davizon-Castillo et al., 2020). However, there is no evidence to date that platelets undergo synthesis of platelet receptors de novo. Platelets are released with a complete suite of receptors, some of which are held in intracellular storage granules (e.g., P-selectin) or sequestered within the complex open canalicular membrane system of platelets (e.g., GPIb-IX-V, αIIbβ3) (Selvadurai and Hamilton, 2018). Platelet receptors transmit molecular signals by various mechanisms, which result in the secretion of granule contents. Platelet receptor activation also leads to positive and negative feedback mechanisms that regulate platelet function. The following

sections will briefly describe primary platelet receptors.

1.3.5 Integrin αIIbβ3

For platelets to perform haemostasis functions, several integrins play essential roles by enabling firm platelet adhesion to the subendothelial matrix (collagen, laminin, fibronectin adhesion involves α2β1, α6β1 and α5β1 respectively) and aggregation. Integrin αIIbβ3, also known as GPIIb/IIIa and CD41/CD61, is the most abundant receptor on the platelet with approximately 80,000 copies per human platelet (Shen et al., 2013, Li et al., 2010). The complex is formed via a calcium-dependent association of the αIIb and β3 subunits, and there are four Ca2+ binding sites within its structure. The αIIb subunit is found only on platelets and megakaryocytes and is thus an ideal surface marker of platelets in flow cytometry experiments using whole blood. It is a heterodimer made of two units α and β which combine to form a binding pocket that can recognise arginine-glycine- aspartic acid (RGD) sequences which are commonly found within several matrix and plasma proteins, including fibrinogen, von Willebrand factor (VWF) collagens, laminins, vitronectin, and fibronectin. Fibrinogen is the primary ligand for αIIbβ3 (Springer et al., 2008).

Disorder in the synthesis of either αIIb and/or β3 subunit can arise from the point, missense, nonsense, or frameshift mutations. These mutations hinder functions of platelets like adhesion, spreading, and aggregation, which give rise to the rare blood disorder of Glanzmann's thrombasthenia (GT) (Nurden and Caen, 1974, Zhou et al., 2018). Immobilized agonists like collagen or VWF, when bound to their receptors GPVI and GPIb-IX-V, or soluble agonists ADP, thromboxane (TXA2) when attached to G-protein-coupled transmembrane domain receptors (GPCRs) inside out signaling commences. These signalling pathways activate a series of kinases which phosphorylate important residues withinnplatelet cytoskeletal proteins, including talin, actin, myosin, tubulin and kindlin. Several of these proteins engage with the integrin αIIbβ3 cytoplasmic tail and orchestrate conformational changes to the extracellular domain of this integrin, which increases receptor affinity and avidity for the major ligand fibrinogen (Nieswandt et al., 2009). Similarly, when soluble fibrinogen binds to activated αIIbβ3, a series of intracellular signaling events is initiated, which leads to stable platelet adhesion, degranulation, and spreading by reorganising the cytoskeleton, enabling thrombus formation (Huang et al., 2019).

1.3.6 GPVI

During the late 1990s, after the discovery, cloning and affirmation that GPVI was the

primary platelet receptor for collagen, this receptor has been the subject of an enormous body of research (Andrews, Arthur, & Gardiner, 2014; Nieswandt & Watson, 2003; Perrella, Nagy, Watson, & Heemskerk, 2021). GPVI is present on megakaryocytes and the membrane of platelets. Between 4000-6000 copies are found on the surface of human platelets (Best et al., 2003; Nieswandt & Watson, 2003). GPVI belongs to a superfamily of immunoglobulin (Ig)-like receptors and is expressed on the platelet surface with two extracellular Ig-like domains, an *O*-glycosylated mucin-like stalk, a transmembrane domain, and a cytoplasmic tail (Miura et al., 2002). GPVI was cloned in 1999 (Clemetson, Polgar, Magnenat, Wells, & Clemetson, 1999; Moroi & Jung, 2004) and is the major collagen receptor on platelets that signals, however this requires cooperation with other molecules. GPVI has emerged as a major receptor for fibrin with implications for thrombus stability (Mammadova-Bach et al., 2015). GPVI and the Fragment constant (Fc) receptor γ chain (FcRγ) are non-covalently linked. The FcRγ chain has an immunoreceptor tyrosine-based activation motif (ITAM) sequence, critical for signalling triggered by GPVI engagement (Rayes et al., 2019). Upon binding of ligand to GPVI, the ITAM of FcRγ is phosphorylated, which leads to the activation of spleen tyrosine kinase (Syk) (Moroi and Jung, 1997, Zheng et al., 2001). GPVI-FcRγ-Syk signaling starts inside out signaling, which activates integrin αIIbβ3, which improves stable interaction with collagen that enhances aggregation (Dütting et al., 2012). Studies show that activation of GPVI can also be triggered by mAbs directed again GPVI and that this activation is fortified by cross-linking.

GPVI has continued to entrance the research community as this receptor is emerging as a leading candidate for antithrombotic therapy (Gawaz et al., 2014; Mackman, Bergmeier, Stouffer, & Weitz, 2020). Because GPVI is likely to play only a minor role in venous haemostatic responses and that it is platelet-specific, means that targeting this receptor may generate only mild bleeding diatheses without additional side effects.

1.3.7 Platelet FcγRIIA

FcγRIIA is a low affinity receptor for IgG. Like GPVI it is a member of the Ig-like superfamily of signalling receptors. FcγRIIA is found on human but not murine platelets and is also present on neutrophils, macrophages, monocytes, and some dendritic cells (Anania, Chenoweth, Wines, & Hogarth, 2019; Vogelpoel, Baeten, de Jong, & Den Dunnen, 2015). Its role on platelets is likely to be related to the innate immune system role of platelets and work has shown that antibody cross-linking activates FcγRIIA (Lu, Mold, Du Clos, & Sun, 2018). That the anti-GPVI mAbs cause activation of platelets which can be prohibited by

immunoblocking FcγRIIA (Mohammad Al-Tamimi et al., 2009) indicates that GPVI and FcγRIIA are closely aligned on the platelet surface. Platelets also play a role in antimicrobial activities and in these settings engagement of FcγRIIA by microorganisms triggers release of different chemokines and cytokines (Scherlinger et al., 2018). Studies have shown that activation of FcγRIIA under pathological conditions can result in autoimmune diseases and thrombosis (Patel, Michael, Naik, & McKenzie, 2021). Treatment of ITP patients bearing an antiplatelet autoantibody by ablating FcγRIIA and other Ig receptors using intravenous gamma globulin (IVIg) can be efficacious, but this is a very restricted treatment due to the potential for adverse effects (Nakar & Bussel, 2009). In the research setting, mAb IV.3 is an anti-FcγRIIA function blocking antibody. The mechanism by which this antibody works is that it identifies residues of amino acids 132-137 within the second Ig-like domain of FcγRIIA (Ramsland et al., 2011). Activation of human platelet FcγRIIA takes place by various factors, including anti-platelet antibodies and pathogens (Arman and Karuel, 2015). This pathological activation leads to different autoimmune disease, including ITP and HIT (Gardiner et al., 2008; Kelton et al., 1988; Reilly et al., 2011).

1.3.7 GPIb-IX-V Complex

For platelets to perform normal haemostasis, adhesion of platelets is crucial. GPIb- IX-V is the receptor for VWF, and it enables the circulating platelets to adhere to the site of injury (Chen and LÓPEZ, 2005, Andrews et al., 2003). GPIb-IX-V complex association with actin filaments in cytoskeleton stabilise the adhesion and aggregation, and it is also the second most abundant receptor on a platelet surface (Auton et al., 2010, Zhang et al., 2015). It is composed of four subunits GPIbα, GPIbβ, GPIX, and GPV, in a ratio of 2:4:2:1 (Li and Emsley, 2013). The binding site for VWF A1 lies within the extracellular domain of GPIbα, while the binding site for Filamin A is in the cytoplasmic region. Filamin A facilitates the linkage of GPIbα with actin filaments (Nakamura et al., 2006).

1.4 Platelets glycans

Complex mucin-type carbohydrates such as N-linked glycans (N-glycans) and O-linked glycan (O-glycans) alter the platelets glycoproteins (Kauskot and Hoylaerts, 2012, Wilhelm et al., 2014). Sialic acid cap both glycans (Li et al., 2015). Sialic acid belongs to the family of acidic monosaccharides. The visible position and negative charge on the sialic acid make them an important player in controlling cellular and molecular interlinkage, crucial in pathological and physiological mechanisms (Xu et al., 2009). Losing Sialic acid is believed to be one of the significant factors determining the platelets' life span (Pereira et al., 1999,

Sørensen et al., 2009). Desialylation is the process in which, under different conditions, sialic acid is cleaved from the glycans (Yule et al., 2020). Desialylation also plays a role in intrinsic apoptosis and platelet activation (Chen and Lopez, 2005, van der Wal et al., 2010). The removal of sialic acid, termed desialylation, triggers the elimination of platelets in the liver (Grewal et al., 2013) by specialised liver macrophages bearing asialoglycoprotein receptors, which are also known as Ashwell-Morell Receptors (AMR) (Grewal et al., 2008, Rumjantseva et al., 2009, Sørensen et al., 2009). Li and colleagues showed in mouse studies that those platelets on which O-glycans are absent have reduced life span and are readily cleared in the liver, the cause of which is might be faulty sialylation. They also confirmed the Kupffer cells have a crucial role in this process, along with the asialoglycoprotein receptor (Li et al., 2017).

1.5 Neuraminidases / Neuraminic acid

Neuraminidase (NEU), or glycoside hydrolase, is an enzyme that cleaves sialic acid from glycolipids and glycoproteins (Gubareva and Mohan, 2020). Neuraminidases are the major enzymes that start the process of desialylation (Monti et al., 2010). There are four isoforms of neuraminidase designated NEU1, NEU2, NEU3, and NEU4, which cleave α2,6 and/or α2,3 glycan linkages (Bonten et al., 1996) and release sialic acid residues. The NEU isoforms exhibit a level of substrate specificity; however, significant differences in their location also contribute to substrate specificity. For example, NEU1 is found in lysosomes and targets sialic residues on glycoproteins. In contrast, NEU2 and NEU3 are located in the cytosol and on cell membranes, explicitly cleaving glycosphingolipids (gangliosides) within neuronal cell plasma membranes (Mozzi et al., 2012 Ha et al., 2004).

NEU4 is associated with mitochondria, and the unique character of this enzyme is that it cleaves sialic acid groups from glycoproteins and gangliosides (Seyrantepe et al., 2004, Bosmann et al., 1972). On resting platelets, NEU1, NEU2, and NEU4 are located on the cell membrane and in platelet mitochondria and α-granules. Initiation of GPIbα clustering by VWF exposure activates platelet NEU found on the plasma membrane but this stimulus is not sufficient to mobilise neuraminidase present within platelets (van der Wal et al., 2020).

1.6 Platelet Desialylation

Desialylation of platelet receptors results in clearance of these platelets by Ashwell Morell receptors in the liver (Spillemaeker et al., 2018, Grozovsky et al., 2015). Interestingly the

interaction of VWF with GPIbα will also promote platelet receptor desialylation within a few minutes (Vincent et al., 2018).

Treatment of washed platelets or PRP with neuraminidase results in increased exposure of galactose residues (de Graaf and Metcalf, 2011). In various studies, platelets destruction in ITP is driven by a mechanism that is Fc- independent, primarily via antibodies targeting GPIbα, the second most abundant platelet receptor and the ligand-binding subunit of the GPIb-IX-V complex. Interestingly ITP patients with antibodies against GPIbα are often resistant to traditional IVIg treatment (Webster et al., 2006b, Nieswandt et al., 2000, Peng et al., 2014) and steroid therapy (Zhu et al., 2015).

Ligand-GPIbα engagement initiates signaling, activating αIIbβ3, causing platelets to aggregate (Ozaki et al., 2005, Du, 2007, Gardiner et al., 2010a). GPIbα is heavily glycosylated (Jamieson et al., 1979) with N and O-linked glycosaminoglycans topped with sialic acid residues, contributing ~64% of the total sialic acid content of the platelet (Solum et al., 1980). GPIbα desialylation was previously reported in chilled platelets, which were rapidly cleared by the liver immediately after transfusion (Jansen et al., 2012, Wandall et al., 2008, Sorrentino et al., 2015, Hoffmeister et al., 2003). This was shown to be due to alteration in the glycans of GPIbα sufficient to cause clearance of the platelets in an Fc-independent manner (Hoffmeister, 2011, Hoffmeister et al., 2003). Exposure of platelets to GPIbα antisera can initiate platelet activation (Li et al., 2010). Still, further investigation needs to assess whether the binding of autoantibody to GPIbα causes platelet desialylation and ultimately clearance of platelets by Fc-independent mechanisms. This type of approach may help identify the suitable treatment regime for an individual ITP patient (Li et al., 2015).

1.7 Platelets and Neuraminidase

Removing sialic acid residues (desialylation) is very important for the clearance of aged platelets (Grozovsky et al., 2010). In addition, platelet desialylation is linked to platelet activation and programmed cell death (van der Wal et al., 2010, Chen and LÓPEZ, 2005, Quach et al., 2018). Platelets from ITP patients exhibit desialylation (Li et al., 2015) mediated by neuraminidases. There are four neuraminidase isoforms discussed in Section 1.5.4.

Previous studies detected an increased NEU1 function in platelets after cold storage and in patients with ITP having antibodies against GPIbα (Jansen et al., 2012). After VWF-mediated clustering of GPIbα, sialic acid was removed from platelets (Gitz et al., 2012).

This was prevented by including 2-deoxy-2-3-didehydro-N- acetylneuraminic acid (DANA), a non-specific NEU inhibitor. Reduced levels of NEU activity were recorded, illustrating that NEU play a part in platelet desialylation when platelets are activated via GPIbα.

1.8 Platelet life cycle and the regulatory role of glycans

Modification of platelet glycan is one factor that can lead to platelet removal, and loss of sialic acid starts the clearance of murine platelets circulating in the blood (Hoffmeister, 2011). The platelet surface features heavily glycosylated GPs, with the most abundant being GPIb-IX-V. Removal of GPIb-IX from platelets can contribute to an 80% decrease of sialic acid per unit area of the platelet membrane. GPIbα glycan has a crucial role in initiating platelets clearance. The cleavage of sialic acid makes them available to bind to AMR in the liver, and in this way, platelets are cleared off the blood circulation (Rumjantseva and Hoffmeister, 2010). The Aswell Morell receptor belongs to the C-type family of lectins in the liver, which identifies, binds, and clears asialoglycoproteins (Grewal et al., 2008). When platelets are circulating in the blood, VWF does not attach to it but, when platelets become static at the site of vascular injury and change shape, only then will VWF bind to the GPIbα (Denis et al., 1998). GPIbα juxtamembrane mechanosensory domain experiences uncoiling when a pulling force is applied by blood flow while the ligand-binding domain is attached to the A1 domain of the VWF. This will result in signaling and desialylation, which will end up in the clearance of the platelets (Zhang et al., 2007).

1.9 Platelet clearance

For maintenance of a stable platelet count, a healthy human body produces 10^{11} platelets (Lefrançais et al., 2017). Production must be balanced with platelet clearance. In platelets, just like nucleated cells, the process of apoptosis is dependent on the balance between antiapoptotic and proapoptotic balance. Few of the Bcl-2 family proteins, including Bcl-2, Bcl-xL, and Bcl-w, are present on human platelets and murine (Zhang et al., 2007, Kodama et al., 2011). Studies have found a new mechanism of platelet clearance that is by antiplatelet antibodies against platelet receptors. In ITP patients, surface glycoproteins are targeted by the antibodies and cleared by the Fc-dependent mechanism by macrophages (Harrington et al., 1990). Recent studies have shown that infusing monoclonal antibodies that identify the N-terminus of ligand-binding domains of GPIbα will rapidly deplete platelets from animals (Becker and Miller, 1989, Cauwenberghs et al., 2000).

1.10 Immune Thrombocytopenia

1.10.1 Introduction

Immune thrombocytopenia (ITP) is an acquired autoimmune condition described by rapid elimination of platelets induced by anti-platelet auto antibodies against integrin αIIbβ3, α2β1, and GPIbα of the GPIb-IX-V complex (Morodomi et al., 2020). It is the most common of platelet based autoimmune diseases. Patients with ITP usually present with unexplained mild bleeding (Sarah M. Hicks, Coupland, Jahangiri, Choi, & Gardiner, 2020)(Boulware and Refaai, 2020). Investigations of these patients show isolated thrombocytopenia with a platelet count that has dropped to less than 100 x 10^9/L. One implication is an increased bleeding risk (Cooper and Ghanima, 2019). Different factors are considered responsible for the ITP infection, drugs, and genetics (Swinkels et al., 2018). Gardiner and colleagues identified a rare case of anti-GPVI mediated ITP in which they showed that primary platelet activation is not only caused by these antibodies but also results in shedding of platelet surface GPVI, causing considerable clinical platelet dysfunction that results in mild bleeding at platelet counts not usually cause bleeding in ITP patients (Gardiner et al, 2008a). The main antigen targeted by platelet reactive CD4+ T cells is αIIbβ3 (Kuwana et al. 2001). The antibodies in an ITP patient's blood bind to platelets and opsonise the surface, leading to early platelet destruction by FcγR facilitated phagocytosis in the reticuloendothelial system and the spleen (Semple et al., 2010).

1.10.2 Prevalence of ITP

For the diagnosis of ITP, the platelet count should be below the lower threshold of 100 × 10^9/L (Rees et al. 2003). Estimation of current data shows that the annual occurrence of ITP is 3.2–3.9 per 100 000 and prevalence at 20.3 per 100 000 (Marieke Schoonen et al., 2009). Approximately 4.5% of the Caucasian population is affected by ITP (Hayter and Cook 2012), and ITP is more prevalent in females, who comprise 57–66% of total ITP patients (Portielje et al., 2001). ITP in children is an acute pathology that generally resolves with time. In adults, the chronic form of ITP persists in up to 80% of individuals for more than one year (Deane et al., 2010).

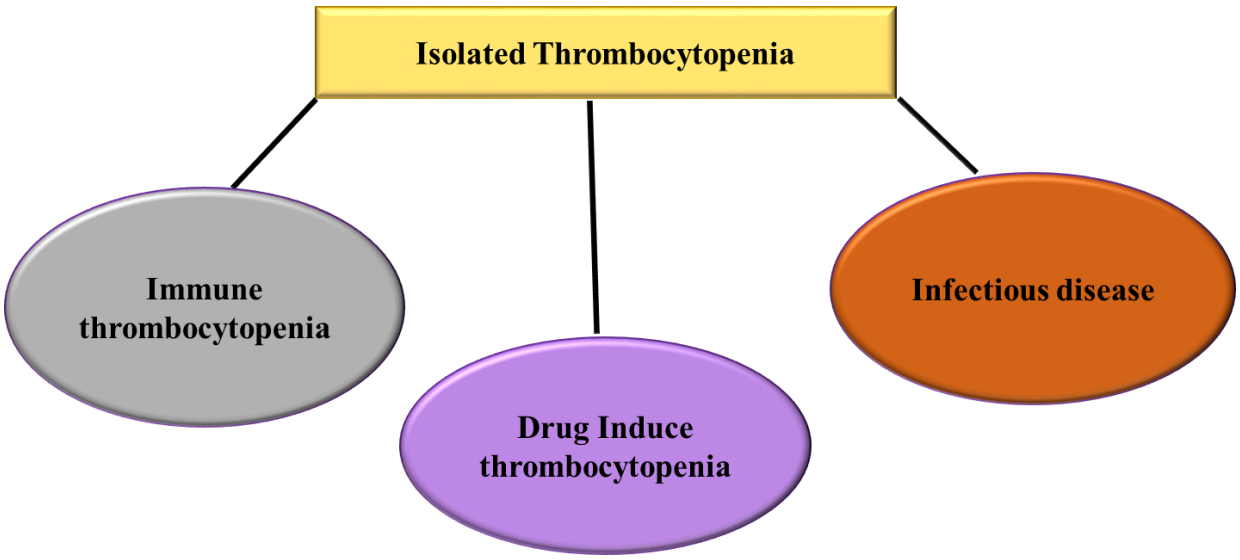

Figure 1.3: Immune thrombocytopenia

Immune thrombocytopenia is a disorder of unknown causes which is mainly thought to be caused by the destruction of platelets by autoantibodies; this destruction of platelets is conceivably T-cell–mediated, which not only cause destruction of circulating platelets but also target the platelet production site bone marrow (Cines and Blanchette, 2002). ITP is diagnosed by excluding all other causes of thrombocytopenia (George et al., 1996), which means eliminating all the reasons, which are the non- immune, presence of other autoimmune diseases, and thrombocytopenia induced by drugs (Cines and Blanchette, 2002).

1.10.2 Pathophysiology

The regulation of immune system function is observed in ITP, like other autoimmune diseases like lupus, rheumatoid arthritis, etc. The critical feature of the pathophysiology of the ITP is defects in cellular immunity, and the result is the production of antiplatelet antibodies by autoreactive B lymphocytes (S. M. Hicks, Lee, Ali, Choi, & Gardiner, 2020)(Kuwana and Ikeda, 2005). In ITP, binding anti-platelet antibodies to platelet glycoprotein and eliminating them is the most recognised mechanism of platelet clearance, along with this sialic acid cleavage and the programmed death (McMillan, 2007). Enhanced depolarisation of mitochondrial membrane activates caspase3 and P-selectin on the platelets surface membrane, which are the markers of platelets death (Goette et al., 2016). Li and colleagues showed that plasma from the ITP patients induces cleavage of the sialic acid from the glycoproteins of platelets, which shows the anti- autoantibodies of GPIb-IX (Li et al., 2015). Autoantibodies can activate platelets, which trigger the release of neuraminidase that cleaves sialic acid from the platelet's glycoproteins (Li et al., 2017).

Marini and colleagues confirmed that anti-αIIbβ3 autoantibodies in the plasma of ITP patients could induce cleavage of sialic acid in healthy platelets glycoproteins (Marini et al., 2019). Megakaryocytes are the precursor of platelet production when autoantibodies are produced by the immune system that binds to αIIbβ3, and GPIb-IX also contribute to inhibiting proplatelet formation (Lev et al., 2014, Iraqi et al., 2015). IgG segment from plasma of the ITP patients hampers the megakaryocytes spreading and adhesion. The presence of anti αIIbβ3 autoantibody prevents the interaction of fibrinogen with megakaryocytes, while autoantibody against GPIb-IX-V weakens the spreading and binding of megakaryocytes to VWF (Grodzielski et al., 2018).

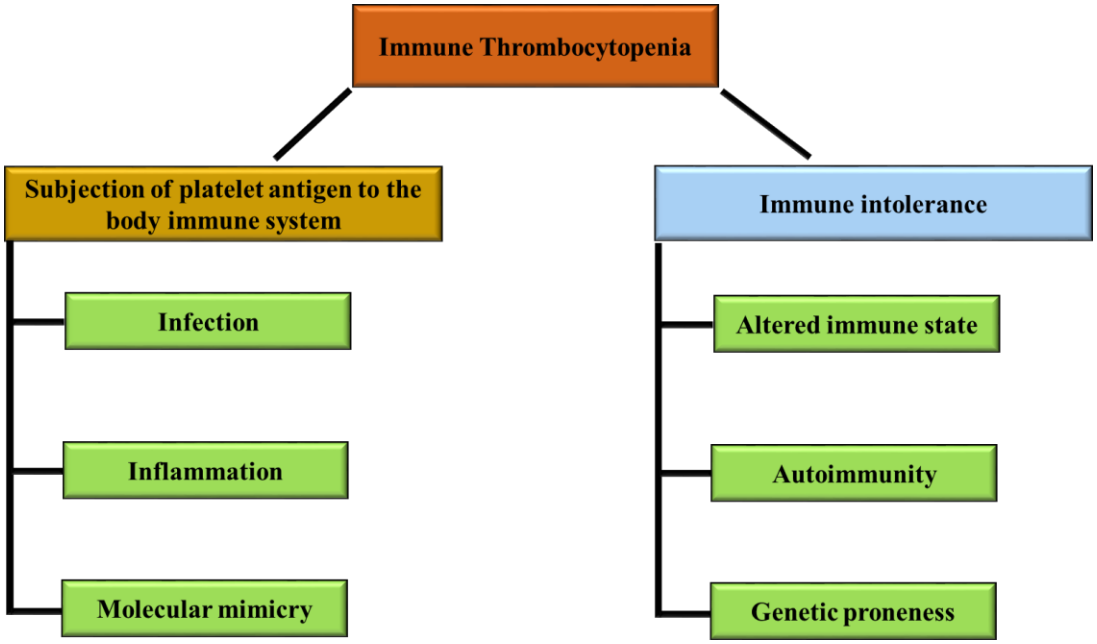

Figure 1.4: Causes of Immune thrombocytopenia (ITP)

Immune thrombocytopenia (ITP) is a complex pathogenesis syndrome. Self-tolerance is lost after which, T helper cells (Th) help produce autoantibody (LeVine and Brooks, 2019). In response to the infection, the body produces antibodies that confuse platelets with pathogens, which destroys platelets in the blood and bone marrow by impairing the production of platelets in the megakaryocytes (Shrestha et al., 2020).

1.10.3 Fc Dependent Mechanisms of Platelet Clearance

In ITP, Ag-specific IgG antibodies mark the platelets followed by phagocytosis in the liver and spleen mediated by FcγR (Kuwana et al., 2002). A study based on the anti-αIIbβ3 antibodies shows that subclasses of these antibodies vary among patients (Chan et al., 2003). B cell clones produce autoantibodies against an antigen on platelets surface (McMillan, 2000). IgG antibodies attach to the platelet receptors, and then the FcRγ

mediate phagocytosis of platelets in the liver and spleen (Kuwana et al., 2002). Different patients have multiple IgG subclasses for GpIIb/IIIa, which vary during the disease (Chan et al., 2003).

1.10.4 Fc Independent Mechanism of Platelet Clearance

Several studies reveal that platelet elimination in ITP can be Fc independent. In immune thrombocytopenia, the primary cause of platelet clearance is the autoantibodies that target its glycoproteins. Experiments by Nieswandt and co-workers show that intact monoclonal anti-GPIb-IX antibody and its F (ab) portion can start a reduction in platelet count in murine models (Renné et al., 2005).

Mechanisms of thrombocytopenia	
Fc dependent	**Fc independent**
• Antibodies target αIIbβ3 and α2β1	• Antibodies target GPIbα
• Fc portion binds macrophages	• Fab mediated desialylation
• Splenic clearance	• Liver clearance

Figure 1.5: Fc dependent and Fc independent mechanisms of platelet destruction

Ashwell Morell receptors (AMR) on liver cells are involved in Fc independent destruction of platelets. Antibody binding to GPIbα triggers platelet desialylation which are recognised by AMRs of liver cells, and cleared (Li et al., 2015). 70 to 80% of patients have antibodies against αIIbβ3. 20 to 40% of patients have antibodies against GPIbα. Some patients have antibodies against both antigens. Making the diagnosis even more challenging, antiplatelet antibodies are not detectable in a significant number of patients (Go et al., 2007).

1.10.5 Molecular mimicry of viruses

Molecular mimicry is used to describe mechanisms by which specific pathogens mimic autologous cell features or peptide sequences from host proteins to escape the immune system resulting in the cross-activation of Tor B cells against the body's proteins (Kohm et al., 2003). Hepatitis C Virus-infected patients can have autoantibodies that target peptide sequences in the platelet β3 integrin subunit. The production of these autoantibodies results from an immune reaction against one or more peptides present in the HCV core envelope protein (Rajan et al., 2005). Similarly, in Human Immune Deficiency Virus infection related thrombocytopenia, autoantibodies target specific peptide sequences such as amino acids 49-66 within the platelet β3 integrin subunit (Nardi et al., 1997). A distinctive characteristic of these antibodies is their capability to generate reactive oxygen species through the initiation of 12- lipoxygenase and NADPH oxidase mechanisms, resulting in platelet fragmentation (Nardi et al., 2004).

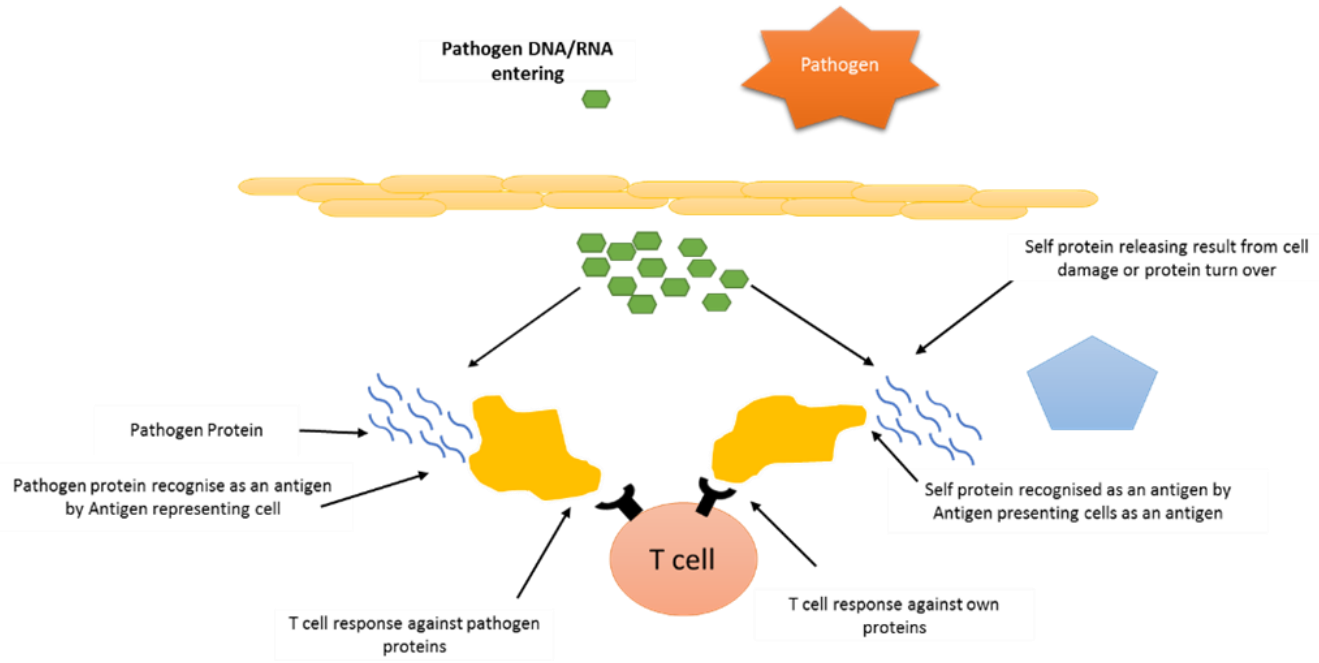

Figure 1.6: Molecular mimicry of viruses

Hepatitis C virus (HCV) and Human Immune Virus (HIV) have structural peptides that mimic GPIIIa, which confuse the body's immune mechanism and encourage the production of antibodies against GPIIIa. Viruses mimic platelets proteins to escape the host immune response (Aster, 2009).

1.10.6 The autoimmune mechanism in ITP

The onset of ITP has no apparent underlying cause but seems to develop through loss or perturbation of B- and T-cell immune tolerance via diverse mechanisms. An abnormal T cell response, driven by splenic T follicular helper cells, stimulates the proliferation and differentiation of autoreactive B cells. Altered T-helper cell ratios and changes to attendant cytokines, such as interleukin (IL)-17, have also been reported to contribute to ITP pathology (Audia et al., 2017). Cytotoxic T cells target platelets in the periphery and affect megakaryocyte proplatelet formation in the bone marrow niche (Olsson et al., 2003, Li et al., 2007). In ITP often, a single glycoprotein is recognised by an autoantibody. However, in some ITP patients, more than one platelet glycoprotein is targeted by autoantibodies (He et al., 1995), predominantly of the IgG class (Hoemberg et al., 2011). Antiplatelet antibodies are synthesised by B cells with special modification in light and heavy chains to adapt to the new foreign elements that confront them (Roark et al., 2002). Splenic follicular T helper cells aid in B cell differentiation and synthesis of antiplatelet autoantibodies by secreting IL- 21 through the interaction of CD4/CD40L (Audia et al., 2014). The cytokine BAFF which increases the survival of B cells and stimulates spleen cells is present at high levels in ITP patient serum (Thai et al., 2016).

T cells also play roles in ITP by producing antibodies against platelets glycoproteins. The isotype switching of antibodies is crucial, causing significant destruction of platelets (Roark et al., 2002). The cytokine profile shows elevated levels of IFN-γ and IL-2 levels in ITP patient serum (Semple et al., 1996). Th1 cell polarization is increased in CD4+ T cells, which links with an increase in CD8 and IFNγ in ITP patients (Audia et al., 2013) and reduced peripheral Th2 cells and Tc2 cells (Stasi, Del Poeta, et al. 2007) along with decreasing number and function of CD25, FOXp3, and Tregs (Ling et al., 2007). Results in a complete derangement of T cell tolerance and the onset of a set of autoimmune responses.

1.11 Diagnostic Challenges

ITP diagnosis is a major clinical challenge, as the current diagnosis is one of exclusion and relies overly on platelet count and of the taking of a careful personal and family history of bleeding (Hicks et al, 2020). Thrombocytopenia can also occur in subordinate forms with systemic lupus erythematosus, antiphospholipid syndrome, lymphoproliferative disorders, human immunodeficiency virus, and hepatitis C virus and therapy with drugs such as heparin and quinidine (Hicks et al, 2020; Diz-Küçükkaya et al., 2001). Currently, there are no specific diagnostic tests available to distinguish ITP from other causes of

thrombocytopenia, and methods to evaluate platelet function and bleeding propensity have been standardised for platelet numbers greater than 100 x 10^9 platelets/L. Bone marrow examination is generally not performed in ITP patients (Provan et al., 2010). The monoclonal antibody immobilization of platelet antigens (MAIPA) assay is designed to identify ITP specific antibodies and is available but not routinely used. The information ascertained is variable and unreliable, and the benefit of determining the nature of the autoantibody is not clear up to this point. Antiplatelet autoantibodies are undetectable in up to 25% of ITP patients (Wang et al., 2014).

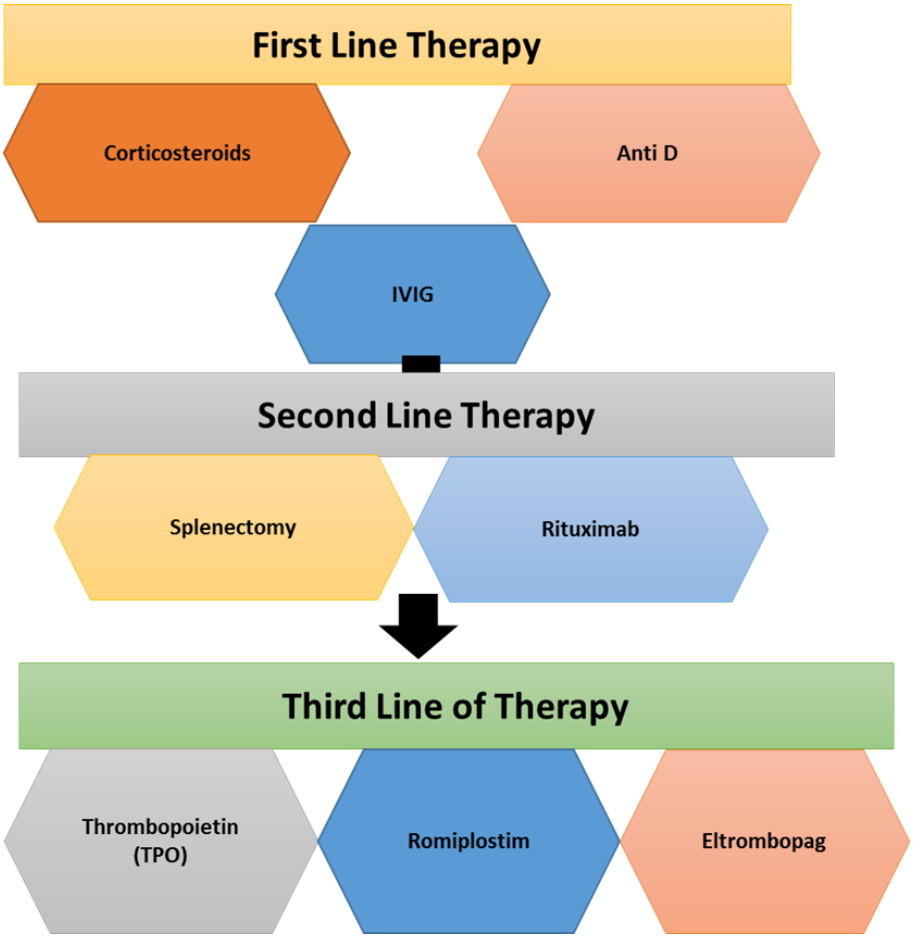

Figure 1.7: Treatment of immune thrombocytopenia

Flow Chart showing the first, second, and third lines of therapy for ITP patients. No single treatment system is suitable for all patients (George 2000). There is a need for individualised therapy (Portielje et al., 2001). Standard treatments for ITP are mainly based on immunosuppression, immunomodulation, or removal of a primary site of platelet destruction (splenectomy). To improve patient outcomes and design and apply more effective therapies, it is essential to understand molecular events leading to platelets destruction (Pang and Lazarus, 2010, Sollazzo et al., 2011).

1.12 Significance of Project

Immune thrombocytopenia (ITP) is a chronic, immune-mediated disease characterised by a transient or long-lasting decrease in platelet counts. However, this is a rare disease; it is the most prevalent autoimmune disorder involving blood cells, afflicting 2 - 5 in 100,000 children per year and 3 in 100,000 adults per year. This is equivalent to approximately 2000 - 2500 adult cases of ITP in Australia each year. For patients between 18-65 years, the incidence of ITP increases with age and is slightly higher in women than men. While the acute form of ITP is benign and self-limited, the chronic form of the disease is accompanied by permanent thrombocytopenia. More than 7 million people worldwide have the chronic form of this disease. The available resources limit the diagnosis and treatment of ITP. Primary ITP is diagnosed by exclusion, with isolated thrombocytopenia as the only genuinely universal feature. Patients with ITP experience numerous clinical severe consequences, including significantly higher rates of infection, bleeding, anemia, debilitating fatigue, and psychological changes. Clinical investigation of patients with ITP varies widely but generally consists of a peripheral blood count and a peripheral blood smear evaluation. Treatment typically comprises conventional first-line therapies that affect the immune system (IV gamma globulins, corticosteroids), followed by splenectomy as a second management line. While newer therapies are emerging, a lack of clinical diagnostic tools and limited financial resources of the individual patient and the health care system strongly impact the choice of treatment. ITP as a syndrome has not been well categorised. Every individual patient has their unique genetic makeup and disease etiology. Ideally, each individual with ITP requires personalized and customized management and therapy. This project will focus on the development of new approaches which can help address some of these diagnostic issues.

1.13 Hypothesis

Platelets from patients with primary ITP show differences in receptor levels which can be quantified by flow cytometry approaches.

1.2 Aims

- To establish and apply research laboratory-based assays to quantify platelet receptor levels in patients with low but normally sufficient platelet numbers.
- Assess receptor levels in healthy platelet samples that have been exposed to neuraminidase treatment.

Chapter 2 Materials and methods

Table: 1 List of general laboratory reagents

Reagents	Composition
Acid citrate dextrose (ACD)	11.9 mM Tri-sodium citrate (TSC), 10.9 mM citric acid, 15.54 mM glucose in distilled water.
Citrate- glucose-saline (CGS)	33.3 mM glucose, 12.92 mM TSC, 123.2 mM NaCl, pH 7.0 dissolved in distilled water.
Tyrode's buffer	5.6 mM glucose, 137 mM NaCl, 2.7 mM KCl, 0.5 mM $MgCl_2$, 1.4 mM $CaCl_2$, 0.36 mM Na_2HPO_4, 9.4 mM $NaHCO_3$ pH 7.4.
Ethylenediaminetetraacetic acid (EDTA)	10 x tris saline buffer pH 7.4, EDTA 37.2 g/200 mL, 20 mL concentrated NaOH solution dropwise added till EDTA is wholly dissolved in distilled water.
Tris Saline (TS)	NaCl 150 mM, Tris (hydroxymethyl) amino methane 10 mM in distilled water, pH adjusted to 7.4.
FACS Buffer	10 mM Tris-HCl, 0.15 M NaCl, 5 mM EDTA pH 7.4
Phosphate Buffer Saline (PBS)	KCl 2.7 mM, NaCl 137mM, Na2HPO4 10 mM in distilled water pH 7.4.
Acrylamide	Sigma-Aldrich (St. Louis, Missouri, USA), catalogue #A8887
Bovine Serum Albumin (BSA)	Sigma-Aldrich (St. Louis, Missouri, USA), catalogue #A1933
Cell Lysis Solution	Qiagen (Hilden, Germany), catalogue # 158906
Citric Acid	AnalaR, BDH Chemicals (Port Fairy, Victoria, Australia), catalogue # 10081
D-Glucose, C6H12O6	Univar, Ajax Finechem (Taren Point, New South Wales, Australia), catalogue # 783
Dimethyl Sulphide (DMSO)	Sigma-Aldrich (St. Louis, Missouri, USA), catalogue #D2650
Ethylenediaminetetraacetic	AnalaR, BDH (Kilsyth, Victoria, Australia), catalogue # 10093

Glacial Acetic Acid, H3COOH	AnalaR NORMAPUR, VWR Chemicals (Radnor, Pennsylvia, USA), catalogue # 20104.334
Methanol	Labchem, Ajax Finechem (Taren Point, New South Wales, Australia), catalogue # AJA5005
N,N,N',N'- Tetramethylethylenediamine (TEMED)	VWR (Radnor, Pennsylvania, USA), catalogue 443083G
Potassium Chloride, KCl	Univar, Ajax Finechem (Taren Point, New South Wales, Australia), catalogue # 383
Rbc Lysis Solution	Qiagen (Hilden, Germany), catalogue # 158902
Sodium Chloride, NaCl	Chem-Supply (Gillman, South Australia, Australia), catalogue # SA046
Sodium Dodecyl Sulphate (SDS)	Sigma-Aldrich (St. Louis, Missouri, USA), catalogue #L3771
Sodium Hydrogen Carbonate, NaHCO3	Univar, Ajax Finechem (Taren Point, New South Wales, Australia), catalogue # 475
SuperSignal ELISA Pico Chemiluminescent Substrate	Thermo Fisher Scientific (Waltham, Massachusetts, USA), catalogue # 37069
Tris Base (Trizma)	Sigma-Aldrich (St. Louis, Missouri, USA), catalogue #T1503
Tween-20	BiXtra, Sigma-Aldrich (St. Louis, Missouri, USA), catalogue #P7949
Agarose	Invitrogen, Thermo Fisher Scientific (Waltham, Massachusetts, USA), catalogue # 16500500
Sodium Hydrogen Carbonate, NaHCO3	Univar, Ajax Finechem (Taren Point, New South Wales, Australia), catalogue # 475

Table 2 Platelet agonists used in light transmission aggregometry

Agonist	Concentration	Source
Adenosine 5’ diphosphate (ADP)	3, 10 and 30 µM	Sigma-Aldrich (St.Louis, Missouri,USA), catalogue # 01905-1G-F
Arachidonic acid	0.5 and 1 mM	Sigma-Aldrich (St.Louis, Missouri,USA), catalogue # 506-32-1
Collagen (Kollagenreagens Horm suspension)	1 and 3 µg/mL	Takeda Austria (Linz, Austria), catalogue #1130630
Collagen-related-peptide (CRP), GCO-[GPO]10-GCOG-NH2	1 and 3 µM	CRP crosslinked in-house using succinimidyl 3-(2-pyridyldithio) propionate (SPDP)
Ristocetin	1.5 mg/mL	Ristocetin (Helena Biosciences, Gateshead, UK) Ref#5372

Table 3: Reagents used for Lectin studies

Reagent	Stock Concentration	Working Conc.	Use	Source
O-Sialoglycoprotein Endopeptidase (OSGE)	2.4 mg/mL	80 μg/mL	Cleaves N-terminal extracellular portion of GPIbα	Cedarlane, Ontario, Canada
Neuraminidase	10 U/mL	0.2 U/mL	Remove terminal sialic acid residues	Sigma, Germany
N-acetyl neuraminic acid, 2,3-dehydro, 2- deoxy sodium salt (DANA)	10 mM	1 mM	Inhibits all 3 neuraminidases isoforms	Sigma, Germany

Table 4: Reagents for platelet surface receptor quantification in whole blood

Antibody	Target	Final Conc. μg/mL	Company
PE-IgG1 isotype	Control antibody	1.2 5	Mouse IgG1 control PE IC002P R&D systems, Minnesota USA
FITC-IgG1 isotype	Control antibody	2	Mouse IgG1 FITC isotype control (B11/6) ab91356 Abcam, Melbourne Australia
APC IgG2b isotype	Control antibody	0.1	Mouse IgG2b APC IC0041A R&D systems, Minnesota USA
PE-CD41	αIIb integrin subunit	1	Ms mAb to CD41 IgG1 (MEM-06) PE ab134372 Abcam, Melbourne Australia
1G5-AF488	GPVI	1	Conjugated in-house
antiADAM10	ADAM10	0.1	Anti-h ADAM10 APC mouse IgG2b 1C1427A R&D systems, Minnesota USA
FITC-AK2	GPIbα	4	CD42b (AK2) FITC mouse IgG1 MAI-82266 Invitrogen, USA
PE-CD9	tetraspanin CD9	1.2 5	Anti-h CD9 PE mouse IgG2b FAB 1880P R&D systems, Minnesota USA
FITC-RCA1		5	Fluorescein labeled Ricinus Communis Agglutinin 1 VEFL1081 Vector Labs, USA
FITC-WGA		5	Anti-wheat germ agglutinin, cat# FL-1021, Burlingame, Vector Labs, USA

Table 5 Reagents for flow cytometry analysis of PRP or washed platelets

Antibody	Stock conc μg/mL	Final conc μg/mL	Details
PE-IgG1 isotype	25	0.25	Mouse IgG1 control PE IC002P R&D systems, Minnesota USA
FITC-IgG1 isotype	50	1.5	Mouse IgG1 FITC isotype control (B11/6) ab91356 Abcam, Melbourne Australia
APC IgG2b isotype	10	0.1	Mouse IgG2b APC IC0041A R&D systems, Minnesota USA
PE-CD41	20	0.2	Ms mAb to CD41 IgG1 (MEM-06) PE ab134372 Abcam, Melbourne Australia
1G5 anti-human GPVI monoclonal	100	1	Conjugated to AF488 in-house
ADAM10	10	0.1	Anti-h ADAM10 APC mouse IgG2b 1C1427A R&D systems, Minnesota USA
FITC-AK2	100	3	CD42b (AK2) FITC mouse IgG1 MAI-82266 Invitrogen, USA
PE-CD9	25	0.25	Anti-h CD9 PE mouse IgG2b FAB 1880P R&D systems, Minnesota USA
FITC-RCA1	5	5	Fluorescein labeled Ricinus Communis Agglutinin 1 VEFL1081 Vector Labs, USA
FITC-WGA	5	5	Anti-wheat germ agglutinin, cat# FL-1021, Burlingame, Vector Labs, USA

2.1 Blood sample collection

Blood was collected from consented drug-free healthy donors age between 18 to 60 years under a protocol approved by the Australian National University Human Research Ethics Committee (Protocol 2016/317). Blood was drawn by a skilled phlebotomist using a 19-gauge needle connected to luer-lock syringes containing anticoagulant. The initial draw of 5 mL blood was used for serum isolation. Then syringes containing 3.2% (w/v) tri-sodium citrate (TSC) at a ratio of 1 volume TSC to 9 volumes of blood were attached for isolation of platelet-rich plasma (PRP). For some experiments, blood was drawn into 1.8 mg/mL ethylenediaminetetraacetic acid (EDTA) or acid citrate dextrose (ACD) (11.9 mM TSC,10.9 mM citric acid, 15.54 mM glucose), the anticoagulants used for isolating blood for washed platelets. Blood was also collected from patients after the provision of informed consent (ACT Health HREC approval ETH.2.17.029), presenting with primary ITP as outpatients at the Canberra Hospital (platelet count < 100 x 10^9/L) or as hospital in-patients with platelet count less than 100 x 10^9/L. ITP diagnosis was determined by clinical assessment. The patient's blood was collected into 3.2% trisodium citrate or EDTA vacutainers (Becton Dickinson Franklin Lakes USA).

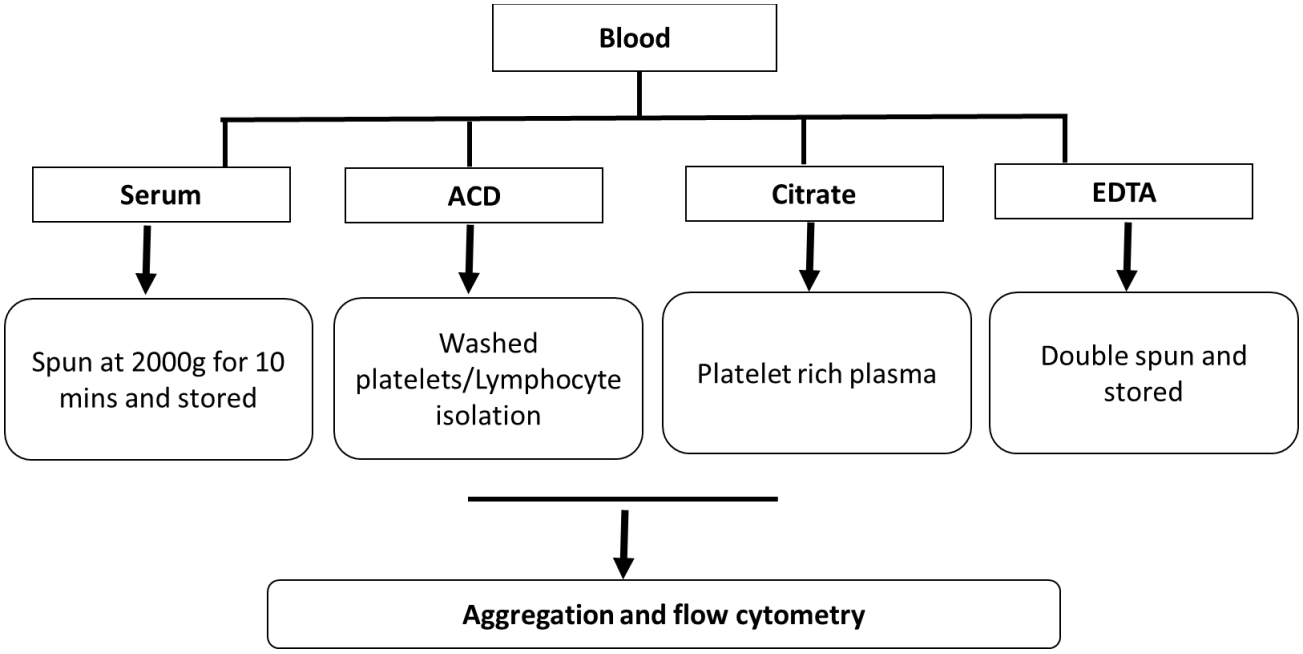

Figure 2.1: Blood collection and processing

Blood was collected with or without anticoagulant and spun at 2000g to collect serum or platelet-rich plasma (PRP). Washed platelets were prepared from the blood collected in ACD, while the sample taken in the citrated tube was used for the whole blood or PRP flow cytometry analysis. Remainder serum and EDTA-plasma were stored in 1 mL barcoded

cryovials at -80^{o}C for batch analyses and biobanking purposes.

2.1.1 Blood count and platelet count in whole blood, washed platelets and PRP

After collection, blood was thoroughly mixed by inverting the tubes gently, and then enumeration of cells using a whole blood count was performed using an automated blood analyser (Abbott Cell-Dyne Emerald 22). All blood count values for healthy donors and for patients were stored in the laboratory database.

2.1.2 Preparation of platelet-rich plasma (PRP)

Blood in anticoagulant was transferred to a 10 mL flat bottom plastic tube (Technoplas Pty Ltd, Adelaide, South Australia) and centrifuged at 110g for 20 mins at room temperature (RT), maximum (max) acceleration, no brakes (Beckman Coulter Allegra X-12R benchtop centrifuge, California USA). The upper PRP phase was collected, preventing buffy coat, and transferred to a 15 mL V-bottom falcon tube (Falcon Corning, Tamaulipas, Mexico).

2.1.3 Preparation of washed platelets (WP) from whole blood

Blood collected from healthy donors by a 19-gauge winged needle into luer-lock syringe containing ACD anticoagulant PRP was prepared as described in section 2.1.1. The upper phase was collected and then combined into a 50 mL V-bottomed tube (Corning Falcon, Corning, New York, USA) centrifuged at 1270 g for 15 minutes with max acceleration and brake to pellet the platelets. Platelet poor plasma (PPP) was removed, and the platelet pellet was gently resuspended in 10 mL of 33.3 mM glucose, 12.92 mM TSC, 123.2 mM NaCl, pH 7.0 (CGS), in this way, platelets were washed by centrifugation/resuspension in CGS twice. Before the third centrifugation step, an aliquot (10 μL) was removed to perform a cell count. After the third centrifugation step, to obtain the washed platelets, the platelet pellet was resuspended in Tyrode's buffer (5.6 mM glucose, 137 mM NaCl,2.7 mM KCl, 0.5 mM $MgCl_2$, 1.4 mM $CaCl_2$, 0.36 mM NaH_2PO_4, 9.4 mM $NaHCO_3$ pH 7.4). The platelet count was adjusted to a count of 5 x10^8 platelets/mL. For some experiments, levels of binding of *Ricinus communis* agglutinin I (RCA), a galactose-binding lectin from castor beans was evaluated by mixing an optimised amount of FITC-conjugated RCA or fluorescently labeled antibodies against GPIbα or GPVI with washed platelets or PRP for one hour. Samples were then evaluated for antibody and lectin binding in a flow cytometer using gating

strategies similar to those described for whole blood (section 2.2).

2.1.4 Isolation and storage of platelet-poor plasma (PPP)

The plasma after the double spun was transferred to 1.5 mL centrifuge tubes and was centrifuged twice at 21,000 g for 5 mins at RT using a microcentrifuge (IEC Micromax, Thermo Electron Corporation, Waltham, Massachusetts, USA). Aliquots of 0.5 mL of this material was labelled PPP and was transferred to barcoded cryovials (Fluidx, BioTools, Logan, Queensland, Australia) at -80^{o}C for analysis of plasma contents and for biobanking purposes.

2.2 SDS-PAGE (Polyacrylamide Gel Electrophoresis)

2.2.1 Preparation of platelet lysate

To prepare samples for electrophoresis, washed platelet suspensions were mixed with equivalent volumes 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.

2.2.2 SDS polyacrylamide gel casting and electrophoresis

The casting of 16 x 20 cm gel was performed using a Protean II xi system (Bio-Rad Laboratories, Hercules, California, USA). The apparatus was assembled according to the manufacturer's instructions. With the final concentration 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, newly made), depending on the percentage required varying amount of 30%(w/v) stock solution of acrylamide was prepared to 35mL using Milli-Q H2O. In the already assembled Protean II xi system, the resolving gel was poured and topped with Milli-Q H2O and let at RT overnight for polymerization.

The next day stacking gel was prepared with final concentration 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. Before pouring the stacking gel, Milli-Q H2O was removed, and a 1.5 mm thick 15-lane Teflon comb (Bio-Rad Laboratories, Hercules, California, USA) was inserted.

After the setting of the stacking gel, it was transferred into the cooling core in the center of the Protean II xi Cell (Bio-Rad Laboratories, Hercules, California, USA), the lower chamber of which was already filled with running final buffer concentrations of (25 mM Tris, 190 mM glycine, 0.1% (w/v) SDS, pH8.3,). After inserting the gel sandwich, the upper chamber was also filled with the running buffer, and the comb was gently removed. Along with the protein, ladder samples were stuffed in the wells. Applying the constant current of 0.03 A at the voltage of 50-60, gel was run for the whole day, or this can be adjusted for the entire night run or according to the gel composition.

2.2.3 Coomassie staining

After electrophoresis, the gel plates were separated. The gel was immersed in a fixative solution containing 10% (v/v) glacial acetic acid and 50% (v/v) methanol and was left for one hour at room temperature on a rocker. To visualise proteins, the gel was then submerged for 1 h in 0.1% (w/v) Coomassie Brilliant Blue R stain solution in 50% (v/v) methanol, 10% (v/v) glacial acetic acid, which had been filtered through Whatman Grade 2 filter paper. Destaining was performed using a destaining solution comprising 10% (v/v) glacial acetic acid and 10% (v/v) methanol until a transparent background was seen. The gel was then imaged using a ChemiDoc MP Imaging System (Bio-Rad Laboratories, Hercules, California, USA) with the associated Image Lab software (version 5.0, Bio-Rad Laboratories, Hercules, California, USA).

2.3 Western blotting

To evaluate the presence or absence of platelet receptors, samples of platelet lysates were prepared and subjected to gel electrophoresis (section 2.5), followed by western blotting with appropriate antibodies. A piece of 0.2 μm polyvinylidene difluoride (PVDF) membrane (Hybond P, Amersham-GE Healthcare, Chicago, Illinois, USA) was activated in 100% methanol then immersed in the transfer buffer (25 mM Tris-HCl pH 8.3 containing 20% (v/v) methanol and 190 mM glycine). The gel was positioned onto the membrane in a sandwich and loaded into a Trans-Blot Cell (Bio-Rad Laboratories, Hercules, California, USA) containing ice-cold transfer buffer. The electro transfer of protein onto the membrane was performed for 1 hour at 100 V. Membranes containing platelet proteins were blocked by immersion in 5% (w/v) fat-free skim milk in 20 mM Tris-HCl, pH 7.6 containing 137 mM NaCl, and 0.1% (v/v) Tween-20 (TBS-T) for 1 h. The membrane was then incubated for

one hour or overnight at 4oC with 1 □g/mL anti-human GPVI cytoplasmic tail polyclonal antibody in TBS-T containing 5% bovine serum albumin with agitation. To remove the surplus antibody, the membrane was washed for 40 minutes with 4 changes of TBS-T with constant agitation. The membrane was then incubated with optimal concentrations of HRP-conjugated secondary antibody (generally 1:1000 dilution) at room temperature for 1 hour. To visualise bound antibodies, the enhanced chemiluminescence (ECL) substrate (Amersham-GE Healthcare, Chicago, Illinois, USA) was applied to the membrane for different exposure times, and images were captured using a ChemiDoc MP Imaging System and Lab version 5.0 software.

2.4 Evaluation of platelet receptors in whole blood samples from patients and healthy donors by flow cytometry

Due to well recognised difficulties involved with isolating PRP fractions from patients with very low platelets counts, an assay using whole blood collected in 3.2% TSC (Section 2.1) was developed for the analysis of platelet receptor surface levels by fluorescence-activated cell sorting (FACS).

All cohorts (Table 6) were compared to a contemporaneously collected healthy control. Samples were collected, processed, and analysed within 3 hours of collection. According to the protocol developed, 20 μL of whole blood was pipetted into 5 mL round bottom flow cytometry tubes (Falcon Corning, Tamaulipas Mexico) and diluted by addition of 80 μL FACS buffer (10 mM Tris-HCl, 0.15 M NaCl, 5 mM EDTA pH 7.4) for the binding of antibodies. Antibodies (detailed in table 4) were added to the appropriate test and control tubes; one tube for each individual was left unstained. The samples were incubated with primary antibodies; 1 μg/mL anti-human GPVI monoclonal antibody, 1G5, 1 μg/mL Alexa Fluor 488-conjugated anti-human GPVI monoclonal antibody, 1G5, or 0.6 μg/mL PE-conjugated mouse anti-CD62P monoclonal antibody, AK4, 3μg/mL anti-human antibody, 0.6 μg/mL PE-Conjugated mouse IgG1 isotype control and protected from light for 30 minutes. Secondary antibody Alexa Fluor 488 conjugated (1 μg/mL goat anti mouse Ig) was used for unconjugated 1G5, and was set for further 30 mins incubation. after staining, and incubation platelets samples were diluted by addition of 150 μL of FACS buffer to each tube. Antibody binding which was indicative of platelet surface protein levels were measured on a FACS Calibur flow cytometer (Becton Dickinson biosciences USA), acquiring 10,000 platelets events. Flow cytometry analysis was performed using FlowJo version 10.4 (FlowJo, LLC, Ashland, Oregon, USA).

Table 6: Patient demographic data

Parameter	Result
Date range of collection	**Oct 2016 – August 2018**
Number of patients with Plt <100 x 10^9/L	**10**
Primary ITP	**8 (6F, 2M)**
Secondary ITP	**0**
Non-ITP thrombocytopenia	**2 (2F)**
Thrombotic microangiopathy	**0**
Platelet dysfunction	**1**
Malignancy	**0**
Drug Induced thrombocytopenia	**0**
?HIT/?VITT	**0**
Undefined	**1**
Healthy donors	**10 (7F, 3 M)**

F = female, M = male

2.5 Analysis of soluble GPVI by enzyme-linked immunosorbent assay (ELISA)

PPP was isolated as described in section 2.1.4 and was used for the measurement of sGPVI levels by an enzyme-linked immunosorbent assay (ELISA) as described (M. Al-Tamimi et al., 2009). For measurement of sGPVI, wells of a flat-bottomed 96-well white microtiter plate (Nunc MaxiSorp, Thermo Fisher Scientific, Waltham, Massachusetts, USA) were coated with 100 µL of 1 µg/mL rabbit polyclonal anti-human GPVI extracellular domain antibody in 10 mM carbonate coating buffer. It was made sure that the pipette tip was not touching the base of the plate. The plate was covered and was incubated at 4°C for an hour or overnight. After incubation plate was washed six times with a wash buffer and pat dried. 200 µL of 1% BSA in PBS (blocking buffer) was pipetted into each well and incubated at room temperature for 1 hour. After incubation, the plate was washed six times with PBS, pH7.4, containing 0.2% (v/v) Tween-20 (PBS-T), afterward blocked with 1% (w/v) BSA in PBS (200µL each well) for 1 h at RT and rewashed six times with PBS-T and dried.

PRP treated with NEM 10% (v/v) and supernatant from GPVI-depleted PPP in PBS was

prepared to use in every plate for the generation of the standard curve against which the levels of sGPVI in test samples were to be compared. To detect variation among the plates, as an internal control, all tests included samples of plasma and serum with known sGPVI levels. For these internal controls of serum and plasma coefficient of variation was 3.8% and 10.9%. All the samples, including the controls, were diluted 10-fold in PBS. After incubation for 1 h at RT, the wells were washed six times with PBS-T, and 100μL of 1 μg/mL murine anti-human GPVI monoclonal antibody was added to the wells and was incubated for 1 h at RT. After 1 h, the wells were washed six times with PBS-T and 100μL of 2.6 μg/mL HRP conjugated polyclonal rabbit anti-mouse secondary antibody (affinity-absorbed against human and bovine Ig, stock at 1.3 mg/mL, Dako) in PBS was added. It was incubated again for 1 h following washed 6 times with PBS-TSuperSignal ELISA Pico Luminol/Enhancer and SuperSingal Pico Stable Peroxide solution (Pierce) equal parts were mixed and 100μL were added to each well and incubated for 1 h. After 1 h incubation light emission was measured on Tecan Infinite 200Pro (Männedrof, Zürich, Switzerland) plate reader.

2.6 Measuring platelet aggregation responses

Lumi-aggregometry was performed using undiluted PRP collected as described in section 2.1.1 using a light transmission aggregometer (Chronolog, Havertown USA) according to standard laboratory procedures (Andrews et al., 2001, Arthur et al., 2005, Gardiner et al., 2008a, Qiao et al., 2012). A panel of agonists and the concentrations used are described in Table 2.

The aggregometer was switched on an hour before starting the experiment so that it warmed up to 37ºC. PPP was prepared as described in section 2.1.2 and pipetted into a reference cuvette and was inserted into the reference chamber of the lumi- aggregometer. In each cuvette, 350 μL PRP was pipetted, and a stir bar was added. PRP was incubated for 2 minutes at 37ºC in the aggregometer incubation wells. After incubation, the cuvette was transferred to the sample chamber of the aggregometer. Light transmission was electronically recorded and adjusted to a baseline (0% transmission reading). The sample was permitted to stabilise for 1 minute, after which appropriate concentrations of selected agonists were added, and light transmission was recorded for up to 15 minutes.

For experiments described in Chapter 3, donor platelets were washed as described in section 2.1.3 and resuspended in Tyrode's buffer to a platelet concentration of 5 x10^8 platelets/mL. Aliquots of 0.2 mL of platelets (10^8 platelets) were placed in cuvettes

containing a stirrer bar and inserted into the aggregometer. Aggregations were triggered by addition of 0.2 mL containing 2 μg/mL collagen-related peptide (for testing purposes) or 0.2 mL control or patient serum. To evaluate the contributions of GPVI and FcγRIIA to the observed aggregations, some platelet suspensions were pretreated with 1 mM NEM or 10 μg/mL anti-FcγRIIA mAb IV.3 before addition of serum. To verify the presence of aggregation activity in NEM-treated platelet-serum suspensions where aggregation had not been triggered by addition of serum, 10 mg/mL ristocetin was added to the suspension.

2.7 Quantification of surface-level receptors by flow cytometry

Using whole blood flow cytometry, levels of platelet receptors (Table 8) on circulating platelets were measured in thrombocytopenic samples in this Chapter. This is a critical research laboratory approach that could be developed as an additional means to evaluate platelet phenotype in patients where ITP is suspected as they are experiencing mild or moderate bleeding issues together with a low platelet count. Altered levels of GPIbα, GPVI, or P-selectin may signal the presence of an activating anti-platelet antibody. Levels of the αIIb and β3 integrin subunits are generally stable and consistent in samples from patients with thrombocytopenia but normal platelet size.

Gating strategy for whole blood samples from patients and healthy donors

Flow cytometry was performed on the samples prepared as described in section 2.2. PE-labeled CD41 was used as a platelet marker. A platelet gate was identified using forward scatter (FSC) and side scatters (SSC) properties (Figure 4.1) and then verified by back gating using CD41 positive samples. A similar approach was performed for patient samples (often from patients with significant thrombocytopenia) to confirm the correct gate. The geomean of positive events for each respective antibody were measured and compared with values gained using a same-day healthy donor. Example histogram profiles are shown in Figure 4.2, with black markers indicating positive events shown. Of note, a peak that coincided with a negative control peak was often observed when patient samples were mixed with antibodies against GPIbα, GPVI, and CD41 consistent with a proportion of the platelets circulating with minimal amounts of antigen on the surface and implying that these receptors had been lost from the surface of circulating platelets.

2.8 Statistical approaches

All statistical analysis was performed using GraphPad Prism Version 7.03 (GraphPad Software, San Diego, California USA). Flow cytometry data were analysed using FlowJo 10.2 software (FlowJo, LLC Oregon USA). The relevant statistical tests used to determine significance are shown in each figure legend. Data was assessed for normality distribution using the Shapiro-Wilk normality test and generally non-parametric tests were applied as the data was not normally distributed around the mean. Two or more group comparisons were performed using one-way ANOVA with Kruskal-Wallis correction for multiple comparisons. For the comparison of two variables, a simple linear regression analysis was used, and for variables with non-linear values, a non-linear regression was performed. Results were considered significant if $p<0.05$.

Chapter 3 Using new laboratory approaches to characterise an antiplatelet antibody in an ITP patient with unexplained bleeding

3.1 Introduction

As discussed in detail in section 1.7, ITP is a relatively common autoimmune mucocutaneous bleeding disorder, characterized by isolated thrombocytopenia in which anti-platelet autoantibodies, primarily against αIIbβ3 and GPIb-IX-V, cause mononuclear macrophage-mediated platelet destruction, chiefly in the spleen (Zufferey et al., 2017). A signature characteristic of ITP is the repeated occurrence of relapsing/remitting thrombocytopenia (Cines et al., 2009); however, significant bleeding events are rarely observed in individuals while the platelet count is maintained above 30 x 10^9/L. If bleeding out of step with the platelet count is observed, this may indicate an unusual underlying pathology resulting in loss of platelet function.

The occurrence of anti-GPVI autoantibodies in ITP is uncommon, possibly because of challenges involved in detecting and characterising the autoantibody. Nonetheless, several reports in the literature describe anti-platelet autoantibodies that target GPVI (Sugiyama et al., 1987, Boylan et al., 2004); however, the molecular mechanism linking the occurrence anti-GPVI autoantibodies with thrombocytopenia and accelerated clearance of platelets from the circulation remains to be defined. Boylan and colleagues demonstrated in a single patient that the autoantibody bound to platelet GPVI and hypothesized that this binding caused the observed depletion of GPVI from the platelet surface and associated mild thrombocytopenia (Boylan et al., 2004). A similar effect has been reported in mice (Bergmeier et al., 2004) or monkeys (Takayama et al., 2008) treated with anti-GPVI antibodies, where transient thrombocytopenia and removal of platelet surface GPVI was observed. The mechanism of reduction of GPVI in these reports involved receptor shedding and/or receptor internalization. It is worth noting that as mouse platelets lack FcγRIIa, there are likely differences between murine and human platelets as human platelets will be able to engage antiplatelet autoantibodies via both the fragment variable (Fv) and Fc portions of the antibody.

3.2 Case report

A 41-year-old female with a long-standing history of ITP presented to a hematology clinic associated with our laboratory to investigate a platelet function defect characterized by an isolated reduction of collagen-induced aggregation that had been detected by light transmission aggregometry (LTA). The cause of the platelet function defect was postulated to be secondary to an acquired autoantibody in the setting of ITP or to an inherited glycoprotein (GP) VI mutation. The platelet defect's undefined nature and clinical significance had delayed surgery in the past; hence, the diagnosis was necessary to inform treatment and predict bleeding risk.

ITP had been diagnosed nineteen years prior following mucocutaneous bleeding associated with a platelet count of 10 x 10^9/L. Subsequent complete blood count examinations, bone marrow biopsy, and response to immunosuppression were consistent with this diagnosis. In the past, the patient had received pulsed, high dose corticosteroid and intravenous immunoglobulin followed by Dapsone for a relapse of severe thrombocytopenia (platelet count 2 x 10^9/L). This approach had achieved a complete response, and Dapsone was later ceased electively. No other treatment for ITP had been required following that period.

At our initial consultation, the patient’s complete blood count demonstrated mild thrombocytopenia with no other abnormalities evident on the peripheral blood film. ITP screening blood tests excluded a secondary acquired cause for thrombocytopenia.

3.3 Results

Platelet function studies were requested before elective laparoscopic pelvic surgery due to bruising symptoms that were not consistent with the degree of thrombocytopenia (platelet count on the day of collection was 91 x 10^9/L). Results of the LTA confirmed a platelet function defect with absent platelet responses to collagen (1.25 μg/ml), with only a slow and reduced response to this agonist at a higher concentration (6 μg/ml). Normal aggregation was noted with adenosine diphosphate (2.5 μM) and arachidonic acid (1.6 mM). An isolated platelet defect in response to collagen was confirmed (Figure 3.1).

Platelet flow cytometry demonstrated normal surface expression of GPIb-IX, integrin αIIbβ3, GPIX, and α2β1 with significantly reduced expression of the collagen receptor GPVI (Figure 3.2). Next-generation sequencing was performed and excluded a mutation in the coding sequence of GPVI (data not shown). At the request of the consulting

haematologist, our laboratory was asked to evaluate levels of GPVI in aliquots of platelets prepared from blood samples provided by the patient after the provision of informed consent. Further, we were requested to investigate for any evidence of an anti-GPVI autoantibody.

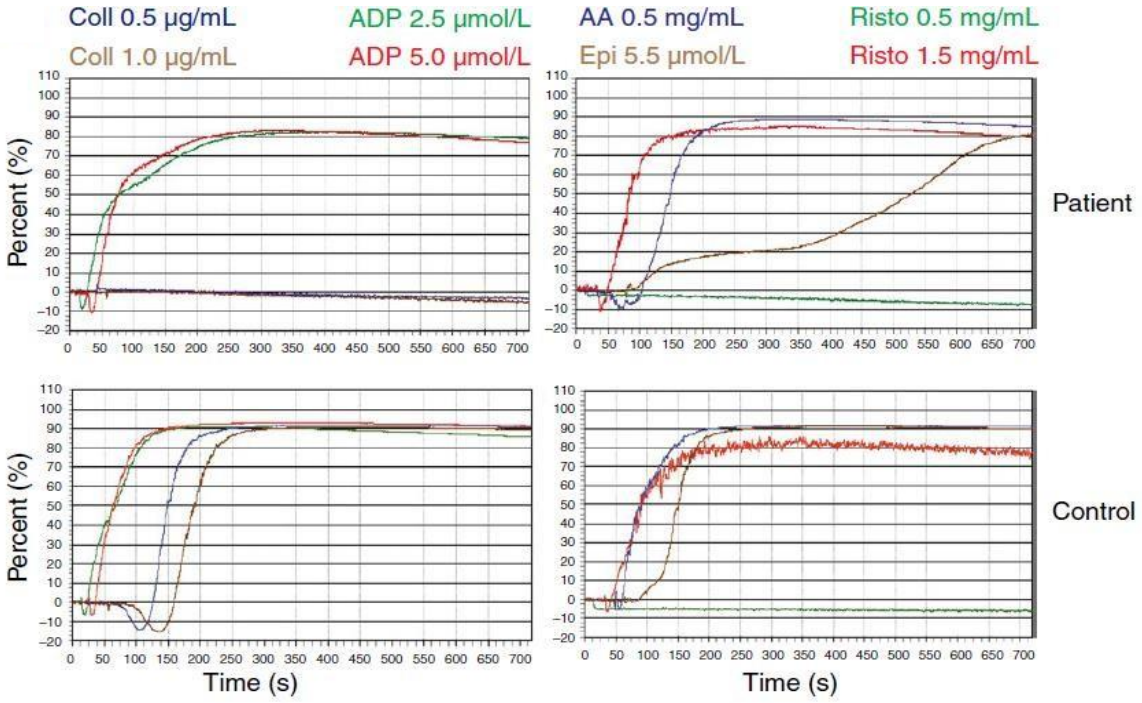

Figure 3.1 Patient platelet function.

LTA using patient (upper panels) or healthy donor (lower panels) PRP reveals that patient platelets fail to respond to 0.5 μg/ml or 1.0 μg/ml collagen (blue and brown curves, left-hand panel) but aggregate normally in response to 2.5 μM and 5 μM adenosine diphosphate (green and red curves, left-hand panels). Patient platelets demonstrate normal aggregation (right hand panels) in response to 0.5 mg/ml (green) and 1.5 mg/ml (red) ristocetin, 0.5 mg/ml arachidonic acid (blue, right hand panel) and 5.5 μM epinephrine (brown curve, right hand panel). PRP prepared from a healthy donor on the same day responded to all agonists. This data was generated by Dr. David Rabbolini and colleagues at Royal North Shore Hospital, Sydney, Australia.

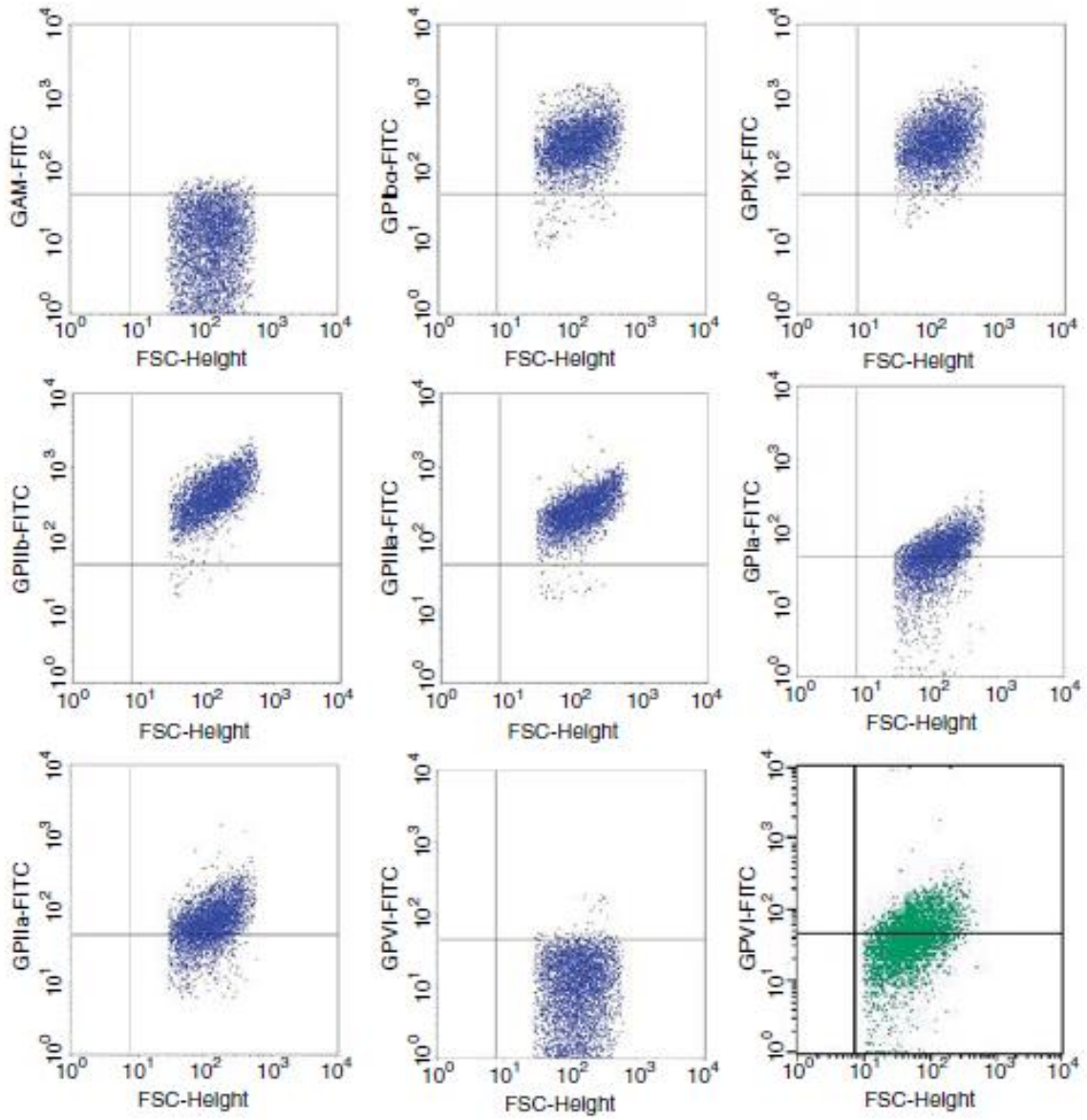

Figure 3.2 Flow cytometric evaluation of receptors on patient platelets.

Levels of platelet receptors were evaluated in samples of PRP prepared from a healthy donor (green dot-plot) or the patient (blue dot plots). The patient platelets showed levels of GPIbα, GPIX, and the integrin subunits, αIIb, β3, α2, and β1 that were within normal ranges found for healthy donors (data not shown). Levels of GPVI were significantly reduced compared to levels observed using healthy donor PRP (green dots). GAM-FITC = FITC conjugated goat anti-mouse-IgG (control for secondary antibody binding). This data was generated by Dr. David Rabbolini and colleagues at Royal North Shore Hospital, Sydney, Australia.

A possible explanation for the isolated loss of responsiveness to collagen by LTA could be the absence of GPVI on the surface of the platelets, but that GPVI was sequestered within the platelets. To confirm that GPVI was not present anywhere in the platelets (i.e., sequestered within the open canalicular system and not detected by flow cytometry), we evaluated washed platelet lysate samples prepared from blood isolated from the blood of the patient. Platelets were collected in ACD, washed as described in Section 2.1.3, then lysed and frozen before shipment to ANU. Samples of patient serum were also shipped to ANU for further evaluation.

Platelet lysates from the patient or a healthy donor (Section 2.1.4) were fractionated by polyacrylamide gel electrophoresis (Section 2.5), and separated proteins were transferred to nitrocellulose (Section 2.5.1). Using an antibody raised against the cytoplasmic tail of human GPVI (Gardiner et al., 2007), it was possible to probe the nitrocellulose membrane and evaluate levels of intact and metalloproteolytically cleaved GPVI (Figure 4.6). As an extra control, healthy donor washed platelets were also treated with 5 mM N-ethylmaleimide (NEM), which is a known activator of metalloproteinases (Huovila et al., 2005) to create the remnant 10 kDa fragment of GPVI produced by the action of metalloproteinases (Gardiner et al., 2007).

Figure 3.3 left panel shows a portion of the acrylamide gel that has been stained for protein (Section 2.10). The purpose of this panel is to demonstrate approximately equal loading of protein from the patient or healthy donor platelet lysates either untreated or treated with 5 mM NEM. Figure 3.3 right panel visualized bands generated after probing the nitrocellulose membrane with anti-GPVI cytoplasmic tail antibody. The position of intact (~60 kDa) and remnant fragment (~10 kDa) of GPVI are indicated.

Regardless of the amount of protein loaded onto the gel and transferred to the membrane, there was no evidence of intact or remnant GPVI bands in lanes containing platelet lysates prepared from patient blood samples. In contrast, lanes containing platelet lysates prepared from healthy donors (control, NT) displayed a band of intact GPVI lost after the platelets were treated with NEM (control +NEM). Lower molecular weight bands representing the remnant GPVI fragment are also indicated. Overall, this data strongly suggests that the lack of responsiveness to collagen observed in samples from the patient is due to an absence of surface GPVI (Figure 3.2) and that GPVI is not sequestered within the patient platelets (Figure 3.3). The lack of a remnant GPVI cytoplasmic tail fragment is likely to be due to the fact that the cytoplasmic tail of GPVI is not stabilised within the membrane by any association with cytoskeletal proteins and so has either been lost during

the processing of the platelets for gel electrophoresis or has been destroyed within platelet lysosomes as part of the normal protein clearance mechanisms.

To determine the presence of anti-GPVI antibodies and elucidate their mechanism of action against platelets, the platelet-activating activity of the patient serum was evaluated as described in sections 2.3 and 2.4. Aggregation of donor platelets by patient serum was demonstrated by mixing in a 1:1 volume/volume with washed donor platelets (Figure 3.4A). Rapid aggregation was noted, blocked by pre- incubation of donor platelets with either 10 µg/mL IV.3 (a function-blocking monoclonal antibody against FcγRIIa) or by pre-treatment of donor platelets with 1 mM NEM (to rapidly induce metalloproteolytic shedding of GPVI as shown in Figure 3.3). NEM- treated platelets remained responsive to ristocetin, demonstrating that GPIb-IX-V and associated signaling pathways were functional, indicating that the aggregating property of patient serum required functional FcγRIIa and intact GPVI (Figure 3.4A). These data are consistent with patient serum containing an anti-GPVI autoantibody that caused platelet activation requiring intact GPVI and functional FcγRIIa.

Flow cytometry of donor platelets incubated for 3 hours with control or patient serum was performed to evaluate whether patient serum could induce loss of platelet surface receptors (GPVI and others) from donor platelets. Expression levels of platelet surface receptors on donor platelets were monitored by flow cytometry. Incubation with a patient but not control serum resulted in the significant generation of microparticles, and so all measured platelet receptors (αIIbβ3, GPIbα, CD9, and GPVI) were reduced. However, GPVI was highly susceptible to lose by this treatment, with 79% receptor clearance occurring during the experimental time frame (Figure 3.4B). Taken together, these data confirmed a rare occurrence of an anti-GPVI autoantibody that engages both GPVI and FcγRIIa, leading to platelet activation and aggregation. The observed loss of GPVI is probably via immunoreceptor tyrosine-based activation motif (ITAM)-mediated metalloproteolytic shedding as described now in a number of other isolated cases (Nurden et al., 2009, Moroi et al., 1989, Boylan et al., 2004).

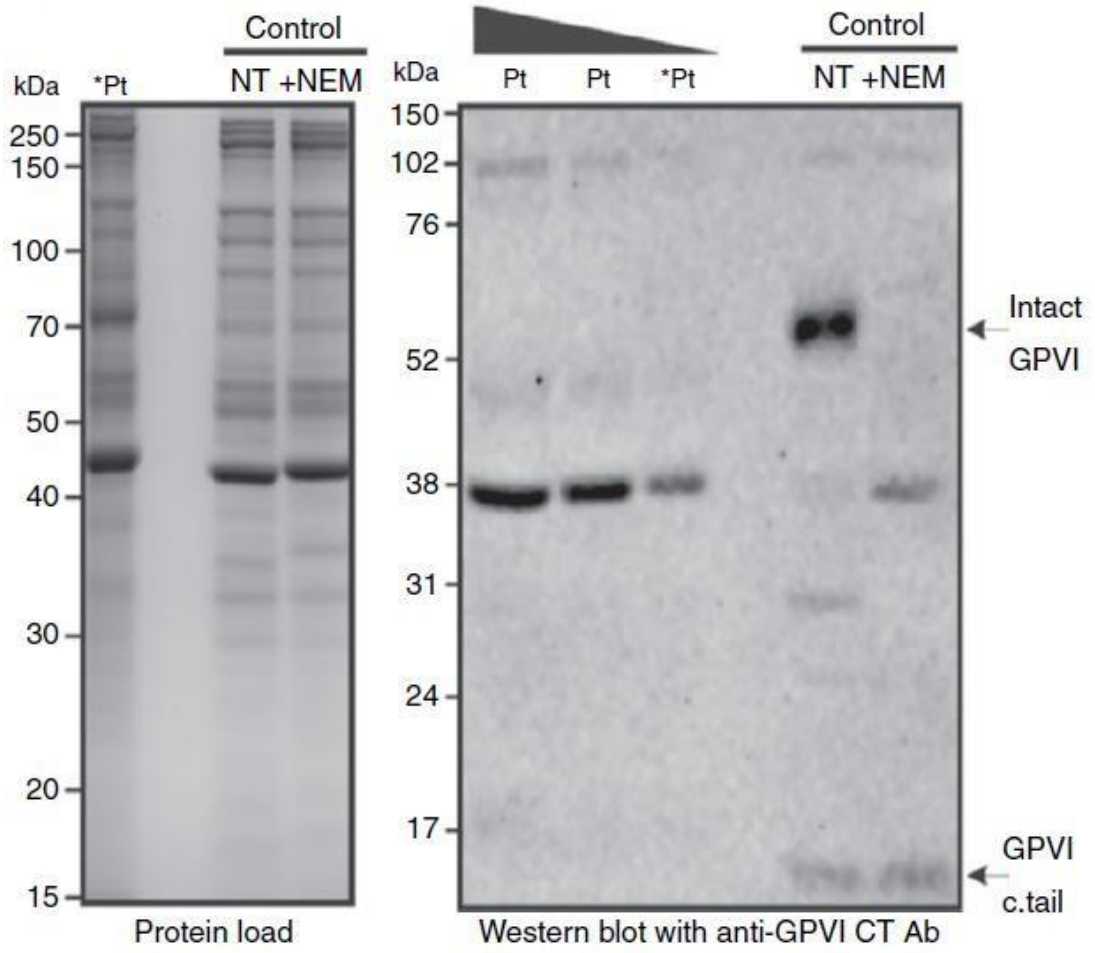

Figure 3.3 Analysis of platelet lysates for intact and remnant shed fragment of GPVI by western blot.

Platelets from a healthy donor (control) or the ITP patient (Pt) were isolated and washed, then lysed in detergent containing buffer (Section 2.1.4) and subjected to polyacrylamide gel electrophoresis on a 5-20% gradient acrylamide/SDS gel (Section 2.5). The left-hand panel shows a portion of the gel subjected to protein staining with Coomassie Blue (Section 2.5.2) and shows equivalent protein loading. The right panel shows that intact GPVI (~60 kDa) and a remnant GPVI tail fragment (~10 kD) were detectable by western blot (Section 2.11) using an anti-GPVI cytoplasmic tail antibody in non-treated (NT) washed platelet lysates from a healthy donor (control) but not at three different loadings of platelet lysates from the patient (Pt; lanes labeled *Pt were loaded with equivalent volumes of lysate). Treatment of control platelets with 5 mM NEM for 15 minutes resulted in a loss of intact GPVI due to metalloproteolytic shedding (Gardiner et al., 2007) enabled detection of a ~10 kDa band (GPVI c.tail). Ms. Christine Lee obtained this data in the Gardiner laboratory.

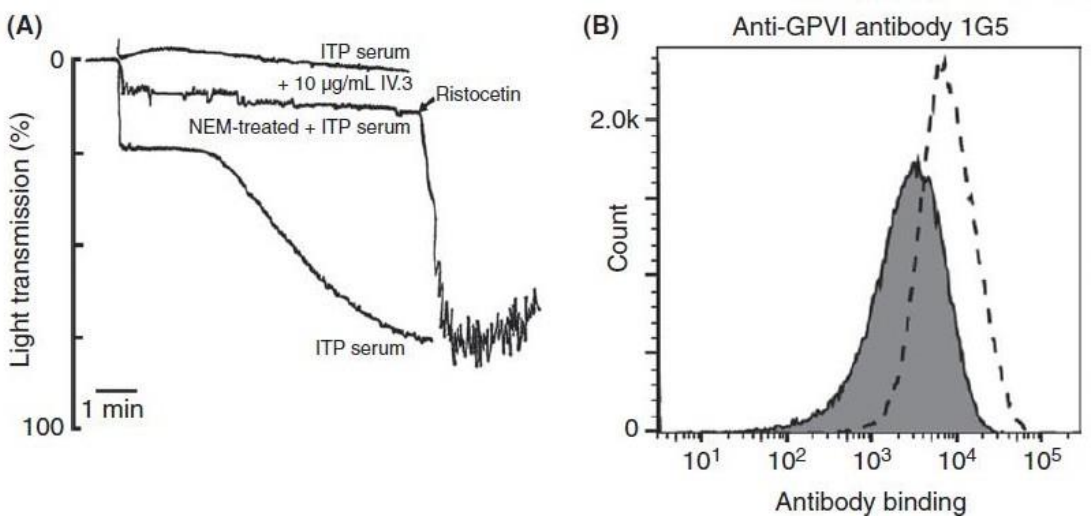

Figure 3.4 Patient serum aggregates donor washed platelets in a FcγRIIa-and GPVI-dependent manner.

Washed platelets were prepared as described in section 2.1.3, and light transmission aggregometry was performed. (**A**) Patient serum was mixed with an equal volume of donor platelets or donor platelets pre-treated with 10 µg/mL FcγRIIa-blocking antibody IV.3 or 1 mM NEM to remove GPVI. NEM-treated platelets did not aggregate in the presence of patient serum but aggregated upon addition of 1.5 mg/mL ristocetin. (**B**) Flow cytometry (section 2.2) of donor platelets using anti-GPVI monoclonal antibody 1G5 to detect GPVI after 3 hours' incubation at 37°C with an equal volume of patient serum (filled histogram) or control serum (open histogram).

3.4 Discussion

Primary ITP has acquired thrombocytopenia that is not associated with systemic triggers such as viral infection, connective tissue disorders, drug therapies, or lymphoproliferative neoplasm. Thrombocytopenia ensues following platelet destruction that is mediated by the production of platelet-specific IgG (most commonly targeting platelet αIIbβ3 and GPIb-IX) by B-lymphocytes, as well as reduced platelet production secondary to T-lymphocyte mediated platelet and megakaryocyte destruction.

GPVI is an integral platelet surface receptor expressed in a non-covalent complex with the platelet Fc receptor γ chain (FcRγ). The surface expression and function of GPVI depend on this association (Bergmeier et al., 2004). The binding of GPVI by agonists that include collagen, fibrin, laminin, collagen-related peptide, and snake venom proteins induce structural changes of both GPVI and FcRγ, leading to phosphorylation of Src and Syk tyrosine kinases (Rayes et al., 2019). These, in turn, initiate an intracellular signaling pathway causing eventual shape change and activation of integrin αIIbβ3 with resultant platelet aggregation mediated by αIIbβ3 binding of von Willebrand factor or fibrinogen.

Autoantibodies to GPVI have been reported in ITP (Moroi et al., 1989, Gardiner et al., 2008a, Boylan et al., 2004, Nurden et al., 2009, Sugiyama et al., 1987, Arai et al., 1995) and as in the patient described in this chapter, activate platelets in a GPVI/FcγRIIa dependent manner. The subsequent loss of platelet surface GPVI causing reduced responsiveness to collagen is likely mediated through autoantibody engagement of GPVI via the variable region of the autoantibody and next engagement of platelet FcγRIIa by the F-crystallisable (Fc) portion of the autoantibody. This triggers activation of ITAM pathways emanating from both GPVI and FcγRIIa, resulting in metalloproteolytic shedding of GPVI by ADAM10 on platelets (Gardiner, 2018). It is intriguing to speculate that current therapies targeting components of the ITAM signaling pathway, including Bruton's tyrosine kinase inhibitors and inhibitors of Syk in other disease pathologies (Denzinger et al., 2019, Series et al., 2019, Poole, 2011), may have relevance for future treatment of ITP.

The diagnosis of ITP is made following the exclusion of secondary causes of thrombocytopenia, as there is no single diagnostic assay (Miltiadous et al., 2020). Routine use of antiplatelet antibody assays, such as the monoclonal antibody immobilization of platelet antigens assay (MAIPA), that measures antibodies bound to specific platelet glycoproteins for the diagnosis ITP is generally not recommended due to lack of reported sensitivity (50%-80%) and specificity (80%) of this platform in the setting of ITP (Brighton et al., 1996). The standard MAIPA platform measures antibodies bound to platelet

antigens, αIIbβ3 and GPIb-IX. It does not include antibodies to α2β1 and GPVI, although the latter have both been described in cases of ITP. The sensitivity and specificity of this assay may likely be improved by adding these additional antigenic targets into standard MAIPA platforms, thereby validating its use for diagnostic purposes. Newly emerging techniques that do not rely on MAIPA approaches may also hold great possibilities for characterizing antiplatelet autoantibodies in the setting of ITP (Porcelijn et al., 2018). The identification of anti-platelet antibodies at diagnosis has been reported to be associated with a higher likelihood of disease chronicity and bleeding symptoms (Grimaldi et al., 2014, Tao et al., 2017, Webster et al., 2006a). Therefore, the utility of these assays prospectively could also potentially impact treatment and monitoring strategies.

Major bleeding, including intracerebral haemorrhage is rare in ITP and occurs predominantly in patients with platelet counts below 10×10^9/L (Lee and Kim, 1998). A platelet count of less than 30×10^9/L is generally regarded as a threshold for treating ITP (Cuker and Cines, 2012), and several newer treatments aim to increase platelet levels rapidly. Thrombopoietin (TPO) regulates thrombopoiesis by activating TPO receptors on the megakaryocyte cell surface, resulting in increased platelet production (Hitchcock and Kaushansky, 2014). Two second-generation TPO agonists, Eltrombopag and Romiplostim, are approved for the treatment of ITP (Ghanima et al., 2019). Intriguingly Romiplostim treatment may directly upregulate the expression of platelet GPVI through receptor-mediated demethylation of the *GP6* promoter (Kanaji et al., 2005) and in the past normalized GPVI levels and restored platelets responses to collagen by LTA in a patient with anti-GPVI mediated ITP following 6 months of treatment with Romiplostim have been reported (Gardiner et al., 2010b). The current case presented in this Chapter illustrates that anti-GPVI antibodies can impair receptor function, contributing to increased mucocutaneous bleeding symptoms at platelet counts above the threshold of 30×10^9/L. Taken together, therefore, identification of anti- GPVI antibodies at diagnosis may aid treating clinicians to elect to pursue closer surveillance schedules or even embark on “disease”-specific therapies with TPO agonists at platelet counts higher than the accepted threshold.

Finally, this chapter's data demonstrates the advantage and utility of genetic-based testing in uncharacterized platelet disorders. Here, the absence of a *GP6* mutation suggested that an acquired rather than inherited platelet function defect was responsible for the loss of GPVI expression. Moreover, it provided reassurance to the patient who had concerns regarding transmission of a possible clinically significant variant to offspring.

In summary, a rare case of anti-GPVI-mediated ITP was described. The autoantibody triggered platelet activation and caused a loss of platelet surface GPVI leading to a clinically appreciable platelet dysfunction with mild bleeding symptoms at platelet counts not usually associated with bleeding in ITP. Anti-GPVI mediated ITP may be more prevalent than expected, as LTA is generally not performed for bleeding symptoms associated with ITP, and probably more significantly, GPVI is not currently included in standard MAIPA assays. The inclusion of GPVI as a protein target, together with other known antigenic markers (α2β1), may improve the diagnostic utility of this assay, provide prognostic information, and facilitate individualized treatment approaches in ITP.

Chapter 4 New approaches to evaluate platelet function in samples from patients with thrombocytopenia

4.1 Introduction

Diagnosis of immune thrombocytopenia (ITP) and prediction of response to therapy remain significant and constant challenges in haematology. In patients with ITP, the platelet count is frequently used as a surrogate marker for disease severity and determines the need for therapy. Although there is a clear link between thrombocytopenia and haemostasis, a direct correlation between the extent of thrombocytopenia and bleeding symptoms, especially at lower platelet counts, is lacking. Thus, bleeding in ITP is heterogeneous, unpredictable, and nearly always based on many risk factors beyond the platelet count. Developing an evidence-based, validated risk stratification model for ITP treatment is an important goal in the ITP community. This chapter develops 2 new laboratory approaches to evaluate the various pathobiologies of ITP that may inform such a model.

The clinical diagnosis of immune-based platelet disorders relies on the accurate collection of family and history of bleeding and clinical laboratory findings and, if possible, additional specialist laboratory data. When a specific test is not readily available, for example, in clinics away from tertiary centers, clinical best judgment is used to make a presumptive diagnosis and initiate treatment (Bendapudi et al., 2017, Cuker et al., 2018). It is not always possible to differentiate these disorders at the presentation time, and the diagnosis is often challenging due to over-lapping features. Typically, many possibilities are considered together, and a general approach towards management is used, based mainly on prioritising safety by using the clinical acumen of the treating physician (Kelton et al., 2018). Poor access to specialized diagnostic modalities can impair timely clinical decision-making. Hence, diagnostic reclassification is not uncommon, and retrospectively it becomes more apparent that some patients may have received unnecessary medical or surgical interventions (Reisi, 2020, Okan et al., 2002, de Moerloose et al., 1987, Arnold et al., 2017).

Routine clinical evidence relevant to platelet dysfunction may include analysis of platelet count and size or microscopic examination of blood smears. Platelet functional testing is more problematic. Tests may include: skin bleeding time, the time taken to stop bleeding after an incision (not now widely used in Australia); platelet aggregometry that measures the rate and extent of platelet activation and aggregation in vitro in platelet-rich plasma

(PRP) or citrated whole blood (CWB) in response to ristocetin, collagen, ADP or other agonists; measurement of shear-dependent platelet function in whole blood in response to ADP or other agonists in devices such as PFA- 100; or analysis of VASP phosphorylation downstream of ADP receptor engagement. However, these tests may suffer from insensitivity, poor standardization, be too time-consuming or labour intensive, be expensive, and/or lack practical algorithms or guidelines for interpretation (Harrison and Lordkipanidzé, 2013, Aradi et al., 2015). Notably, many of these methods are not applicable for immune or non-immune thrombocytopenia (low platelet count) or macrothrombocytopenia (large or giant platelets).

Engagement of the platelet surface by antibodies targeting platelet proteins can lead to platelet activation. This is most readily seen in heparin-induced thrombocytopenia (HIT), where autoantibodies against platelet factor 4/heparin form an immune complex that can engage the antigen on the platelet surface via Fab regions and simultaneously interact with FcγRIIa on the platelet via the Fc portion resulting in platelet activation (Greinacher, 2009). The binding and activation of FcγRIIa require that the pathological antibody binds to its antigenic target in an appropriate orientation that permits Fc interaction with platelet FcγRIIa. To what extent ITP-related antiplatelet autoantibodies trigger metalloproteolysis, and other platelet activations and degranulation events remain to be determined. However, the Fc portion of platelet-associated IgGs are likely to mediate platelet clearance by engaging with Fc receptor on platelets to induce activation and on monocytes and macrophages to trigger engulfment (Zufferey et al., 2017).

Receptors on the platelet enable interaction of platelets with the surrounding environment and coordinate platelet responses after interaction with agonists and inhibitors. Granules in the platelets along with the receptors, control platelets, detailed cellular individuality, for example, signal transduction, shape change, and other essential functions (Al-Zube et al., 2009). Despite being anuclear, platelets can carry out protein synthesis (Davizon-Castillo et al., 2020) which may implicate de novo production of tissue factor and cytokines and growth factors from activated platelets as part of a pathological process.

A flow cytometric assay was developed to measure the levels of receptors present on the surface of circulating platelets from healthy donors and a cohort of patients with ITP. The receptors chosen included the αIIb integrin subunit which plays a critical role in signaling and platelet aggregation and is a primary antigenic target in ITP. Platelets are the only cells

that express this integrin, with fifty to eighty thousand copies per platelet. The GPIb-IX-V complex, which contains GPIbα the primary platelet receptor for VWF, is expressed as twenty-five thousand to fifty thousand copies per platelet. It is the second most abundant receptor on platelets after integrin αIIbβ3 (Burkhart et al., 2012) and is also a major antigenic target in ITP. GPVI is also platelet-restricted and is the platelet signalling receptor for collagen, laminin, fibrin, and fibrinogen (Mammadova-Bach et al., 2015, Rayes et al., 2019). CD9 belongs to the tetraspanin group of proteins, which bear four membrane spanning domains expressed on many cell types, including platelets (Boucheix et al., 1991). CD9 is a twenty-four kDa protein composed of four transmembrane proteins. A lack of CD9 has been noted in people with Bernard Soulier Syndrome, implying that this tetraspanin has a molecular link with the GPIb-IX- V complex (Qiao et al., 2015).

4.2 Results

Table 7: Platelet surface receptors

Surface protein	Role in platelets
GPIIbIIIa CD41/CD61 αIIbβ3	• Platelet specific integrin • Most abundant platelet adhesion receptor. • Binds to fibrinogen, fibrin, von Willebrand Factor (vWF) and fibronectin promotes platelet aggregation. (Bennett et al,.1990)
GPIbα	• Second most common platelet receptor. • GPIbα is instrumental in the initiation and propagation of both hemostasis and thrombosis. (McFadyen and Kaplan, 2015) • Metalloproteolytically shed constitutively from the platelet surface
GPVI	• Crucial role in the collagen-induced activation and aggregation of platelets. (Moroi et al,. 2004) • Metalloproteolytically shed from activated platelets
CD9 (a Tetraspanin)	• Linked to the expression levels of GPIb-IX-V, • May regulate platelet metalloproteinase activity (ADAMs activity)
ADAM10	• Platelet surface metalloproteinase • Cleaves GPVI on platelets • Ubiquitous expression • Cleaves adhesion proteins, growth factors, cytokines on surface of cells. (Moss et al., 2004)

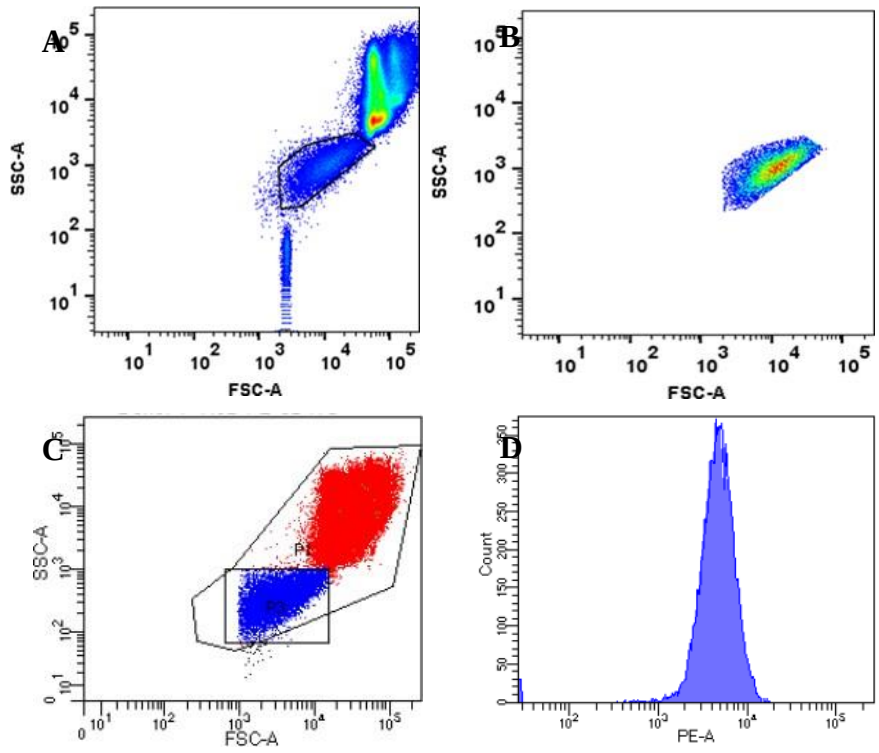

Figure 4.1 Identifying the platelet population in whole blood by flow cytometry.

Platelet flow cytometry gates were set as described in section 2.2. Examples of FSC/SSC dot plots for citrated whole blood (A) before and (B) after identifying the platelet gate using FSC/SSC parameters characteristic of platelets are shown. The gate and population are shown in (B) were routinely verified to be platelets by backgating a whole blood sample mixed with a PE-conjugated platelet-specific antibody recognising CD41 (αIIb integrin subunit). Platelets are identified as αIIb positive (blue events in panel C), differentiating the platelet population from leukocytes and red blood cells (red events in panel C). Panel D shows a histogram of PE-CD41 antibody-labeled platelets

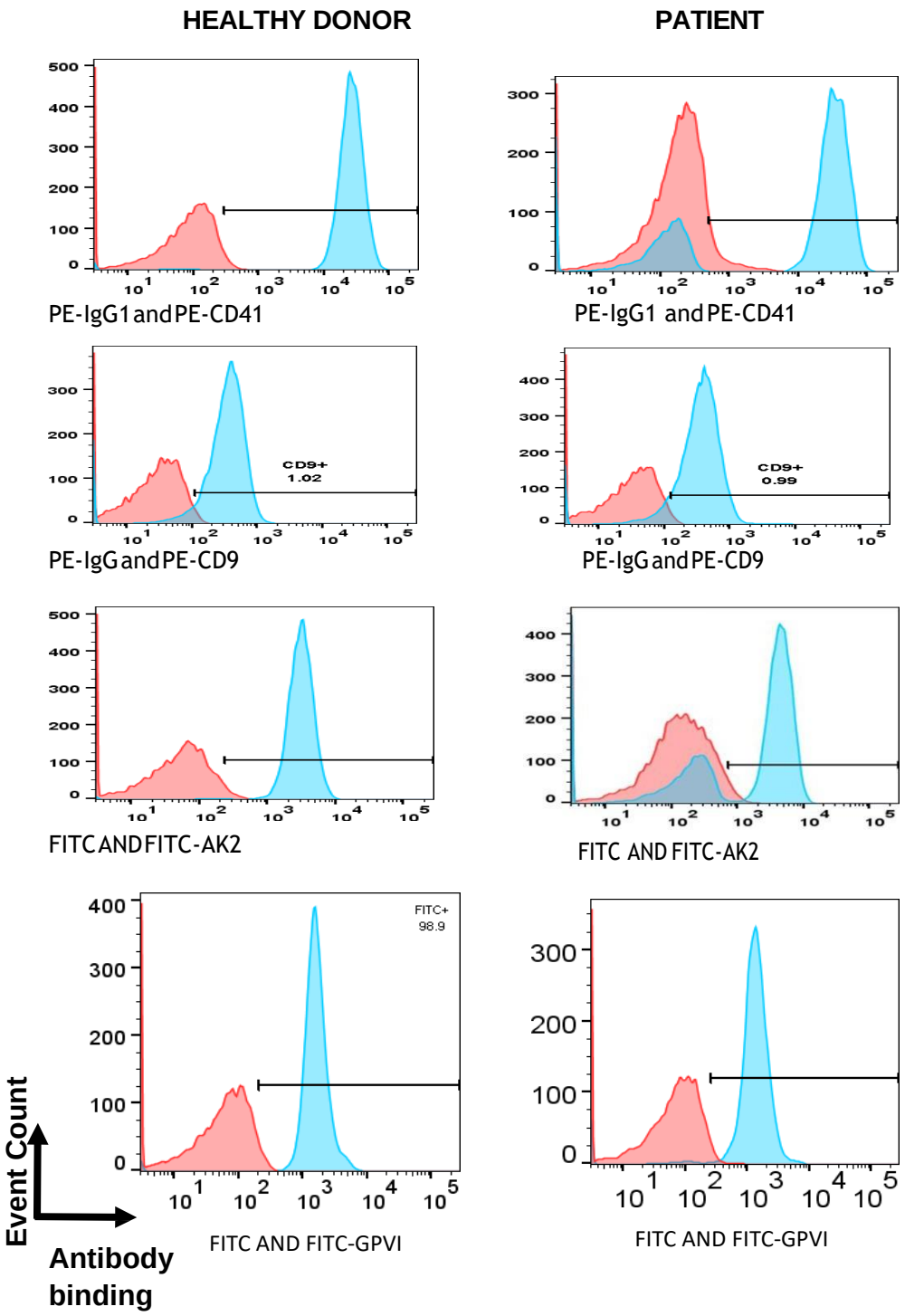

Figure 4.2: Detecting levels of proteins on platelets in whole blood samples.

Example of histograms obtained using citrated whole blood from a healthy donor (left panels) or a patient with ITP (right panels). The binding of a control antibody (red) or the test antibody (blue) is shown. Note dual blue populations of platelets differentially expressing levels of specific proteins in patient samples. PE-CD41 (anti-αIIb), PE-CD9 (anti-CD9), FITC-AK2 (anti-GPIbα) and FITC-GPVI (anti-GPVI).

The relative platelet count and size determined in whole blood, together with surface expression levels of GPIbα, GPVI, αIIb integrin, and CD9 determined by flow cytometry in whole blood, for healthy and thrombocytopenic donors are summarised in Table 9. The thrombocytopenia group are presented in the table and in subsequent figures as TOTAL (n = 10) as well as ITP (n = 8) and TCP (n = 2) subgroups. For some of the analyses, more sample data was available. The data in Table 9 represent patients for which a near-complete dataset was obtained. From Table 9, the following points can be made: First, as expected, the thrombocytopenia group and ITP and TCP subgroups show significantly lower platelet count (Figure 4.3) and increased platelet size as determined by mean platelet volume measurement (Figure 4.4) compared with platelets from the healthy donors.

Interestingly, the ITP individuals show apparent differences in MPV relative to both the healthy group and the thrombocytopenia group. New reticulated platelets display an elevated MPV, which may reflect enhanced platelet production in the ITP group that was not observed in the TCP group, hinting at MPV possibly providing a clue to the clinician regarding disease pathology. This readout is part of a currently available standard clinical measures (platelet count, MPV) suite. Second, analysis by flow cytometry was shown to apply to the study of receptor surface levels in thrombocytopenia, and there was a significant deficiency of GPIbα in the entire thrombocytopenia group compared with healthy donors or non-ITP thrombocytopenia, compared with the other receptors (Figure 4.5).

Table 8. Analysis of platelet parameters in a subset of blood samples from healthy donors and thrombocytopenic patients.

		Thrombocytopenia		
	Healthy donors	**TOTAL**	**ITP**	**TCP**
n	**6-8**	**10**	**8**	**2**
Platelet count (x 10^9/l)	226 ± 52	36 ± 35	30 ± 18	60 ± 85
(Range)	(161 – 344)	(0 – 121)	(13 – 69)	(0 – 121)
Mean Platelet Volume (fl)	7.3 ± 0.4	9.6 ± 1.5	10 ± 1.2	7.8 ± 0.6
(Range)	(6.5 – 7.7)	(8.2 – 11.8)	(8.2 – 11.8)	(7.4 – 8.2)
Platelet Receptors				
Surface GPIbα	2099 ± 323	2329 ± 844	2575 ± 623	1344 ± 1129
(Geomean)	(1627 – 2494)	(546 – 3376)	(1766 –3376)	(546 – 2143)
(Range)				
Surface GPVI (Geomean)	1207 ± 416	1080 ± 945	1080 ± 945	830*
(Range)	(874 – 1849)	(334 – 2631)	(120 – 2631)	
Surface αIIb (Geomean)	2733 ± 350	2919 ± 958	3112 ± 981	2146 ± 158
(Range)	(2247 – 3380)	(2034 – 4602)	(1780 –4602)	(2034 – 2258)
Surface CD9 (Geomean)	29 ± 10	31 ± 6	30 ± 5	32 ± 11
(Range)	(18 – 46)	(22 – 40)	(22 – 39)	(24 – 40)

*one measurement of GPVI was lost

Statistical analyses of each of the variables in each of the cohorts are shown as part of subsequent figures in this Chapter.

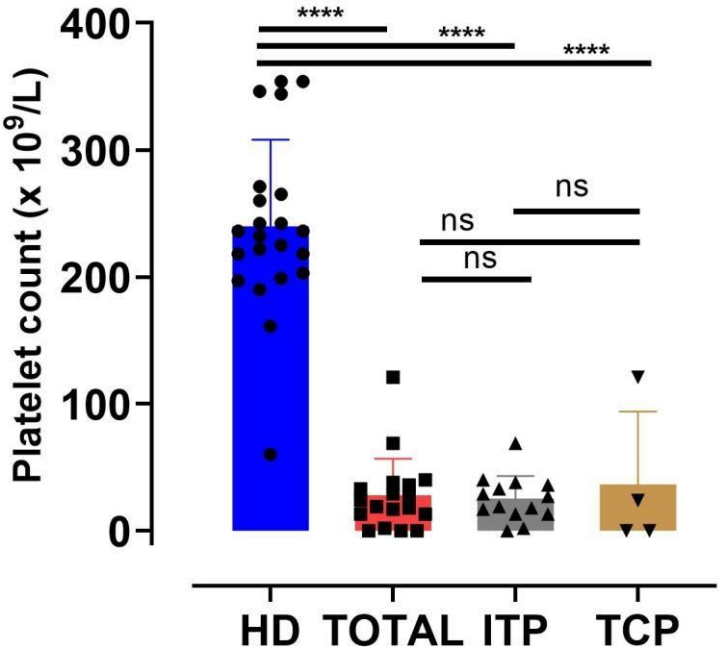

Figure 4.3. Platelet count in thrombocytopenia.

Comparison of individual platelet counts in whole blood from n=24 healthy donors and all thrombocytopenia (TOTAL, n=18), or in sub-groups of thrombocytopenia that were non-ITP (TCP n=4) or subsequently confirmed ITP (n=14). As expected, there are observed differences between healthy and thrombocytopenia, but not between non-ITP and ITP. Statistical analysis was carried out using Prism software (version 6, GraphPad, 1-way ANOVA with corrections for multiple comparisons). Error bars represent mean ± SD; ns, non-significant. ****, ***, ** and * reflect P<0.0001, P<0.005, P<0.001 and P<0.05 respectively

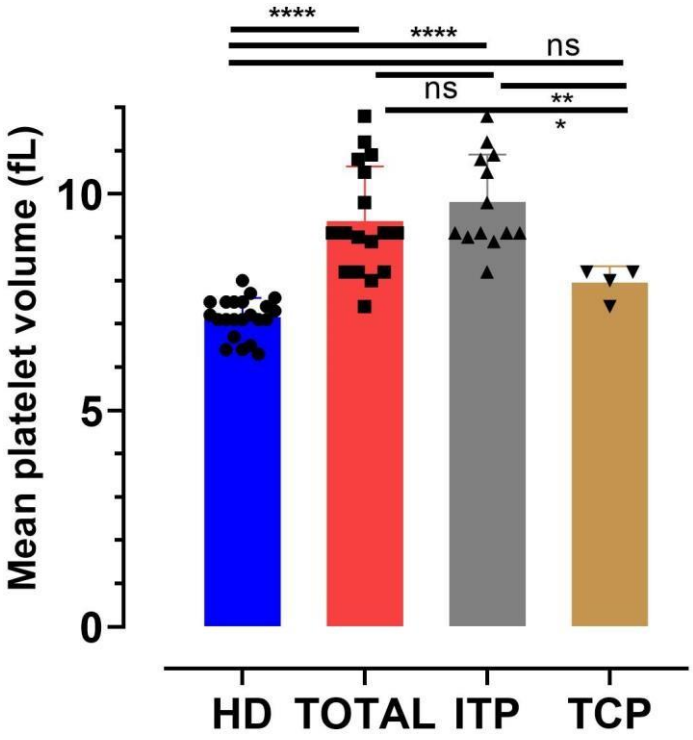

Figure 4.4. Mean platelet volume (MPV) in thrombocytopenia.

Comparison of individual MPV in blood from n=24 healthy donors and all thrombocytopenia (TOTAL, n=18), or in sub-groups of thrombocytopenia that were non-ITP (TCP n=4) or subsequently confirmed ITP (n-14). As expected, there are observed differences between healthy and thrombocytopenia, but not between non- ITP and ITP. Statistical analysis was carried out using Prism software (version 6, GraphPad, 1-way ANOVA with corrections for multiple comparisons). Error bars represent mean ± SD; ns, non-significant. ****, ***, ** and * reflect P<0.0001, P<0.005, P<0.001 and P<0.05 respectively

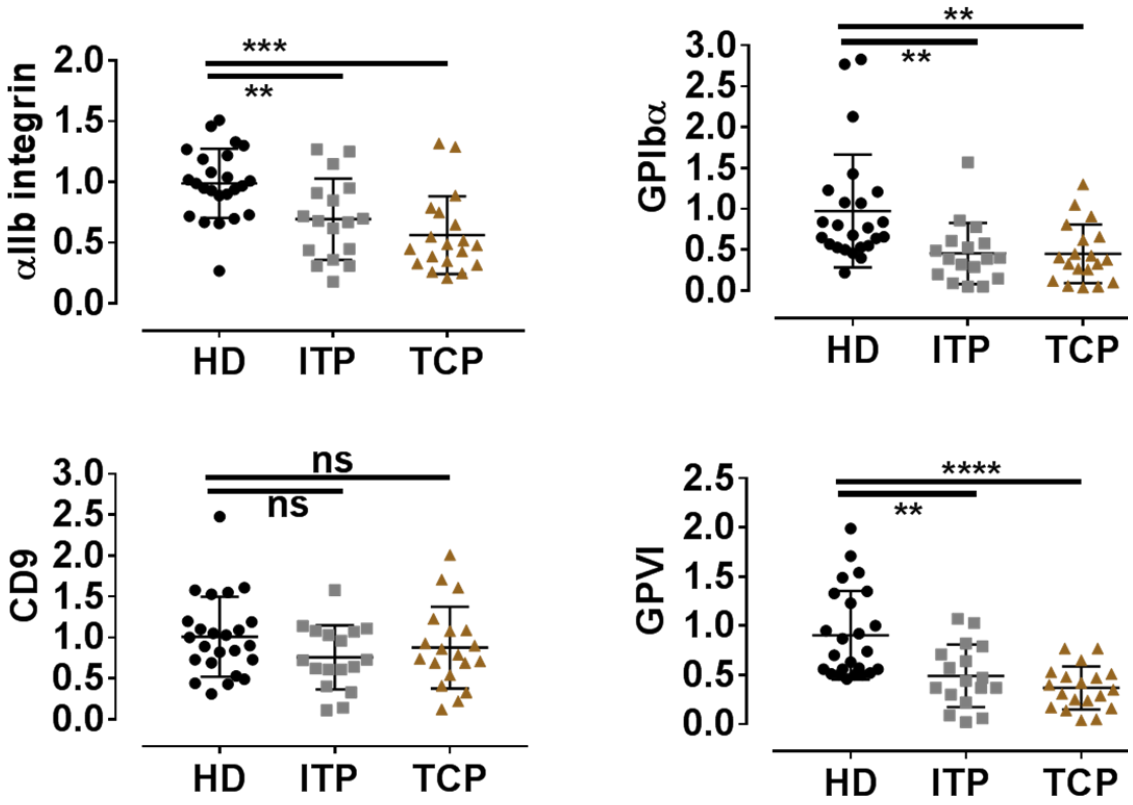

Figure 4.5: Variable levels of αIIbβ3, GPVI, and GPIb on circulating platelets from patients with low platelet counts.

Platelet receptor levels in citrated whole blood samples were analysed by flow cytometry as described in Section 2.2. Levels of αIIb integrin subunit (top left), GPIbα (top right), CD9 (lower left), and GPVI (lower right) are shown. Data were normalised by dividing each mean fluorescence intensity by the average of the control data for each receptor and analysed using 1-way ANOVA with corrections for multiple comparisons. ****, ***, ** and * reflect P<0.0001, P<0.005, P<0.001 and P<0.05 respectively. HD- Healthy Donors (n=23) ITP- Immune Thrombocytopenia Patients (n=17) TCP- Thrombocytopenic Patients (n=19).

Samples from 17 primary ITP patients or 19 TCP patients with non-autoimmune thrombocytopenia and 23 healthy donors were obtained. Surface GPIbα, αIIb integrin and GPVI levels were notably lower in patients with low platelet count than healthy controls analysed on the same day (Figure 4.5). Levels of tetraspanin CD9 and ADAM10 (not shown) remained within normal ranges for both ITP and TCP patient samples. While the mechanisms by which these receptors are regulated *in vivo* remain unclear, several platelet receptors can be metalloproteolytically shed from platelets, particularly upon engagement of the platelet antigen by an antiplatelet autoantibody. Being able to assess one or more

shed ectodomain fragments in plasma using enzyme-linked immunosorbent assays (ELISA) offers flexibility in sample acquisition and storage for batch analysis. Notably, a small number of ITP patients who presented with bleeding were shown to have an anti-GPVI autoantibody (Chapter 3 and (Gardiner et al., 2008a)), with evidence of platelet activation and metalloproteolysis of GPVI, resulting in the release of the GPVI ectodomain (sGPVI) and loss of collagen-related platelet function. Whether other platelet autoantibodies, particularly antibodies against GPIbα, also trigger shedding of platelet receptors, and whether monitoring the loss of receptor ectodomains by flow cytometry of patient platelets in whole blood or measurement of one or more shed ectodomain fragments in patient plasma have any value in assessing the presence of a platelet-activating antibody are open research questions. For example, a significant increase in soluble GPVI as quantified by ELISA signaled the presence of an acquired anti-GPVI autoantibody (Nurden et al., 2009, Gardiner et al., 2010b). These measurements explained the loss of collagen responsiveness in platelet aggregation assays and the observed bleeding that could not be demonstrated by the platelet count (Gardiner et al., 2008a, Moroi et al., 1989). Figure 4.6 shows levels of sGPVI in a small number of HD, ITP, and TCP samples. Levels of sGPVI in the healthy donor cohort were 18.19 ± 13.18 ng/ml, n = 18, which is in excellent agreement with published data (19.7 ± 8.1 ng/ml, n=159) for a healthy cohort measured as part of a stroke study (Al-Tamimi et al., 2011). While a significant amount of variability in sGPVI levels was observed, no difference in plasma sGPVI levels was observed in this cohort, possibly reflecting the heterogeneity of these groups of patients.

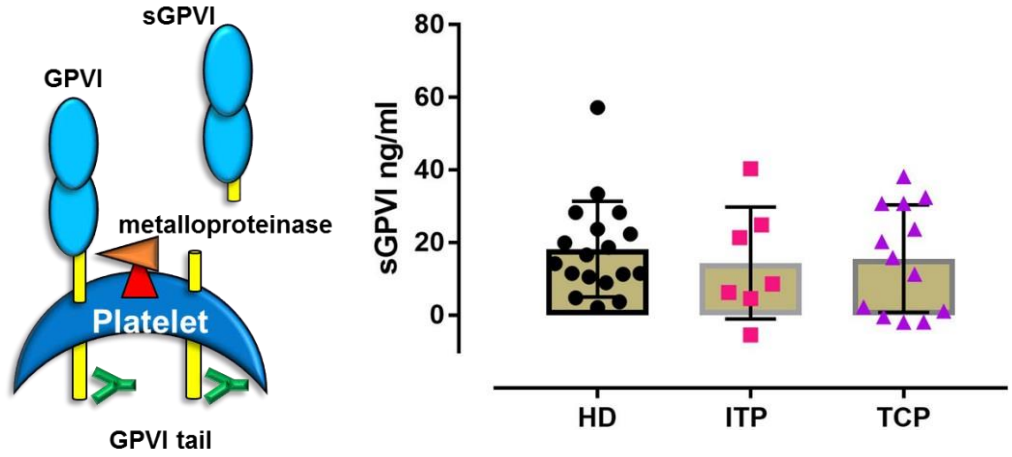

Figure 4.6: sGPVI levels in plasma samples from healthy donors and patients with thrombocytopenia.

A schematic illustrates the generation of the ectodomain fragment of GPVI in response to platelet activation events (left-hand panel). Plasma sGPVI was evaluated in samples from healthy donors (HD) or patients diagnosed with ITP (ITP) or thrombocytopenia (TCP) as described in Section 2.3 using an established ELISA. Data were analysed using 1-way ANOVA with corrections for multiple comparisons. No statistical significance between any of the groups was observed.

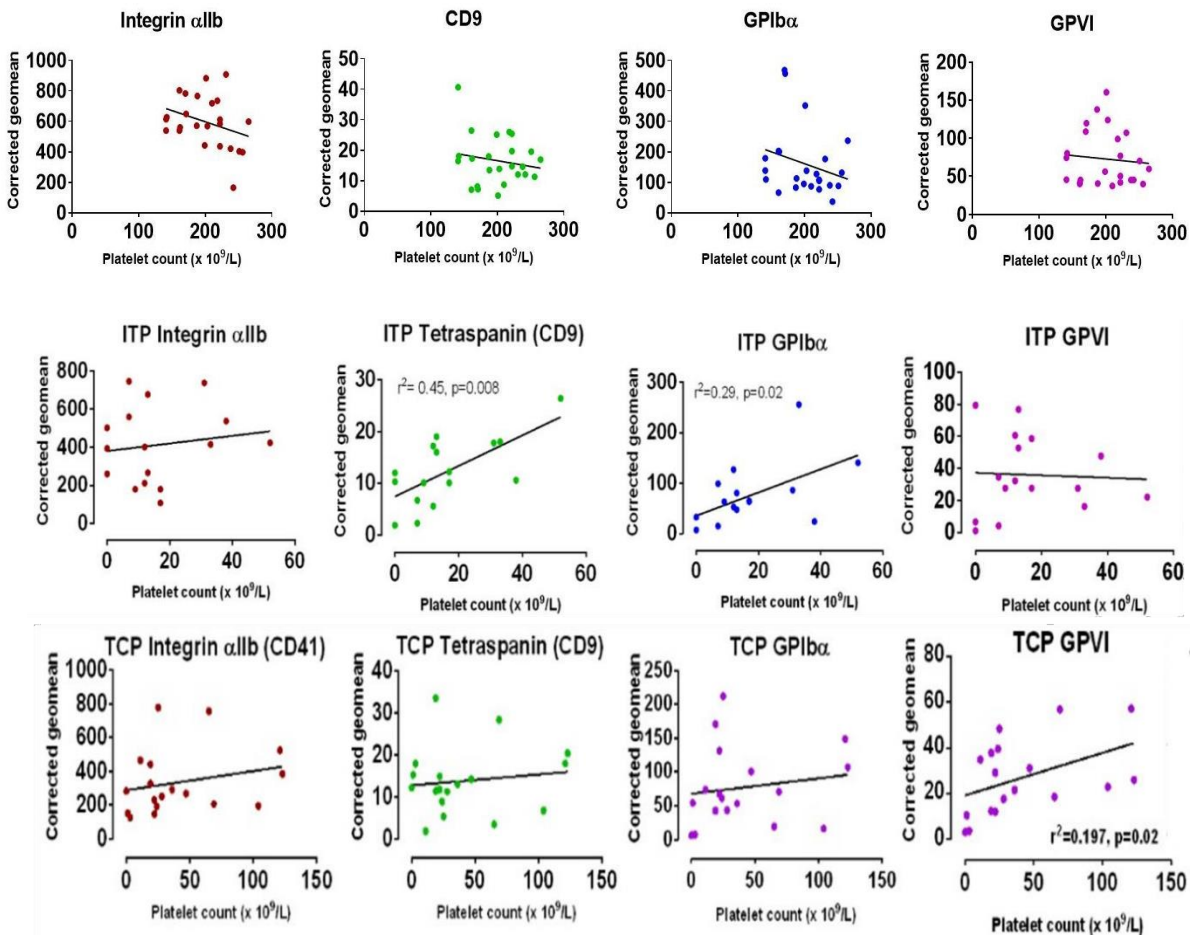

Figure 4.7: Correlation between platelet surface receptor levels and platelet count.

Platelet receptor levels in citrated whole blood samples were analysed by flow cytometry as described in section 2.2. GPIbα and CD9 show a mild level of correlation with platelet count in samples from people with ITP (middle panel) that is less apparent in individuals with thrombocytopenia (TCP, lower panel). Levels of receptors on healthy donor platelets (upper panel) showed no correlation with platelet count. Statistical analysis was carried out using Prism software (version 6, GraphPad, non-linear and linear regression analysis).

In a linear regression analysis, no relationship was observed between any of the measured platelet surface receptors in healthy donors and platelet count; however, there were mild correlations observed between platelet count and GPIbα and CD9 in ITP and GPVI in TCP (Figure 4.7). A mild correlation was identified between platelet count and GPVI in TCP. Interestingly, low GPIbα and CD9 levels have previously been shown to be linked to Bernard Soulier Syndrome, a form of thrombocytopenia caused by the congenital lack of expression of the GPIb-IX-V complex (Zhu et al., 2015, Qiao et al., 2015). The differences

between these patients groups may relate to different mechanisms of pathology by which the platelets are destroyed and aged. These patient groups may relate to other mechanisms of pathology. From this data, it can be concluded that GPVI, GPIbα , and CD9 are linked with low platelet count in specific pathological scenarios.

4.3 Detection of platelet desialylation by flow cytometry

To set up an assay that could detect increased platelet desialyation, and to evaluate whether platelet desialylation could be monitored in a flow cytometer, PRP or washed platelets were treated with NEU1 and then exposed galactose residues resulting from removal of sialic acid were quantified by using *Ricinus communis* agglutinin I (RCA), a galactose-binding lectin from castor beans. Increased RCA binding is indicative of sialic acid removal (Revilla et al., 2019). As a negative control, platelets were also treated with NEM to remove sialomucin GPIbα and GPVI. Figure 4.8 shows that RCA is bound to untreated PRP and washed platelets, and this binding was increased by treatment with increasing amounts of NEU1. Neuraminidase was shown to be effective in the presence and absence of plasma, and NEU1 treatment did not alter levels of GPIbα or GPVI. RCA binding could be diminished by NEM-mediated shedding of GPIbα and GPVI (~50% and 75% respective reduction in receptor levels), indicating that a portion of RCA binding was via these receptors (predominantly GPIbα) and that neuraminidase treatment increased RCA binding. This data suggests that a flow cytometry assay could potentially be used to evaluate the release of NEU1 due to exposure of platelets to anti-platelet autoantibodies, particularly antibodies targeting GPIbα. This remains to be addressed experimentally.

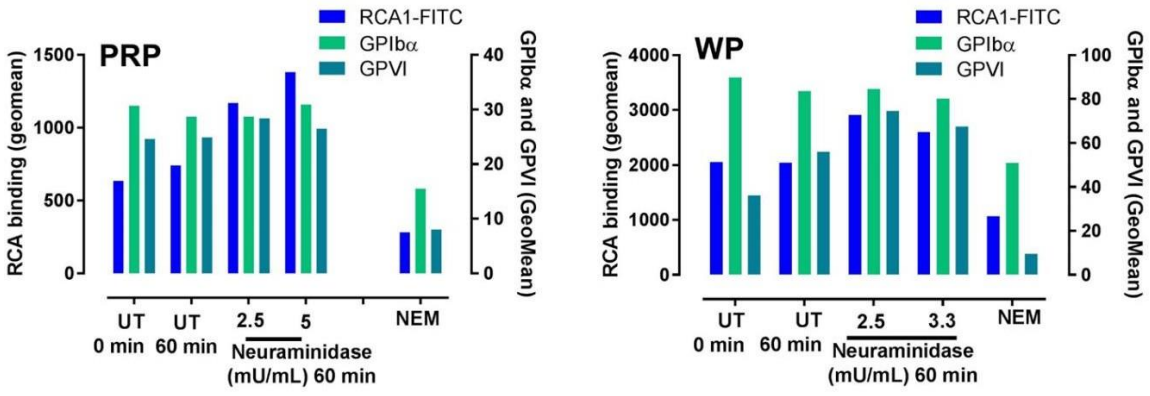

Figure 4.8: Detection of desialylation (increased RCA lectin binding) together with respective levels of GPIbα and GPVI by flow cytometry.

PRP or washed platelets (WP) were left untreated (UT) or treated with 2.5, 3.3 or 5 mU/ml neuraminidase, or 1 mM NEM for 1 h, then levels of GPIbα and GPVI and RCA binding were measured by flow cytometry.

4.4 Assessment of levels of RCA binding and levels of platelet receptors in healthy donors

To begin to assess whether RCA binding could act as a surrogate marker for platelet receptor desialylation, levels of each of GPVI and GPIbα in PRP isolated from healthy donors were compared with RCA binding capacity (Figure 4.9). A mild but significant correlation was found between RCA binding and relative protein levels of each of GPIbα and GPVI.

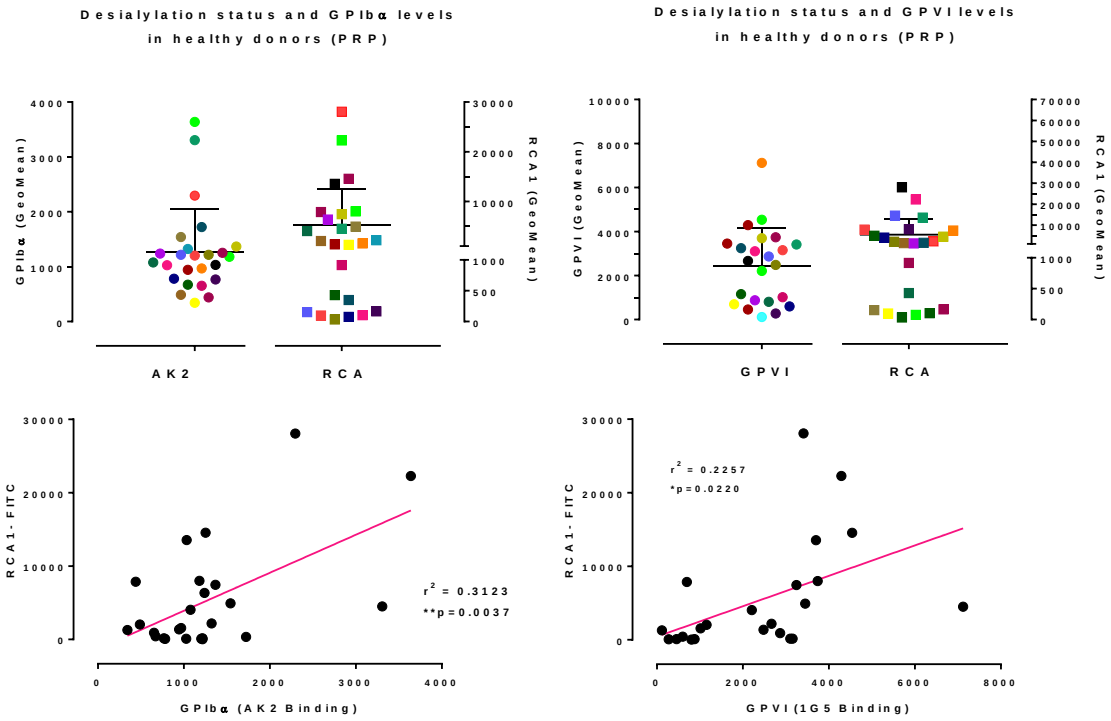

Figure 4.9 Correlation of RCA binding with surface levels of platelet receptors.

Using flow cytometry, RCA's binding levels to platelets in citrated PRP isolated from healthy donors were correlated with levels of GPIbα (left panels) and GPVI (right panels). Respective r^2 values of 0.3123 and 0.2257 were obtained that were shown to have significance (p=0.0037 and 0.022, respectively). Colours indicate data obtained with the sample from a unique donor.

4.5 Discussion

Receptors on the platelet enable interaction of platelets with the surrounding environment and coordinate platelet responses after interaction with agonists and inhibitors. Using flow cytometry, surface levels of GPIbα, αIIb integrin and GPVI levels were evaluated and found to be diminished in patients with low platelet count compared to healthy controls analysed on the same day. There was no relationship between any of the measured parameters and platelet count in healthy donors. Levels of CD9 remained within normal ranges for both ITP and TCP patient samples. In ITP patients, lower levels of the platelet adhesion receptors and the main fibrinogen receptor on platelets may reflect the presence/activity of anti-platelet autoantibodies, and loss of these receptors will likely contribute to the bleeding propensity observed in these patients. This is an important avenue of future research.

Incorporating flow cytometry and platelet function assays utilising flow cytometry will provide additional means of assessing patients at risk of bleeding in the future. The whole blood flow cytometry assay is rapid and easy to perform, with minimal handling of the samples. It could be suitable for standardisation in a clinical haematology facility. Reduction in platelet surface receptors may indicate platelet activation and signaling the presence of antiplatelet autoantibodies in ITP. GPVI and GPIbα may be used to examine mechanisms of cell receptor shedding and may predict bleeding in ITP patients. Platelet protein surface levels thus may stratify ITP patients for appropriate therapies and may help evaluate bleeding risk.

Regression analysis showed a correlation between GPIbα and CD9, platelet count in ITP samples, and between GPVI and platelet count in TCP samples. This implies differences in the mechanism of thrombocytopenia between groups. sGPVI levels in plasma from HD, ITP, and TCP patients show no significant differences. This is somewhat surprising given that platelets from ITP and TCP patients showed low surface levels of GPVI and should be further investigated in ‘add back’ experiments using washed donor platelets and ITP patient plasma.

In two proof-of-principle experiments, the utility of RCA lectin to differentially bind to platelets was demonstrated. A contribution from GPIbα and GPVI for RCA binding (receptor desialylation) was shown. These data begin to explore some of the assays

that could be used to dissect the mechanisms at play in ITP mediated by autoantibody engagement at the platelet surface.

Chapter 5 Discussion and concluding remarks

The overall purpose of these studies was to enable training in blood collection and preparation, platelet aggregation, and flow cytometry. Then the principal aim was to evaluate applications of these approaches and experimentally address a hypothesis regarding the consequences of the action of different antiplatelet antibodies in vitro. Chapter 3 describes a series of research laboratory-based experiments performed using samples of platelets or platelet lysates or serum from a patient with ITP and bleeding that was out of step with the platelet count. This resulted in the description of an anti-GPVI autoantibody that was actively causing shedding of GPVI from the surface of the patient's platelets. Unfortunately, there was no plasma sample available from this patient to enable the measurement of sGPVI. Still, flow cytometry studies and western blot analysis demonstrated a loss of GPVI, which likely explained the bleeding propensity in this patient.

Chapter 4 aimed to develop and apply an assay that monitored platelet receptor levels in patients with newly diagnosed ITP intending to stratify patients based on platelet receptor quality. Whilst the numbers of patients included in this study did not permit evaluation of responses to ITP therapies, the key finding of the work, that circulating platelets in ITP patients display fundamental deficiencies in the two primary adhesion-signaling receptors GPIbα and GPVI, as well as a reduction in levels of the primary platelet receptor for fibrinogen, αIIbβ3. Future research endeavors will evaluate the utility of analysis of platelet receptor expression in vivo or monitoring platelet reactivity or exposure of platelets to antiplatelet antibodies in the setting of ITP.

To understand the pathology of ITP, there is a strong need to develop new tools to better diagnose disease aetiology and pathology for individual therapy. Diagnosis at a molecular level, for example, by detecting the presence of specific antibodies against a platelet receptor, may revamp and improve the diagnosis of ITP and aid in clinical decisions regarding therapy. Flow cytometry of platelets in whole blood is a reliable option for detecting signature events such as platelet receptor shedding that may define the presence of platelet receptor-specific antibodies. Further, the flow cytometer can provide information on platelet quality and platelet function in a setting with a meager platelet count, and routine clinical approaches fail.

Platelet receptors are fundamental to the haemostatic properties of platelets. GPIbα is a receptor for VWF, and GPVI binds collagen and fibrin, subsequently activating intracellular signaling cascades that culminate in platelet aggregation formation. Reduction in one or more of the platelet receptors in a setting where an autoantibody is present can also be a very useful tool for diagnosing the ITP. The loss of GPIbα from the surface of platelets can be readily detected by flow cytometry even in samples from patients with severe thrombocytopenia and is an ideal alternative to measurement of the shed ectodomain fragment GPIbα (glycocalicin), which is challenging. In contrast to GPIbα, which is constitutively shed at a low rate, glycoprotein GPVI on circulating platelets remains intact and is metalloproteolytically shed in response to ligand engagement, or if targeted by auto antibody generally via FcγRIIa binding the Fc portion of the antibody.

The cleaved GPVI ectodomain fragment is thus a marker of platelet engagement and activation and can be used as a biomarker to diagnose ITP or other pathological conditions (Al-Tamimi et al., 2012). Pathological shedding of platelet receptors GPVI and GPIbα may contribute to platelet dysfunction and increased bleeding risk. Recently the utility of measuring sGPVI as a predictor of bleeding risk was demonstrated in patients with heparin-induced thrombocytopenia (Pishko, Andrews, Gardiner, Lefler, & Cuker, 2020), and platelets receptor loss is a signature event in sepsis. It can be linked to an elevated risk of bleeding (Montague et al., 2018).

Autoantibody-initiated activation and clearance of platelets is a feature of ITP (Zeng et al., 2012), and hence reduced platelet receptor levels may reflect specific autoantibody presence. Improving our ability to characterise the nature of the pathological autoantibody in ITP may help decide on individual therapy for a patient with refractory ITP. Specifically, the presence of autoantibodies targeting GPIbα can trigger the desialylation of platelets and result in clearance via the liver (Li et al., 2015). A recent study shows that the mechanosensory domain of GPIbα is unfolded upon the desialyation, and in this way, platelets may be quickly cleared from the blood (Wang et al., 2020). Indeed, plasma from patients with ITP can activate healthy donor platelets and cause desialylation of glycoprotein GPIbα (Li et al., 2015). Data shown in Chapter 4 begins to establish a method by which levels of desialylation of the platelet surface could be evaluated. Future development of this assay would include precisely measuring levels of desialylation of GPIbα using multicolour flow cytometry and a panel of antibodies.

The relative contributions of platelet consumption and platelet production to the clinical observation of thrombocytopenia are an ongoing clinical problem. These studies will provide a new molecular analysis of platelet receptors and platelet turnover and establish new clinical tools for monitoring platelet receptors relevant to platelet function. Diagnosis and treatment of ITP are limited by the available resources, and primary ITP is diagnosed by exclusion, with isolated thrombocytopenia as the only genuinely universal feature. Patients with ITP experience numerous serious clinical consequences, including significantly higher infection rates, bleeding, anemia, debilitating fatigue, and psychological changes. Clinical investigation of patients with ITP varies widely but generally consists of a peripheral blood count and a peripheral blood smear evaluation. Treatment typically comprises conventional first-line therapies that affect the immune system (IV gamma globulins, corticosteroids), followed by splenectomy as the second management line. While newer therapies are emerging, a lack of clinical diagnostic tools and limited financial resources of the individual patient and the health care system strongly impact the choice of treatment. ITP as a syndrome has not been well categorised.

Every individual patient has their unique genetic makeup and disease etiology. Ideally, each individual with ITP requires personalized and customized management and therapy. An approach to individualized treatment with understanding how disease pathology can inform clinical decisions and management of ITP and possibly explain an individual's resistance to specific therapies. Designing a new approach to easily and rapidly detect anti-GPIbα autoantibodies, for example by flow cytometric evaluation of inhibition of binding of a panel of anti-GPIbα mAbs, or by searching for signalling events that are signatures of GPIbα engagement such as loss of calmodulin or 14-3-3ζ binding will help improve diagnosis and treatment of ITP. This has extra interest as ITP patients with autoantibodies against GPIbα but not αIIbβ3 are resistant to IVIg treatment and also may be well placed for treatment with a neuraminidase inhibitor such as oseltamivir (Revilla et al., 2019; Shao et al., 2015). More recent therapies using recombinant or synthetic forms of thrombopoietin aim to increase platelet production and are used as second-line therapies or in combination with first-line therapy (Miltiadous et al., 2020). Presently, treatment decisions are based on clinical presentation and on patient choices with minimal laboratory evaluations and consideration of the specific mechanism of pathogenesis that contributes individually in every patient with ITP (Grodzielski et al., 2019). Research studies such as these will ultimately be helpful in the management of therapeutic approaches; similarly, clinical decisions can be informed by these attributes, and these attributes may be predictive of

response to therapy.

Limitation of these studies

The work described here involved setting up a protocol and pipeline of analysis from inception with almost no baseline protocol to help. Hence these studies and the associated findings should be considered with a number of limitations in mind. First, the numbers of patient samples that were able to be collected within the stated time frame of 3 h were limited in number. Whilst these samples were clinically curated by an expert in the diagnosis of ITP and we can be certain that the primary ITP samples were indeed that, the small numbers in each of the cohorts significantly hampers interpretation. Second, a significant amount of the clinical data was unable to be collected prior to thesis writing and submission. The ability to review the clinical parameters alongside the biochemical and platelet molecular analyses would have enhanced this study and increased the power and breadth of the findings. Third, a number of samples were either lost or produced unusable data due incorrect settings being employed on the flow cytometer or incorrect data analysis steps. Fourth, the severely thrombocytopenic samples (platelet counts below 5 x 10^9/L) still presented major challenges for analysis by flow cytometry due to the extended period of collection time to obtain even 5000 events in the platelet gate resulting in significant amounts of non-platelet events being collected in the same gate. Finally portions of the study were performed where there were significant challenges in accessing laboratories in order to complete laboratory experiments (particularly for work destined to be part of Chapter 4) due to the COVID19 pandemic.

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